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Edited by

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METHODS IN MOLECULAR BIOLOGY ™

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Edited by

Matthew J. Peirce, PhD and Robin Wait, PhD

Kennedy Institute of Rheumatology Division,
Faculty of Medicine, Imperial College London, UK

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Editor
Matthew J. Peirce
Kennedy Institute of Rheumatology Division
Faculty of Medicine
Imperial College London
UK
m.peirce@imperial.ac.uk

Robin Wait
Kennedy Institute of Rheumatology Division
Faculty of Medicine
Imperial College London
UK
r.wait@imperial.ac.uk

Series Editor
John M. Walker
University of Hertfordshire
Hatfield, Herts
UK

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Preface

The membranes surrounding cells and organelles constitute their interface with the local environment. The functions of membrane proteins include cell/cell and cell/extracellular matrix recognition, the reception and transduction of extracellular signals, and the transport of proteins, solutes and water molecules. Abnormal membrane protein expression has profound biological effects and may, for example, underlie phenotypic and functional differences between normal and tumour cells. Moreover the accessibility, particularly of plasma proteins traversing the plasma membrane of cells, makes them of particular utility to the therapeutic intervention in disease. Indeed, it is estimated that of all currently licensed pharmaceuticals, approximately 70% target proteins resident in the plasma membrane.

In theory, unbiased technologies such as proteomics have the power to define patterns of membrane protein expression characteristic of distinct states of cellular development, differentiation or disease, and thereby identify novel markers of, or targets for intervention in, disease.

However, although about 25% of open reading frames in fully sequenced genomes are estimated to encode integral membrane proteins, global analysis of membrane protein expression has proved problematic. Membrane protein analysis poses unique challenges at the level of extraction, solubilization, and separation in particular, and to a lesser extent of identification and quantitation. These challenges have, however, fostered creativity, innovation, and technical advances, many of which are brought together in *Membrane Proteomics.*

Two-dimensional gel electrophoresis (2DE) can resolve mixtures containing many hundreds of components but membrane proteins are often severely underrepresented in 2DE protein patterns because of factors including their size, low abundance, and hydrophobicity. Thus, several chapters describe modifications to standard 2DE protocols; novel combinations of chaotropes, and detergents or solution-phase isoelectric focussing that improve recovery of membrane proteins. Other chapters discuss other electrophoretic methods such as blue-native gel, free-flow, and 16-BAC electrophoresis, which lend themselves to the resolution of membrane proteins yet are not widely used.

Several protocols focus specifically on the problems associated with the low abundance of membrane proteins; an array of protocols for the specific enrichment of plasma membrane proteins using organic solvents or detergents, chemical labelling of lysine or cysteine residues or proteolytic cleavage of PM proteins is presented. The selective enrichment of proteins derived from membranes of the nucleus, mitochondria, and lipid rafts are also covered.

The limitations of 2DE have spurred the development of alternative, nongel based approaches to the resolution of membrane proteins. One approach is to generate a proteolytic digest of a membrane protein sample, the component peptides of which are then resolved by multidimensional chromatography prior to MS/MS. By analysing peptides derived from membrane proteins, rather than the proteins themselves, many of the problems associated with hydrophobicity and large size of membrane proteins are avoided.

Such approaches have proved extremely useful for the study of membrane proteins not least because they lend themselves to techniques such as ICAT and iTRAQ, which enable the relative amounts of particular peptides in similar samples (e.g., resting versus activated cells) to be compared. Several chapters address both the application of such peptide "shotgun" approaches to various types of membrane protein and their combination with ICAT and iTRAQ experiments to enable peptide quantitation.

Another way to obviate the technical obstacles to proteomic examination of membrane proteins is to use an in silico approach. For sequenced genomes such efforts can be extremely informative and we have included several chapters describing how publicly available bioinformatic tools can be used to predict membrane proteins and can be combined with other unbiased approaches, such as transcriptomic analysis, to acquire information regarding the membrane protein complement of a particular cell at a particular time.

In practice, a strategy can be devised for the analysis of virtually any individual membrane protein; the challenge is to find methods capable of simultaneous analysis of large and diverse populations of such proteins. At the moment there seems to be no *single* technology platform which enables global membrane proteome analysis, and it is necessary to apply methods in combination.

This need for methodological pluralism is reflected in the diversity of procedures included in *Membrane Proteomics.* It is intended as a laboratory bench resource which provides a comprehensive toolkit of proven methods, contributed by leading experts in the field, for investigators wishing to apply state of the art membrane proteomic methodologies in their own research programs.

Mathew J. Peirce, PhD
Robin Wait, PhD

Contents

Contributors

PHILIP C. ANDREWS • *Department of Biological Chemistry, University of Michigan, Ann Arbor, MI, USA*

ARJEN Q. BAKKER • *Aimm Therapeutics, Amsterdam, Netherlands*

HENRY BIGELOW • *CUBIC, Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY, USA*

RALF J. BRAUN • *GSF-National Research Centre for Environment and Health, Institute of Human Genetics, Munich, Germany*

SHANE C. BURGESS • *Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, MS 39762, USA*

GEORGINA S. BUTLER • *Centre for Blood Research, University of British Columbia, Vancouver, BC, Canada*

XUEQUN CHEN • *Department of Biological Chemistry, University of Michigan, Ann Arbor, MI, USA*

ANDREW P. COPE • *Kennedy institute of Rheumatology Division, Faculty of Medicine, Imperial College London, London, UK*

AMANDA M. COOKSEY • *Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, MS 39762, USA*

GIAN MARIA D'AMICI • *Department of Environmental Sciences, University of Tuscia, Viterbo, Italy*

MATTHEW N. DAVIES • *Department of Crystallography, Birkbeck College, London, UK*

SIMON J. DAVIS • *Nuffield Department of Clinical Medicine and MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK*

RICHARD A. DEAN • *Centre for Blood Research, University of British Columbia, Vancouver, BC, Canada*

EDWARD J. EVANS • *Nuffield Department of Clinical Medicine and MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK*

HENRIK EVERBERG • *Department of Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, Luund, Sweeden*

ELIZABETH A.L. FAIRLEY • *The Wellcome Trust Centre for Cell Biology and Institute of Cell Biology, University of Edinburgh, Edinburgh, UK*

DARREN R. FLOWER • *Edward Jenner Institute, Newbury, UK*

LAURENCE FLORENS • *The Stowers Institute for Medical Research, Kansas City, MO, USA*

LEONARD J. FOSTER • *UBC Centre for Proteomics, University of British Columbia, Vancouver, BC, Canada*

CECILE A.W. GEUIJEN • *Genmab BV, Utrecht, Netherlands*

MICHAEL B. GOSHE • *North Carolina State University, Department of Molecular and Structural Biochemistry, Raleigh, NC, USA*

KRISTEN M. GLENISTER • *Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, Australia*
ROBERT J.A. GOODE • *Joint Proteomics Laboratory, Ludwig Institute for Cancer Research & The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia*
DAVID W. GREENING • *Joint Proteomics Laboratory, Ludwig Institute for Cancer Research & The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia*
NIKLAS GUSTAVSSON • *Department of Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, Lund, Sweden*
LAWRENCE HENE • *Nuffield Department of Clinical Medicine and MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK*
S. Hussain I Abidi • *Nuffield Department of Clinical Medicine and MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK*
CHRISTIAN G. HUBER • *Department of Environmental Sciences, University of Tuscia, Viterbo, Italy*
HONG JI • *Joint Proteomics Laboratory, Ludwig Institute for Cancer Research & The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia*
NORBERT KINKL • *Technical University Munich, Institute of Human Genetics, Munich, Germany*
KELLI G. KLINE • *Department of Pharmacology, University of Colorado School of Medicine, Aurora, CO, USA*
NADIA KORFALI • *The Wellcome Trust Centre for Cell Biology and Institute of Cell Biology, University of Edinburgh, Edinburgh, UK*
JOHN DE KRUIF • *Merus BV, Utrecht, Netherlands*
LIANG LI • *Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada*
JUSTIN W. LIM • *Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, Australia*
ROMMEL A. MATHIAS • *Joint Proteomics Laboratory, Ludwig Institute for Cancer Research & The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia*
FIONA M. MCCARTHY • *Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, MS 39762, USA*
SRIJEET K. MITRA • *North Carolina State University, Department of Molecular and Structural Biochemistry, Raleigh, NC, USA*
HANS GERD NOTHWANG • *Abteilung Neurogenetik, Institut für Biologie und Umweltwissenschaften, Oldenburg, Germany*
CHRISTOPHER M. OVERALL • *Centre for Blood Research, University of British Columbia, Vancouver, BC, Canada*
MATTHEW J. PEIRCE • *Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, London, UK*
THIERRY RABILLOUD • *Laboratoire, d'immunologie, DRDC/ICH, INSERM, Grenoble, France*
BURKHARD ROST • *Columbia University Center for Computational Biology and Bioinformatics, Irvine Center for Cancer Research, New York, NY, USA*

JENS SCHINDLER • *Abteilung Neurogenetik, Institut für Biologie und Umweltwissenschaften, Oldenburg, Germany*
ERIC C. SCHIRMER • *The Wellcome Trust Centre for Cell Biology and Institute of Cell Biology, University of Edinburgh, Edinburgh, UK*
RICHARD J. SIMPSON. *Joint ProteomicS Laboratory, Ludwig Institute for Cancer Research & The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Parkville, VIC, Australia*
DEREK SMITH • *University of Victoria Proteomics Centre, Vancouver Island Technology Park, Victoria, BC, Canada*
ROSEMARY L. SPARROW • *Research Unit, Australian Red Cross Blood Service (ARCBS), Melbourne, Australia*
SELENE K. SWANSON • *The Stowers Institute for Medical Research, Kansas City, MO, USA*
FOLKE TJERNELD • *Department of Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, Lund, Sweden*
MARIUS UEFFING • *GSF-National Research Center for Environment and Health, Institute of Human Genetics and Technical University Munich, Institute of Human Genetics, Munich, Germany*
MAI VUONG • *Nuffield Department of Clinical Medicine and MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK*
ROBIN WAIT • *Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, London, UK*
NAN WANG • *Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada*
JACEK R. WIŚNIEWSKI • *Department of Proteomics and Signal Transduction, Max-Planck Institute for Biochemistry, Martinsried, Germany*
CHRISTINE C. WU • *Department of Pharmacology, University of Colorado School of Medicine, Aurora, CO, USA*
BRYCE YOUNG • *Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada*
HANS ZISCHKA • *GSF-National Research Center for Environment and Health, Institute of Toxicology, Munich, Germany*
LELLO ZOLLA • *Department of Environmental Sciences, University of Tuscia, Viterbo, Italy*

Part I

In Silico Methods for Prediction of Membrane Protein Hydrophobicity and Topology

Chapter 1

Online Tools for Predicting Integral Membrane Proteins

Henry Bigelow and Burkhard Rost

Abstract

We identify and describe a set of tools readily available for integral membrane protein prediction. These tools address two problems: finding potential transmembrane proteins in a pool of new sequences, and identifying their transmembrane regions. All methods involve comparing the query protein against one or more target models. In the simplest of these, the target "model" is another protein sequence, while the more elaborate methods group together the entire set of transmembrane helical or transmembrane beta-barrel proteins. In general, prediction accuracy either in identifying new integral membrane proteins or transmembrane regions of known integral membrane proteins depends strongly on how closely the query fits the model. Because of this, the best approach is an opportunistic one: submit the protein of interest to all methods and choose the results with the highest confidence scores.

Key words: Membrane protein structure prediction, transmembrane helix, transmembrane beta barrel, hidden Markov model, neural network, remote homolog detection, proteome searching.

1. Introduction

Experimentalists often work with proteins of unknown or partially known structure or function. For transmembrane proteins, there are two types of predictions that can be made. The more common type is identifying transmembrane regions and orientation of a protein already known or suspected to be integral to the membrane. Less common is the problem of identifying all potential integral membrane proteins in a pool of proteins. A variety of online services to solve both problems are available, but it is often time-consuming to find them and especially to discern which methods are reliable and how to interpret the results. Here we provide a guide to aid experimentalists in this endeavor.

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1.1. Basic Concept of Alignments

At a basic level, all methods work by the same paradigm. The simplest of these is BLAST. BLAST aligns the query sequence with each target sequence in a database. The alignment algorithm assigns a score to each alignment of query and target using a 20×20 matrix of scores called a "substitution matrix." The substitution matrix quantifies how often proteins whose sequences are aligned, based on known structure, have the same or different amino acids at each position. The alignment score involves summing substitution matrix values along with scores associated with gaps. Finally, taking all these alignments, a score threshold identifies a subset as target homologs.

1.2. Homology-Transfer through Alignments

Available experimental information for any of the targets can be transferred to the query (homology-transfer). For example, if one of the targets (database proteins) is experimentally known to be a transmembrane helical (TMH) protein, the homologous query is likely to also be a TMH protein. Moreover, if particular regions of a target protein are known to be TMHs, the regions in the query aligned to these regions are also likely to be TMHs. Of course, both inferences are subject to the accuracy of the alignment and the similarity between the two proteins.

As with all elements of living things, protein sequences originate from an evolutionary process of divergence and selection, creating a tree of proteins related in hierarchical fashion. Extending this idea to the homology search, a query protein can be compared to an entire family of related target proteins that are prealigned. Often, where a query might not have apparent similarity to any individual target protein in a family, it may have similarity to the target family taken as a whole. Essentially all advanced methods implement this idea.

1.3. Improved Profile-Based Alignment Methods

A well-known example of this extension is PSI-BLAST *(1)*, which works as follows. First, the query is searched against a database of individual sequences using ordinary BLAST, resulting in a set of query-target alignments. Next, the query and set of target proteins are aligned to each other in a single multiple-sequence alignment. The frequencies of each amino acid as occurring in the columns of the multiple-sequence alignment are calculated, resulting in a set of 20-element vectors, one for each position in the original query. This statistical representation, called a position-specific score matrix (PSSM) can be seen as a substitution matrix, custom designed for each position in the query protein. In subsequent rounds, the PSSM, rather than the original query, is searched against the original database of individual sequences. For statistical reasons, conserved regions tend to be more influential in scoring subsequent alignments, allowing for improved detection of more diverged sequences.

Similar to PSI-BLAST, Pfam *(2)* uses multiple-sequence alignments. There are two differences, however. First, while PSI-BLAST iteratively re-queries a database of individual sequences with a PSSM, Pfam is the inverse: it is a database of protein families, and the individual query protein is aligned against each family in the database. Second, while PSI-BLAST uses PSSMs to represent a protein family multiple sequence alignment, Pfam uses a hidden Markov model (HMM). An HMM extends the idea of position specific substitution scores to include gap insertion and deletion scores that are also position specific. These are possible to derive from the original multiple-sequence alignment by observing how many aligned proteins contain insertions or deletions relative to the query protein at each position in the query. As in PSI-BLAST or BLAST, the query protein is aligned against each HMM in the Pfam database and assigned an E-value comparable to BLAST E-value, representing the expected number of matches as good or better occurring by chance. Since HMM-based alignment methods are often more sensitive than BLAST or PSI-BLAST, they may succeed in finding a homologous family.

BLAST, PSI-BLAST, and Pfam are very general methods capable of identifying sequence or family homologs of virtually any kind of protein, including specific kinds of membrane proteins. For integral membrane protein prediction however, another generalization yields further improvement.

1.4. Two Major Classes of Transmembrane Proteins: TMB and TMH

Integral membrane proteins come in two general structural classes. Transmembrane alpha helical (TMH) proteins span the plasma membrane in one or more alpha helices in alternating direction (**Fig. 1.2**). TMH proteins can be found in plasma membranes, organelle membranes, archaea, and bacteria, except the outer membranes of Gram-negative bacteria. Transmembrane beta-barrel (TMB) proteins reside exclusively in outer membranes of Gram-negative bacteria, atypical Gram-positive ("mycolata") bacteria, mitochondria, and chloroplasts (**Fig. 1.1**). TMBs form an "all-next-neighbor" beta-barrel in the membrane, meaning each transmembrane strand neighboring in sequence is physically adjacent in the beta-sheet forming the barrel. N- and C-termini always reside in the periplasm, dictating an even number of transmembrane strands. Examples range from 8 (OmpX) to 22 (FepA) transmembrane strands.

1.4.1. Specific Prediction Methods

Methods designed to predict TMH or TMB proteins in general are built on each class taken as a group. Because of the diversity in specific structure (different numbers of transmembrane helices or strands), it is impossible to derive a single multiple sequence alignment for such a class. Instead, these methods extract features in common to all TMH or all TMB proteins without the need for explicit multiple-sequence alignment. Technically, this is

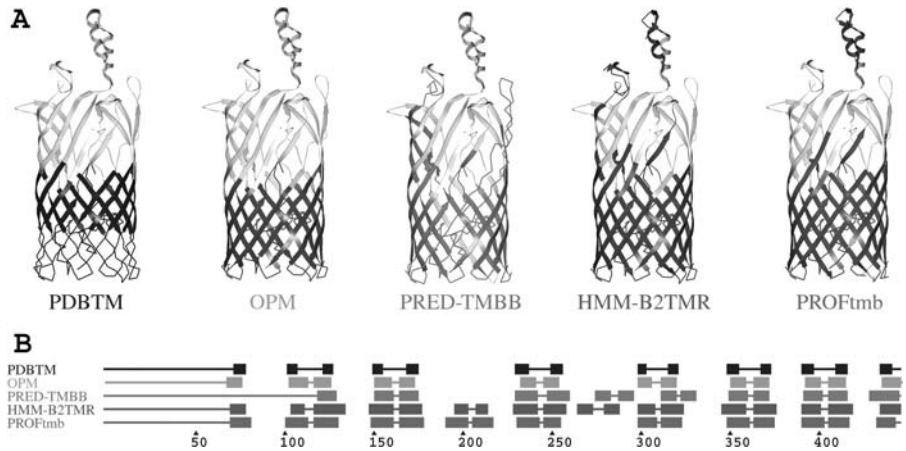

Fig. 1.1. TMB per-residue prediction methods. Per-residue predictions from the three best-performing TMB methods are displayed for long-chain fatty acid transport protein from *E. coli*, both linearly and mapped onto the 3D structure (PDB: 1T16 *(10)*). PDBTM and OPM, both 3D structure-based estimates of relative membrane orientation, are used for comparison against per-residue predictions. Note that the agreement between the methods for this example is not representative. **A.** 3D structure illustration. Inner loops, TM-β-strands, and *outer loops* are depicted. Note that none of the programs actually predicts such a 3D *ribbon plot*, instead the actual predictions are as shown in **B**. **B**. Linear display. *Inner loop*, TM-β-strands, and *outer loop* are depicted respectively by a *thin line, thick line*, and no lone. 3D structure images rendered by GRASP *(47)*.

achieved by assigning one of a set of discrete labels to each position in each sequence, based on its structure. For example, the set of labels T, I, and O can be assigned one per residue to each TMH sequence, identifying the transmembrane helices, inner and outer loops. From the resulting set of labeled protein sequences, a general model (also often a HMM) can be derived that recognizes features common to all labeled protein sequences. Such general models are potentially able to detect TMH or TMB proteins even further diverged from any sequence homolog, (perhaps an example of a previously undiscovered subfamily), than sequence-alignment based methods such as PSI-BLAST and Pfam.

A different homology approach is exemplified in the PROSITE (*3*) and PRINTS *(4)* databases. They contain a set of local sequence patterns defined by strong association with a specific protein function or structure. Because protein function and structure can be modular, some of these patterns may be found within a collection of proteins differing in overall structure. Others are very well correlated with overall structure despite their sequence-local nature. For identifying TMH or TMB proteins, several patterns prove useful (see Methods Section). Potentially, such patterns may be conserved in a protein whose overall sequence is so diverged from any homologs as to be unidentifiable by alignment-based methods.

In general, all methods relying on alignment of proteins work optimally in aligning proteins in a specific similarity range

corresponding to the range of sequences from which they are derived. In a degenerate sense, BLAST can be thought of as searching a database of "models" consisting of individual sequences. It is optimized to find close-range homologs. PSI-BLAST and Pfam build statistical models from multiple alignments of very similar sequences, and they work best to find medium-range homologs. TMH and TMB-specific methods are single statistical models built from a diverse set of TMH or TMB proteins only related by broad structural category. Thus, they are optimized to find long-range homologs.

Optimal results of each of these methods will be obtained fortuitously when the query happens to have a single sequence homolog, homology to a sequence family, or homology to a structure family. It is impossible to know in advance which if any of these will be the case. Because of this, we recommend an opportunistic approach: run all prediction methods and select those giving highest confidence scores. We provide a guide for obtaining as much relevant information about your protein as possible, and some general principles for interpreting the information.

This guide is in three parts. Firstly, we describe how to obtain a quick, comprehensive set of homology-based information and possible experimental information about your protein, and how to use it to identify whether it is an integral membrane protein. Secondly, we describe those methods suitable for screening an entire set of proteins for potential TMH or TMB proteins. Thirdly, we present the methods for predicting which residues in a known or suspected transmembrane protein are in the membrane, and the overall orientation in the membrane. For quick reference, we provide a list of selected programs (**Table 1.1**) and databases (**Table 1.2**).

2. Methods

2.1. Determining If Your Protein Is Integral to the Membrane

There are TMH- or TMB-specific and general methods available. The general methods are motif- and domain-based, and potentially identify the protein as one of a subtype of TMH or TMB proteins. TMH- or TMB-specific methods are designed to identify features common to all TMH (or all TMB) proteins, and do not identify subtypes. InterProScan is a portal that allows querying the general methods at once. UniProt provides a comprehensive view of previously analyzed results on many proteins and accompanying experimental information on structure or function.

2.1.1. TMB-Specific Methods

BOMP (β-barrel outer membrane protein predictor), TMB-HUNT, and PROFtmb are specially designed to identify TMB proteins in a pool. They have all been evaluated for accuracy in

Table 1.1
Selected programs

Method	Scope	Service	URL	References
BLAST and PSI-BLAST	general	WP	www.ncbi.nlm.nih.gov/BLAST	*(1, 15)*
TMHMM	TMH	PR3, S	www.cbs.dtu.dk/services/TMHMM	*(16)*
PiMohtm	TMH	PR3	www.predictprotein.org	*(17)*
Phobius	TMH	PR3, SP, S	phobius.cgb.ki.se	*(18)*
HMMTOP	TMH	PR3	www.enzim.hu/hmmtop	*(19)*
MEMSAT	TMH	PR5	bioinf.cs.ucl.ac.uk/psipred	*(20)*
Split4	TMH	PR2	split.pmfst.hr/split/4	*(21)*
PRED-TMBB	TMB	PR3	bioinformatics2.biol.uoa.gr/PRED-TMBB	*(22, 23)*
HMM-B2TMR	TMB	PR3	gpcr.biocomp.unibo.it	*(24)*
PROFtmb	TMB	PR3, S	rostlab.org/services/proftmb	*(25)*
TMB-HUNT	TMB	S	www.bioinformatics.leeds.ac.uk	*(26, 27)*
BOMP	TMB	S	www.bioinfo.no/tools/bomp	*(28)*
SignalP	SP	SP, S	www.cbs.dtu.dk/services/SignalP	*(29)*
Pfam	domain	WP	www.sanger.ac.uk/Software/Pfam	*(2)*
Superfamily	domain	WP	supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY	*(30)*
Panther	domain	WP	www.pantherdb.org	*(31)*
SMART	domain	WP	smart.embl-heidelberg.de	*(32, 33)*
PROSITE	motif	WP	ca.expasy.org/prosite	*(3)*
PRINTS	motif	WP	umber.sbs.man.ac.uk/dbbrowser/PRINTS	*(4)*

Selected programs and databases for identification and per-residue prediction of integral membrane proteins.
Scope*. TMH, TMB**: built on a representative collection of TMH or TMB proteins. **motif**: built on short sequence motifs associated with particular function or structure. **domain**: built on medium to long sequence regions of particular structure. ***Service. Per residue predictions PRn: all residues are assigned to one of a number of discrete structural states; **PR2**: TM, non-TM; **PR3**: TMB: TM-strand, extra cellular loop, and periplasmic loop. TMH: TM-helix, cytoplasmic loop, and noncytoplasmic loop. **PR5**: PR3, but distinguishing non-TM portions of helical overhang on both sides. **SP**: Signal peptide and cleavage site prediction. **S**: suitable for whole-proteome screening; these methods all allow multiple-sequence submission and have been evaluated for accuracy and coverage in whole protein discrimination. **WP**: whole protein prediction of individual proteins.

discriminating TMBs from background. Unfortunately, a definitive comparison is complicated by the fact that the evaluations are all done on different data sets. It is recommended that you submit your query to all three and scrutinize the results. Taking a consensus of predictors has been found consistently to yield better accuracy than relying on one individual predictor.

Table 1.2
Selected databases

Database	Common name/description	URL	References
GO	Gene Ontology	www.geneontology.org	*(34)*
PIR	Protein Information Resource	pir.georgetown.edu	*(6)*
PDB	Protein Data Bank	www.rcsb.org/pdb/Welcome.do	*(35)*
InterPro	Database of Protein Families, Domains and Functional Sites	www.ebi.ac.uk/interpro	*(36)*
SCOP	Structural Classification of Proteins	scop.mrc-lmb.cam.ac.uk/scop	*(37–39)*
InterProScan	Scanning of InterPro Database	www.ebi.ac.uk/InterProScan	*(40, 41)*
UniProt	Universal Protein Resource	www.pir.uniprot.org	*(42)*
OPM	Orientations of Proteins in Membranes	opm.phar.umich.edu	*(43)*
PDBTM	Protein Data Bank of Transmembrane Proteins	pdbtm.enzim.hu	*(44, 45)*
MPtopo	Membrane Protein Topology Database	blanco.biomol.uci.edu/mptopo	*(46)*

2.1.2. TMH-Specific Methods

Of the six TMH-specific methods, only TMHMM has been rigorously evaluated for accuracy in discriminating TMH proteins from others. While all methods implicitly predict whether a protein is a TMH by the presence of one or more predicted TM-helices, since the others are not evaluated for accuracy, it is not recommended to use them to screen a pool for potential TMH proteins.

2.1.2.1. InterProScan

InterProScan submits your query to up to 13 individual predictors at once. Go to InterProScan, make sure all **Applications to Run** are selected, paste your sequence, and **Submit**. When results are returned, select **Table View** to see the individual scores associated with each hit. Individual scores are unfortunately not in any standard units. Though a thorough statistical comparison between different scoring systems has not been done, we will discuss this issue in the subsequent paragraphs.

2.1.2.2. UniProt

UniProt joins together all sequences from SWISS-PROT, TrEMBL *(5)*, and PIR *(6)* (Protein Information Resource). Release 6.0 of September 2005 contains 2,299,834 sequences (see www.ebi.uniprot.org/support/docs/rel_notes/relnote6.0.

html). Each protein is linked with a set of prerun predictions and annotations in databases in an advanced searchable framework. Results of searches contain links to the original sources of prediction and annotation.

1. Go to UniProt. Select **Searches/Tools –> Blast**. Select **UniProtKB** in the pull-down menu of databases to search. Paste your query and **Submit**.
2. You will see a table with several columns. Select checkboxes of *all* proteins with convincing alignments. The goal is to discover homologs with associated structural or functional information. Though your query may have several very close homologs, only those with associated experimental information are useful. Therefore, it is best to be liberal here, say to E-value ≤ 1.0. If you have a particular region of interest of your protein, you can base this choice on suitable position and length of alignment overlap, shown graphically in the **Alignment** column. Clicking on the colored bar in that column pops up the BLAST alignment.
3. To view complete information, you must first re-query UniProt with the IDs of your selected homologs. To do this, select **Save Options –>Table**, and extract the list from the resulting tab-separated file.
4. Select **Searches/Tools** -> **Useful Tools/Links –> Batch Retrieval (PIR)**. Select database **UniProtKB**, and define Entry IDs as **UniProt KB ID**, paste your list of IDs and **Search**.
5. Now, you will have a table of all homologs with complete information. Click **Display Options** and move all columns into **Columns in Display**, then select **Apply**.

2.1.3. Analyzing the Results

Looking at your InterProScan and UniProt results, your protein has hopefully matched homologs with associated structural or functional annotation in databases PIR, PDB, InterPro, SCOP, and Gene Ontology (GO). Matches to alignment-based models of interest are Pfam, SMART, Superfamily, and TigrFAM. Finally, functional motif databases are PROSITE and PRINTS.

The goal is to identify whether your query is indeed a TMH or TMB. But, it is not always easy to determine how closely associated these features are with integral membrane status. Because of the diversity of integral membrane proteins, there is no simple way to distinguish which annotations or features definitively identify with integral membrane status. Therefore, it will be necessary to do a careful reading of the descriptions, available through the links on the InterProScan and UniProt results tables. In the subsequent discussions, we describe some of these in more detail. For PROSITE we present our own quick analysis of particular motifs closely associated with TMH or TMB proteins.

2.1.3.1. Pfam

As discussed earlier, Pfam is a database of HMMs, with each HMM built from an alignment of sequence-related proteins. As of December 2005, Pfam contains 8,183 families. Of all known protein sequences, 94% match at least one Pfam family. Its main purpose is to identify to what family or families a protein of unknown structure or function belongs. To search Pfam, one submits the protein of interest, and just as in BLAST, it is aligned to each HMM and significant alignments are reported with an accompanying $\log_2$-odds (or 'bits') and E-value scores. The Pfam E-value is comparable with a BLAST E-value. Bits is the $\log_2$ (logarithm of base 2) of the odds of the sequence being a true match. For example, a bits score of 3 means the protein is 8 times more likely to be a true match than false match (8/9, or 89% chance of being a true match).

Pfam families are categorized as "family," "domain," "motif," and "repeat" and have an accompanying average length of alignment. Family and domain type families are most closely associated with structure. Families are organized into clans, one of which is "outer membrane beta-barrel." There is no corresponding clan covering TMH associated Pfam models, but a keyword search "membrane" reveals many relevant families.

Using Pfam to determine integral membrane status is complicated by the possibility that TMH or TMB proteins may contain N- or C-terminal domains or extramembrane loops that form domains also found in soluble proteins.

2.1.3.2. SMART

Similar to Pfam, SMART is a collection of HMMs built from seed alignments. SMART focuses exclusively on protein domains, and describes them as "extensively annotated with respect to phyletic distributions, functional class, tertiary structures, and functionally important residues." A large fraction of SMART domains will overlap with Pfam.

2.1.3.3. Superfamily

Similar to Pfam and SMART, Superfamily is a database of HMMs built on multiply aligned protein sequences. While Pfam uses similar sequences, Superfamily groups together sequences that have no detectable homology but are structurally similar at the SCOP superfamily level of structural classification. Because these HMMs are built from more diverse groups of sequences, they may have greater power to detect TMH or TMB proteins evolutionarily more distant from known homologs than Pfam. Depending on whether your sequence has a close or remote homolog, you may see reliable hits to any or all of these HMM-based databases.

2.1.3.4. Gene Ontology (GO)

According to their homepage, "the Gene Ontology project provides a controlled vocabulary to describe gene and gene product attributes in any organism." It is useful in computer searches

in which the existing words typically used to describe a given function are imprecise or ambiguous. Using the Gene Ontology ***(GO)***, the European Bioinformatics Institute, in a project called GOA *(7, 8)* (Gene Ontology Annotation), manually assigns GO terms to existing proteins in UniProt based on the literature, thus allowing comprehensive searches for proteins by GO terms.

2.1.3.5. PROSITE

PROSITE is a database of usually short sequence patterns tightly associated with function or overall protein structure. The patterns are all evaluated for their predictive power of protein class. For example, PROSITE pattern [LIVMFYC]-{A}-[HY]-x-D-[LIVMFY]-[RSTAC]-{D}-x-N-[LIVMFYC](3), called "Tyrosine protein kinases specific active-site signature" detects 97.9% of all known protein tyrosine kinases at 95.4% accuracy. Since each PROSITE pattern is designed to identify specific elements of function or structure, it would be useful to know if any of these happen to correlate well with TMH or TMB proteins (**Tables 1.3 and 1.4**).

2.1.3.6. PROSITE Motifs Specific for Integral Membrane Proteins

Since PROSITE motifs are defined by association to a specific function or structure which is often local, some motifs are found in more than one overall protein structure. Many other motifs are well correlated with overall structure, and are potentially useful in identifying integral membrane proteins.

To estimate how well each motif correlates with integral membrane protein structure, we did the following. We prepared lists of TMH and TMB proteins by querying UniProt as follows. For TMBs, we queried with **GO**: "integral to membrane" **AND GO:** "outer membrane," returning 3,464 proteins. For TMH the query was **GO:** "integral to membrane" **NOT GO:** "outer membrane," returning 309,360 proteins. Technically, we carried out these queries by parsing a file called gene_association.goa_uniprot from the GOA project at http://www.ebi.ac.uk/GOA/goaHelp.html since TMH proteins exceeded the download limit.

With the lists of known TMH and TMB proteins, we counted, for each PROSITE motif, the number of TMH (or TMB) proteins containing the motif, (true positives) and the number of non-TMH (or non-TMB) proteins containing the motif (false positives). We calculated the accuracy of a given motif in identifying TMH (TMB) proteins, selecting those patterns with a significant number of true positives at a given accuracy (**Tables 1.3 and 1.4**). This is the same procedure for PROSITE pattern accuracies but taken with respect to TMH or TMB proteins. Since our lists of TMH and TMB will be incomplete due to missing GO annotation, the accuracies are necessarily lower bound estimates.

Table 1.3
TMH-specific PROSITE motifs

TP	FP	Minimum accuracy (%)	Accession	PROSITE motif description
303	0	100	PS50928	ABC transporter integral membrane Type-1 domain profile.
212	0	100	PS00236	Neurotransmitter-gated ion-channels signature.
209	0	100	PS50261	G-protein coupled receptors family 2 profile 2.
209	0	100	PS00077	Heme-copper oxidase catalytic subunit, copper B binding region signature.
1,273	5	100	PS51003	Cytochrome b/b6 C-terminal region profile.
1,326	9	99	PS51002	Cytochrome b/b6 N-terminal region profile.
265	2	99	PS50999	Cytochrome oxidase subunit II transmembrane region profile.
2,136	20	99	PS50262	G-protein coupled receptors family 1 profile.
209	2	99	PS50855	Cytochrome oxidase subunit I profile.
250	4	98	PS00232	Cadherin domain signature.
646	11	98	PS50850	Major facilitator superfamily (MFS) profile.
253	5	98	PS50268	Cadherins domain profile.
205	6	97	PS00238	Visual pigments (opsins) retinal binding site.
334	11	97	PS00154	E1-E2 ATPases phosphorylation site.
2,122	95	96	PS00237	G-protein coupled receptors family 1 signature.
274	14	95	PS50857	Cytochrome oxidase subunit II copper A binding domain profile.
271	14	95	PS00078	CO II and nitrous oxide reductase dinuclear copper centers signature.
263	46	85	PS00216	Sugar transport proteins signature 1.
256	45	85	PS50929	ABC transporter integral membrane Type-1 fused domain profile.
223	97	70	PS50109	Histidine kinase domain profile.
245	113	68	PS00217	Sugar transport proteins signature 2.
305	192	61	PS50853	Fibronectin Type-3 domain profile.
993	754	57	PS50835	Ig-like domain profile.
461	417	53	PS01186	EGF-like domain signature 2.
209	204	51	PS00109	Tyrosine protein kinases specific active-site signature.

(continued)

Table 1.3 (continued)

TP	FP	Minimum accuracy (%)	Accession	PROSITE motif description
292	289	50	PS00290	Immunoglobulins and major histocompatibility complex proteins signature.
355	355	50	PS50026	EGF-like domain profile.
418	441	49	PS00022	EGF-like domain signature 1.
215	321	40	PS00152	ATP synthase alpha and beta subunits signature.
337	522	39	PS00142	Neutral zinc metallopeptidases, zinc-binding region signature.
317	1,301	20	PS50893	ATP-binding cassette, ABC transporter-type domain profile.
333	1,412	19	PS00211	ABC transporters family signature.
338	1,809	16	PS50011	Protein kinase domain profile.
326	1,800	15	PS00107	Protein kinases ATP-binding region signature.

PROSITE motifs specific for TMH proteins.
TP: True Positives; the number of TMH proteins containing the motif. **FP**: False Positives; the number of non-TMH proteins also containing the motif. **Minimum Accuracy**: TP/(TP + FP), a *lower bound* estimate of the probability that an unknown protein containing the motif in question is a TMH. It is a *lower bound* because some TMH proteins will be missing the appropriate GO terms, and will incorrectly be considered false positives in these lists (when in fact they should be true positives).

Similar analysis can be done for PRINTS but we did not perform this. Since Pfam, SMART, and Superfamily domains tend to match long (>100 residues) stretches of sequence, they tend to correlate well with overall protein structure. We advise simply to read the descriptions of each domain your protein may match.

2.1.4. Overall Recommendation

Taking all information you can gather from both homology-based models and individual sequence homology to any proteins with useful annotation, decide which annotations you trust based on the respective E-values and other scores given by the search methods. Read the descriptions carefully, keeping in mind the possibility that integral membrane proteins contain motifs also found in soluble proteins. In general, matches to short regions of your protein are less reliable indicators of overall protein structure. This includes Pfam families of the "motif" and "repeat" types. Hopefully, this first step will give a very comprehensive view of what kind of domains or motifs your protein contains and what kind of protein it is likely to be.

Table 1.4
TMB-specific PROSITE motifs

TP	FP	Minimum accuracy (%)	Accession	PROSITE motif Description
39	0	100	PS00576	General diffusion Gram-negative porins signature.
36	0	100	PS00558	Eukaryotic mitochondrial porin signature.
34	0	100	PS01151	Fimbrial biogenesis outer membrane usher protein signature.
10	0	100	PS00695	Enterobacterial virulence outer membrane protein signature 2.
9	0	100	PS00694	Enterobacterial virulence outer membrane protein signature 1.
46	7	87	PS01068	OmpA-like domain.
5	1	83	PS00835	Aspartyl proteases, omptin family signature 2.
5	1	83	PS00834	Aspartyl proteases, omptin family signature 1.
46	18	72	PS51123	OmpA-like domain profile.
28	46	38	PS01156	TonB-dependent receptor proteins signature 2.
10	23	30	PS00439	Acyltransferases ChoActase / COT / CPT family signature 1.
10	23	30	PS00440	Acyltransferases ChoActase / COT / CPT family signature 2.
2	16	11	PS01098	Lipolytic enzymes 'G-D-S-L' family, serine active site.
28	332	8	PS00430	TonB-dependent receptor proteins signature 1.
3	42	7	PS50304	Tudor domain profile.
4	83	5	PS50255	Cytochrome b5 family, heme-binding domain profile.
4	88	4	PS00191	Cytochrome b5 family, heme-binding domain signature.
3	76	4	PS50209	CARD caspase recruitment domain profile.
43	1,359	3	PS00013	Prokaryotic membrane lipoprotein lipid attachment site.
3	242	1	PS50084	Type-1 KH domain profile.
5	467	1	PS50005	TPR repeat profile.
5	496	1	PS50293	TPR repeat region circular profile.

PROSITE motifs specific for TMB proteins. Abbreviations as in **Table 1.3**.

2.2. Screening a Pool of Sequences for Integral Membrane Proteins

A few methods identify potential integral membrane proteins among a pool of sequences. They have been evaluated for accuracy and coverage and allow multiple sequence submission. BOMP and PROFtmb screen for Gram-negative bacterial TMB proteins, while TMB-HUNT additionally screens for eukaryotic TMB proteins and those of atypical Gram-positive bacteria. TMHMM and Phobius screen for TMH proteins.

2.2.1. TMB Screening Methods

BOMP scores sequences on a 0–5 scale. The special score 0 means that BOMP itself did not predict the query protein to be a TMB but it had one or more known TMB homologs. The scores 1–5 represent varying qualitative degrees of confidence in the prediction. BOMP is extremely fast, screening about one protein per second. It can be run with or without using BLAST for additional information (we recommend running with BLAST). Precomputed predictions for entire genomes are available for download.

TMB-HUNT discriminates TMBs from non-TMBs based on whole-protein amino acid composition. It is also extremely fast and runs with or without the use of BLAST internally. Unfortunately, multiple-sequence submission is only available when it is run without BLAST, which is less accurate. TMB-HUNT reports for each protein an odds ratio and E-value calculated from the odds ratio. Although it is not the same formula as the BLAST E-value, the authors claim it can be interpreted the same way.

PROFtmb is much slower, requiring about 4 minutes per protein. It builds a PSI-BLAST profile for each query, allowing user-specified parameters. Internally, each protein is assigned a bits score in log odds units. For technical reasons, the traditional E-value measure cannot be calculated; instead, a so-called Z-score is reported. The Z-score is the number of standard deviations the query bits score is separated from the average bits scores of background proteins of similar length in a test set. Also reported are the accuracy and coverage for the given Z-score. The accuracy can be interpreted as the probability that the given query protein is a TMB.

2.2.2. TMH Screening Programs

Of the six TMH prediction programs, only TMHMM is evaluated for accuracy in discriminating TMH from non-TMH proteins. It reports the position and orientation of each predicted helix and the expected number of residues in TM-helices. While there is no note on the Website, in the original work they recommend using the expected number of residues, with a cutoff at 18, as the criterion for whole-proteome screening. For a test set of 160 TMH proteins and 645 soluble proteins, only 0.5–1% of the soluble proteins are predicted to have over 18 residues in TM-helices and only 1 or 2 TMH proteins (1%) were predicted to have 18 or

fewer TM-helical residues. They caution that signal peptides are often mispredicted as TM-helices.

Phobius combines TMHMM and SignalP to optimize discrimination between signal peptides and transmembrane helices, and prediction of protein orientation (N-terminal out or in) using the fact that presence of a cleavage site for a TMH protein is equivalent to N-terminal out orientation. While Phobius accuracy in whole-protein discrimination hasn't been formally measured, the results have been thoroughly compared with TMHMM. It is likely that Phobius is an improvement over TMHMM for the purpose of whole-protein screening. Choosing **short** output format, a single table is provided with columns **TM** (number of predicted TM-helices), **SP** (Y=signal peptide present; N=absent), and **Prediction** (positions and orientations of each predicted TM-helix). The resulting list of proteins can easily be screened in the **TM** column. If more than one version of the sequence is available, the longer precursor sequence should be submitted. Polyphobius is also available on the Website, although it isn't documented in the original paper. It first builds a multiple-sequence alignment using BLAST before running the model.

2.3. Per-Residue Predictions

Per-residue prediction is the prediction of each residue to be in a structural state. For TMH proteins, it is usually one of three states TM-helix, cytoplasmic loop, and noncytoplasmic loop. For TMB proteins, they are TM-strand, extracellular loop, and periplasmic loop. Such prediction is not provided explicitly by Pfam, PROSITE, or any of the family-, domain-, or motif-specific methods. The methods described below do provide such a prediction. In the rare case that your protein has close homology to a target protein of known structure, you will get a more accurate prediction simply by transferring structural annotation to your query through the alignment. In this case, you may use 3D structure-based methods to estimate position and thickness of the membrane bilayer relative to the protein. These methods include MPTopo (Membrane Protein Topology), PDBTM (Protein databank of Transmembrane Proteins), and OPM (Orientations of Proteins in Membranes). They are also used as reference standards for evaluating accuracy of per-residue prediction methods that predict TM regions from sequence alone. Low-resolution experiments also reveal transmembrane regions, which are annotated in the FEATURES Section of the UniProt record (click on the UniProt ID) or "FT" fields in the flat file (go to **Save Options –> Flatfile**).

2.3.1. TMB Proteins

A recent evaluation by Bagos et al. *(9)* evaluated per-residue prediction accuracy of 11 TMB methods. They used 20 TMB PDB structures as a reference set, and defined just the bilayer-spanning region (as defined by PDBTM) of each TM β-strand.

Three methods were found most accurate by far, especially in distinguishing between periplasmic and extracellular loops. They were PRED-TMBB, HMM-B2TMR, and PROFtmb. All three are HMMs that provide three-state (TM-strand, extracellular loop, and periplasmic loop) per-residue prediction, probability plot roughly indicating the confidence in the prediction for the given residue, and an overall score indicating how likely the query is to be a TMB. Importantly, PROFtmb was designed to predict the entire span of each TM-β-strand including extra cellular extensions. Thus, the choice of evaluation criteria did not match the intended use for this method. Also, it is apparent that the membrane thickness is estimated differently between OPM and PDBTM (**Figs. 1.1 and 1.2**), leading to significant differences in the inferred position of protein TM regions along the sequence.

2.3.2. Specific Recommendations

PRED-TMBB allows three decoding schemes. In the independent evaluation, posterior decoding gave the best performance, closely followed by n-best. The Viterbi decoding option was not listed among the top predictors at all, and we recommend against using it. Similarly, HMM-B2TMR and B2TMR (NN-based) are both available, but only HMM-B2TMR scored well in the evaluation. Use of NN-based B2TMR is, therefore, not recommended. Also, note that PROFtmb is designed to predict the entire span of TM-β-strands, including any possible extramembrane extensions.

2.3.3. Interpretation of Results

For all the three methods, results consist of a three-state per-residue prediction, and a graphical output of state probabilities at each sequence position. Positions predicted with high probability according to the graph are more likely to be correct although the correlation hasn't been rigorously quantified in any of the models. PROFtmb is designed to predict the position of entire transmembrane strands including extracellular and periplasmic extensions, while the other two are designed to predict just the transmembrane portion of strands.

2.3.4. Overall Confidence Score

PRED-TMBB gives a whole protein score for which lower is better. The authors do not provide any estimate of probability of TMB according to the score, but simply state that a score *below* the threshold of 2.995 is probably a TMB. PROFtmb provides a Z-score, defined as the number of standard deviations from the average non-TMB protein of similar length (discussed above).

If you already know your protein is TMB and only need to know the positions of transmembrane strands, the overall score is still useful. A higher score indicates a better fit to the model, which always indicates more accurate prediction of positions of transmembrane strands. Also, a tell-tale sign of an unreliable prediction is a poorly shaped probability graph in which the predicted

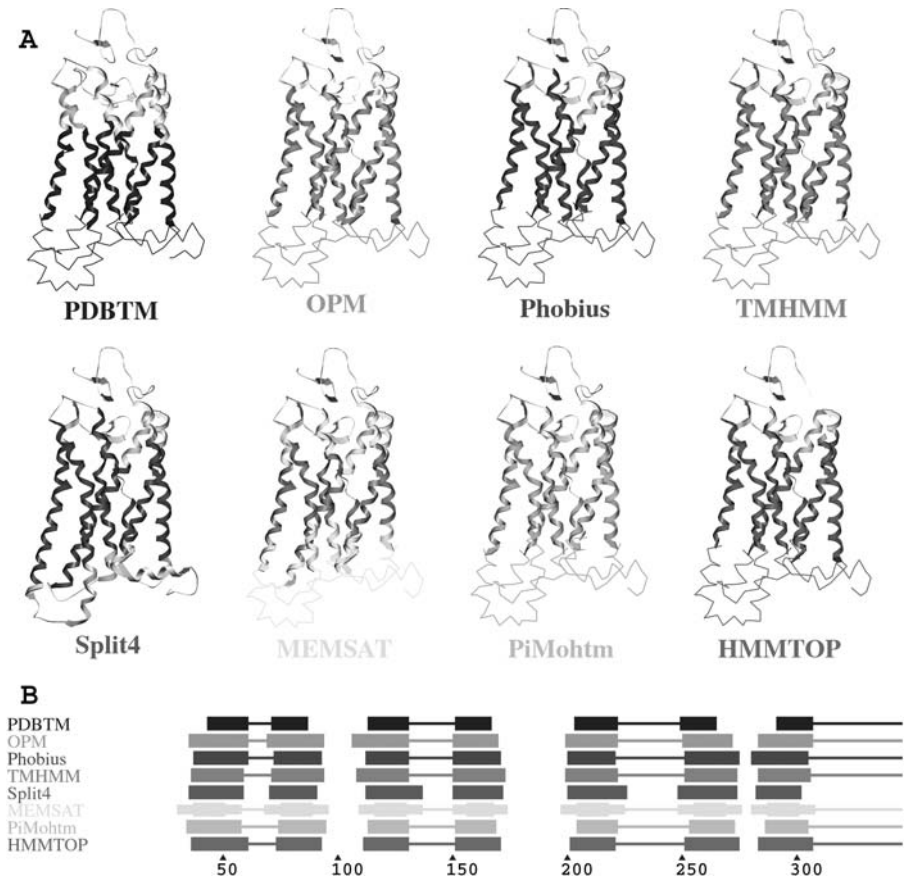

Fig. 1.2. TMH per-residue prediction methods. Per-residue predictions from the six best-performing TMH methods are displayed for cow rhodopsin linearly and mapped onto the 3D structure (PDB: 1GZM *(14)*). PDBTM and OPM are used as in **Fig. 1.1**. Note that the agreement between the methods for this example is not representative. **A**. 3D structure illustration. *Inner loops*, TM-helices, and *outer loops* are depicted. Note that none of the programs actually predicts such a 3D *ribbon plot*, instead the actual predictions are as shown in **B**. **B**. Linear display. *Inner loop*, TM-helix, and *outer loop* are depicted respectively by a *thin line, thick line*, and no lone. For MEMSAT, predicted TM-*helix caps* are depicted as an intermediate thickness line and *darker ribbons* on the structure. Note that all methods except Split4 distinguish *inner and outer loops*. 3D structure images rendered by GRASP *(47)*.

transmembrane strands do not have near 100% probability for a majority of their length.

2.4. TMH Proteins

Two studies comparing accuracies of available methods reveal overlapping results. In 2002, Chen et al. *(11)* tested a set of 28 methods using the positions of TM-helices, estimated by MPtopo, from 36 PDB structures as a reference set. The three most accurate methods were PiMohtm (then known as PHD-htm), HMMTOP, and TMHMM. They all predicted 80% of residues correctly as being either TM-helix or non-TM-helix. The most recent study in June 2005 by Cuthbertson et al. *(12)* used 25 TMH PDB structures. They used the DSSP algorithm *(13)*

to define alpha-helical secondary structure, manually joining two helices if they were separated by one or two kinks. Unlike the 2002 study, no attempt was made to define a transmembrane region. Instead, the entire length of the helix including regions likely outside of the bilayer, were considered "TM-helix" for the purpose of evaluation. They reported the most accurate methods (by two-state accuracy) as Split4 (85.2%), TMHMM (83.3%) and MEMSAT (82.6%).

Comparison of 3D structure-based estimation of transmembrane regions by PDBTM and OPM reveals differences that have significant effect on the inferred position of TM-helices (**Fig. 1.2**). The different per-residue prediction methods have a tendency to over- or under-predict helices on the inside or outside end. Unfortunately, a detailed analysis of particular strengths and weaknesses of individual predictors is not available. All predictors except Split4 distinguish inner from outer loops, while MEMSAT further distinguishes transmembrane portion of the helix from extramembrane helical overhangs.

As with TMB predictors, PiMohtm, Split4, and TMHMM provide per-residue probability plots that aid in distinguishing the relative reliability in the prediction at different locations in the sequence (not shown).

3. Conclusion

The main strategy in homology search is to be opportunistic. One cannot know in advance whether the query will turn out to have a close, medium, or remote homolog. Various methods are optimized to search for homologs in different ranges of similarity, from BLAST (closest) to Pfam (medium) to the set of TMB- or TMH-specific methods (remote). Because of this, all of these methods must be tried and the prediction with the most reliable scores chosen. In the case, where more than one prediction is equally reliable, take a consensus method. Lastly, continue to check methods every few months as they are steadily improving, as are the underlying databases of structural and functional annotation.

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Chapter 2

In Silico Identification of Novel G Protein Coupled Receptors

Matthew N. Davies and Darren R. Flower

Abstract

G-protein coupled receptors (GPCRs) are a superfamily of membrane integral proteins responsible for a large number of physiological functions. Approximately 50% of marketed drugs are targeted toward a GPCR. Despite showing a high degree of structural homology, there is a large variance in sequence within the GPCR superfamily which has lead to difficulties in identifying and classifying potential new GPCR proteins. Here the various computational techniques that can be used to characterize a novel GPCR protein are discussed, including both alignment-based and alignment-free approaches. In addition, the application of homology modeling to building the three-dimensional structures of GPCRs is described.

Key words: G-protein coupled receptors, Orphan GPCRs, bioinformatics, proteochemometrics, homology modeling.

1. Introduction

The G-protein coupled receptors (GPCRs) are a large and diverse multigene superfamily of integral membrane proteins that perform many important physiological functions *(1–3)*. The GPCRs are responsible for the transduction of a remarkable diversity of endogenous extracellular signals into an intracellular response. They act primarily through their activation of ubiquitous guanine nucleotide-binding regulatory (G) proteins. When activated, the receptor associates with a heterotrimeric G-protein complex causing the exchange of GTP for GDP bound to Gα followed by dissociation of Gα-GTP from $G\beta_{\gamma}$, which frees the Gα to couple to the effector enzyme. More than one sort of GPCR can interact with more than one kind of G-protein, creating a complex

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system involving a variety of mechanisms. GPCR receptors control or affect physiological processes as diverse as neurotransmission, cellular metabolism, secretion, cellular differentiation, and inflammatory responses as well as taste, smell, and vision *(4)*. Malfunctions in their expression can result in ulcers, allergies, and migraine attacks. Approximately 45% of marketed drugs target a cell membrane receptor, and most of these are GPCRs *(5–7)*. However, these drugs only affect around 30 receptors, *(8)*. Thus, considering the large repertoire of GPCRs within the human genome, this superfamily remains a source of drug targets that are unparalleled in their potential.

The GPCR superfamily can be divided into six classes (*see* **Table 2.1**). These are the Class A–Rhodopsin-like, which account for over 80% of all GPCRs, Class B–Secretin-like Class C–Metabotropic glutamates, Class D–Pheromones, Class E cAMP receptors and the frizzled/smoothened family. Class A can further be classified into subfamilies and subsub families based upon the function of the GPCR receptor and on the specific ligand that binds it. There are also over a hundred "orphan" GPCR receptors that show the properties of a GPCR receptor but for which there is no defined ligand or function (*see* **Note 1**). It is possible to identify a receptor's natural ligand by various experimental techniques. Such techniques include antagonistic antibodies, application of antisense DNA technologies, and transgenic animal studies. However, in order to focus such work one can make use of various information *in silico* approaches in order to characterize a GPCR receptor and suggest its potential function.

Table 2.1
GPCR Classification. A hierarchy of classes, sub-families and sub-sub-families

Class	Sub-families	Sub-sub-families
Class A	Amine	Acetylcholine
Class B	Peptide	Adrenoreceptors
Class C	Hormone	Dopamine
Class D	(Rhod)opsin	Histamine
Class E	Olfactory	Serotonin
	.	
Frizzled/Smoothened family	.	Octopamine
	.	
	Orphans	Trace amine

2. Methods

2.1. Sequence Analysis

Various programs are freely available on to the Web that can be used to characterize the protein sequence to establish whether it is, in reality, a GPCR receptor and, if it is, where it might belong with the GPCR protein superfamily. A lack of homology within the GPCR superfamily and difficulties in aligning the protein sequences make this sort of analysis problematic for GPCR identification. Nevertheless, the programs are simple and straightforward to use and may provide guidance for more sophisticated predictive techniques.

2.1.1. BLAST Search

The most obvious and straightforward approach to characterizing a protein is to run a standard BLAST search using WU-BLAST2 (Washington University Basic Local Alignment Search Tool Version 2.0) *(9)*. WU-BLAST2, located at (http://www.ebi.ac.uk/blast2/), uses an amino acid sequence in FastA format as a query to search a nonredundant protein database for related sequences. The degree of relatedness is calculated by generating gapped alignments of the protein sequences together with statistical significance estimates. The BLOSUM matrix assigns a probability score for each position in an alignment that is based on the frequency with which that substitution is known to occur among consensus blocks within related proteins. BLOSUM62 is a general-purpose matrix and is the default choice in WUBLAST2 and one of the best available matrices for detecting weak protein similarities. Other supported options include the matrices PAM30, PAM70, BLOSUM80, and BLOSUM45. The output will list proteins that closely resemble the submitted sequence in descending order of expectation or "E-values." The lower the E value, the more significant the score and thus the higher predicted relatedness between the submitted sequence and the database entry. The E-value threshold can be changed from the default value of 10 to a setting of 1. Although hits with E-values much higher than 0.1 are unlikely to reflect true sequence relatives, it is useful to examine hits with lower significance (E-values between 0.1 and 10) for short regions of similarity. It is likely that the majority of the suggested proteins that show homology will also be GPCR receptor sequences. If the sequence shows a high degree of homology with a different protein family, it is possible that it is not a GPCR receptor protein.

2.1.2. Motif-Based Searching

Some types of low-complexity sequences may not be detected by the filtering option in BLAST. For example, coiled-coil and transmembrane regions need to be detected using the

appropriate programs outside of BLAST. While BLAST searches may identify generic similarities between proteins, a motif-based approach focuses on specific traits unique to families or subfamilies. PROSITE (http://www.expasy.org/prosite/) *(10)* and Pfam databases (http://www.sanger.ac.uk/Software/Pfam/) *(11)* can both be used to classify based on motifs that they share with a known protein family. The fastest way to examine these databases is to use the ISREC ProfileScan Server (http://www.swbic.org/origin/proc_man/pfscan/search/PFSCAN_form.html).

It is recommended that you check all four of the database boxes and set the sensitivity by selecting "include weak match" in the drop menu. Again, the protein sequence is entered in the FastA format. The protein's name must be entered into the query's title box. This results page also begins by providing information about the query sequence, the parameters that were set, and the databases that were searched. The hits from each site are color coded and, in addition, any significant matches are preceded by a red exclamation mark. The line for each hit shows the normalized score (if it is available), the raw score, the position in the query where the match occurs for that hit, the name of the profile, and the name/description of the hit sequence in the database. Only hits from the PROSITE Profiles, Pfam, and Gribskov collection databases have normalized scores. These scores have an inverse-log relationship to the E-value. A normalized score of 10 corresponds to an E-value of around 1×10^{-2}, any score above this may indicate a very significant match. Hits from the PROSITE patterns databases do not show a normalized or raw score. However, if an exact match to a pattern in the database is found in the search sequence, the score is given as 1. If a pattern is found in the query that is only similar to one in the database a 0.1 score is given and will only be seen if weak matches are allowed. These scores, along with the name/description of the hits, indicate whether or not there are any families or domains related to the query in the databases searched. There are also diagnostic "fingerprints" for rhodopsin-like GPCRs, which have been developed based on common patterns of conservation within the seven transmembrane regions *(12, 13)*. The fingerprint approach can be used to design protein "signatures" at different levels of the GPCR superfamily (*see* **Note 2**).

2.1.3. Transmembrane Prediction

All GPCR receptors contain the seven alpha helices that make up the transmembrane bundles. These helices may be predicted by a similar method of analysis to the sequence similarity search. Identifying the transmembrane regions (TM1-7) also identifies the composition of the rest of the GPCR receptor. This sequence will contain the three extracellular loops (EL1-3),

three intracellular loops (IL1-3), as well as the protein termini. It can, therefore, be broken down into:

N termini-TM1-IL1-TM2-EL1-TM3-IL2- TM4-EL2- TM5-IL3- TM6 -EL3-TM7-C termini

Any evidence of there being seven clearly show that TM regions within the sequence is a good indication of it being a GPCR receptor. It is not necessary for the regions to be equidistant from each other within the sequence as the length of both the intracellular and extracellullar loops can be of extremely variable length. Prediction of Transmembrane Regions and Orientation (TMpred) (http://www.ch.embnet.org/software/TMPRED_form.html), uses a combination of several statistical preference matrices, derived from an expert-compiled data set of membrane proteins, to predict the location of transmembrane helices. The default settings for the minimum and maximum transmembrane lengths are 17 and 33, these should be altered to be just beyond the 25–35 amino acid range commonly observed for the GPCR transmembrane regions. The TMHMM Server version. 2.0 Prediction of transmembrane helices in proteins (http://www.cbs.dtu.dk/services/TMHMM/) uses a FastA format and breaks down the sequence into the most probable distribution compared to known GPCRs. The program is a novel method to model and predict the location and orientation of alpha helices in membrane—spanning proteins. It is based on a hidden Markov model (HMM) with an architecture that corresponds closely to the biological system. The close mapping between the biological and computational states allows the programs to infer which parts of the model architecture are important to capture the information that encodes the membrane topology, and to gain a better understanding of the mechanisms and constraints involved. Models are estimated both by maximum likelihood and a discriminative method. The output details each of the TM regions within the sequence identified by the HMM. Other transmembrane prediction programs include TMpred *(14)*, TopPred II *(15)*, PRED-TMR2 *(16)*, TMHMM 2.0 *(17)*, HMMTOP 2.0 *(18)*, and TM Finder *(19)*. It is sensible to seek a consensus between several different algorithms.

2.1.4. GPCR Subfamily Classifier

A slighty more sophisticated approach is used by the GPCR Subfamily Classifier algorithm (http://www.soe.ucsc.edu/research/compbio/gpcr-subclass/) *(20)*. This program uses Support Vector Machines (SVMs) to transform protein sequences into fixed-length features vectors. SVMs use a mathematical tool known as a kernel function, which measures

the similarity between the examples. The algorithm learns to distinguish between subfamily members and nonmembers by making several passes through a training set. The vector of trained weight can then be used to make a prediction for a novel protein sequence. A sequence can only be submitted in the FastA format, an example of which is given in the website. There are no parameters that can be determined by the user (*see* **Note 3**).

3. Alignment Independent Classification

Conventional bioinformatics determines information about a protein sequence through alignment or by comparing the sequence with previously determined motifs. As mentioned in **Section 2**, while this approach is certainly valid, it may not necessarily be the most effective form of analysis when dealing with the problem of GPCR identification. Firstly, the sequence length of the GPCR superfamily varies between 290 and 834 amino acids in length, meaning that many of the subfamilies cannot be effectively aligned. It is also important to bear in mind that the accepted GPCR classification system is based not on sequence homology but by the ligand to which the receptor is bound. There are, therefore, examples within the GPCR superfamily of convergent/divergent evolution where apparent sequence similarity between receptors is not reflected in the classification scheme. Alignment-independent classification systems that use the physiochemical properties of the protein to determine differences between the subfamilies are an alternative approach to a motif-based system.

3.1. Proteochemometrics

Proteochemometrics takes a different approach by analyzing 26 separate physicochemical properties of the protein *(21)* (*see* **Note 4**). From these properties, five "z values" can be empirically determined for all 20 amino acids. The z1 value accounts for the amino acid's lipophilicity and is determined by thin layer chromatography (TLC) variables, log P, and the nonpolar surface area. A large negative value corresponds to a lipophilic amino acid while a large positive value corresponds to a polar/hydrophilic amino acid. Steric properties are accounted for by the z2 values, which summarizes the residue's steric bulk/polarizability. In this case, a large negative value corresponds to a lower molecular weight and small surface area while a large positive value corresponds to a higher molecular weight and large surface area. The z3 value describes the polarity of the amino and is determined by the log P values and nonpolar surface area. A lipophilic amino acid corresponds to a large negative value while a polar/hydrophilic amino acid has a large positive value. The electronic effects,

determined by electronegativity, heat of formation, electrophilicity, are described by the z4–5 values. These five values are calculated for each amino acid in the sequence, generating a matrix that provides a purely numerical description of the protein's character. AutoCross Co-variance is used to normalize the uneven size of the z matrices and then Principal Component Analysis (PCA) and Partial Least Squares (PLS) are performed in order to provide a classification system for the various classes of protein. Key to the ACC approach is balancing the two factors of Maxium lag, L, and the degree of normalization, p. The lag is a summation, over the sequence, of the product of Z values for all pairs of two amino acids separated by the defined lag value. This summation is then normalized by the number of terms in the summation. Optimal parameter values were experimentally determined in terms of total classification accuracy by trying various combinations.

3.2. Self-Organizing Maps

Self-organizing maps (SOM) *(22)* are also calculated by using z values. SOM are Artificial Neural Networks (ANNs) that perform unsupervised learning to distinguish one protein family from another. Unlike PCA, which relies upon establishing linear relations, SOM can accommodate nonlinear relations into its algorithm. The SOMs can locate samples from an input space to particular "neurons" in a 2D lattice through an adaptive process. The output space is 50 by 50 neutrons on a square lattice. The learning process causes closely located neurons to become responsive to similar input data. Sequences from the same family are expected to form a cluster although it cannot be assumed that the clusters will be visually recognized on the SOM output map. In order to make a family map, it is necessary to determine a family area that contains the most frequent "activator" family samples. The classification method is essentially a neural network method that uses the weight space vector of neurons. The overall performance of the map can be assessed using ROC values (sensitivity and specificity) as well as calculating the total accuracy of prediction.

4. Three-Dimensional Structures of GPCRS

4.1. Homology Modelling

Homology modeling is a technique that takes the amino acid sequence of an unknown structure and the solved structure of a homologous protein and computationally mutates each amino acid in the solved structure into the corresponding amino acid from the unknown structure. The newly generated structure can be optimized by the use of molecular dynamics simulations. This technique cannot realistically be used to identify a GPCR sequence but if the sequence has been classified then generating a

3D structure of the protein can be useful in trying to characterize ligands that might bind the receptor.

In conventional homology modeling, one or more templates can be used. Therefore, although target and templates are likely to be correctly aligned if sharing more than 40% identity, they need to be realigned if they are in the "twilight zone" sharing less than 30% identity. However, there are very few high-resolution structures of membrane proteins available in the Protein Data-Bank compared to the number of globular protein structures. This is due to the difficulties of over-expression, purification, and concentration of membrane proteins. The only experimentally determined structure of a GPCR is that of bovine rhodopsin, which was elucidated to 3.5Å using X-ray crystallography *(23)*. Very few members of the GPCRs are sufficiently homologous to bovine rhodopsin to have more than 40% identity; the vast majority are within the twilight zone. Despite this, all GPCRs show a common pattern of hydrophobicity and similarity of structure even when there is sequence divergence. For this reason, the bovine rhodopsin structure has frequently been used as the basis for GPCR homology modeling and has been used to generate structures that have successfully been used in virtual screening experiments.

The structure of GPCRs contain seven highly conserved transmembrane segments of 25–35 consecutive residues of high-calculated hydrophobicity, which form seven α-helices in a flattened two-layer structure. Conventional homology modeling is of no use if, as is most typically the case, there is low-sequence similarity between the unknown protein sequence and that of bovine rhodopsin. However, the transmembrane sequences can be docked together using the bovine rhodopsin structure as a scaffold so that hydrophobic faces are orientated into the membrane phase and hydrophilic faces point into the lumen of the protein. Hydrophobic profiles of multisequence alignment of GPCRs can be used to assign helical transmembrane regions. For example, the program WHATIF *(24)* (http://swift.cmbi.kun.nl/whatif/) can be used. This presupposes that the locations of the transmembrane regions within the protein can be identified (see **Section 2.1.3**).

The next stage is to incorporate the extracellular and intracellular loops as well as the termini of the molecule into the transmembrane scaffold. The loops are far harder to model because not only will there be low homology between bovine rhodopsin and the protein sequence but also the respective loops may be of an entirely different length. It is for this reason the termini and loops are usually added in an extended conformation. The program DRAWBRIDGE *(25)* builds loop regions onto protein structures using the conformational propensities of amino acids to generate novel candidates for protein loop regions. End

constraints are satisfied using a random tweak method. There are also limitations to the accuracy of loop modeling software and it is advisable to further optimize the structure if possible.

4.2. Molecular Modeling Simulations

Molecular modeling is broadly defined as creating three-dimensional representations of molecular systems. In this instance, we can use molecular modeling for the purposes of improving what is essentially a crude representation of a GPCR structure (*see* **Fig. 2.1a–b**). Energy minimization can be applied to a molecular system in order to try to identify a low-energy state by optimizing the energetic interactions between its component atoms. The interactions are controlled by an energetic force field that contains terms for bond lengths, bond angles, dihedral angles, and nonbonded interactions such as electrostatic and van der Waals. AMBER *(26)* and CHARMM *(27)*. By imposing penalties for atomic interactions that deviate from the optimum values, hence raising the required energy of the system, the simulations will tend toward a lower-energy state. The simulation is run over a series of time steps (usually approximating to one femtosecond) after which the pair wise energy interactions between atoms are reevaluated and the next time step occurs. Over a series of time steps, unrealistic atomic interactions are eliminated and the system reaches an energy minima (see **Note 5**).

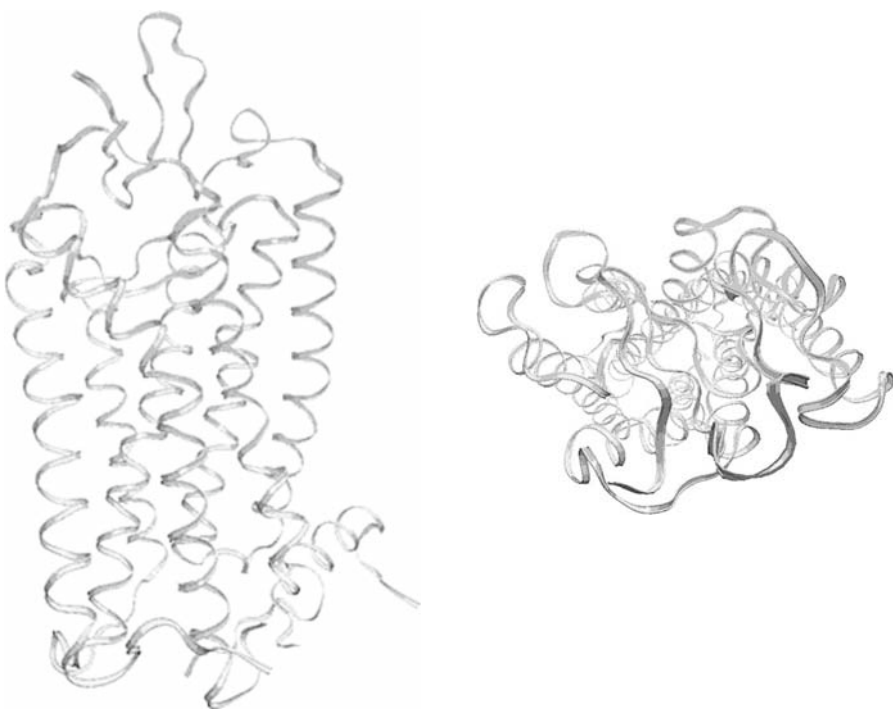

Fig. 2.1a–b. The crystal structure of bovine rhodopsin.

It is important to stress that it is the entire system, and not just the protein, that is minimized. Energy-minimization simulations once took place *in vacuo*, with no other atoms present but the protein itself, now they take place *in aqua* where the protein is surrounded by water molecules. A transmembrane protein like GPCR is ideally represented through the presence of a lipid bilayer. Representations of optimized bilayers are publicly available and it is possible to incorporate a GPCR structure into a bilayer *(28)*. All bad contacts between the protein and its surrounding lipids must be eliminated. Lipid midpoint plane (LMP) corresponds to contacts of the hydrophobic plane *(29)* (*see* **Note 6**). The protein-ligand complex should be fully solvated on both the extracellular and intracellular side and an energy minimization simulation was used to optimize the protein structure. The reliability of the models can be assessed with PROCHECK *(30)* or WHATCHECK *(31)*.

4.3. Virtual Screening

The primary interest in developing and refining homology models of GPCR proteins is their potential use in searching for potential agonist and antagonists for the receptor. Virtual screening is a computational method, which aims to select the most promising compounds by docking a succession of molecules within the homology model in order to calculate which has the highest affinity for the ligand-binding cavity. Commonly used docking programs include Gold *(32)*, Dock *(33)*, and FlexX *(34)*. The technique has previously been applied only to experimentally determined high-resolution structures. However, there has been an increased interest in whether or not homology models could be used as the basis of virtual screening, especially for GPCRs where there is only a single structure available *(35)*. In spite of the difficulties in generating the models, described in **Section 4.1**, virtual screening studies have shown to be effective at identifying ligands with antagonistic properties (this is less the case with agonists, possibly suggesting that the bovine rhodopsin structure is the inactive form of the GPCR) *(36)*.

5. Notes

1. Many orphan receptors have been classified as displaying "Class A-like properties" but have not been assigned any specific function. Fredrikkson et al., *(37)* have suggested possible functions for orphan receptors based on phylogenetic analysis of the family.
2. As the number of known members for a family expands, it becomes harder to define precisely the fingerprints; therefore, atypical members of the GPCR family cannot necessarily be identified in this way.

3. Regrettably, the program has not been updated since March 2002 and does not incorporate sequences or classes added since that time into the algorithm.
4. These properties are the molecular weight; van der Waals volume, heat of formation, energy of the highest occupied molecular orbital, energy of the lowest unoccupied molecular orbital, log *P*, R polarizability, absolute electronegativity, absolute hardness, total molecular surface area, polar molecular surface area, number of hydrogen bond donors, number of hydrogen bond acceptors, indicator of positive charge in the side chain, indicator of negative charge in the side chain, NMR R-proton shift at *p*D 2, 7, and 12.5 and 7 descriptors representing thin-layer chromatographic mobilities using different stationary and mobile phases.
5. A more effective version of this simulation is simulated annealing, where the energy of the system is first raised very high and then gradually reduced allowing the system to overcome local energy gradients and eventually reach the global energy minima rather than a local one.
6. The hydrophobic regions of the transmembrane bundle will position themselves in the middle of the bilayer so the maximum hydrophocity will be present in the plane of the membrane.

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Chapter 3

Transcriptome-Based Identification of Candidate Membrane Proteins

Edward J. Evans, Lawrence Hene, Mai Vuong, S. Hussain I. Abidi, and Simon J. Davis

Abstract

A full understanding of leukocyte responses to external stimuli requires knowledge of the full complement of proteins found on their surfaces. Systematic examination of the mammalian cell surfaces at the protein level is hampered by technical difficulties associated with proteomic analysis of so many membrane proteins and the large amounts of starting material required. The use of transcriptomic analyses avoids challenges associated with protein stability and separation and enables the inclusion of an amplification step; thus allowing the use of cell numbers applicable to the study of sub populations of, for example, primary lymphocytes. Here we present a transcriptomic methodology based on Serial Analysis of Gene Expression (SAGE) to recover an essentially complete and quantitative profile of mRNA species in a particular cell. We discuss how, using bioinformatic tools accessible to standard desktop computers, plasma membrane proteins can be identified *in silico*, from this list. While we describe the use of this approach to characterise the cell surface protein complement of a resting $CD8^+$ T-cell clone, it is theoretically applicable to any cell surface, where a suitable pure population of cells is available.

Key words: Serial Analysis of Gene Expression (SAGE), T lymphocyte, plasma membrane protein, transcriptomics.

1. Introduction

Cell function and particularly the cell's response to its environment is dependent on the ensemble behaviour of many different proteins at the cell surface. Many techniques are available to identify sets of such proteins that are important on individual cells or are involved in a particular process. Ideally, such techniques might identify proteins directly from preparations of the

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cell membrane, as described elsewhere in this volume. However, such techniques are not yet sufficiently advanced to guarantee the identification of *complete* sets of proteins. An alternative is to use gene expression technologies capable of identifying all the components involved, and extracting those at the cell surface retrospectively, using bioinformatics.

The most commonly used methods for gene expression analysis are cDNA or oligonucleotide microarrays. Such 'closed' architecture systems, however, do not currently give global transcriptome coverage. In the light of uncertainty regarding the actual number of transcribed regions in the human genome *(1–3)*, and the technical difficulties associated with using whole genome tiling arrays, it may be some time before all human genes can be confidently sampled in this way. For other higher eukaryotes, this may take far longer still. Instead, 'open' gene expression technologies can be used. Many of these techniques are based on the sequencing of short tags created from pooled transcripts. Having compared at least serial analysis of gene expression (SAGE); *(4)*) and Massively parallel signature sequencing (MPSS; *(5)*), we consider LongSAGE (a variation of the original technique; *(6)*) the best technique to identify complete transcriptomes (L.H., Sreenu Vattipally, M.V., E.J.E., and S.J.D., unpublished).

Here we describe a protocol for the generation of a SAGE library from a cell of interest and bioinformatic procedures firstly to identify a list of transcripts of interest from comparisons of such libraries and secondly to identify membrane bound proteins from this list of candidates. The SAGE protocol given is adapted from the originally published method *(4)* and all of these methods were developed, in the first instance, for the analysis of leukocyte cell surfaces. They were successfully used to identify a set of proteins on the surface of a resting cytotoxic T lymphocyte (CTL), which appears to represent all those not also found on non-haematopoietic cells *(7)*. The bioinformatic methods described here are also those originally used for this type of analysis and require only basic programs found on most desktop computers. For larger scale or more complex analyses, alternative high-throughput bioinformatic methods are currently being developed.

2. Materials

2.1. The SAGE Method

In addition to standard molecular biology reagents, the following materials are required. Alternatively, the I-SAGE or I-SAGE Long kits from Invitrogen (Carlsbad, CA) can be used (*see* **Note 1**).

1. Oligonucleotides:
   oligodT primer: 5′-biotin-TTTTTTTTTTTTTTTTTTTT-3′
   ditag PCR primers: 5′-biotin-GGATTTGCTGGTGCAGTACA-3′
   5′-biotin-CTGCTCGAATTCAAGCTTCT-3′
   M13F-AP primer: 5′-GCTATTACGCCAGCTGGCGAAAGGGGGATGTG-3′
   M13R-AP primer: 5′-CCCCAGGCTTTACACTTTATGCTTCCGGCACG-3′
   M13 Forward primer: 5′-GTTTTCCCAGTCACGAC-3′
   M13 Reverse primer: 5′-CAGGAAACAGCTATGAC C-3′
2. *Nla III* (New England Biolabs, Hitchin, UK): 10 U/μl; must be stored at −80°C at all times; for digestion buffer add BSA to a final concentration of 100 μg/ml (provided at 10 mg/ml) to the buffer ('NEB buffer 4') supplied (*see* **Note 2**).
3. LoTE buffer: 3 mM Tris-HCl pH7.5, 0.2 mM EDTA.
4. Streptavidin M-280 Dynabeads (Dynal, Bromborough, UK).
5. SAGE adaptors (*see* **Note 2**)
   Adapter A (anneal the following two oligonucleotides):
   5′-TTTGGATTTGCTGGTGCAGTACAACTAGGCTTAATAGGGACATG-3′
   5′-PO_3-TCCCTATTAAGCCTAGTTGTACTGCACCAGCAAATC-amino C7-3′
   Adapter B (anneal the following two oligonucleotides):
   5′-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATG-3′
   5′-PO_3-TCCCCGTACATCGTTAGAAGCTTGAATTCGAGCAG-amino C7-3′
6. High Concentration T4 DNA Ligase (Invitrogen): 5 U/μl; supplied with 5x ligation buffer for the reaction.
7. *Bsm FI* (New England Biolabs); 2 U/μl; supplied with NEB buffer 4 and a 100x solution of BSA to make the digestion buffer.
8. Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs): 5 U/μl; supplied with 10x*E.coli* buffer for the reaction (*see* **Note 2**).
9. Ampliwax PCR Gem 100 beads (Applied Biosystems, Foster City, CA).
10. AmpliTaq (Applied Biosytems): 5 U/μl.
11. BV Buffer: 166 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH8.8, 67 mM $MgCl_2$, 100 mM β-mercaptoethanol.
12. Spin-X Plastic Centrifuge Tubes (Corning Lifesciences, Acton, MA).

13. pZERO-1 vector (Invitrogen): provided at 1 μg/μl in TE buffer, pH7.5.
14. Sph I (New England Biolabs); 5U/μl; supplied with NEB buffer 2 as the digestion buffer.
15. ElectroMax DH10B cells (GibcoBRL)
16. Zeocin (Invitrogen): 100mg/ml in water
17. BioTaq (Bioline, London, UK): 5U/μl; supplied with a 10x NH_4-based PCR reaction buffer.
18. Exonuclease I (New England Biolabs): 20U/μl.
19. Shrimp Alkaline Phosphatase (Roche Applied Science, Indianapolis, IN): 1U/μl

2.2. Basic SAGE Data Analysis

1. SAGE program group software. Available from the Molecular Genetics Laboratory at the Johns Hopkins School of Medicine for non-profit academic use, via www.sagenet.org/protocol/index.htm
2. Publicly available SAGE data for comparison. This can be downloaded from a number of sources, including:
   www.sagenet.org/resources/data.htm
   http://cgap.nci.nih.gov/SAGE/
   ftp://ftp.ncbi.nih.gov/pub/sage/
   ftp://genome-ftp.stanford.edu/pub/yeast/data_download/systematic_results/SAGE/
   http://bloodsage.gi.k.u-tokyo.ac.jp/
3. Linker tags lists. These can be downloaded from www.t-cell biology.org/sage. Original lists courtesy of the National Cancer Institute Center for Bioinformatics.
4. SAGEmap tag-to-gene mappings. These can be downloaded from ftp://ftp.ncbi.nlm.nih.gov/pub/sage/mappings/ according to species and restriction site. So for human standard SAGE libraries constructed using *Nla III*, as described here, download file SAGEmap_Hs_Nlalll_10_best.gz
5. Example SQL queries for use in Microsoft Access can be downloaded from www.t-cellbiology.org/sage including those for:
   Linker removal and normalisation
   Linking sample data to mapping data

2.3. LongSAGE Data Analysis Using the Human Genome Sequence

1. Bioperl modules to interface with the Ensembl Perl API. Instructions for installation can be found at http://www.ensembl.org/info/using/api/api_installation.html
2. The Phred software package. This is available from the University of Washington, free of charge to academic users. See http://www.phrap.org/consed/consed.html#howToGet
3. Tag extraction Perl scripts were adapted from those written at the Marine Biological Laboratory, Woods Hole, USA by the lab of A.G. McArthur. They are available at www.t-cellbiology.org/sage

4. Ensembl data. Full analysis requires the download of the full-core files containing the full genome and all gene predictions. A flat file can be downloaded from Ensembl (http://www.ensembl.org/info/data/download.html) or the Perl API can be used to query the Ensembl MySQL database directly as in the scripts available at www.t-cellbiology.org/sage

2.4. Identification of Transcripts of Interest

1. Example SQL queries for use in Microsoft Access can be downloaded from www.t-cellbiology.org/sage including those for:
   Comparison of two libraries
   Calculation of p values

2.5. Identification of Transcripts Encoding Known Cell Surface Proteins in the Set

1. The Gene Ontology Database (GO). This can be downloaded at
   http://www.geneontology.org/GO.downloads.shtml
2. SAGE Genie Gene-to-Tag mapping data. Databases for short or long SAGE with varying degrees of mapping confidence can be downloaded from ftp://ftp1.nci.nih.gov/pub/SAGE/HUMAN/. For automatic derivation of tags for genes selected from GO, we use the 'best_tag' dataset.
3. Example SQL queries for use in Microsoft Access can be downloaded from www.t-cellbiology.org/sage including those for
   Comparison of gene list to SAGE Genie
   Linking this list to your library
4. List of Cluster of Differentiation (CD) antigens linked to Entrez Gene IDs. This can be obtained from www.hcdm.org or http://www.uniprot.org/docs/cdlist

2.6. Identification of Transcripts Encoding Other Membrane Proteins in the Set

1. URLs for Web-access to or downloading programs used:

BioMart:	http://www.biomart.org/biomart/martview
BLAST:	http://www.ncbi.nlm.nih.gov/BLAST/download.shtml
ORF Finder:	http://www.ncbi.nlm.nih.gov/gorf
InterPro:	http://www.ebi.ac.uk/InterProScan
SMART:	http://smart.embl-heidelberg.de
SignalP:	http://www.cbs.dtu.dk/services/SignalP
TMHMM:	http://www.cbs.dtu.dk/services/TMHMM/
CAP3:	http://seq.cs.iastate.edu/download.html

3. Methods

3.1. The SAGE Method

This method was used to produce standard SAGE libraries from lymphocyte cell lines. For genomic analysis of the resulting

tags, LongSAGE is the preferred technique (*see* **Note 2**). This protocol is derived from the original SAGE protocol *(4)* and incorporates the adaptations of Powell *(8)*. SAGE libraries can now also be produced using the 'I-SAGE' and 'I-SAGE Long' kits from Invitrogen (*see* **Note 1**). A useful figure, depicting a schematic representation of the SAGE method, was available at the time of writing at the following URL: http://sciencepark.mdanderson.org/labs/ggeg/sage_fig1_zoom.htm

1. Prepare cDNA from the cell of interest by your usual method, but using a 5′ biotinylated oligo-dT primer (*see* **Note 3**).
2. Digest the cDNA with *Nla III* in digestion buffer for 90 min at 37°C.
3. Ethanol precipitate the digested DNA in the presence of glycogen: add one tenth the sample volume of 3 M sodium acetate, two volumes of 100% ethanol and 1 μl of 20 mg/ml glycogen and centrifuge for 30 min at 13,000 rpm and 4°C. Remove supernatant and wash DNA pellet with 300 μl 70% ethanol by centrifugation for 5 min at 13,000 rpm and 4°C. Remove the supernatant, air dry the pellet and resuspend in LoTE buffer (*see* **Note 4**).
4. Separate the DNA into two halves, mix Streptavidin M-280 Dynabeads with each half and incubate at room temperature for 30 min, with occasional mixing to allow biotinylated DNA to bind to the beads.
5. After washing, mix the first pool of beads with SAGE adaptor A and the second pool with SAGE adaptor B. Each reaction mix should comprise of the bead pool, 20 μl LoTE, 10 μl adaptor at 0.2 μg/μl and 8 μl 5x ligase buffer. Incubate at 50°C for 2 min, then at room temperature for 15 min, before addition of 2 μl high-concentration T4 DNA ligase. Incubate at 16°C for a further 2 hours with occasional mixing.
6. After washing the beads, add 85 μl LoTE, 1 μl *Bsm FI*, 10 μl of the supplied buffer and 2 μl BSA solution (*see* **Note 2**) and incubate for 90 min at 65°C for digestion, mixing occasionally.
7. Careful remove the magnetic beads using the magnet provided.
8. Ethanol precipitate the SAGE tags that were cleaved from the beads in the presence of glycogen as before and resuspend in 11 μl LoTE.
9. Mix 10 μl DNA, 5 μl 10x*E.coli* buffer, 3 μl dNTPs (25 mM), 31 μl deionised water and 1 μl of the Klenow fragment and incubate at 25°C for 30 min to fill in cleaved ends (*see* **Note 2**).

10. Again ethanol precipitate the DNA and resuspend it in 6 μl LoTE.
11. Mix 2 μl of each SAGE tag pool (A and B) with 1.2 μl 5x ligase buffer and 0.8 μl high concentration T4 DNA ligase and ligate overnight at 4°C (*see* **Note 2**).
12. Extract the resulting ditags with phenol-chloroform and ethanol precipitate them once again.
13. Amplify ditags by PCR: Place Ampliwax beads into a tube containing a reaction mix of 10 μl BV buffer, 6 μl DMSO, 6 μl dNTPs (25 mM), 4 μl of each biotinylated ditag PCR primer (A and B) at 175 ng/μl, and 56 μl deionised water. Heat at 70°C for 3 min to create wax seal. Add 10 μl deionised water, 2 μl AmpliTaq and 2 μl of the ligated ditags above the wax. After initial denaturation for 1 min at 95°C, run 25 cycles of the following three steps: 95°C for 30 seconds, 55°C for 1 min, 70°C for 1 min. Complete the programme with a final extension at 70°C for 5 min. The number of reactions required depends on the size of library you desire. In our hands, 150 reactions is sufficient for a final library of ~75,000 tags.
14. Retrieve polymerase chain reaction (PCR) products from beneath the reformed wax beads, then clean the DNA by phenol chloroform extraction and ethanol precipitation.
15. Resuspend the pellet in 360 μl LoTE, add loading dye and load the DNA onto 1.5 mm thick 12% PAGE gels (29:1 acrylamide:bis-acrylamide) using 20 μl per well. Run these gels for 3 hours and stain with ethidium bromide for 15 min. Excise the ~100 bp bands (linkers containing ditags, ~80 bp band contains empty linkers) and split between nine 0.5 ml tubes. Pierce the top and bottom of these tubes with a 19 gauge needle and place them inside 1.5 ml tubes. Centrifuge at 10,000 rpm for 3 min and add 300 μl LoTE. Incubate tubes overnight at 4°C and then for 15 min at 65°C. Transfer the mixture to a Spin-X column and centrifuge at 13,000 rpm for 2 min.
16. Cleave the amplified 100 mer ditags with *Nla III* for 1 hour at 37°C in a reaction containing 121 μl sample, 15 μl 10x NEB buffer 4, 1.5 μl 100x BSA and 12 μl *Nla III*.
17. Extract cleaved DNA with phenol-chloroform, ethanol precipitate and resuspend in 32 μl LoTE (*see* **Note 4**). Add 7 μl loading dye and run the mixture on a 0.75 mm thick 12% (29:1) PAGE gel using 13 μl per lane. Excise the 26 bp ditag band, separating it from undigested and partially digested products.
18. Split the bands between two tubes and extract the DNA as before, although following centrifugation, the acrylamide can be incubated in LoTE for 2 hours at 37°C rather than being left overnight.

19. Add streptavidin-coated Dynabeads to remove any undigested material, as before, and remove carefully.
20. Ethanol precipitate the 26 bp ditags (*see* **Note 4**) before ligating into concatemers overnight at 16°C in a reaction containing 7 μl DNA, 2 μl 5x ligase buffer and 1 μl high concentration T4 DNA ligase.
21. Purify two ranges of concatemers: 0.5–1.0 kb and 1.0–1.5 kb from a 8% (37.5:1) PAGE gel using the same protocol as before but incubating the acrylamide after centrifugation with LoTE for 2 hours at 37°C and then for 15 min at 65°C.
22. Extract the purified concatemers with phenol-chloroform and ethanol precipitation.
23. Clone these concatemers into the pZERO-1 vector, which had been cleaved with Sph I and treated with alkaline phosphatase under standard conditions.
24. Transform ElectroMax DH10B cells with the concatemer containing vectors by electroporation. Grow the bacteria on agar plates containing Zeocin, pick colonies and grow individual colonies overnight in low salt LB broth in 96-well plates.
25. Add glycerol to the cultures to a final concentration of 15% and store at –20°C (*see* **Note 5**).
26. Amplify the sequence in each clone by PCR in 18 μl reactions containing 2 μl 10x PCR buffer, 0.625 μl $MgCl_2$ at 50 μM, 0.166 μl dNTPs at 25 mM each, 1 μl of each primer (M13F-AP and M13R-AP, 4 μM each), 0.25 μl BioTaq and 14 μl deionised water. After initial bacterial lysis for 15 min at 95°C, run 18 cycles of the following three steps: 95°C for 30 s, 60°C for 30 s, 65°C for 1 min.
27. Treat PCR products with Exonuclease I and shrimp alkaline phosphatase for 15 min at 37°C and heat inactivate the enzymes for 30 min at 70°C.
28. Sequence treated PCR products directly by fluorescent dideoxy sequencing using standard M13 forward and reverse primers according to your standard protocol.

3.2. Basic SAGE Data Analysis

1. Use the SAGE program group *(4)* to identify ditags from the concatemer sequences, exclude duplicate ditags (which are likely to have arisen from PCR bias) and produce a list of unique 10 bp tag sequences together with their frequency of observation. This software also allows you to keep a record of the 11th base in the tag for future cross-referencing (*see* **Note 6**). Export this list to the Microsoft Access database package. Public SAGE databases required for future comparisons should be downloaded as text files (see Materials), imported into Access and treated in the same way as the libraries generated in house.

2. In Microsoft Access, exclude tags that may be derived from linker sequence (see Materials). Simultaneously, calculate normalised values of tag abundance (e.g. counts per 100,000 tags if all your libraries are smaller than 100,000 tags or counts per million if some libraries are larger). SQL code for a query to perform these two tasks is available (see Materials). Now link the tags from the library to the Tag to Unigene SAGEmap *(9)* database or to an alternative source of gene information (*see* **Note 7**).
3. For more accurate identification of genes associated with tags of interest (*see* **Section 3.3**), manual curation of the automatically generated matches may be necessary. Apparent Unigene matches should be checked to ensure that the tag did not only match a single EST, 5′ ESTs (i.e. tag was not truly at a 3′ most *Nla III* site), ESTs which had been incorrectly clustered or those which appeared to contain single base errors compared to the true transcript (i.e. the sequence represented by the mRNAs and the majority of ESTs). Matches should also be checked to ensure that they have the correct 11th base, where this was identified by the SAGE program group for that tag.
4. SAGE tags representing known genes used as a test set for the depth of expression analysis can also be derived automatically from a list of Unigene accession numbers using the SAGEmap data in Access. The same manual curation procedures should be applied to ensure accurate tag-to-gene mapping.

3.3. LongSAGE Data Analysis Using the Human Genome Sequence

When analysing LongSAGE libraries, the basic procedures described earlier can also be used. However, the increased length of the tags also allows a direct analysis using the entire human genome. Particularly for the very large libraries necessary for complete transcriptome analysis, this is most easily performed using the Perl programming language to access the Ensembl database *(10)* using the Perl API they provide. For this, LongSAGE data should be stored in a MySQL database. Thus, a completely new approach to data analysis should be used, summarised briefly here.

1. Sequencing runs from LongSAGE libraries should initially be processed with Phred *(11)* to remove poor quality bases that are likely to introduce sequencing errors (*see* **Note 8**).
2. Extract SAGE tags from the resulting sequence. Perl scripts to achieve this step are available (see Materials and **Note 9**).
3. Import the resulting tags into a MySQL database and remove those that may be derived from linker sequence (*see* **Section 2.2**). Normalise tag counts to tags per million (tpm).
4. Download public LongSAGE libraries for comparison (*see* **Section 2.2**) and import these into the MySQL database

using the same normalisation and linker removal procedures as for your data.

5. Genome and transcriptome data should be extracted from Ensembl as follows. Extract the tag (i.e. 17 bp) following every NlaIII restriction site on either DNA strand from each human chromosome (using unmasked data) and from the mitochondrial genome. For each tag, extract its start position on the chromosome, which strand it is on and whether it occurs within or downstream of an annotated gene (predicted or confirmed by evidence). We used a window of up to 5,000 bases downstream of the end of each known gene. From this gene information, each tag can be classified as occurring outside a known gene, close to the 3′ end of a known gene, in an exon, in an intron or across a boundary (i.e. the tag is partly in both an exon and an intron). In addition to this genomic analysis, the known transcriptome should be analysed by extracting tags, in the same way, from the sense and anti-sense strands of all transcripts in the Ensembl Genes dataset. If tags overlap the 3′ end of a transcript, they can be extended along the genome unless the transcript is predicted to contain a polyA site (as defined in *(12)*), then adenosines should be added to the 3′ end of the tag to complete its length. Repeat this procedure for the EST-Genes dataset. This transcriptomic analysis identifies tags that overlap splice junctions, and therefore are not found in the genome.
6. The information from the Ensembl tag extractions described in the preceding discussions must be collated by calculating the frequency of occurrence for each tag, both in the genome and transcriptome. Each tag can then be classed as a single match, multiple match, no match or very common match (more than 20 hits) to the genome. Further analysis of very common matches is complex and, in general, unproductive. If the tag matches the genome a few times, it can be annotated as matching multiple genes if there are genes close to two or more matches; but if only once genomic occurrence is close to an annotated gene, then the tag is assigned to this gene. In addition to genomic classification, annotate each tag accord to whether it matches a known transcript in Ensembl Gene or Ensembl ESTGene (in the sense or anti-sense direction) or as failing to match the transcriptome. Note tags that map differently to the genome and transcriptome as these are likely to indicate splice junctions.

3.4. Identification of Transcripts of Interest

It is not easy to carry out detailed analysis of every transcript expressed in a cell, therefore, some method is required to identify transcripts of interest. As some proteins with important functions are poorly expressed, and because the correlation between RNA

and protein expression levels is poor *(13)*, transcripts of interest cannot be identified by tag abundance alone. Instead, comparisons to other SAGE libraries can be used to do this. In many expression studies, similar cells or samples are compared where there is only one small difference, thus comparison identifies transcripts responsible for this difference. Such studies are usually best performed using micro arrays to compare several samples. However, to identify a large set (e.g. all membrane proteins) of new genes important for the function of a cell, the expression library can be compared to one or more libraries made from cells of an unrelated tissue type. The aim of this procedure is to exclude transcripts encoding housekeeping and other universally expressed proteins without removing transcripts of potential interest. For example, in the initial use of this technique *(7)*, a cytotoxic T-cell library was compared to libraries from cerebellum, ovary epithelium and several unrelated tumours rather than to other leukocytes. This ensured that important pan-leukocyte antigens, such as CD45, were not excluded from analysis.

1. Download appropriate control SAGE libraries and run the basic SAGE analysis on them as earlier.
2. Combine your library with these control libraries in your chosen database application, ensuring no tags from any of the libraries are lost in the final comparison table.
3. Calculate ratios of the normalised tag abundance in your library to that in each control library. Tags absent in a library must be assigned an arbitrary value for this. In general we suggest using a normalised value of one, that is, one per 100,000 if you are comparing small libraries or 1 per million when comparing large libraries. These ratios give an initial indication of tags derived from transcripts differentially expressed between your library and the control libraries.
4. Run statistical analysis of the differential gene expression between the libraries to determine whether observed differences in expression are significant, that is, whether they can be explained by random sampling effects only. Various statistical tests have been used for this (*see* **Note 10**), and many SAGE analysis packages will do these calculations. However, if you do not use any proprietary software, we recommend the test devised by Audic and Claverie *(14)* specifically for digital gene expression studies. In this method, the probability of an observed increased abundance of a tag in your library compared to a control library occurring at random due to sampling effects (the p-value) is given by:

$$1 - \sum_{y=0}^{y=y_{ob}} \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}}$$

where N_1 and N_2 are the sizes of the control library and, your library, respectively, and x and y_{ob} are the counts of this particular tag in the control library and your library, respectively (*see* **Note 11**). Depending on the application, tags whose p values when compared with all the control libraries are less than either 5 or 1% could be defined as a set of interest.

5. The application of biological understanding to a series of library comparisons can be used to fine tune the set of transcripts identified. For example, in our analysis of a cytotoxic T-cell, so as not to exclude pan-leukocyte antigens, initial comparisons were made to unrelated brain libraries. The set of transcript identified in this way included many genes important for cell proliferation as a T-cell clone proliferates far more rapidly than cells in a solid organ. In order to remove tags from these transcripts, which were not of interest to that particular study, a panel of tumour SAGE libraries was used (*see* **Note 12**). Comparison to a single tumour may have excluded transcripts of genuine interest either because of their expression in the cell from which the tumour was derived or their aberrant expression following oncogenesis. By using a panel, and excluding transcripts expressed as highly in at least two tumours as in the cell of interest, those involved in proliferation were removed without other transcripts being affected.

3.5. Identification of Transcripts Encoding Known Cell Surface Proteins in the Set

The first step in identifying membrane proteins in either the entire library or a defined set of tags is to identify the known genes encoding cell surface proteins that are expressed.

1. Obtain the list of all proteins classed as encoding cell molecules expressed according to the GO database *(15)*, that is, those in categorises 'integral to membrane' (0016021) and 'plasma membrane' (0005886). For a more precise list, manual curation can be used to remove incorrectly classified genes, for example, those encoding membrane proteins restricted to internal organelles. At the time of our last analysis this reduced the list in GO from 2,818 to 1,776 genes.
2. Using the corresponding UniGene IDs *(16)*, map SAGE tags to these genes in your database system using a pre-compiled database table (*see* **Note 13**).
3. Link this table of tags corresponding to known cell surface proteins to your SAGE library to identify which are expressed in your cell or sample.
4. For particular cell types, other reference sets of cell surface genes are available, such as the CD designations for leukocyte cell surface antigens used in *(7)*. These can also be linked to SAGE tags, using this automated procedure or by manual curation, and used to screen your library for expression.

3.6. Identification of Transcripts Encoding Other Membrane Proteins in the Set

Having identified all the known cell surface proteins expressed in a cell, the remaining transcripts can be screened for other (i.e. uncharacterised) membrane proteins. As this procedure is most accurate when manually curated, it is usually only performed on SAGE tags in a set of interest (*see* **Section 3.3**).

1. Divide the tags in your set into those matching well characterised genes, those matching sequenced but uncharacterised cDNAs, those matching EST sequences and those matching the human genome sequence only, according to the basic SAGE analysis already described. Tags not matching the human genome may represent allelic difference or polymorphisms, or they may cross splice junctions, where cDNA for that splice variant has never been sequenced. However, they are most likely to be sequencing errors.
2. For tags matching well-characterised genes, those linked to membrane proteins should have been identified by the procedures described in the previous section. However, lists of UniGene IDs, Entrez Gene Numbers *(16)* or Ensembl gene numbers can be outputted and searched for transmembrane domains using, for example, Ensembl's installation of BioMart *(17)*.
3. If tags are linked to uncharacterised cDNA sequences, these may still have been analysed automatically and entered into GO. However, these sequences should be screened using the following tools to identify other membrane proteins found in your cell or sample.
4. Firstly, analyse such sequences using BLAST *(18)*. Use BLASTp if a predicted amino acid sequence is defined for the transcript and BLASTx (with the DNA sequence) if not. This identifies related sequences or alternative transcripts that are better annotated,
5. Secondly, sequences should be screened for known domains and motifs and especially for predicted transmembrane domains and signal peptides. This requires amino acid sequence, so if none is given, the longest forward reading open reading frame (ORF) should be identified and translated (e.g. using the NCBI ORF Finder tool). The easiest way to search several databases of known domains and profiles is using Interpro *(19)*, but searches of some specific databases give well-presented results, for example, SMART *(20)*. Prediction of signal peptides and transmembrane domains can be preformed using SignalP *(21)* and TMHMM *(22)*.
6. Tags matching Unigene clusters containing only ESTs can be analysed in a similar manner following assembly of the ESTs into contigs using a programme such as CAP3 *(23)*. Each resulting contig should be checked for the presence of the SAGE tag originally used to identify the cluster and then analysed with BLASTn and BLASTx to identify possible

homologues. Where a clear ORF is present, the domain analysis tools described earlier should be used.
7. Tags failing to match any Unigene cluster or cDNA sequence can be linked only to the human genome. If matching downstream of a known gene, this gene can be analysed for a transmembrane domain as mentioned earlier.
8. For each membrane protein whose expression is indicated by these analyses, expression should be checked using an alternative technique such as micro array analysis, Northern blot or real time PCR.

3.7. Assessing Sets for Completeness and Specificity

The methods described here are mainly for use in the analysis of complete transcriptomes, as other techniques can 'cherry-pick' individual molecules more easily. Whether an analysis is complete will depend on the size of the transcriptome, the expression level of the genes of interest and the size of the SAGE library analysed. Therefore, it is important to be able to quantify the completeness of the set of transcripts identified by each analysis. This can be done using a comparative small reference set of transcripts known to be important in this cell or condition, provided this reference set can be assumed to be representative of the complete set required. The reference set must, therefore, have been identified by an alternative technique that is not limited by the same factors as the new analysis, in particular mRNA expression level. For our analysis of the CTL surface, such a set was readily available because of the large amount of work previously done using protein detection reagents (mainly monoclonal antibodies) to identify the crucial components on the CTL surface for the activation and function of the cell. This group of molecules is very unlikely to be biased towards those whose transcripts are more highly (or weakly) expressed, or towards those whose mRNAs have other features affecting their detectablility by SAGE for two reasons. Firstly, there is a low correlation between protein and mRNA expression levels *(13)*, and secondly, the importance of these molecules does not simply reflect their expression level, but rather the nature of their ligands, their binding properties and their signalling capability. In order to use the methods described here to identify the complete set of genes encoding membrane proteins in a particular cell and condition with any degree of confidence, it is necessary to identify a similar reference set and show that they are all identified by the method used. In particular, this demonstrates whether the library is of sufficient size and whether the statistical comparisons to other libraries are too stringent. A further reference set of transcripts definitely not involved would allow the specificity of the procedure to be assessed. Additional comparisons or more stringent tests could then be used to refine the technique to exclude more of the false positives without excluding any of the set known to be involved.

4. Notes

1. The protocol for creating a SAGE library described here does not *require* a specialist kit, however, for those new to SAGE, we recommend the use of the I-SAGE or I-SAGE Long kits, which provide all the reagents necessary for generation of a number of libraries. Each library can be produced from cDNA preparation to storage of bacterial colonies in 7 days. If using these kits, the following tips may help to optimise efficient production of high-quality libraries.
   i) Before using the kit, total RNA must be extracted from cells. We recommend cell lysis in TRIzol reagent (Invitrogen) according to the standard protocol. When sample is limited, the protocol can be scaled down to fit a 1.5 ml tube.
   ii) We recommend the use of phase-lock tubes (Flowgen,) for small-scale (1.5 ml tube) separation of solvent from aqueous phase. We used these tubes for small scale TRIzol extraction of total RNA and all phenol/chloroform extractions in the SAGE protocol.
   iii) Where possible, use a 'mastermix' of reagents used for multiple reactions to ensure they are as uniform as possible.
   iv) For all ethanol precipitations, after adding mussel glycogen, ammonium acetate and 100% ethanol to the DNA, make sure you vortex the mixture for 10 seconds. This ensures the DNA is well mixed and increases the amount recovered.
   v) When washing ethanol precipitated DNA pellets, use 2x 1 ml washes with cold 70% ethanol. To dry pellet, pulse at 2,000 rpm for 5 seconds and use a thin nose pipette to remove excess ethanol before air-drying for 5 min at room temperature. It should be possible to see the DNA pellet 'fizz' into solution as it is resuspended.
   vi) When ligating bead-bound cDNA to the SAGE adapters, first make a 2x mix without adapters, aliquot 16 μl into two tubes labelled A and B, then add 1.5 μl of the correct adapter to the two tubes and finally add each mix to one half of the beads.
   vii) After *Bsm FI / Mme I* digestion to remove adapter-tags from cDNA-beads and removal of the tag-containing supernatant from the beads, do not discard the beads. Instead, add 500 μl wash buffer D to them. This sample can then be used in a check PCR to show the efficiency of digestion. If digestion is complete, no PCR product should be seen in this bead sample.
   viii) Prior to *Nla III* digestion of the 100 bp (130 bp in LongSAGE) ditags, remove 0.5 μl of the ditags from

the 126 µl pool to use as an undigested sample on the check gel. Then, after 1 hour of the *Nla III* digestion, remove 5 µl of the digest from one tube to run a 12% polyacrylamide analytical gel alongside the undigested sample, but continue the *Nla III* digest until gel results are seen to ensure maximum digestion and hence a higher yield of 26 bp (34 bp for Long SAGE) ditags. Once digestion is complete, the three tubes can be combined into two for ethanol precipitation, allowing better balancing in the centrifuge. Ethanol precipitation is then done on 2×300 µl sample rather than 3×200 µl using 133 µl 7.5 M ammonium acetate, 3 µl mussel glycogen and 1 ml 100% ethanol.

ix) When eluting the 26 bp (34 bp in Long SAGE) ditags from the gel slices, do so overnight at 4°C (rather than 2 hours at 37°C).

x) Leave the ligation of ditags to form concatemers at 16°C for *at least* 2 hours.

xi) We find the 500–800 bp band of concatemers (as excised from the gel) gives the most reproducible sequencing results and the optimal number of tags per concatemer. Concatemers running larger on the gel are in fact far more variable in length and often give poor-sequence readouts.

xii) When re-suspending the concatemer DNA pellets (before cloning into the pZero vector) do so in 12 µl LoTE rather than 6 µl. Ligate concatemers into the pZero vector using all 12 µl, with 2 µl 10xbuffer, 1 µl vector, 1 µl Ligase and 3 µl water. Incubate at 16°C overnight rather than for a few hours.

2. As noted in the text, to enable genomic analysis of the resulting tags, LongSAGE is the preferred technique. However, the protocol described here is for regular SAGE. Long SAGE libraries are best made using the I-SAGE Long kit (*see* **Note 1**) but the protocol here can be adapted for Long SAGE as follows:

   i) The Long SAGE linkers must be used in place of those for regular SAGE. These contain the *Mme I* site.

   ii) Cleavage of adaptor ligated cDNA is with *Mme I* (New England Biolabs) in place of *Bsm FI*

   iii) Klenow fragment digestion prior to ligation into ditags is no longer necessary, as *Mme I* leaves 2 base 3′ overhangs than can ligate together.

3. As with all work using RNA or small quantities of DNA, the utmost care must be taken to avoid contamination of samples. Gloves should be used at all times and new reagents should be opened for each SAGE library produced. In particular, SAGE libraries should not be made in laboratories where ditags have previously been amplified by PCR as the

amount of ditags present post-PCR means small amounts of contamination are very significant when compared to the very small amount of ditags produced pre-PCR for the second library. Segregation of pre-PCR work in a separate 'PCR clean room' is the best solution to minimise the chance of cross library contamination.

4. Because of the small size and quantity of DNA being purified, all ethanol precipitations must be done with great care in the presence of mussel glycogen to ensure a substantial pellet. Particular care should be taken when purifying the 26 bp (34 bp in Long SAGE) ditags, when an ultracentrifuge should be used to ensure maximum DNA recovery.
5. In the preparation of large libraries, many bacterial colonies (and hence concatemers) are required. We find storage of the bacteria as glycerol stocks is the most stable format to enable them to be sequenced plate by plate, although the process of picking, growing and freezing the colonies is laborious. We have stored up to 1000 96-well plates from a single large library for up to 6 months and continue to obtain good quality sequence from them, with no apparent tag bias in later samples (as judged by following the average GC content of tags over time).
6. Standard SAGE tags are theoretically 11 bases long – not including the CATG of the *Nla III*. However, because *Bsm FI* cuts at variable positions, usually only 10 bases are kept from each tag. The SAGE program group software follows this convention, but keeps a record of the 11th base in the tag. If *Bsm FI* cut short, this base will actually belong to the other tag in the ditag so it is not completely reliable. However, when a tag is sequenced multiple times, in the majority of cases the 11th base will be the same. If this is so, it can be used to confirm or refute gene matches for that tag.
7. We used SAGEmap for our initial Tag-to-Gene matching because it was the most comprehensive mapping available at the time of our analysis – giving all possible matches for a tag. This produces many 'false positives' so many tags appear to match multiple genes. Manual curation removes many of these errors, enabling an optimal mapping of tags of interest. If a fully automated procedure is required, SAGE Genie is a better mapping resource (e.g., *see* **Section 3.5**). Another alternative is the direct matching of tags to the genome followed by analysis of the genome at each matching location to search for possible genes. This approach is only feasible for Long SAGE tags as in **Section 3.3**.
8. When choosing a Phred score cut-off that indicates acceptable quality data, one has to balance the need to remove unreliable sequences against the danger of losing genuine data. Analysis of sequences of a similar length to SAGE ditags led Prosdocimi et al. *(24)* to conclude that low Phred

settings allowed the optimal ratio of genuine sequences retained to errors removed. Therefore, sequences with Phred scores of 10 and above should be kept.

9. The extraction of SAGE tags from raw sequence data involves a number of steps, all carried out by the Perl scripts described in **Section 2.3**. First base calls in the sequence with Phred scores below the cut-off (*see* **Note 8**) are replaced with N (for an ambiguous base). Then sequences are searched for *Nla III* sites. These sites should flank ditags. Each putative ditag sequence is checked for length. We allow ditags between 40 and 44 bases for LongSAGE (28 and 32 bases for regular SAGE). The set of correct length ditags is assessed for duplication. Because the chance of the same pair of tags ligating together is low, duplicate ditags are likely to represent bias in the PCR step and are discarded. The first 17 bases (not including the *Nla III* site itself; 10 bases in regular SAGE) of the ditag from each end are extracted as the tag sequences. Any tag sequence containing an ambiguous base call is discarded. The final output consists of a table of unique tag sequences and the times each is found.
10. Monte-Carlo simulations can be used for an accurate analysis of the differential expression of individual tags but are computationally expensive, and therefore, inappropriate for repeated analysis of all tags in a library against other libraries. Several other tests have been applied to digital expression data including the general χ^2 statistic, the χ^2 2×2 test, Fisher's 2×2 test and tests designed by Audic and Claverie (AC test; *(14)*) and Greller and Tobin *(25)*. The power, specificity and robustness of these different tests have been compared by several authors *(26–28)*. Surprisingly the χ^2 statistic was found to be the most efficient for the comparison of expression levels in multiple samples. For pair wise comparisons, the 2×2 Fisher test is accurate but conservative, resulting in few false positives but comparatively many false negatives. In addition, the theoretical basis for using this test is weak – it depends on the production of a 2×2 table as follows:

	Library 1	**Library 2**	
Transcript X	n_{11}	n_{12}	$N_1.$
All other tags	n_{21}	n_{22}	$N_2.$
	N_1	N_2	N..

n_{ij} is the tag count for gene X ($i = 1$) or all other tags ($i = 2$) in library j. $N_i.$ and N_j are the marginal totals for rows and columns, respectively, and N. is the total number of tags in the two libraries. This is inappropriate for SAGE because: (1) 'All other tags' represent a different group of

tags in each tissue, so is not a consistent category. (2) The test requires that marginal frequencies are fixed a priori – this is reasonable for the columns (the totals are the numbers of tags sequenced) but not for the rows because these totals depend on the intrinsic transcript expression level (*a priori* unknown). The χ^2 2×2 test uses a similar table and is also accurate but conservative. Theoretically, it again groups 'all other tags' together and, in addition, it is valid only where the expected number of tags is ≥5.

The AC statistic is specifically designed for digital gene expression profiles (originally EST sequencing); it is less conservative and can be used at all expression levels. We, therefore, favour this test here.

11. The factorials involved in calculating the AC statistic can become very large and sometimes cannot easily be calculated. However, where they are large, expected frequencies will also be large and so a simple χ^2 2×2 test becomes valid and is likely to be most effective.
12. The tumour SAGE libraries used in this analysis were derived from five brain tumours (astrocytoma, glioblastoma, medulloblastoma, oligodenroglioma and recurrent ependyoma), a breast ductal carcinoma, a colon adenocarcinoma, a gastroesophageal junction adenocarcinoma, a pancreatic adenocarcinoma, a prostate tumour (including stroma and epithelium) and two ovarian cancers (carcinoma and cystadenoma).
13. We believe the best resource for this purpose is SAGE Genie *(29)*. To assess the quality of the tag-to-gene mapping achieved by such an automated process, we compared such mappings with mappings we had already manually curated for 374 genes encoding leukocyte cell surface proteins (the 'CD antigens'). SAGE Genie contained mappings for 364 of the genes, and in 309 cases it identified the same optimum SAGE tag as we had previously chosen.

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Part II

Extraction and Purification of Membrane Proteins

Chapter 4

Separation of Thylakoid Membrane Proteins by Sucrose Gradient Ultracentrifugation or *Blue Native-SDS-PAGE* Two-Dimensional Electrophoresis

Gian Maria D'Amici, Christian G. Huber, and Lello Zolla

Abstract

Generally, a combination of two or more chromatographic and/or electrophoretic methods is conducted to separate membrane protein complexes. Here we describe how thylakoid membrane protein complexes from the photosynthetic apparatus can be successfully separated by two main steps: preparative methods that enable purification of membrane protein complexes in the native (intact) form, and analytical methods that allow resolution of each membrane protein. Thus, separation of intact supercomplexes was achieved by solubilisation of the sample using mild detergents followed either by sucrose gradient ultracentrifugation or by blue native gel (BNG) electrophoresis. Complexes, thus, recovered were then resolved further using either reversed phase liquid chromatography or SDS-PAGE respectively.

Key words: Chloroplast, thylakoid, photosystem membrane proteins, sucrose gradient ultracentrifugation, Blue Native gel electrophoresis, *Arabidopsis thaliana*, *Spinacia olearcea Hordeum vulgare*.

1. Introduction

The difficulties in the investigation and separation of membrane protein complexes originate from their nature as membrane proteins. They are multi-subunit complexes, very hydrophobic and have single or several transmembrane domains, or closely associate with the membrane. Such membrane protein complexes contain many cofactors and, inevitably, lipids. Keeping the membrane protein complex intact is a prerequisite for preparative separation, but not for analytical separation. Consequently, the separation methods for research on membrane proteins, and their complexes, fall into two categories: preparative and analytical

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separation. The first aims to purify the membrane protein complex from membrane fractions while retaining its native form, mainly to characterize its *function*. The second aims to *catalogue* the constituents of the membrane protein complex, usually on a small scale.

Thylakoid membrane proteins present in chloroplasts represent a good model for optimising analytical methods suitable for separation and identification of membrane proteins. Moreover, they can achieve sufficient quality (high purity, high homogeneity, monodispersity, etc.) to enable crystallisation of samples as well as more traditional analysis of membrane proteins. The photosynthetic apparatus contains two main protein complexes the PSI and PSII pigment-protein complexes which are embedded in the thylakoid membrane of plants, algae and cyanobacteria and are made up of about 50 different subunits *(1,2)*. Both photosystems consist of a central core complex containing highly hydrophobic proteins, with between 5 and 11 transmembrane helices, and other small proteins. In total, the thylakoid membrane contains a mosaic of different membrane-embedded proteins which are highly hydrophobic (GRAVY indexes ranging between -0.4 and $+1.5$), and range in molecular weight between 4 and 85 kDa.

Two-dimensional electrophoresis has been used for membrane protein separation but the iso-electric focussing step is especially problematic. Many attempts have been made to resolve such problems, for example, introduction of new chaotropes, surfactants, reductants or supporting matrices, but without complete success *(3)*. Blue native gel electrophoresis, as an alternative first dimension to isoelectric focusing (IEF), has proved to be a good method to allow complete separation of each protein by SDS-PAGE in the second dimension. As an additional alternative first dimension, sucrose gradient ultracentrifugation in the presence of a mild detergent gives good results. Because of its flexibility and suitability for proteins having a very wide range of molecular masses and hydrophobicities, both methods are generally applicable in the analysis of complex mixtures of membrane proteins.

2. Materials

Protocols here described are suitable for thylakoid membrane isolation from chloroplasts of spinach (*Spinacia olearcea*), Arabidopsis (*Arabidopsis thaliana*) and barley (*Hordeum vulgare*).

2.1. Homogenisation of Leaves and Isolation of Intact Chloroplasts

1. Grinding buffer: 330 mM sorbitol, 20 mM tricine, 5 mM EGTA, 5 mM EDTA, 10 mM Na_2CO_3, 0.1% (w/v) BSA, 1.9 mM ascorbic acid.
2. 40% Percoll solution: mix 4 mL Percoll (Sigma, Poole, UK) with 6 mL of grinding buffer, plus 3.30 mg of ascorbic acid

and 1.30 mg of L-gluthatione reduced. Pour the resulting 10 mL of 40% Percoll solution into a ultracentrifuge tube of 50 mL (*see* **Note 1**).
3. Intact chloroplast storage solution: the grinding buffer but without 0.1% (w/v) BSA.
4. Miracloth (Calbiochem, San Diego, CA, USA).
5. Swelling rotor (Beckman Coulter model SW28 or similar).

2.2. Isolation of Thylakoid Membranes from Intact Chloroplast

1. Hypotonic buffer: 50 mM sorbitol, 5 mM tricine, pH 7.8, EDTA 10 mM, 0.5% (v/v) protease inhibitor cocktail for plant cell (Sigma, Poole, UK).

2.3. Separation of Thylakoid Membrane Complexes

2.3.1. Sucrose Gradient and Protein Separation by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

1. DDM solution: 2% (w/v) n-dodecyl-β-D-maltoside (DDM) (Sigma, Poole, UK,) (*see* **Note 2**).
2. Light sucrose solution: 0.1 M sucrose, 5 mM tricine, pH 7.8, 0.03% (w/v) DDM.
3. Heavy sucrose solution: 1 M sucrose, 5 mM tricine, pH 7.8, 0.03% (w/v) DDM.
4. Gradient maker connected with peristaltic pump.
5. Swelling rotor (Beckman Coulter model SW41 or similar).
6. Protein C-4 columns (250 × 4.6 I.D. mm) containing 5-μm porous butyl silica (Vydac, Hesperia, CA, USA).
7. HPLC: sample injection valve with 50 μL sample loop and UV detector. Mobile phase A: 99.9% acetontrile (LC-MS Chromasolv grade, Sigma, Poole, UK); mobile phase B: 99.9% water (LC-MS Chromasolv grade, Sigma) (A): 99.9% (v/v) Acetonitrile LC-MS CHROMASOLV (Sigma, Poole, UK,) 0.1% trifluoroacetic acid (TFA).
   Phases (B): 99.9% (v/v) water LC-MS CHROMASOLV (Sigma, Poole, UK,) 0.1% trifluoroacetic acid (TFA).

2.3.2. Separation of Thylakoid Membrane Proteins by Two-Dimensional Electrophoresis

1. Acrylamide/bis solution: T 49.5% C 3%. This is a neurotoxin when unpolymerised and so care should be taken not to receive exposure. Use a fresh solution degassed by helium for 20 min.
2. Gel buffer: 150 mM bis/tris-HCl, pH 7.0 (*see* Note **3**), 1.5 M *ε*-amino-*n*-caproic acid. Store at 4°C.

2.3.2.1. First Dimension: Blue Native Gel (BNG)

3. Glycerol solution: 75% (w/v) glycerol. Store at 4°C.
4. Cathode buffer without dye: 50 mM tricine, 15 mM bis/tris-HCl, pH 7.0. Store at 4°C.
5. Cathode buffer containing dye: 50 mM tricine, 15 mM BisTris-HCl, 0.01% (w/v) Brilliant blue G (Sigma, Poole, UK). Store at 4°C.
6. Anode buffer: 25 mM bis/tris-HCl, pH 7.0. Store at 4°C.

7. Washing buffer: 330 mM sorbitol, 50 mM bis/tris-HCl, pH 7.0, 250 μg/mL Pefabloc (Roche, Indianapolis, IN, USA). Store at −20°C.
8. 25BTH20G buffer: 20% (w/v) glycerol, 25 mM Bis/Tris-HCl, pH 7.0, 250 μg/mL Pefabloc. Prepare fresh solution for each use.
9. Solubilisation buffer: 20% (w/v) glycerol, 25 mM Bis/Tris-HCl, pH 7.0, 250 μg/mL Pefabloc, 2% (w/v) n-dodecyl-β-D-maltoside (DDM). Prepare fresh solution for each use.
10. Comassie Blue solution: 5% (w/v) Brilliant blue G, 100 mM bis/tris-HCl, pH 7.0, 30% (w/v) sucrose, 500 mM *ε*-amino-*n*-caproic acid. Store at −20°C.
11. N,N,N,N′-Tetramethyl-ethylenediamine (TEMED).
12. Fresh 5% (w/v) ammonium persulfate solution.
13. Gradient maker connected with peristaltic pump.

2.3.2.2. Second Dimension: 6 M Urea Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Acrylamide/bis solution: T 50% C 1.33%. Use a fresh solution degassed by helium for 20 min.
2. Separating buffer: 1.5 M Tris-HCl, pH 8.8. Store at room temperature.
3. Stacking buffer: 0.5 M Tris-HCl, pH 6.8. Store at room temperature.
4. SDS solution: 20% (w/v) sodium dodecyl sulphate (SDS). Store at room temperature.
5. Running solution (10X): 250 mM tris, 1.9M glycine, 1% (w/v) SDS. Store at 4°C.
6. N,N,N,N′-Tetramethyl-ethylenediamine (TEMED).
7. Fresh 10% (w/v) ammonium persulfate solution.
8. Second dimension incubation buffer: 6 M urea, 4.3% (w/v) SDS, 25% (v/v) glycerol, 140 mM Tris-HCl, pH 6.8, 5% (v/v) *β*-mercaptoethanol. Use a fresh solution.
9. Sealing buffer: 0.5% (w/v) agarose, 192 mM glycine, 0.1% (w/v) SDS, 25 mM Tris. Heat to 50–100°C. Store at 4°C.

3. Methods

3.1. Homogenisation of Leaves and Isolation of Intact Chloroplast

1. Wash 30 g of spinach leaves with deionised water and remove excess of water (*see* **Note 4**). Remove the midrib veins with sharp scissors and cut the leaves into small (1–3 cm) pieces.
2. Add 120 mL of grinding buffer and homogenise with a blender (*see* **Note 5**).
3. Filter the macerate through four layers of Miracloth and divide the filtrate between four 50 mL super-centrifuge tubes (*see* **Note 6**).
4. Centrifuge the tubes for 3 min at 200 × g (4°C).
5. Discard the white pellet and transfer the supernatant into four clean tubes and centrifuge for 7 min at 1,000 × g (4°C).

6. Discard the supernatant and re-suspend the green pellet with 1 ml of grinding buffer (*see* **Note 7**).
7. Carefully overlay the chloroplast suspension on top of 40% Percoll solution (*see* **Note 8**). Centrifuge for 6 min at 1,700 × g (4°C) using a swelling rotor like SW28 model by Beckman Coulter. Discard the upper phase and re-suspend the pellet in 0.5 mL of intact chloroplast storage solution.

3.2. Isolation of Thylakoid Membranes from Intact Chloroplast

1. Re-suspend the green intact chloroplast pellet with 30 mL of hypotonic buffer directly into a super-centrifuge tube of 50 mL. Centrifuge for 10 min at 10,000 × g (4°C). Discard the supernatant. Repeat the centrifugation twice. The green pellet contains the thylakoid membranes (*see* **Note 9**).

3.3. Separation of Thylakoid Membrane Complexes

3.3.1. Sucrose Gradient and Protein Separation by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

1. Re-suspend the green thylakoid pellet with the hypotonic buffer so that the chlorophyll concentration, determined according to the method of Porra et al. *(4)*, is 2 mg chlorophyll *per* mL.
2. Mix light and heavy sucrose solutions by a gradient maker into a 12 mL ultra-centrifuge tube to get a gradient sucrose solution (*see* **Note 10**).
3. Mix 2 mL of the thylakoid suspension with 2 mL of DDM solution; so that the final concentration for chlorophyll is 1 mg/mL and DDM 1% (w/v), respectively (*see* **Note 11**). Stir the final 4 mL solution at 0–2°C for 10 min.
4. Centrifuge the suspension at 20,000 × g for 10 min (4°C) to pellet the insoluble material. Collect the supernatant and discard the pellet.
5. Load 500 μL of supernatant (corresponding to 500 μg of chlorophyll) onto 12 mL ultra-centrifuge tube containing 0.1–1 M sucrose gradient solution.
6. Centrifuge at 260,000 × g for 19 h (4°C) using a swelling rotor such as SW41 model from Beckman Coulter.
7. Collect each of the five green bands (**Fig. 4.1**) using a syringe. Measure the chlorophyll concentration according to the method of Porra et al. In order to prepare the sample for high-performance liquid chromatography (HPLC), dilute a portion of sucrose green bands with 100% acetonitrile to give a final volume of more than 50 μL with a chlorophyll concentration (0.5 μg/μL).
8. Pre-equilibrate the Vydac C4 column C-4 columns with 38% (v/v) aqueous acetonitrile solution containing 0.1% (v/v) TFA for 30 min and load each sample onto it using a 50 μL loop.
9. Elute the sample using a linear gradient from 38 to 55.4% (v/v) acetonitrile in 22 min, followed by 3 min isocratic elution with the eluent containing 55.4% acetonitrile, followed by a second gradient of 55.4–61.8% acetonitrile in 8 min and

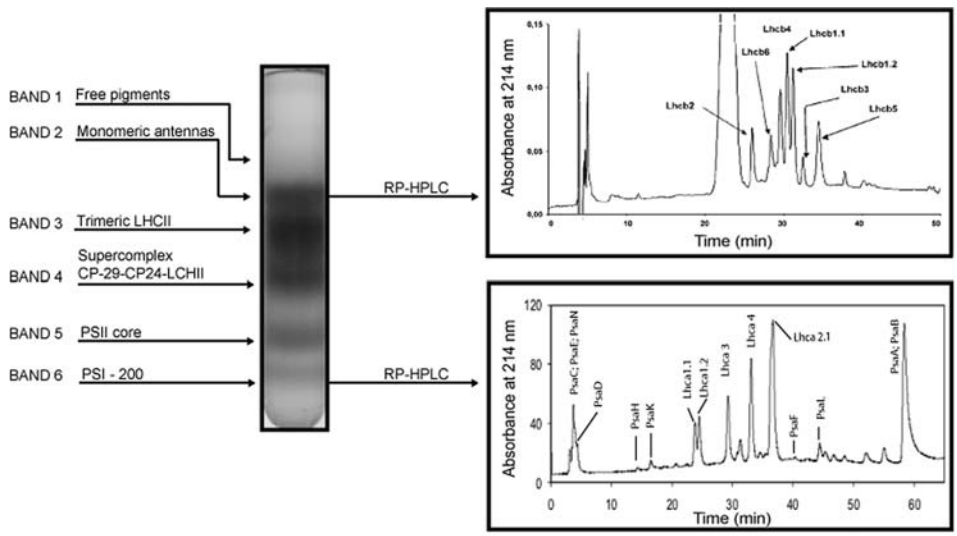

Fig. 4.1. Isolation of photosynthetic membrane proteins from plant leaves. Fractionation in 0.1–1 M sucrose gradient and RP-HPLC separation.

by a third gradient of 61.8–95% acetonitrile in 1 min. The final part of the curve up to 95% acetonitrile is used for washing out hydrophobic contamination. Set UV detector wavelength at 214 nm (*see* **Note 12**).

3.3.2. Separation of Thylakoid Membrane Proteins by Two-Dimensional Electrophoresis

3.3.2.1. First Dimension: BNG

1. Gel casting.
   Prepare a 0.75-mm thick (*see* **Note 13**), 5–12% gradient gel by mixing (with a gradient maker connected with a peristaltic pump) the light solution (T 5%, 0.96 mL acrylamide/bis solution, 3.17 mL gel buffer, 0.64 mL glycerol solution, 4.65 mL MQ water, 9 μL TEMED, 50 μL 5% (w/v) ammonium persulfate solution) with heavy solution (T 12.5%, 2.4 mL acrylamide solution, 3.17 mL gel buffer, 2.55 mL glycerol solution, 1.30 mL MQ water, 50 μL 5% (w/v) ammonium persulfate solution). After casting, overlay the gel with Milli-Q (MQ) water. The running gel should polymerize in about 45 min. After polymerisation, remove the water and prepare the stacking gel (T 4%) by mixing 0.81 mL acrylamide/bis solution, 3.33 mL gel buffer, 5.73 mL MQ water, 30 μL TEMED, 150 μL 5% ammonium persulfate solution. Use about 5 mL of this quickly to rinse the top of the gel and then pour the stack and insert the comb. The stacking gel should polymerise in about 30 min.
2. Sample preparation. (*see* **Note 14**).
   Take a 500 μL eppendorf. Add 300 μL of washing buffer to 100 μL of the thylakoid suspension, at chlorophyll concentration of 2 mg/mL. Vortex for 10 s. Centrifuge at 3,500 × g for 2 min (4°C). Discard the supernatant and suspend the green pellet with 25BTH20G buffer to the final total volume of 200 μL (at final chlorophyll concentration of

1 mg/mL). Vortex the sample for 10 s. Add to 200 µL of the 25BTH20G thylakoid suspension 200 µL of solubilisation buffer. Vortex it for 10 s and place on ice for 3 min. Remove insoluble material by centrifugation at 18,000 × g for 15 min (4°C). Recover the supernatant and discard the pellet. Mix the supernatant (approximate chlorophyll concentration of 0.5 mg/mL) with 0.1 volumes of Comassie blue solution and vortex it for 3 min. Load about 30–40 µL of the final solution (at approximately 0.5 µg/µL of chlorophyll concentration).

3. Running conditions (*see* **Note 15**).
   Complete the assembly of the gel unit and connect to the power supply. Pour the cathode buffer containing dye and the anode buffer, respectively, in the upper and lower chamber of the gel unit. Run the gel at 4°C using the following voltages: 50 volts for 1 h, 60 volts for 2 h, 85 volts for 16 h, 110 volts for 50 min, 160 volts for 75 min, 200 volts for 50 min 250 volts until the end of run. Stop the run when the dye front reaches the bottom of the gel.

3.3.2.2. Second Dimension: 6 M Urea Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. This protocol is for gels cast using the Protean II XI cell system (Bio-Rad, Hercules, CA, USA) with 165 × 155 mm gels. It is critical that the glass plates for the gels are scrubbed clean with a detergent after use and rinsed extensively with distilled water. They can be kept clean until use in a plastic rack in 30% (v/v) nitric acid. They will just need rinsing (distilled water then 95% ethanol) to remove the acid and air-dry.
2. Prepare a 1.0 mm, 15% gel by mixing 9.95 mL acrylamide/bis solution, with 7.6 mL of separating buffer,11.9 g of urea, 5.85 mL of MQ water, 0.66 mL SDS solution, 75 µL 10% (w/v) ammonium persulfate solution and 11.25 µL TEMED. Pour the gel solution (about 26 mL), leaving space for a stacking gel, and overlay with isobutanol. The gel should polymerise in about 45 min. When polymerisation is complete, pour off the isobutanol and rinse the top of the gel twice with water.
3. Prepare the staking gel by mixing 1.2 mL acrylamide/bis solution with 2.5 mL of stacking buffer, 3.61 g of urea, 3.45 mL of MQ water, 0.2 mL SDS solution, 50 µL 10% (w/v) ammonium persulfate solution and 5 µL TEMED. Pour the gel solution (about 4 mL), leaving space to load the first dimension gel strip, and overlay with isobutanol. The stacking gel should polymerise in about 30 min.
4. Cut the BNG strip using a scalpel and put it into a Petri plate, containing 15 mL of fresh second dimension incubation buffer. Incubate the strip for 30 min with gentle agitation (use a rocking platform).
5. Place the strip on the stacking gel (*see* **Note 16**). No space should be between the BNG strip and the stacking gel.

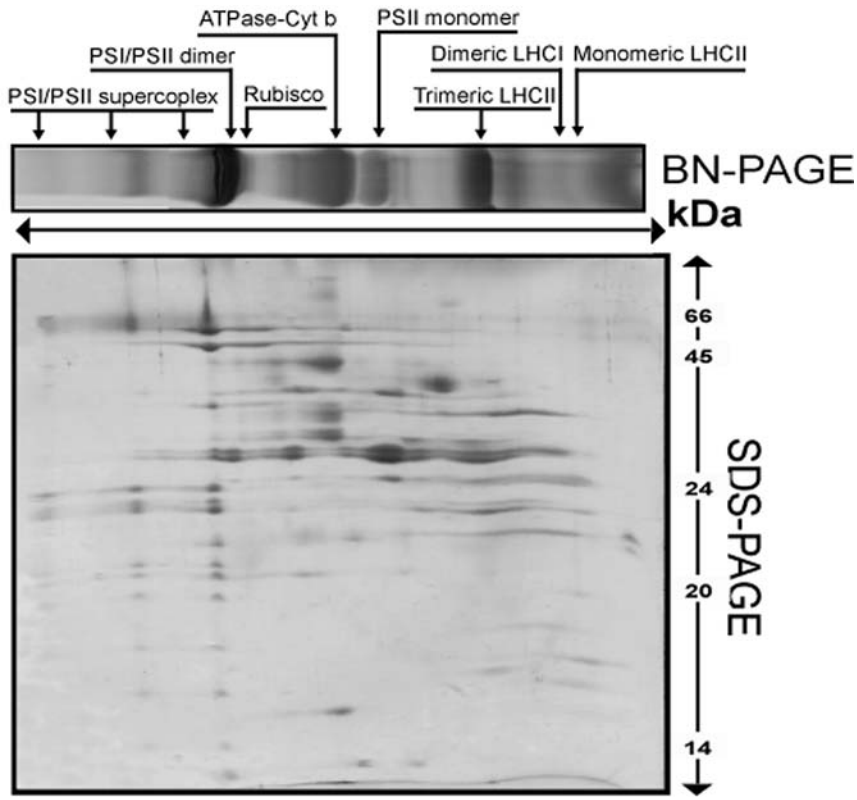

Fig. 4.2. Isolation of photosynthetic membrane proteins from plant leaves. 2-D-BNG/SDS map of spinach thylakoid membranes.

Overlay the strip with about 1 mL of hot (50–100°C) sealing buffer. After 5 min the sealing buffer should be a gel.

6. Prepare the running buffer by diluting 200 ml of running solution (10x) with 1.8 L of MQ water. Complete the assembly of the gel unit and connect to the power supply. If a cooling system is available for the gel unit then set the temperature at 13°C. The gel runs at 11 mA overnight then during the day at 300 V, until the dye front reaches the bottom of the gel.
7. The protein spots can be visualised using Coomassie R-250, colloidal Coomassie and also by silver staining (**Fig. 4.2**) *(5)*.

4. Notes

1. Prepare 10 mL of 40% Percoll solution for every 6 mL of chloroplast suspension.
2. After stirring for 30 min at 37°C, centrifuge the clear DDM solution at 10,000 × g for 2 min. The presence of a white pellet suggests a bad DDM solubilisation. For a good membrane protein extraction from thylakoid membranes, a homogenous DDM solution is required.

3. The pH value of BNG solutions must be checked at 4°C.
4. All steps should be performed in the dark and at 4°C. Use pre-cooled buffers, blender and centrifuge tubes (50 mL). Tube surfaces should be kept scrupulously clean. For optimal yield of intact chloroplasts the plant leaves must be kept in the dark at 4°C over night before the preparation in order to avoid starch accumulation. In this procedure, we use 30 g of leaves. For different amounts scale the quantities accordingly.
5. Optimal chloroplast isolation requires a different ratio of grinding buffer/leaves in grams: Arabidopsis 4:1, Barley 5:1, Spinach 4:1. Process the leaves with 4 blender strokes (within 5 seconds) to homogenise with minimal production of froth.
6. To facilitate the filtration, use a spoon. In the ultra-centrifuge tube the fill volume should not exceed 2/3 of the tube total volume (approximately 35 mL for each tube).
7. Both intact and broken chloroplasts are present in the green pellet. Thus, it is very important to re-suspend the pellet very gently using a little brush to avoid foaming and to get a uniform suspension.
8. Use 10 mL of 40% Percoll solution for every 6 ml of chloroplast suspension in a 50 mL ultra-centrifuge tube. After the centrifugation, the broken chloroplast will form a large green band on the top of the Percoll layer, whereas the intact chloroplast will sediment to the bottom as a small green pellet. The green pellet is very fragile; therefore, carefully remove all the upper phases and the Percoll layer by using a Pasteur pipette. Re-suspend the green pellet in 500 μL of the storage solution and snap freeze in liquid nitrogen. You can store the solution at −80°C. For functional assays, the chloroplast suspension should be kept in the dark on ice and the intact chloroplast should be used as soon as possible because activity is lost rapidly. You can estimate the intact chloroplast total chlorophyll according to the method of Amon et al. *(6)*.
9. Suspend both chloroplast green pellet and thylakoid pellet using a small brush ensuring a uniform suspension.
10. Use 5.5 mL of light solution and 5.5 mL of heavy solution in order to have a final sucrose gradient solution of 11 mL in an ultra-centrifuge tube of 12 mL.
11. This is a crucial step because the protein super-complex solubilisation of the thylakoid membrane takes place. Use 2 mL of thylakoid suspension and 2 mL of DDM solution into a 5 mL picker. During the stirring keep the picker in ice and avoid foaming.
12. Analysis of the fractions by RP-HPLC-ESI-MS revealed that band 2 contained a mixture of the protein components of the minor and monomeric major PSII antenna systems, whereas band 3 essentially contained the protein components of the

trimeric major PSII antenna system. Band 4 contained the super-complex CP-29-CP24-LCHII, band 5 contained the reaction centre complex of PSII, and finally, band 6 the subunits of PSI. In the first band, there are the plant pigments *(7)*. Similar separations were obtained for the different species (arabidopsis, spinach and barley).

13. This protocol is for gels cast using the Protean II XI cell system and gels of 165 × 155 mm but is easily adaptable to minigels. Prepare light and heavy solution in two 50 mL centrifuge tubes. Keep solutions in ice until gel casting. Before gel casting by gradient maker, mix the solutions well using a vortex. The BNG should be cast the day before the running. Store the gel at 4°C in MQ water and covered with Para-Film.
14. During all sample preparation steps, it is very important to work in a cold room (4°C) and in the dark. A homogeneous suspension between the thylakoid green pellet and 25BTH20G buffer is required. Note that the 200 μL of final volume should include the pellet volume plus the 25BTH20G buffer, in order to obtain a final chlorophyll concentration of 1 mg/mL *(8)*.
15. Run the gel in a cold room (4°C). If a cooling system is available for the gel unit, then set the temperature to 4°C. Start the run using cathode buffer containing the dye. When you change the voltage to 160 volts, use the cathode buffer without dye until the end of the run.
16. You can use a gel spacer of 0.75 mm to facilitate loading the BNG strip.

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Chapter 5

Extraction of Yeast Mitochondrial Membrane Proteins by Solubilization and Detergent/Polymer Aqueous Two-Phase Partitioning

Henrik Everberg, Niklas Gustavsson, and Folke Tjerneld

Abstract

Identification and characterization of membrane proteins is of increasing importance in modern proteomic studies. It is of central interest to have access to methods that combine efficient solubilization with enrichment of proteins and intact protein complexes. Separation methods have been developed based on nondenaturing detergent extraction of yeast mitochondrial membrane proteins followed by enrichment of hydrophobic proteins in aqueous two-phase system. Combining the zwitterionic detergent Zwittergent 3-10 and the nonionic detergent Triton X-114 results in a complementary solubilization of proteins, which is similar to that of the anionic detergent sodium dodecyl sulfate (SDS) but with the important advantage of being nondenaturing. Detergent/polymer two-phase system partitioning offers removal of soluble proteins, which can be further improved by manipulation of the driving forces governing protein distribution between the phases. Integral and peripheral membrane protein subunits from intact membrane protein complexes partition to the detergent phase while soluble proteins are found in the polymer phase. A protocol is presented which combines nondenaturing solubilization of membrane proteins with extraction in detergent/polymer two-phase system for application in proteomic studies as a mild and efficient method for enrichment of membrane proteins and membrane protein complexes.

Key words: Membrane proteins, detergents, solubilization, aqueous two-phase systems, proteomics.

1. Introduction

The traditional method for separation of complex protein mixtures in proteomic research is two-dimensional gel electrophoresis (2DE) based on charge-dependent separation (isoelectric focusing, IEF) in the first dimension and size-dependent separation (sodium dodecyl sulfate poly-acrylamide gel electrophoresis,

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SDS-PAGE) in the second dimension. Membrane proteins are, however, poorly compatible with this methodology due to their hydrophobic properties and are known to aggregate and precipitate when focused at their isoelectric point in the first dimension *(1, 2)*. Thus, transfer to the second dimension SDS-PAGE is impeded and consequently membrane proteins will not be quantitatively represented on the final 2DE gel if present at all. The strategies that are currently used in membrane proteomics are often based on high-resolution separation of peptides by liquid chromatography in combination with tandem-mass spectrometry (LC-MS/MS) *(3–5)*. Although this approach has been successful in identifying large numbers of membrane proteins, a major drawback derived from the peptide-based nature of the approach is that protein-specific information such as size, iso-electric point, isoforms, posttranslational modifications, and protein-protein interactions, such as for multi-subunit membrane protein complexes, is lost in the proteolysis step *(6)*. Other separation methods relying on chromatography or one-dimensional SDS-PAGE suffer from low resolution and require sample prefractionation for successful characterization of membrane proteomes. Alternative methods for separation of membrane proteins are needed, therefore, to facilitate and develop membrane proteomic research.

A procedure is presented here for initial extraction of membrane proteins (solubilization) followed by enrichment through partitioning in a detergent/polymer aqueous two-phase system. In the solubilization step, a zwitterionic detergent (Zwittergent 3-10) is combined with a nonionic detergent (Triton X-114) and after optimizing experimental conditions in terms of detergent/protein ratio; this combination was shown to be both quantitatively and qualitatively comparable to the solubilization efficiency of sodium dodecyl sulfate (SDS), but with the important advantage of being nondenaturing. Thus, it will be possible to obtain information about protein-protein interactions and to further prefractionate enriched protein complexes after using the solubilization protocol described here.

The optimized membrane solubilization is compatible with membrane protein enrichment by detergent/polymer aqueous two-phase partitioning (**Fig. 5.1**). Many nonionic detergents will form aqueous two-phase systems when mixed with a polymer in sufficient concentrations and proteins distribute between them depending on the inherent properties of the proteins and the phase components *(7, 8)*. Detergent/polymer two-phase systems can be designed to increase the partitioning of soluble proteins to the polymer phase by manipulating the thermodynamic driving forces governing protein partitioning between the phases. In this protocol, electrostatic interactions drive the partitioning of soluble proteins in to the aqueous phase while hydrophobic interactions drive the partitioning of membrane proteins in to the detergent phase. This results in an enrichment of membrane

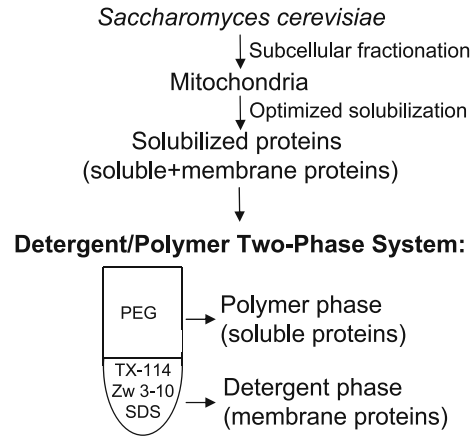

Fig. 5.1. Enrichment strategy. Mitochondria from *S. cerevisae* were isolated by ssubcellular fractionation.The isolated mitochondria were solubilized fractionated in a detergent/polymer aqueous two-phase system formed by addition of polymer and salt to the detergent solubilized mitochondrial membranes. Membrane proteins partitions to the detergent (*bottom*) phase and the bulk of soluble proteins to the polymer (*top*) phase at the chosen conditions.

proteins in the detergent phase *(7–9)* and the detergent/polymer two-phase system we have developed for this application will be described subsequently.

The method described in this chapter has been utilized for initial extraction and enrichment of membrane proteins and membrane protein complexes in mitochondria from the yeast *Saccharomyces cerevisiae*. The method can also be combined with further prefractionation by ionexchange chromatography *(7)*, separation by SDS-PAGE and LC-MS/MS analyses *(9)*.

2. Materials

The protocol described here applies to preisolated mitochondria from commercially processed *S. cerevisiae*. This membrane system represents a relatively small proteome from a fully sequenced organism, which contains besides both soluble and membrane-bound proteins, a number of well-known multisubunit membrane protein complexes.

2.1. Preparation of Mitochondria from Saccharomyces cerevisiae

1. Mitochondria isolation buffer (MIB): 20 mM HEPES-KOH, pH 7.4, 0.6 M mannitol. Complete protease inhibitor cocktail, (Boehringer Mannheim GmbH, Germany), 1 tablet/50 ml buffer. Prepare MIB fresh at use.
2. Dounce homogenizer with loose fitting plunger.
3. Glass beads 450–600 microns (Sigma-Aldrich, St Louis, MO, USA).

4. Beadbeater homogenizer (BioSpec Products Inc. Bartlesville, OK, USA).

2.2. Solubilization

1. Detergent stock solutions (% w/w): 20% Zwittergent 3-10 (Calbiochem, San Diego, CA, USA) and 2% Triton X-114 (Sigma-Aldrich, St Louis, MO, USA), (*see* **Note 1**). Store at +4°C.
2. Tris-HCl stock: 100 mM, pH 9.0.
3. Refrigerated centrifuge with rotor for 200 µl and capacity for 100,000×g.
4. Sodium dodecyl sulfate stock: 10% w/w (Merck, Darmstadt, Germany). Store at room temperature.
5. Bicinchoninic acid (BCA) protein determination assay reagent A and B (Pierce, Rockford, Il, USA).

2.3. Detergent/Polymer Two-Phase Partitioning

1. Polyethylene glycol (M_r: 40,000, Serva, Heidelberg, Germany) 25% (w/w) stock solution. Store at +4°C.
2. Detergent stock solutions: 100% (w/w) Triton X-114; 20% (w/w) Zwittergent 3-10; 100 mM SDS.
3. Glycine–NaOH stock: 200 mM, pH 10.0.
4. Maximum recovery 1.5 ml tubes (Axygen, Union City, CA, USA) (*see* **Note 2**).
5. Refrigerated centrifuge for Eppendorf tubes (1,000×g).

2.4. Sample Concentration and Clean-Up

The isolated phases after two-phase partitioning require concentration and removal of polymers and detergents before separation by SDS-PAGE (*see* **Note 3**).

1. SDS-PAGE clean-up kit (GE Healthcare, Uppsala, Sweden).
2. Dithiothreitol (Sigma-Aldrich, St Louis, MO, USA).

3. Methods

3.1. Preparation of Mitochondria from Saccharomyces cerevisiae

This protocol is adapted from McAda et al. *(10)* and all experiments are performed at +4°C.

1. Pressed yeast (400 g) is suspended in 200 ml ice cold MIB, using a dounce homogenizer.
2. The yeast suspension is homogenized 5 × 20 s with 1 min rest on ice between each treatment, using a Beadbeater homogenizer filled to 2/3 with glass beads.
3. The homogenized cell suspension is pooled and the suspension is centrifuged at 3,500×g for 10 min and the pellet is discarded. This is performed three times in total to remove cell debris.
4. The supernatant is centrifuged for 20 min at 17,000×g, to pellet the mitochondria.

5. The pellet is washed once in ice cold MIB and **Step 4** is repeated.
6. The mitochondrial pellet is resuspended in a minimal volume of MIB (~5 ml) and stored in aliquots at −80°C until use.

3.2. Solubilization

1. Mitochondria (0.5 mg total protein) are homogenized by freeze thawing twice (−80°C) to disrupt membrane structures and release the soluble fraction into the solution (*see* **Note 4**).
2. Add 20 µl Tris-HCl buffer from stock solution (10 mM final concentration).
3. Add 60 µl Zwittergent 3-10 from stock solution (20% w/w) to obtain the desired detergent/protein ratio (30 mmol detergent/g protein) (*see* **Note 5**).
4. Add 56 µl TX-114 from stock solution (2% w/w) to obtain the desired detergent/protein ratio (5 mmol detergent/g protein).
5. Add 64 µl water to obtain the right final detergent/protein ratio and a solubilization volume of 200 µl.
6. Incubate with gentle agitation at +4°C for 30 min.
7. Centrifuge at 100,000×g for 45 min to pellet any unsolubilized material and recover the supernatant.
8. The pellet is resolubilized by vortexing in 50 µl 2% SDS at room temperature until totally dissolved.
9. The amount of protein in the supernatant (and the pellet) is determined using the BCA protein assay (*see* **Note 6**).

3.3. Detergent/Polymer Two-Phase Partitioning

The supernatant after optimized detergent solubilization is transferred to a two-phase system premixed in Maximum recovery 1.5 ml tubes (Axygen, Union City, CA, USA) and the total weight of each system is 0.5 g (*see* **Note 7**).

1. Add 0.075 g TX-114 (100% stock solution) to obtain final concentration of 15% (w/w).
2. Add 0.080 g PEG 40000 (25% (w/w) stock) to obtain final concentration of 4%.
3. Add 0.025 g Glycine-NaOH pH 10 (200 mM stock) to a final concentration of 10 mM (*see* **Note 8**).
4. Add 0.015 g SDS (100 mM stock) to a final concentration of 3 mM (*see* **Note 9**).
5. Add 0.138 g water.
6. Finally add 0.167 g of the supernatant after solubilization (6% (w/w) Zwittergent 3-10) to reach a final concentration of 2% (w/w) Zwittergent 3-10 (*see* **Note 10**) and the desired total system weight of 0.5 g.
7. Mix thoroughly by inverting the tube until the system is opaque and phase separation is achieved.
8. Incubate for 30 min at +4°C with gentle agitation.

9. Speed up phase separation by centrifugation at 1,600×g for 15 min.
10. Isolate the phases using a Pasteur pipette (*see* **Note 11**).
11. The protein amount in each phase is determined by the BCA-assay (*see* **Note 6**) and the partitioning coefficient is calculated as the total protein concentration in the top phase divided by the total protein concentration in the bottom phase.

3.4. Sample Concentration and Clean Up

1. Perform clean up of sample to remove polymers and detergents using SDS-PAGE clean up kit (Amersham Biosciences) (*see* **Note 12**). Concentrated and cleaned samples can be used immediately or stored at −20°C.
2. Sample is then ready for SDS PAGE and visualization and analysis as required (*see* **Note 13**).

4. Notes

1. As solubilization efficiency might not be comparable between different membrane systems, an initial screening of detergents and combinations of detergents should be performed. We have studied the quantitative solubilization efficiency of one zwitterionic (Zw 3-10), one nonionic (TX-114), one anionic detergent (SDS) (**Fig. 5.2a**) and the qualitative solubilization efficiency of Zw 3-10 and TX-114 combined at optimized conditions (**Fig. 5.2b**). It is essential that tubes used during screening for optimal solubilization conditions are absolutely clean by washing with 70% EtOH and Millipore water to avoid contamination of the sample with other detergents giving false results of solubilization efficiency.
2. To minimize the loss of protein due to adsorption to the test tube walls.
3. This step is performed to be able to transfer as much of the protein content in the isolated samples to the SDS-PAGE and to prevent polymers and detergents disturbing protein migration in the gels.
4. As the important parameter is detergent/protein ratio, volumes or amounts should be scaled accordingly.
5. The relatively high detergent/protein ratio of ~30 mmol/g needed to reach the maximum level of protein extraction for Zw 3-10 (**Fig. 5.2a**) is probably due its high-critical micelle concentration (~40 mM).
6. To minimize the interference of membrane lipids and detergents, all samples are diluted with SDS stock solution to reach a final concentration of 2% SDS. Standard curves are made from known concentrations of BSA prepared in

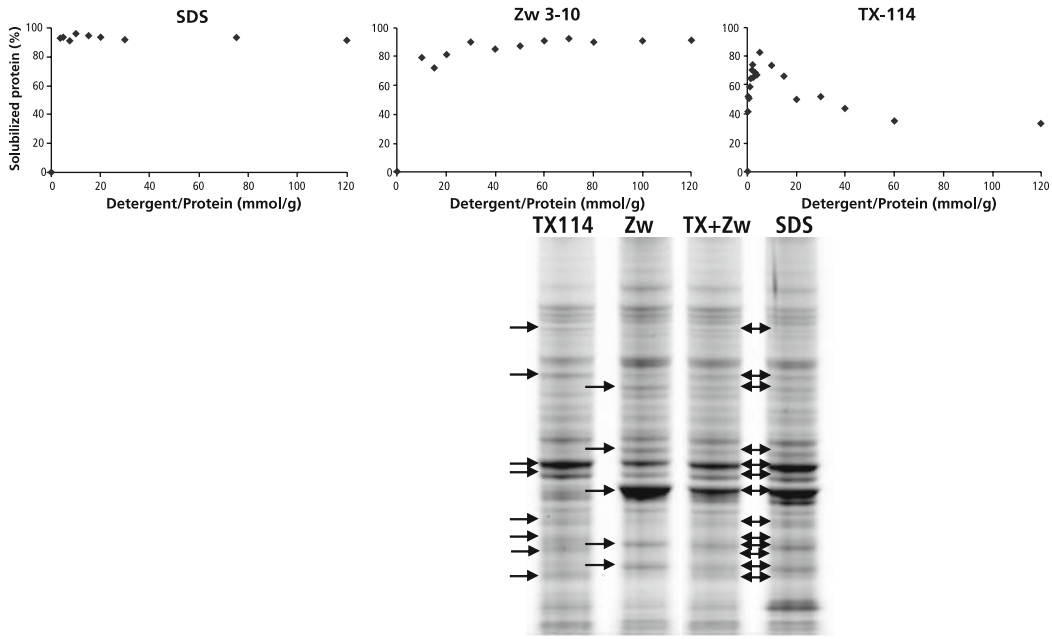

Fig. 5.2. Optimization of solubilization efficiency. (**a**) The quantitative solubilization efficiency was investigated for one ionic (SDS), one zwitterionic (Zw 3-10) and three non-ionic (here represented by TX-114) detergents. The detergent/protein molar ratio was screened and the amount of protein in the supernatant after incubation and centrifugation was measured. (**b**) Complementary detergent solubilization. After incubation with detergent and centrifugation the protein content of the supernatants was separated by SDS-PAGE. The *arrows* indicate a number of membrane proteins more efficiently solubilized by either Triton X-114 (TX114) or Zwittergent 3-10 (Zw). By mixing the detergents (TX+Zw) a complementary protein pattern was obtained. This pattern was also found comparable to the protein pattern obtained after solubilization with SDS.

2% SDS and 1% of the detergent used for solubilization (provided that a dilution series has been made to ensure that increased detergent concentration do not result in increased absorption). The solubilization efficiency is presented in **Fig. 5.2a**. It is defined (**Eq. 5.1**) as the fraction of solubilized membrane protein found in the supernatant divided by the total amount of membrane protein in pellet plus supernatant.

$$\text{Detergent solubilization efficiency (\%)} = \frac{(A-B)}{C+(A-B)} \times 100 \tag{5.1}$$

Here, A stands for total protein amount in the supernatant; B for soluble protein amount in the supernatant; and C for protein amount in the pellet. The soluble protein amount was determined as the amount of protein found in the supernatant when no detergent was added.

7. It is important to get the right proportions of the phase components for successful phase separation and phase vol-

ume ratio. Thus, due to the viscosity of the polymers and detergents they are weighed to obtain the right system composition.

8. The ionic strength of the system should be kept to a minimum to optimize the electrostatic repulsion effect discussed in **Note 9**.
9. Increased partitioning of proteins to the polymer phase is obtained by addition of a negatively charged detergent, SDS, at a concentration below its Critical micelle concentration (CMC) (10 mM). Monomers of SDS get incorporated in the mixed micelles of nonionic and zwitterionic detergents, thus introducing negative charges *(7–9, 11)*. This results in repulsion of negatively charged soluble proteins from the negatively charged mixed micelles in the detergent phase. The denaturing properties of SDS will be insignificant due to the low concentration of SDS monomers in the solution. By raising the pH, increased negative protein net charge leads to stronger repulsion between proteins and negatively charged mixed micelles, which enhances the partitioning of soluble proteins to the polymer-enriched top phase. Membrane proteins are not affected since hydrophobic interactions between membrane proteins and the hydrophobic core of the mixed micelles govern the partitioning of these proteins. The results shown in **Fig. 5.3** demonstrate an increased partitioning of proteins to the polymer phase as an effect of including SDS and increasing the pH of the system from 7 to 10.
10. The concentration of Zwittergent 3-10 should be kept to a minimum but above the CMC to avoid losing membrane protein solubility.

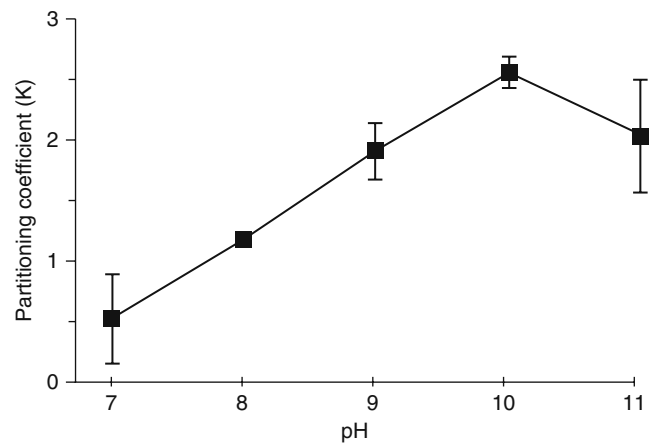

Fig. 5.3. Effect of pH on protein partitioning in the TX-114/PEG aqueous two-phase system. By increasing the pH of the system including SDS, the partitioning coefficient (K = (protein concentration in the top phase)/(protein concentration in the bottom phase)) is shifted from a partitioning predominantly to the detergent phase (*K=0.5*) at pH 7, to a more pronounced partitioning to the polymer phase (*K=2.5*) at pH 10.

11. Be sure to leave the interphase undisturbed to minimize contamination from the bottom phase as the top phase is withdrawn. Discard the interphase and collect the bottom phase with a clean Pasteur pipette
12. The concentration and clean up is performed according to the manufacturers instructions but instead of the final heating step (95°C, 5 min), the samples are incubated at room temperature for 30 min to avoid aggregation of hydrophobic proteins. The pellet was totally dissolved in 25 µl loading buffer by vortexing and incubation in a sonicator bath.
13. We used NuPAGE Bis-Tris pre-cast gradient gels (4–12%) from Novex (San Diego, CA, USA) for SDS-PAGE according to the manufacturer's instructions, stained in colloidal Coomassie brilliant blue and destained in water, followed by tryptic in-gel digestion and LC-MALDI TOF/TOF analysis.

Acknowledgments

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Chapter 6

16-BAC/SDS-PAGE Analysis of Membrane Proteins of Yeast Mitochondria Purified by Free Flow Electrophoresis

Ralf J. Braun, Norbert Kinkl, Hans Zischka, and Marius Ueffing

Abstract

Mitochondria are essential organelles in cellular metabolism. These organelles are bounded by two membranes, the outer and inner membrane. Especially the inner membrane comprises a high content of proteins, for example, the protein complexes of the respiratory chain. High-resolution separation and analysis of such membrane proteins, for example, by two-dimensional gel electrophoresis (2-DE), is hampered by their hydrophobicity and tendency for aggregation. Here, we describe the separation of mitochondrial membrane proteins of *Saccharomyces cerevisiae* by 16-benzyldimethyl-*n*-hexadecylammonium chloride/sodium dodecyl sulfate polyacrylamide gel electrophoresis (16-BAC/SDS-PAGE). This method enables the separation of membrane proteins owing to the solubilizing power of the ionic detergents 16-BAC and SDS, respectively. Mitochondria were isolated from yeast cultures by differential centrifugation and were further purified by free flow electrophoresis (FFE) in zone-electrophoretic mode (ZE). Subsequently, membrane proteins from ZE-FFE-purified mitochondria were enriched by carbonate extraction and subjected to 16-BAC/SDS-PAGE. The resulting protein spot patterns were visualized by a highly sensitive fluorescence stain with ruthenium-II-bathophenantroline disulfonate chelate (RuBP), and by colloidal Coomassie staining. Proteins were identified by Maldi-Tof mass spectrometry and peptide mass fingerprinting.

Key words: Mitochondria, *Saccharomyces cerevisiae*, 16-BAC, 16-BAC/SDS-PAGE, zone electrophoresis, free flow electrophoresis, ruthenium staining.

1. Introduction

Mitochondria are the principal sites of ATP production in aerobic cells and are, therefore, called the "power plants" of the cell. Beside their role in cellular energy metabolism, these organelles participate in a multitude of enzymatic pathways including the metabolism of certain amino acids, heme biosynthesis, and

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iron-sulfur cluster formation *(1)*, and are crucially involved in cellular processes like apoptosis *(2)* and ageing *(3)*.

Classically, mitochondria are described to consist of four compartments, the outer membrane, the intermembrane space, the inner membrane, and the matrix. The outer membrane comprises 32 proteins for *S. cerevisiae* with known functions in the outer membrane although more proteins have been identified by a recent proteome study *(4)*. Among the known outer membrane proteins, components of the protein import machinery (translocase of outer membrane [TOM] complex), the sorting and assembly machinery (SAM) complex, pore-forming components (porin), and proteins that control fusion and fission of the organelle have been described *(4, 5)*. The mitochondrial inner membrane is the cellular membrane containing the highest protein content in eukaryotic cells (75% protein per weight) *(6)*. The inner membrane demonstrates, in contrast to the outer membrane, highly selective permeability and contains numerous transport proteins that control the passage of metabolites such as ADP, ATP, phosphate, and ions such as Ca^{2+} *(6)*. The very high protein content of the inner membrane is a result of the five major multiprotein complexes of the respiratory chain that form supercomplexes, in order to guarantee efficient transduction of the energy of carbon sources into the energy stored in ATP via oxidative phosphorylation *(7)*.

Since membrane proteins are crucially involved in mitochondrial function, analysis of mitochondrial membrane protein extracts is of high importance. In order to perform successful separation of membrane proteins, they must be extracted from their membranous environments into soluble forms and must remain soluble during the separation. However, this is hampered predominantly by their hydrophobicity, resulting in a high tendency for aggregation in aqueous media *(8)*. Aggregation of membrane proteins is a major problem in two-dimensional gel electrophoresis (2-DE; IEF/SDS-PAGE) during isoelectric focusing (IEF), where proteins reach their isoelectric point (pI), which is the point of minimal solubility *(8)*. Nonionic detergents (such as 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propansulfonate [CHAPS]) have been added to the standard urea/thiourea-based solutions used in IEF, in order to improve the solubility of hydrophobic proteins *(9)*. However, these detergents have much lower solubilizing power than ionic detergents such as SDS that is incompatible with IEF at higher concentrations.

Because of the high solubility of SDS-(membrane) protein complexes, SDS-PAGE *(10)* is still widely used for the analysis of protein extracts enriched with membrane proteins, although this one-dimensional method (1D-PAGE) lacks the capability to resolve complex protein samples. Macfarlane

developed a gel electrophoresis system with 16-benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) as cationic detergent that has a similar solubilizing power to the anionic detergent SDS *(11)*. Equivalent to SDS-PAGE, 16-BAC-protein complexes are separated in the polyacrylamide gel (16-BAC-PAGE) according to their size *(11)*. Combination of 16-BAC-PAGE as first and SDS-PAGE as second dimension results in a two-dimensional gel electrophoresis that allows a higher resolution of complex protein mixtures compared to 1D-PAGE *(12, 13)*. Due to slight differences in the separation behavior of detergent-protein complexes between 16-BAC-PAGE and SDS-PAGE, possibly because of different affinities of individual proteins to the ionic detergents, proteins fan out in the second dimension as protein spots along a diagonal. Although the resolution of protein extracts in 16-BAC/SDS-PAGE is lower compared to 2-DE (IEF/SDS-PAGE), this method is, in contrast to classical 2-DE, suitable for the separation of protein extracts enriched with hydrophobic membrane proteins *(12, 14, 15)*.

This chapter describes the analysis of mitochondrial membrane proteins of *S. cerevisiae* by 16-BAC/SDS-PAGE. Mitochondria were prefractionated by differential centrifugation after an enzymatic digest of the yeast cell wall and homogenization

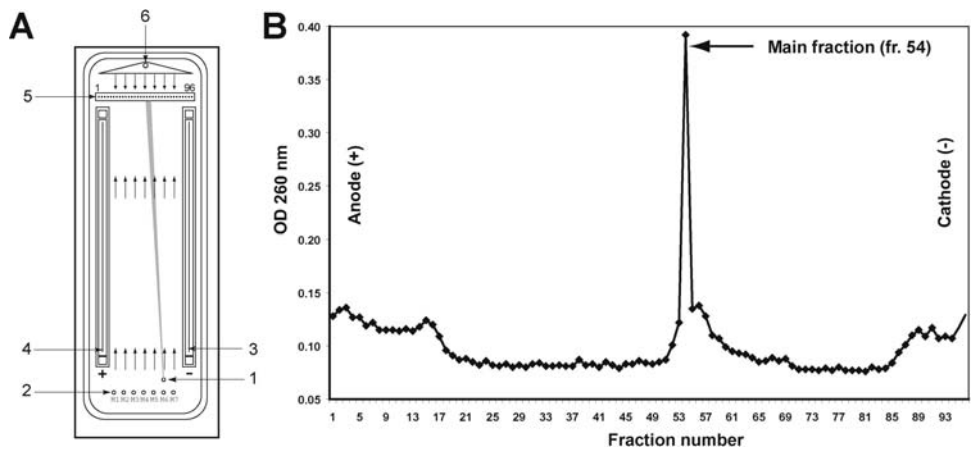

Fig. 6.1. ZE-FFE separation of a mitochondrial preparation isolated by differential centrifugation. Separation chamber (**A**) and ZE-FFE separation profile (**B**). Mitochondria were isolated from stationary yeast cultures (yeast strain KFY437) grown under fermentative conditions (glucose full medium, YPGlc; *see* **Section 3.1**) by differential centrifugation (*see* **Section 3.2**). The mitochondrial extracts were further purified by ZE-FFE (*see* **Section 3.3**). Mitochondria were injected into the electrophoresis chamber via the sample inlet (**A**, no. 1) at the cathodal side (**A**, no. 3) of the chamber, transported with the laminar buffer flow produced by continuous input of separation media via the media inlets (**A**, no. 2) and deflected toward the anode (**A**, no 4). At the end of the electrophoresis chamber, the laminar buffer flow was blocked via the counter flow (**A**, no. 6) and samples were fractionated in a 96-well format (**A**, no. 5). Separation was monitored by measuring the OD_{260} of 200 μL aliquots of the collected 96 fractions and illustrated as separation profile (**B**). Mitochondria with high purity were obtained from fraction number 54 (main fraction) (*see* **Note 30**) by centrifugation.
ZE-FFE separation conditions: (i) separation voltage: 750 v; (ii) media velocity: 400 mL/h; (iii) sample concentration: 2 mg/mL; (iv) sample velocity: 2 mL/h (v) separation temperature 4°C.

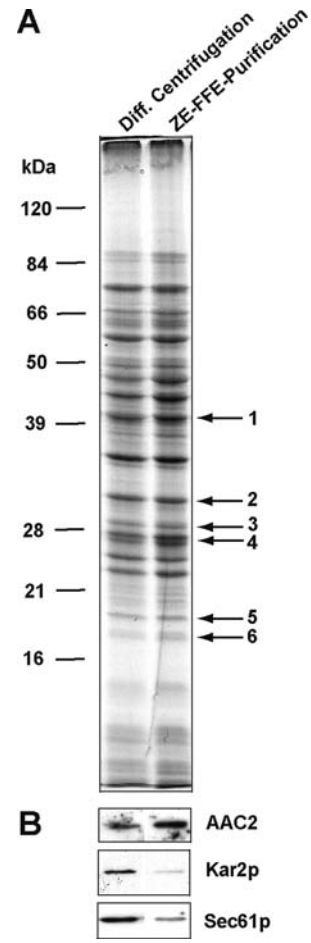

Fig. 6.2. Characterization of the ZE-FFE main fraction. Yeast mitochondria from the ZE-FFE main fraction (*see* **Fig. 6.1**) were analyzed by SDS-PAGE (A, *see* **Section 3.5**) and Western blot (B, ECLplus detection). (**A**) 10 μg mitochondria isolated by differential centrifugation and 10 μg ZE-FFE-purified mitochondria were separated by SDS-PAGE (12%, 18 cm format). Gels were stained with colloidal Coomassie (*see* **Section 3.5.2**). Protein bands were excised (*arrows*) and proteins were identified by Maldi-Tof mass spectrometry: (1) TIM44 (*inner membrane*) and TOM40 (*outer membrane*), (2) malate dehydrogenase (matrix) and pyruvate dehydrogenase (matrix), (3) ATP synthase gamma chain (*inner membrane*), (4) ADP, ATP carrier protein 2 (*inner membrane*), (5) ATP synthase subunit 5 (*inner membrane*), (6) ATP synthase D chain (*inner membrane*). Note that protein bands containing mitochondrial proteins are more pronounced in ZE-FFE-purified mitochondria compared to mitochondria isolated by differential centrifugation, indicating enrichment of mitochondrial proteins and thus purification of mitochondria by ZE-FFE. (**B**) 10 μg mitochondria isolated by differential centrifugation and 10 μg ZE-FFE-purified mitochondria were separated by SDS-PAGE (10%, mini gel format) and proteins were blotted onto PVDF membranes. The protein AAC2 was used as marker for mitochondria (rabbit polyclonal, 1:1000, kind gift of W. Neupert, University of Munich, Germany). The proteins Kar2p (rabbit polyclonal, 1:10000, kind gift of R. Schekman, University of California, USA) and Sec61p (rabbit polyclonal, 1:3000, kind gift of R. Schekman) were used as markers for microsomal luminal and membrane proteins, respectively. Enrichment of AAC2 and depletion of the microsomal proteins Kar2p and Sec61p demonstrate the purification of mitochondria by ZE-FFE.

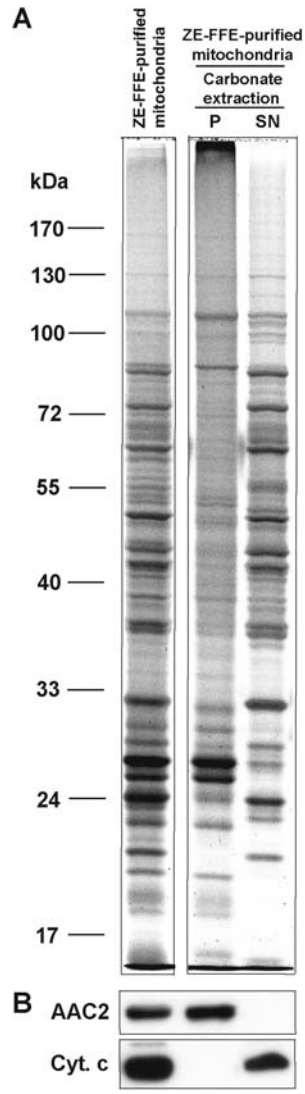

Fig. 6.3. Enrichment of mitochondrial membrane proteins. Yeast mitochondria purified by ZE-FFE (ZE-FFE main fraction, *see* **Section 3.3** and **Fig. 6.1**) were subjected to carbonate extraction (*see* **Section 3.4**). Membrane (P: membrane pellet) and soluble (SN: supernatant) fractions were separated by SDS-PAGE (**A**, *see* **Section 3.5**) and analyzed by Western blot (B, ECLplus detection). (**A**) 10 μg ZE-FFE-purified mitochondria and the equivalent of 10 μg of the membrane and soluble fractions of carbonate-treated mitochondria were separated by SDS-PAGE (10% gel, 18 cm format) and subsequently stained with RuBP (*see* **Section 3.5.1**). Note that distinct protein bands observed in the complete mitochondrial fraction appear either in the membrane or the soluble fraction, indicating the separation of the mitochondrial fraction upon carbonate extraction. (**B**) Western blot analysis of membrane and soluble fractions. SDS-PAGE was performed as in (**A**) but in mini gel format (14%) and proteins were blotted onto PVDF membranes. The proteins AAC2 (rabbit polyclonal, 1:1000) and cytochrome c (Cyt. c, rabbit polyclonal, 1:1000, kind gift of N. Pfanner, University of Freiburg, Germany) were used as markers for an integral mitochondrial membrane protein and a soluble mitochondrial protein, respectively. The soluble protein cytochrome c was found only in the supernatant, whereas the membrane protein AAC2 remained in the pellets of carbonate-extracted mitochondria.

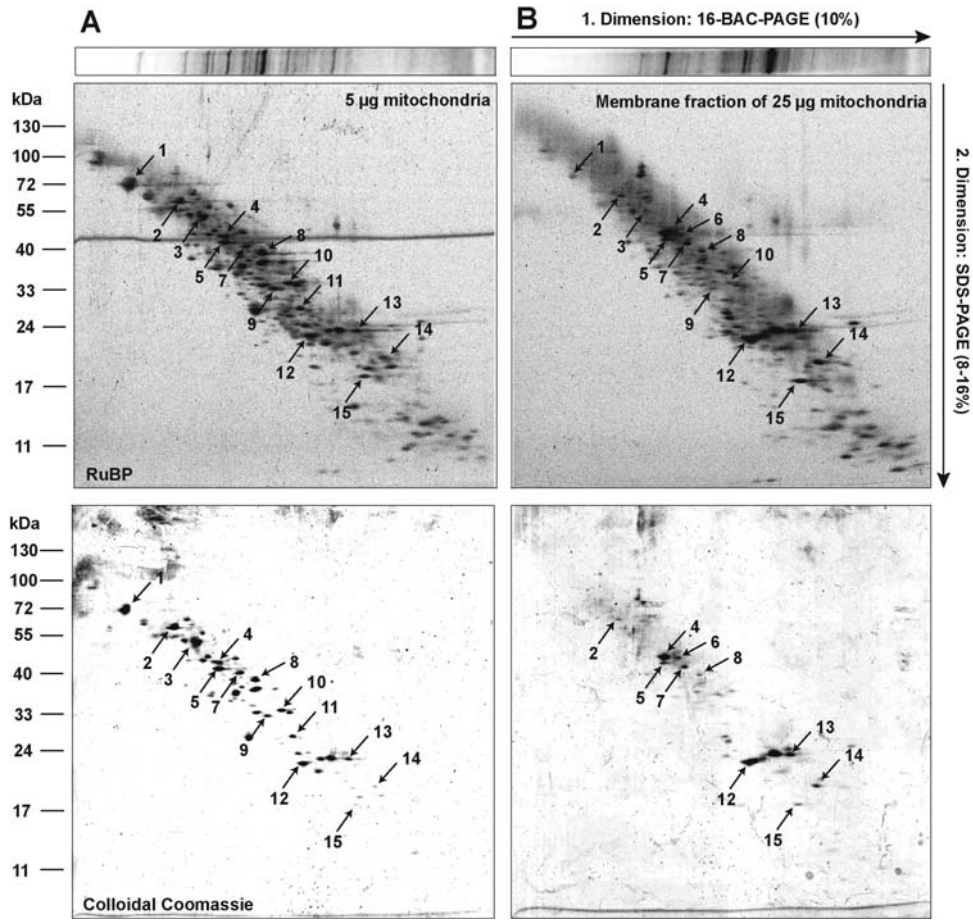

Fig. 6.4. 16-BAC/SDS-PAGE of mitochondrial membrane proteins. Protein extracts of ZE-FFE-purified yeast mitochondria (gel A, *see* **Section 3.3**, **Figs. 6.1 and 6.2**) as well as their membrane fraction obtained by carbonate extraction (gel B, *see* **Section 3.4**, **Fig. 6.3**) were subjected to 16-BAC/SDS-PAGE (*see* **Section 3.6**). 16-BAC gel strips were stained with Coomassie (*see* **Section 3.6**). 16-BAC/SDS gels were stained with RuBP (*see* **Section 3.5.1**, *upper panel*) and subsequently with colloidal Coomassie (*see* **Section 3.5.2**, *lower panel*). Protein spots were identified either by Maldi-Tof mass spectrometry (spots 1–12, 15) or by Western blot using specific antibodies (spots 13–14, ECLplus detection). In gel A 5 µg ZE-FFE-purified mitochondrial extracts were separated. In gel B the membrane fraction of the equivalent of 25 µg ZE-FFE-purified mitochondrial extracts were analyzed. 1: aconitase (matrix, ACON_YEAST), 2: Hsp77/SSC1 (matrix, HSP77_YEAST), 3: Hsp60 (matrix, HSP60_YEAST), 4: Rotenone-insensitive NADH-ubiquinone oxidoreductase (*inner membrane*, NDI1_YEAST), 5: ATP synthase alpha chain (*inner membrane*, ATPA_YEAST), 6: L-galactono-gamma-lactone oxidase (*membrane-embedded*, ALO_YEAST), 7: dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (matrix; ODO2_YEAST), 8: ATP synthase beta chain (*inner membrane*, ATPB_YEAST), 9: isocitrate dehydrogenase [NAD] subunit 2 (matrix, IDH2_YEAST), 10: ketol-acid reductoisomerase (matrix, ILV5_YEAST), 11: pyruvate dehydrogenase E1 component beta subunit (matrix, ODBP_YEAST), 12: voltage-dependent anion-selective channel protein 1 (VDAC-1; outer membrane, VDAC1_YEAST), 13: AAC2 (*inner membrane*, Western blot, rabbit polyclonal, 1:1000), 14: Rieske iron-sulfur protein (RIP1, *inner membrane*, Western blot, kind gift of N. Pfanner), 15: ATP synthase D chain (*inner membrane*, ATP7_YEAST). SwissProt entry names. Note that most soluble proteins (matrix proteins) found in mitochondrial extracts are depleted in membrane fractions, for example, spots 1–3, whereas most membrane proteins accumulate, for example, spots 12–15, as a result of the applied carbonate extraction (*see* **Section 3.4**, **Fig. 6.3**).

(16). Mitochondria were further purified by free flow electrophoresis (FFE) in zone-electrophoretic mode (ZE) (a FFE separation chamber is illustrated in **Fig. 6.1A** and a ZE-FFE separation profile in **Fig. 6.1B**). In ZE-FFE, mitochondria are transported with a laminar buffer flow through the electrophoresis chamber and purified from contaminants, that is, cytosol, microsomes, golgi, and peroxisomes, by specific deflection due to a perpendicular oriented electric field *(17, 18)* (**Figs. 6.1 and 6.2**. ZE-FFE-purified mitochondrial preparations were enriched with membrane proteins by carbonate extraction *(19)* (**Fig. 6.3**) and subjected to 16-BAC/SDS-PAGE (**Fig. 6.4**). Protein patterns of the 16-BAC/SDS gels were visualized by the economic but highly sensitive and mass spectrometry-compatible staining with the fluorescence dye ruthenium-II-bathophenantroline disulfonate chelate (RuBP) *(20, 21)*, allowing the detection of proteins down to 8 ng per spot *(20)*. In order to facilitate excision of protein spots for further identification by mass spectrometry (Maldi-Tof) and peptide mass fingerprinting, gels were subsequently stained with colloidal Coomassie *(22–24)*.

2. Materials

2.1. Yeast Culture Medium

1. YPGlc medium: to prepare 1 L, dissolve 10 g yeast extract, 20 g bacto-peptone in 700 mL dH_2O, adjust the volume to 840 mL to obtain YP medium. Prepare 160 mL of a 25% (w/v) glucose solution in dH_2O. Sterilize both solutions by autoclaving for 20 min at 120°C. Mix the 840 mL YP medium and the 160 mL 25% (w/v) glucose solution to obtain a final glucose concentration of 4% (w/v) (YPGlc medium). Store at room temperature.
2. YPGlc agar plates: add 15 g agar per 840 mL YP medium prior to autoclaving. Before pouring the plates add glucose as described earlier. Store solid plates at 4°C.

2.2. Isolation of Mitochondria by Differential Centrifugation

1. Zymolyase 20T (Seikagaku) or Lyticase (Sigma). Store at 4°C.
2. 1.2 M sorbitol: dissolve 437.24 g sorbitol in 1.2 L dH_2O. Adjust to a total volume of 2 L with dH_2O. Store at 4°C.
3. 100 mM PMSF: dissolve 1.74 g phenylmethylsulfonylfluorid (PMSF) in 100 mL ethanol (p.A. grade) and store in aliquots at −20°C.
4. 1 M potassium phosphate buffer (KPB), pH 7.4: prepare 500 mL of 1 M KH_2PO_4 (68.0 g) and 500 mL of 1 M K_2HPO_4 (87.1 g) in dH_2O. To 500 mL KH_2PO_4 slowly

add the K_2HPO_4 until a pH of 7.4 is reached. Store at room temperature.

5. 1 M Tris-HCl, pH 7.4: dissolve 60.57 g Tris in 450 mL dH_2O, adjust pH to 7.4 with 5 M hydrochloric acid (HCl), and adjust to a total volume of 500 mL. Store at room temperature.
6. 1 M Tris-H_2SO_4, pH 9.4: dissolve 60.57 g Tris in 450 mL dH_2O, adjust pH to 9.4 with 5 M sulfuric acid (H_2SO_4), and adjust to a total volume of 500 mL. Store at room temperature.
7. 500 mM EDTA: dissolve 73.1 g ethylenediaminetetraacetate (EDTA) in 450 mL dH_2O. Adjust pH to 8.0 with 10 M KOH, and add dH_2O to a total volume of 500 mL. Store at room temperature.
8. 1 M DTT: dissolve 3.08 g dithiothreitol (DTT) in 15 mL ddH_2O and add ddH_2O to a final volume of 20 mL. Store 1 mL aliquots at −20°C.
9. Zymolyase Buffer: 1.2 M sorbitol, 20 mM KPB, pH 7.4. To prepare 500 mL, mix 490 mL 1.2 M sorbitol with 10 mL 1 M KPB, pH 7.4. Store at 4°C.
10. Homogenization Buffer: 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2 mM PMSF. To prepare 500 mL, add 5 mL 1 M Tris-HCl, pH 7.4, and 5 mL 500 mM EDTA to 250 mL 1.2 M sorbitol. Adjust to a total volume of 500 mL with dH_2O. Store at 4°C. Immediately before use add 10 mL 100 mM PMSF.
11. SET Buffer: 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2 mM PMSF. Dissolve 42.8 g sucrose in 400 mL dH_2O, add 5 mL 1 M Tris-HCl, pH 7.4, and 5 mL 500 mM EDTA. Adjust to a total volume of 500 mL with dH_2O. Store at 4°C. Immediately before use add 10 mL 100 mM PMSF.

2.3. Purification of Yeast Mitochondria by ZE-FFE

2.3.1. Free-Flow Apparatus

The OCTOPUS instrument from Weber GmbH (Kirchheim, Germany) was used for the elaboration of the present protocol. The instrument consists of an electrophoresis chamber, with dimensions of 500 mm (length), 100 mm (width), and 0.5 mm thickness. The chamber was run in horizontal mode; collection of the separated fractions was done using the counterflow technique. The present protocol should be easily adaptable to the most recent instrumentation, the BD™ Free Flow Electrophoresis System (BD Proteomics, Franklin Lakes, NJ, USA).

2.3.2. Free-Flow Buffers

1. 5x Electrode solution (5x EL): 500 mM acetic acid, 500 mM triethanolamine, pH 7.4. Add 28.6 mL acetic acid and 66.65 mL triethanolamine to 850 mL ddH_2O, adjust pH to 7.4 with 1 M KOH and adjust to a total volume of 1 L. Store at 4°C.

2. Electrode solution: 100 mM acetic acid, 100 mM triethanolamine. To prepare 1 L, add 200 mL 5x EL to 800 mL ddH_2O. Store at 4°C.
3. Stabilization medium: 100 mM acetic acid, 100 mM triethanolamine, 0.28 M sucrose. Dissolve 95.8 g sucrose in 600 mL ddH_2O, add 200 mL 5x EL and adjust to a total volume of 1 L. Store at 4°C.
4. Separation medium: 10 mM acetic acid, 10 mM triethanolamine, 0.28 M sucrose. Dissolve 95.8 g sucrose in 700 mL ddH_2O, add 100 mL electrode solution and adjust to a total volume of 1 L. Store at 4°C.
5. Counterflow medium: 0.28 M sucrose. Dissolve 95.8 g sucrose in 800 mL ddH_2O and adjust to a total volume of 1 L. Store at 4°C.

2.4. Carbonate Extraction

1. 0.1 M sodium carbonate (Na_2CO_3): dissolve 106 mg Na_2CO_3 (p.A. grade) in 10 mL ddH_2O. The pH of the solution should be approximately 11.5. Prepare always freshly and cool down to 4°C before use.
2. 100% (w/v) TCA: dissolve 50 g trichloroacetic acid (TCA) in 50 mL dH_2O.

2.5. SDS-PAGE

1. 1.5 M Tris-HCl, pH 6.8: dissolve 181.71 g Tris in 800 mL ddH_2O. Adjust pH with concentrated HCl to 6.8, and add ddH_2O to a final volume of 1 L. Store at room temperature.
2. 1.5 M Tris-HCl, pH 8.8: dissolve 181.71 g Tris in 800 mL ddH_2O. Adjust pH with concentrated HCl to 8.8, and add ddH_2O to a final volume of 1 L. Store at room temperature.
3. 30% (w/v) acrylamide:bisacrylamide (37.5:1). Store at 4°C. Neurotoxic if unpolymerized, use gloves!
4. 10% (w/v) SDS: dissolve 25 g sodium dodecyl sulfate (SDS) in 250 mL ddH_2O (*see* **Note 1**). Store at RT.
5. TEMED (N,N,N′,N′-tetramethyl-ethylendiamine). Store at room temperature.
6. 10% (w/v) APS: dissolve 1 g ammonium peroxodisulfate (APS) in 10 mL ddH_2O. Store 500 µL aliquots at −20°C.
7. 10x SDS electrophoresis buffer: 250 mM Tris, 1.92 M glycine, 1% (w/v) SDS. Dissolve 151.3 g Tris, 720 g glycine and 50 g SDS in 4 L dH_2O and adjust to a final volume of 5 L (*see* **Note 1**). Store at RT.
8. 5x SDS sample buffer: 5% (w/v) SDS, 250 mM Tris-HCl, pH 6.8, 50% (v/v) glycerol, 500 mM β-mercaptoethanol, 0.5% (w/v) bromophenol blue. Mix 0.5 g SDS, 1.67 mL 1.5 M Tris-HCl, pH 6.8, 5 mL 100% glycerol, 350 µL β-mercaptoethanol and 50 mg bromophenol blue. Add ddH_2O to a final volume of 10 mL and vortex. Store 500 µL aliquots at −20°C.

9. Protein ladder, for example, PageRuler^{TM} Prestained Protein Ladder (Fermentas).

2.5.1. Ruthenium-II-Bathophenantroline Disulfonate Chelate (RuBP) Staining

1. RuBP fixation solution: 30% (v/v) ethanol, 10% (v/v) acetic acid. Mix 500 mL ddH_2O with 300 mL ethanol and 100 mL acetic acid and add ddH_2O to a final volume of 1 L. Store at room temperature for up to 1 week.
2. 20% (v/v) ethanol: Measure 200 mL ethanol and add ddH_2O to a final volume of 1 L. Store at room temperature.
3. 20 mM ruthenium-II-bathophenanthroline disulfonate chelate (RuBP, Tris-(bathophenanthrolinedisulfonate)-ruthenium(II) sodium salt solution) (*see* **Note 2**). Store at 4°C.
4. RuBP destaining solution: 40% (v/v) ethanol, 10% (v/v) acetic acid. Mix 400 mL ddH_2O, 400 mL ethanol and 100 mL acetic acid. Add ddH_2O to a final volume of 1 L. Store at RT for up to 1 week.
5. 5% (v/v) glycerol: mix 50 mL 100% glycerol in 800 mL dH_2O and fill up with dH_2O to a final volume of 1 L. Store at room temperature.

2.5.2. Colloidal Coomassie Staining

1. Colloidal Coomassie fixation solution: 20% (v/v) methanol, 10% (v/v) phosphoric acid. To prepare 1 L, add 200 mL methanol and 100 mL 85% (ortho-) phosphoric acid to 500 mL dH_2O. Add dH_2O to a final volume of 1 L. Store at room temperature.
2. 10% (v/v) phosphoric acid: add 100 mL 85% (ortho-) phosphoric acid to 800 mL dH_2O. Add dH_2O to a final volume of 1 L. Store at room temperature.
3. Colloidal Coomassie equilibration solution: 20% (v/v) methanol, 10% (v/v) phosphoric acid, 10% (w/v) ammonium sulfate. To prepare 1 L, dissolve 100 g ammonium sulfate ($(NH_4)_2SO_4$) in 600 mL dH_2O. Add 200 mL methanol, 100 mL 85% (ortho-) phosphoric acid, and adjust volume to 1 L with dH_2O. Store at room temperature.
4. 2% (w/v) Coomassie G-250 solution: dissolve 2 g Coomassie Brilliant Blue G-250 under stirring in 100 mL hot water. Filter the solution through a fluted filter. Store at room temperature.

2.6. 16-BAC/SDS-PAGE

2.6.1. First Dimension: 16-BAC-PAGE

1. 500 mM potassium phosphate buffer (KPB), pH 2.1: prepare 500 mL of 500 mM phosphoric acid (H_3PO_4, 16.85 mL 85% (ortho-) phosphoric acid add 500 mL dH_2O) and 500 mL of 500 mM potassium di-hydrogen phosphate (KH_2PO_4, 34.0 g in 500 mL dH_2O). To 100 mL 500 mM H_3PO_4 slowly add 500 mM KH_2PO_4 until the pH is adjusted to 2.1. Store at room temperature.

2. 500 mM potassium phosphate buffer (KPB), pH 4.1: to 100 mL 500 mM KH_2PO_4 slowly add 500 mM H_3PO_4 until the pH is adjusted to 4.1. Store at room temperature.
3. 30% (w/v) acrylamide:bisacrylamide (37.5:1). Store at 4°C. Neurotoxic if unpolymerized, use gloves!
4. 2% bisacrylamide (N,N′-methylene-bis-acrylamide, electrophoresis grade). Store at 4°C. Neurotoxic if unpolymerized, use gloves!
5. 250 mM 16-BAC: weigh out 4.45 g 16-benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC). Add ddH_2O to a volume of 50 mL and mix. Store suspension at RT. Immediately before use warm suspension to 60°C and vortex until dissolved.
6. 5% (w/v) Pyronin Y: dissolve 250 mg pyronin Y in 5 mL ddH_2O. Store at room temperature.
7. 1 M DTT: dissolve 3.08 g DTT in 15 mL ddH_2O and add ddH_2O to a final volume of 20 mL. Store 1 mL aliquots at −20°C.
8. 5 mM ferrous sulfate ($FeSO_4$): dissolve 14 mg $FeSO_4 \cdot 5H_2O$ in 10 mL ddH_2O. Prepare always freshly.
9. 80 mM ascorbic acid: dissolve 141 mg ascorbic acid in 10 mL ddH_2O. Prepare always freshly.
10. 30% hydrogen peroxide (H_2O_2). Store at 4°C.
11. Water-saturated *n*-butanol. Shake 1 volume dH_2O and 2 volumes *n*-butanol in a glass bottle and allow separating. Use the upper phase. Store at room temperature.
12. 10x 16-BAC electrophoresis buffer: 500 mM phosphoric acid, 1.5 M glycine, 25 mM 16-BAC. Dissolve 9.9 g 16-BAC and 112.6 g glycine in 950 mL dH_2O. Add 33.7 mL 85% (ortho-) phosphoric acid and finalize to 1 L with dH_2O. Store at room temperature.
13. 100% glycerol. Store at room temperature.
14. Coomassie fixation solution: 40% (v/v) methanol, 10% (v/v) acetic acid. Mix 400 mL methanol and 300 mL dH_2O. Add 100 mL acetic acid and fill up with dH_2O to a final volume of 1 L. Store at room temperature.
15. Coomassie staining solution: 40% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R-250. Dissolve 0.5 g Coomassie Brilliant Blue R-250 in 500 mL Coomassie fixation solution. Store at room temperature.

2.6.2. Second Dimension: SDS-PAGE

1. *see* **Section 2.5** (**steps 1–7, 9**)
2. Equilibration solution: 50 mM Tris-HCl, pH 6.8, 1.5 M urea, 30% (v/v) glycerol, 2% (w/v) SDS. Mix 90 g urea, 20 g SDS, 300 mL 100% glycerol and 33.3 mL 1.5 M Tris-HCl, pH 6.8. Add ddH_2O under stirring to a final volume of 1 L. Store at room temperature (*see* **Note 1**).

3. Reducing equilibration solution containing 1% (w/v) DTT: dissolve 1 g DTT in 100 mL equilibration solution. Prepare solution always freshly (*see* **Note 3**).
4. Alkylating equilibration solution: 4.8% (w/v) 2-iodoacetamide and bromophenol blue. Dissolve 4.8 g 2-iodoacetamide and one tip of bromophenol blue in 100 mL equilibration solution. Prepare solution always freshly (*see* **Note 3**).
5. Equilibration solution containing bromophenol blue. Dissolve one tip of bromophenol blue in 100 mL equilibration solution. Always prepare fresh solution.
6. 0.5% (w/v) agarose in 2x SDS electrophoresis buffer: weigh out 0.5 g agarose and add 100 mL 2x SDS electrophoresis buffer. Store at room temperature. Before use, dissolve agarose by heating the suspension.

3. Methods

3.1. Growth of Yeast Cultures

1. Streak out the yeast strain, for example, KFY417/KFY437, onto an YPGlc agar plate and grow for 3–5 days at room temperature.
2. Inoculate 50 mL YPGlc medium with a full inoculation loop (*see* **Note 4**).
3. Grow the culture overnight at 28°C in a baffled flask, shaking at 130 rpm.
4. Use the overnight culture to inoculate fresh YPGlc medium. Periodically measure the OD_{600} to determine the logarithmic growth phase of the expanded cultures (initial OD_{600} of approximately 0.05–0.10).
5. Isolate mitochondria from the yeast cultures in logarithmic growth phase (*see* **Note 5**).

3.2. Isolation of Mitochondria by Differential Centrifugation

1. Harvest cells (e.g., 5 L) by centrifugation at 2,000 g for 5 min at room temperature.
2. Discard supernatant and resuspend cells in 250 mL dH_2O
3. Centrifuge cells at 2,000 g for 5 min at room temperature and determine the wet weight of the pellet.
4. Resuspend cells in 100 mM Tris-H_2SO_4, pH 9.4, at 2 mL/g wet weight (*see* **Note 6**).
5. Add 10 µL 1 M DTT per mL resuspended cells to obtain a final concentration of 10 mM DTT, in order to reduce disulfide bonds of the yeast cell walls.
6. Incubate cells for 15 min at 30°C in a shaking water bath (*see* **Note 7**).

7. Centrifuge cells at 2,000 g for 5 min at room temperature to remove surplus DTT.
8. Resuspend cells in 1.2 M sorbitol using 2 mL/g wet weight.
9. Centrifuge cells at 2,000 g for 5 min at RT.
10. Resuspend cells in zymolyase buffer at 7 mL/g wet weight. Add 4 mg zymolyase or lyticase per gram wet weight, in order to digest the yeast cell wall enzymatically leading to spheroplasts (*see* **Note 8**). Remove a small aliquot prior to the addition of the zymolyase/lyticase for the spheroplast test.
11. Incubate cells at 30°C in a shaking water bath.
12. Check for spheroplast formation by adding 50 μL cells to 1 mL dH_2O and measure the optical density at 600 nm (OD_{600}). Spheroplasts—in contrast to yeast cells with intact cell walls—will disrupt in hypotonic aqueous solution, leading to a decrease in OD_{600}. Stop incubation when the OD_{600} is 30–50% of the OD prior to the addition of zymolyase/lyticase.
13. Centrifuge the spheroplasts at 1,000 g for 5 min at 4°C (*see* **Note 9**).
14. Resuspend the spheroplasts gently in ice-cold (isotonic) 1.2 M sorbitol and centrifuge at 1,000 g for 5 min at 4°C, in order to remove surplus zymolyase/lyticase (*see* **Note 9**).
15. Resuspend spheroplasts in ice-cold homogenization buffer at 7 mL/g wet weight.
16. Transfer the spheroplast suspension to a glass potter and homogenize with a motor driven Teflon pestle with 10 strokes at 800–1,000 rpm. Avoid foaming of the suspension.
17. Centrifuge the suspension at 1,700 g for 5 min at 4°C, in order to remove cell debris and nuclei.
18. Transfer the supernatant to a new centrifuge tube and centrifuge again at 1,700 g for 5 min at 4°C.
19. Transfer the supernatant to a new centrifuge tube and centrifuge at 17,400 g for 12 min at 4°C, in order to obtain the first crude mitochondrial preparation.
20. Discard the supernatant and resuspend the pellet carefully in ice-cold SET buffer.
21. Centrifuge at 3,000 g for 5 min at 4°C to remove agglomerates.
22. Transfer the supernatant to a new centrifuge tube and centrifuge at 17,400 g for 12 min at 4°C.
23. Resuspend the mitochondrial pellet in a small volume of ice-cold SET buffer and determine the protein concentration using the Bradford protein assay.
24. Adjust the protein concentration to 5 mg/mL with ice-cold SET buffer. Samples can be snap frozen with liquid nitrogen and stored at −80°C or used immediately.

3.3. Purification of Saccharomyces cerevisiae Mitochondria by ZE-FFE

Mitochondria are further purified by ZE-FFE as described in *(17, 18)*.

1. Mitochondrial preparations isolated by differential centrifugation (*see* **Section 3.2**) are used for ZE-FFE purification. During manipulations keep mitochondrial suspensions on ice.
2. Wash the mitochondrial preparations twice in ice-cold separation medium (4°C, 16,000 g, 10 min).
3. Carefully resuspend the mitochondrial pellet in ice-cold separation medium at a concentration of 1–3 mg/mL and adjust the sample to the sample inlet pump (*see* **Note 10**).
4. Set the rate of media delivery (without counterflow) to 300–400 mL/h.
5. Set the voltage to 750 V and switch on the high voltage. Be aware that the current occurring during separation is sufficient to cause severe bodily harm. In case of problems switch off power first!
6. Set the sample pump to a rate of 2 mL/h and turn the sample pump on.
7. Avoid introducing air bubbles via the sample inlet and carefully inspect the entry of the mitochondrial sample in the separation chamber. The sample should enter the laminar media stream without clotting or agglomeration and a major line should be visible that deflects toward the anode (*see* **Note 11**).
8. As soon as the sample reaches the end of the separation chamber or a stable sample deflection is observed, start collecting the sample fractions in a 96-deep-well plate.
9. From the collected sample fractions optical density at 260 nm (OD_{260}) can be measured by means of a 96-well plate reader and separation can be illustrated as separation profiles (*see* **Note 12**).
10. Continue to purify the remaining sample. To ensure a stable separation pattern over the whole sample application time, regularly check the sample volume and the media volumes during the separation run. Inspect the fractionation plate for blocked tubes during the run and avoid introduction of air bubbles.
11. According to the separation profile corresponding main peaks from the deep well plates can be pooled (*see* **Note 13**).
12. Collect the purified mitochondria by centrifugation at 16,000 g for 12 min at 4°C. Samples can be snap frozen in liquid nitrogen and stored at −80°C or used immediately.

3.4. Carbonate Extraction

1. Dilute ZE-FFE-purified mitochondrial extracts (e.g., 10–100 μg) with ice-cold 0.1 M sodium bicarbonate, pH 11.5, to a final concentration of 0.02–0.10 μg/μL.
2. Vortex and incubate the solution for 30 min on ice.

3. Sediment the membranes containing the membrane proteins by ultracentrifugation at 160,000 g for 1 h at 4°C.
4. Remove the supernatant containing the soluble protein fraction.
5. Wash the pellet in carbonate buffer, centrifuge at 160,000 g for 1 h at 4°C and discard the supernatant. Store membrane pellets at −80°C until use.
6. Precipitate proteins from the supernatant of the first ultracentrifugation (soluble protein fraction) by adding 1:10 (v/v) 100% TCA, that is, to 200 µL sample add 20 µL 100% TCA.
7. Vortex and incubate on ice for 30 min and sediment precipitated proteins by centrifugation at 16,000 g for 15 min at 4°C.
8. Discard supernatant and wash the pellet of precipitated proteins with 300 µL acetone (p.A. grade, pre-cooled at −20°C).
9. Centrifuge at 16,000 g for 15 min at 4°C, discard supernatant and dry pellets. Store pellets at −20°C until use.
10. Treat samples (membrane pellets and precipitated soluble protein fraction, respectively) with SDS sample buffer for SDS-PAGE (*see* **Section 3.5**) and Western blot analysis or 16-BAC sample buffer for 16-BAC/SDS-PAGE (*see* **Section 3.6**).

3.5. SDS-PAGE

These instructions are based on the use of the MiniProtean 3 (mini gel format, Bio-Rad) and the Protean II gel system (18 cm format, Bio-Rad) but should be easily adaptable to other formats.

1. Clean glass plates with dH_2O and then with 100% ethanol and put them into the casting chambers for mini gels and Protean II gels, respectively. Use 0.75 mm spacers.
2. Prepare 50 mL SDS separating gel solution (10%) for ten mini gels or two 18 cm gels by mixing 20 mL ddH_2O, 12.5 mL 1.5 M Tris-HCl, pH 8.8, 16.7 mL 30% acrylamide:bisacrylamide, 500 µL 10% (w/v) SDS and 50 µL TEMED. Start polymerization by adding 250 µL 10% (w/v) APS. Pour the gels, leaving space for the stacking gel, and overlay with water-saturated *n*-butanol. Polymerize for 1 h at RT.
3. Prepare 10 mL SDS stacking gel solution (4%) for four mini gels or two 18 cm gels by mixing 7.8 mL ddH_2O, 833 µL 1.5 M Tris-HCl, pH 6.8, 1.33 mL 30% acrylamide:bisacrylamide, 100 µL 10% (w/v) SDS, and 20 µL TEMED. Discard butanol from the separating gels and wash the top of the gels twice with dH_2O. Start polymerization by adding 100 µL 10% (w/v) APS to the stacking gel solution. Overlay the separating gels with stacking gel solution

and insert the combs. Polymerize for 1 h at room temperature (*see* **Note 14**).

4. Prepare 1 L of 2x SDS electrophoresis buffer by diluting 200 mL 10x SDS electrophoresis buffer with 800 mL ddH_2O. Let the buffer cool down to 4°C (*see* **Note 15**).
5. Treat protein extracts in 1x SDS sample buffer for 60 min on ice if dealing with extracts containing membrane proteins (*see* **Note 16**).
6. Place the SDS gels into the electrophoresis tanks. Fill the buffer chambers with 2x SDS electrophoresis buffer. Carefully remove the combs and wash the wells with 2x SDS electrophoresis buffer.
7. Briefly centrifuge protein samples at 16,000 g for 30 s and load sample onto the gel.
8. Start electrophoresis toward the anode with 6 mA per mini gel for 30 min at 4°C and increase the current to 12 mA per mini gel. For 18 cm gels perform electrophoresis overnight with 9 mA per gel at 4°C. Stop electrophoresis after the bromophenol blue dye front reaches the end of the separating gel. Equilibrate the gels in RuBP fixation buffer and stain the gels with RuBP (*see* **Section 3.5.1**). Alternatively, incubate the gels in Western blotting buffer if you want to proceed with Western blot analysis.

3.5.1. RuBP Staining of SDS Gels

1. After electrophoresis incubate the SDS gels for 1 h in RuBP fixation solution.
2. Equilibrate SDS gels for 10 min in ddH_2O.
3. Wash SDS gels four times for 30 min in 20% (v/v) ethanol.
4. Equilibrate SDS gels for 10 min in ddH_2O.
5. Finalize the RuBP staining solution (20% (v/v) ethanol, 200 nM RuBP) by adding 5 µL 20 mM RuBP to 500 mL 20% (v/v) ethanol.
6. Accomplish staining by incubating the gels in the dark for 3 h in RuBP staining solution.
7. Discard RuBP staining solution and incubate in the dark for 3 h in freshly prepared RuBP staining solution.
8. Equilibrate gels twice in the dark for 10 min in ddH_2O.
9. Incubate the gels in the dark overnight in RuBP destaining solution to reduce background staining.
10. Equilibrate the gels twice for 10 min in ddH_2O and digitalize the gels using an appropriate scanner. We used the Fuji FLA-3000 scanner (Laser 473 nm, Filter R675, Pixel size 100 µ, Bits per pixel 8, Sensitivity 1000) (*see* **Note 17**).

3.5.2. Colloidal Coomassie Staining of SDS Gels

1. After staining the gels with RuBP, incubate the gels twice in colloidal Coomassie fixation solution for 15 min under gentle shaking (*see* **Note 18**).

2. Equilibrate the gels three times for 10 min in 10% (v/v) phosphoric acid with gentle shaking (*see* **Note 18**).
3. Incubate the gels in colloidal Coomassie equilibration solution for 15 min with gentle shaking (*see* **Note 18**).
4. Start staining by adding 1.2 mL 2% (w/v) Coomassie G-250 solution per 100 mL colloidal Coomassie equilibration solution. Stain the gels until a steady state is achieved (about 24 h) (*see* **Note 19**).
5. Rinse the gels in dH_2O and digitalize the gels using a scanner.
6. Equilibrate the gels three times for 10 min in dH_2O (*see* **Note 20**).
7. Incubate the gels for 10 min in 5% (v/v) glycerol (*see* **Note 21**).
8. Pack the gels between two wet cellophane foils and dry them at 60°C for 2 h or at RT for 72 h.

3.6. 16-BAC/SDS Polyacrylamide Gel Electrophoresis (16-BAC/SDS-PAGE)

3.6.1. First Dimension: 16-BAC-PAGE

1. These instructions are based on the use of the Protean II gel system (18 cm gel format, Bio-Rad) but should be easily adaptable to other formats.
2. Clean glass plates with dH_2O and then with 100% ethanol and place them into the casting chambers for 18 cm gels. Use 0.75 mm spacers.
3. Prepare 50 mL separating gel solution for two 10% 18 cm gels by mixing 9 g urea, 7.5 mL 500 mM KPB, pH 2.1, 16.7 mL 30% acrylamide:bisacrylamide, 1.98 mL 2% bisacrylamide, 500 µL 250 mM 16-BAC, 80 µL 5 mM $FeSO_4$, 2.5 mL 80 mM ascorbic acid and add ddH_2O to 48.0 mL. Start polymerization by adding 2 mL of a freshly prepared aqueous 1:1200-dilution of a 30% H_2O_2 solution. Pour the gels, leaving space for the stacking gel, and overlay with water-saturated *n*-butanol. Polymerize for 2 h at room temperature.
4. Prepare 20 mL stacking gel solution (4%) for two 18 cm gels by mixing 2 g urea, 5 mL KPB, pH 4.1, 2.66 mL 30% acrylamide:bisacrylamide, 2.34 mL 2% bisacrylamide, 140 µL 250 mM 16-BAC, 17 µL 5 mM $FeSO_4$, 1 mL 80 mM ascorbic acid and add ddH_2O to 19.0 mL. Pour off the butanol and rinse the top of the separating gel twice with dH_2O. Start polymerization by adding 1 mL of an aqueous 1:750-dilution of a 30% H_2O_2 solution to the stacking gel solution. Overlay the separating gels with stacking gel solution and insert the combs. Polymerize overnight at RT (*see* **Note 14**).
5. Prepare 1 L of 16-BAC electrophoresis buffer by diluting 100 mL 10x 16-BAC electrophoresis buffer with 900 mL dH_2O and let the buffer cool down.
6. Prepare 2x 16-BAC sample buffer by mixing 2.25 g urea, 0.5 g 16-BAC, 0.5 mL 100% glycerol and 2.5 mL ddH_2O.

Carefully warm the mixture to 60°C until completely dissolved (do not boil!). Add 375 µL 1 M DTT and 50 µL 5% (w/v) pyronin Y, fill up with ddH_2O to 5 mL. Prepare immediately 1x 16-BAC sample buffer by mixing equal volumes of 2x 16-BAC buffer and ddH_2O (*see* **Note 22**).

7. Treat membranous pellets obtained by carbonate extraction (e.g., the equivalent of 10–100 µg mitochondria, *see* **Section 3.4**) with 50 µL 1x 16-BAC sample buffer for 1 h at RT. Vortex every 15 min (*see* **Note 23**).
8. Place the 16-BAC gels into the electrophoresis tanks. Fill the buffer chambers with 16-BAC electrophoresis buffer. Carefully remove the combs and wash the wells thoroughly with 16-BAC electrophoresis buffer.
9. Centrifuge the samples for 30 sec at 16,000 g. Load the samples onto the 16-BAC gels. Fill up remaining wells with 50 µL 1x 16-BAC sample buffer and start electrophoresis toward the cathode (opposite to SDS-PAGE!) with 25 mA per gel in the cold room. Renew the buffer in the upper buffer chamber after 1 h. Stop electrophoresis after the pyronin Y dye front,that is, the Schlieren line, has nearly completely left the end of the separating gel (*see* **Note 24**).
10. Equilibrate 16-BAC gels in Coomassie fixation solution for 15 min with gentle shaking.
11. Stain the 16-BAC gels by incubation in Coomassie staining solution for 1 h.
12. Remove background staining by incubating the gels in Coomassie fixation solution. Exchange fixation solution until the protein pattern is clearly visibly beside the background staining (usually three times for 15 min).
13. Digitalize gels with a scanner.
14. Either use 16-BAC gels immediately for the second dimension or freeze the gels at −20°C until further use (*see* **Note 25**).

3.6.2. Second Dimension: SDS-PAGE

1. These instructions are based on the use of the multicasting chamber of the Protean II gel system (18 cm format, Bio-Rad) coupled with the gradient maker of the Ettan Dalt II Gel Caster System (GE Healthcare) but should be easily adaptable to other formats.
2. Clean glass plates with dH_2O and then with 100% ethanol and put them into the multicasting chambers for 18 cm gels (Protean II multicasting chamber, Bio-Rad). Use 1.5 mm spacers.
3. Use gradient gels (8–16%) for the second dimension for optimal separation of mitochondrial protein extracts.
4. The following instructions are based on the simultaneous casting of nine 18 cm gels (Protean II) with 1.5 mm spacers using the Ettan Dalt II Gel Caster System (*see* **Note 26**).

5. Prepare 220 mL 8% SDS separating gel solution by mixing 55.0 mL 1.5 M Tris-HCl, pH 8.8, and 58.7 mL 30% acrylamide:bisacrylamide. Fill up with ddH_2O to 220 mL. Degas solution using a vacuum pump (*see* **Note 27**).
6. Prepare 220 mL 16% SDS separating gel solution by mixing 55.0 mL 1.5 M Tris-HCl, pH 8.8, 117.3 mL 30% acrylamide:bisacrylamide, and 15.0 mL 100% glycerol. Fill up with ddH_2O to 220 mL. Degas solution using a vacuum pump (*see* **Note 27**).
7. Add 74 μL TEMED and 740 μL 10% APS to the 220 mL gel solution with the lower acrylamide concentration (8%), mix and fill the solution into the right side of the gradient maker. Remove air bubbles from the tubes and the gradient mixer. Add 74 μL TEMED and 740 μL 10% APS to the 220 mL gel solution with the higher acrylamide concentration (16%), mix and fill the solution into the left side of the gradient maker. Start the pump of the gel caster system that presses the lower concentrated gel solution from below into the casting chamber. Open the connection of the higher concentrated gel solution to the gradient mixer, leading to filling of the casting chamber with progressively higher concentrated gel solution. After the chamber has been filled, overlay the gels with water-saturated *n*-butanol. Polymerize overnight at RT (*see* **Note 28**).
8. Prepare 30 mL SDS stacking gel solution (4%) for two 18 cm gels by mixing 23.4 mL ddH_2O, 2.5 mL 1.5 M Tris-HCl, pH 6.8, 4 mL 30% acrylamide:bisacrylamide, 300 μL 10% SDS and 60 μL TEMED. Discard butanol and wash the top of the separating gels with dH_2O twice. Start polymerization by adding 300 μL 10% APS to the separation gel solution. Pour the gel, leaving space for 16-BAC gel strips and overlay with water-saturated *n*-butanol. Polymerize for 1 h at room temperature.
9. Prepare 2 L of 2x SDS electrophoresis buffer by diluting 400 mL 10x SDS electrophoresis buffer with 1.6 L dH_2O. Let the buffer cool down to 4°C (*see* **Note 15**).
10. Cut out lanes of the 16-BAC gels resulting in 16-BAC gel strips (*see* **Note 29**).
11. Rinse the 16-BAC gel strips with ddH_2O and equilibrate them in equilibration solution for 15 min at RT with gentle shaking.
12. Discard equilibration solution and incubate the gel strips again in equilibration solution for 15 min at RT with gentle shaking. Repeat this step.
13. Discard equilibration solution and equilibrate the gel strips in reducing equilibration solution for 15 min at room temperature with gentle shaking.

14. Discard equilibration solution and equilibrate the gel strips in alkylating equilibration solution containing bromophenol blue for 15 min at room temperature with gentle shaking.
15. If the alkylating equilibration solution turns greenish discard the alkylating equilibration solution and incubate the gels strips in equilibration solution containing bromophenol blue for 15 min at room temperature with gentle shaking (*see* **Note 30**).
16. Pour off the butanol from the SDS stacking gels, rinse the surface of the gels twice with dH_2O and overlay with 2x SDS electrophoresis buffer.
17. Rinse 16-BAC gel strips first with ddH_2O and then with 2x SDS electrophoresis buffer and transfer them on top of the SDS gels. Gently press the strips on top of the SDS gels to remove air bubbles between 16-BAC gel strips and the SDS stacking gels. Remove surplus 2x SDS electrophoresis buffer completely. Pipette 10 μL protein ladder on small strips of Whatman paper. Place the strips next to the 16-BAC strips on top of the stacking gel. Overlay 16-BAC gel strips with agarose dissolved in 2x SDS electrophoresis buffer to fix them (*see* **Note 31**).
18. After solidification of the agarose, place the SDS gels into the electrophoresis tank and fill the buffer chambers with 2x SDS electrophoresis buffer. Perform electrophoresis with 9 mA per gel overnight in the cold room. Stop electrophoresis when the bromophenol blue dye front reaches the end of the gel (*see* **Note 32**).
19. Equilibrate the gels in RuBP fixation buffer and stain the gels with RuBP (*see* **Section 3.5.1**) and subsequently with colloidal Coomassie (*see* **Section 3.5.3**).

4. Notes

1. Since inhalation of SDS powder is toxic, use inhalation protection.
2. We used both, a 20 mM RuBP solution that we prepared according to a published protocol *(21)* and the dye from Fluka (Order-No. 03038) that is currently the only commercially available source of this dye. The results were indistinguishable but lot-to-lot variances cannot be excluded.
3. Equilibration and transfer of 16-BAC strips is critical in 16-BAC/SDS-PAGE. For best results prepare DTT and 2-iodoacetamide solutions immediately prior to use.
4. Yeast cells grown in glucose medium are predominantly fermentative, that is, the mitochondria-dependent respiration takes place at very low levels. Under such conditions yield of mitochondria and the stability of the organelles is reduced

compared to yeast cultures grown in obligatory respiratory medium, such as lactate or glycerol medium.

5. Stationary cultures are more resistant toward zymolyase/lyticase treatment and prolonged incubation times are necessary, thereby decreasing yield and quality of the isolated mitochondria.
6. The pH of the Tris-H_2SO_4 buffer should be 9.4, because enforced reduction of the yeast cell walls with DTT, especially at lower pH, will dramatically decrease the yield and quality of the isolated mitochondria.
7. Do not prolong reduction for more than 15 min, since this will lead to drastically reduced yield and quality of mitochondria.
8. In our hands zymolyase was more efficient in the digestion of yeast cell walls than lyticase.
9. Spheroplasts are prone to disruption. Therefore, they should be centrifuged at low speed and subsequently resuspended with care.
10. Lower concentrations will hamper a visual inspection during the separation; higher concentrations are possible but agglomeration of the sample should be carefully avoided.
11. ZE-FFE can purify mitochondria significantly. However, if the preceding isolation yielded a high amount of broken and severely damaged organelles, large scale clotting will occur and the whole procedure will fail. It is, therefore, important to handle the mitochondria with care, especially resuspension should avoid strong shear forces and mitochondria should be kept on ice during the manipulations. If clotting is visible in the starting sample for ZE-FFE a short spin (e.g., 500 g for 3 min, 4°C) can remove larger aggregates.
12. The progress of the separation can be monitored during the run by collecting 96-deep-well plates and measuring parameters like optical density (OD), absorption of mitochondrial DNA, protein concentration, enzymatic activities and so forth by means of an appropriate reader. Since the volume in the 96-deep-wells will vary, it is advisable to use a multichannel pipette and transfer equal volumes (e.g., 200 μL) of the collected fractions to a separate measuring plate.
13. One critical issue, which has frequently been discussed, is the choice of the two major electrophoresis parameters, that is, the separation voltage and the media flow velocity. We would suggest that these parameters are selected such that the major peak, containing the purified mitochondria, lies in the middle area of the separation profile. Routinely we use 750 volts and a media velocity of 300–400 mL/h. In our experience, increasing the voltage will only shift the mitochondria more toward the anode but not increase separation power. On the

other hand a decrease in media flow velocity will also cause an anodal shift but leads to significant peak broadening, thereby hampering organelle purification.

14. Gels can be stored at 4°C for at least 7 days when wrapped in wet paper and foil.
15. For electrophoresis we use 2x SDS electrophoresis buffer according to (10) due to its higher buffer capacity.
16. Do not heat samples, in order to prevent aggregation of the hydrophobic membrane proteins.
17. Protein patterns can also be visualized, but with decreased sensitivity, by laying the stained gels onto an UV transilluminator.
18. If gels were not prestained with RuBP, incubation and equilibration times should be prolonged for efficient colloidal Coomassie staining: Fixation with colloidal Coomassie fixation solution (**Step 1**), three times for 30 min. Equilibration in 10% (v/v) phosphoric acid (**Step 2**), three times 20 min. Equilibration in colloidal Coomassie equilibration solution (**Step 3**), once for 30 min.
19. If the Coomassie dye strongly precipitates on the gel, repeat **Steps 3 and 4**.
20. Equilibration in dH_2O is necessary if you want to dry the gels. Without equilibration surplus ammonium sulfate will precipitate upon drying.
21. The use of glycerol diminishes the probability of disintegration of the gels when drying them.
22. We strongly recommend using the 2x 16-BAC sample buffer immediately, since urea and DTT are unstable at higher temperatures. Gentle warming of 2x 16-BAC sample buffer is necessary for completes solvation and to avoid precipitation. In 1x 16-BAC sample buffer components stay in solution at room temperature.
23. If you want to apply 16-BAC/SDS-PAGE for lower-concentrated samples, such as total mitochondrial samples, use equal volumes of 2x 16-BAC sample buffer and incubate for 1 h at room temperature.
24. In the beginning the voltage is about 200 v but will rise up to 500 v with this system. Electrophoresis will take about 6 h.
25. Since we observed a marked loss of proteins, we recommend not storing the gels for longer periods in Coomassie fixation solution.
26. Adequate separating gel volumes must be determined for every gel casting system and number of gels cast.
27. SDS was not added here in order to circumvent foaming during the casting procedure. Since we used 2x SDS electrophoresis buffer instead of 1x SDS electrophoresis buffer (*see* **Note 15**), sufficient amounts of SDS, that is, 0.2% instead of 0.1%, were present during SDS-PAGE.

28. Gels can be stored in separating gel buffer (375 mM Tris-HCl, pH 8.8) for at least 7 day at 4°C.
29. This step is highly critical in 16-BAC/SDS-PAGE. Cutting should result in 16-BAC gel strips with smooth borders to ensure optimal contact between 16-BAC gel strips and the SDS stacking gel in the second dimension.
30. The Coomassie fixation solution is highly acidic. Even after several equilibration steps 16-BAC strips might have a pH value below the pH value of the equilibration solution (pH 6.8). Equilibration and transfer of 16-BAC strips are critical in 16-BAC/SDS-PAGE; therefore, we recommend prolonging the equilibration procedure for optimal results.
31. This step is highly critical in 16-BAC/SDS-PAGE. Ensure that the 16-BAC gel strips are fixed free from air bubbles on top of the SDS stacking gel and that the 16-BAC gel strips and the SDS stacking gels are in direct contact. Otherwise, protein spots in the second dimension may demonstrate horizontal streaking effects.
32. In this experiment, the separation profile was found to be quite sharp, that is, the main fraction comprising of only 1 out of 96 fractions. It should be emphasized that the separation profile of yeast mitochondria depends on many factors, such as (i) the yeast strain, (ii) the carbon source (e.g., fermentative *vs.* respiratory media), (iii) the growth phase, (iv) the purity and physical integrity of the prefractionated mitochondria. Mitochondria isolated from yeast cultures grown in fermentative medium (YPGlc) at logarithmic growth phase typically show a broader separation profile, that is, highly pure mitochondria can also be found in neighboring fractions *(25)*. In contrast, mitochondria isolated from yeast cultures grown at logarithmic growth phase in obligatory respiratory medium with lactate as the principal carbon source typically demonstrate a sharper separation profile similar to that shown in **Fig. 6.1B** *(25)*. We recommend assessment of the purity of your obtained fractions by further analyses (e.g., by SDS-PAGE and Western blot, *see* **Section 3.5** and **Fig. 6.2**).

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Chapter 7

Sequential Detergent Extraction Prior to Mass Spectrometry Analysis

Fiona M. McCarthy, Amanda M. Cooksey, and Shane C. Burgess

Abstract

Sequential detergent extraction of proteins from eukaryotic cells has been used to increase proteome coverage of 2D-PAGE. We have adapted sequential detergent extraction for use with the high-throughput non-electrophoretic proteomics method of liquid chromatography and electrospray ionisation tandem mass spectrometry. This method of extraction yields comprehensive proteomes that include up to twice as many membrane proteins as other published methods. Two thirds of these membrane proteins have more than one transmembrane domain and many of these have multiple transmembrane domains. Since sequential detergent extraction (SDE) separates proteins based upon their physicochemistry and sub-cellular localisation, this method also provides useful data about cellular localisation.

Key words: Membrane proteins, fractionation, mass spectrometry, annotation, functional proteomics, MudPIT.

1. Introduction

Traditionally, two-dimensional (2D) polyacrylamide gel-electrophoresis (PAGE) systems have been used for expression proteomics *(1)*. However, many hydrophobic proteins are poorly soluble in electrophoresis buffers and are thus under-represented in gels *(2)*. Non-electrophoretic proteomics, based on liquid chromatography and electrospray ionisation tandem mass spectrometry (LC ESI/MS/MS; also known as multidimensional protein identification technology [MudPIT]; *(3)*, is often used instead of, or to complement, 2-D gel-based proteomics methods. Poor membrane protein solubility may be circumvented with LC ESI/MS/MS because separation can be based on the

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physicochemical properties of more-soluble peptides derived from less-soluble proteins by protease digestion, rather than the poorly soluble intact precursor proteins themselves.

The importance of membrane proteins is clear from genome analyses. One third of all currently described genes code for membrane proteins *(4, 5)*. One half of the mass of the plasma membrane is protein and this proportion is even greater in internal organelle membranes *(6)*. Being able to identify and work with cell membrane proteomes is important because the plasma membrane is a cell's primary interface. The cell membrane is also the cell's interface with the local environment. Membrane proteins direct processes involved in cell adhesion, cell–cell communication, proliferation and differentiation to name a few. In addition to the cell surface, cell organelles also have lipid membranes and proteins in these membranes are essential for intracellular physiology.

A method using a series of detergents to isolate proteins from different cell components was first applied to gel-based proteomics using 2-D gels and then to identify the proteins by mass spectrometry *(7)*. The first detergent, digitonin, interacts with cholesterol to form pores in the cell membrane and extract soluble proteins from the cytosol *(8)*. Triton X-100 solubilises membrane and organelle proteins, and a combination of deoxycholate (DOC) and Tween 40 extracts the soluble nuclear fraction. Nuclear matrix proteins and the more insoluble proteins are solubilised using SDS. Detergents are incompatible with mass spectrometry *(9)*, but the process of gel-electrophoresis removes the detergents prior to mass spectrometry analysis.

We have adapted SDE for high-throughput proteomics using electrospray ionisation tandem mass spectrometry. This allows us to obtain more comprehensive proteomes than possible with 2-D gels. These proteomes also contain representative proportions of membrane proteins *(10)*. We found few membrane proteins are present in more than one detergent fraction and the presence of proteins in multiple fractions creates a profile that can be used as a predictor of protein sub-cellular localisation *(10)*. We used the specific profiles of well annotated proteins (for which large amounts of data exist) to suggest cell locations and functions for poorly described proteins with the same SDE pattern. The SDE method we describe here isolates a larger proportion of membrane proteins than other methods that have been specifically designed to isolate membrane proteins for proteomics *(11)*. SDE is a simple method consistent with the high-throughput paradigm of expression proteomics. It also facilitates functional genome annotation, and allows analysis of proteins with multiple transmembrane domains *(11, 12)*.

2. Materials

The buffers used for SDE are critical to the procedure and improperly prepared buffers, or buffers that have been stored for too long, are the most common cause of problems encountered when applying this method.

Two **base buffers** form the basis of the four SDE buffers. Both of these base buffers can be made in advance and stored at 4°C in the dark for up to 2 months (*see* **Note 1**). Each SDE extraction buffer should be prepared 1 day in advance and stored at 4°C in the dark. However the PMSF (see below) should be added just prior to use. High-grade water is used to prepare all buffers and stock solutions.

2.1. Preparation of SDE Base Buffers and Stock Solutions

1. **Base Buffer 1**: Dissolve 103 g sucrose, and 5.8 g NaCl in 100 ml water. Dissolve 3 g of PIPES separately in a minimal volume (approximately 5–10 ml) of 1 M sodium hydroxide (*see* **Note 2**). Mix the two solutions and add water to make a final volume of 250 ml. Filter sterilise by passing through a 0.45 μm filter and store at 4°C in the dark for no longer than 2 months (*see* **Note 1**). This base solution is used to prepare SDE buffers 1 and 2.
2. **Base Buffer 2**: Dissolve 3 g of PIPES in a minimal volume (approximately 5–10 ml) of 1 M sodium hydroxide by applying a low-to-medium heat while stirring until dissolved (*see* **Note 2**). Add 0.58 g NaCl and 0.2 g $MgCl_2.6H_2O$ and add water to make a final volume of 100 ml. Filter sterilise by passing through a 0.45 μm filter and store at 4°C in the dark for no longer than 2 months (*see* **Note 1**). This base solution is used to prepare SDE buffer 3.
3. Prepare a 0.1 M EDTA stock solution by dissolving 3.36 g EDTA in 100 ml of water and store at room temperature.
4. Prepare a 0.1 M phenylmethylsulfonyl fluoride (PMSF) stock solution by dissolving 174 mg PMSF (*see* **Note 3**) in 10 ml of isopropanol and vortex to dissolution. The PMSF solution is stored at room temperature in the dark as 1ml aliquots and added to SDE buffers immediately prior to use.

2.2. SDE Extraction Buffers and Solutions

1. SDE Buffer 1: To 10 ml of base buffer 1, add 18.75 mg digitonin (*see* **Note 4**) and heat up to boiling, while stirring, until the digitonin dissolves. Allow to cool to room temperature, add 5 ml 0.1 M EDTA stock solution and adjust to 25 ml using SDE base buffer 1. Adjust pH to 6.8 and add water to a final volume of 100 ml. Store at 4°C in the dark until required and add 1ml PMSF immediately prior to use.

2. 10% (v/v) Triton X-100 stock solution: Using a truncated 1 ml pipette, add 5 ml Triton X-100 to 30 ml of water while stirring gently. When the Triton X-100 has dissolved, cool on ice and adjust the pH to 7.4 (*see* **Note 5**). Add water to a final volume of 50 ml.
3. SDE Buffer 2: To 25 ml Base Buffer 1 add 3 ml of 0.1 M EDTA and 5 ml of Triton X-100 stock solution. Adjust the volume to 100 ml using water and store at 4°C in the dark until required. Add 1 ml PMSF immediately prior to use.
4. SDE Buffer 3: Dissolve 0.5 g deoxycholate (*see* **Note 6**) in 50 ml of water. Add 1 ml Tween-40 and mix gently. Add 10 ml of SDE base buffer 2. Adjust pH to 7.4 and add water to a final volume of 100 ml. Store at 4°C in the dark until required and add 1 ml PMSF immediately prior to use.
5. SDE Buffer 4: Add 20 ml of PBS (pH 7.4) and 5 g of SDS (*see* **Note 7**) to 50 ml of water. Check pH and if necessary adjust to 7.4. Add PBS to a final volume of 100 ml. This solution can be stored and used at room temperature to avoid SDS precipitation at 4°C.

2.3. Cell Homogenisation and Fractionation

1. To preserve cell architecture, viable cells are used in SDE. However, it is often necessary to work with frozen tissue and we have applied SDE to extract proteins from frozen spleen samples stored at −80°C *(13)*. We first ground the spleens to powder using a mortar and pestle sitting on dry ice. We then applied the SDE method exactly as described. Although we primarily used SDE to isolate membrane proteins and increase our proteome coverage by increasing the numbers of fractions analysed by 2-D LC MS/MS, we found that the SDE retains its ability to separate proteins by cell compartment.

3. Methods

The following protocol uses ice-cold PMSF-containing buffers, and samples must be kept on ice at all times to minimise proteolysis. For SDE extraction from viable B-lymphocytes we used 10^7–10^8 cells pelleted in a 1.5 ml microfuge tube *(10)*. If desired the procedure can be paused after the addition of SDE buffer 3, and the sample stored at −80°C. Also, when collecting cells from geographically distant sites such as oocytes *(14)* we have cold-shipped SDE buffer 1 to the distant site, added the cells that we wish to process to the buffer and then cold shipped the samples back to the laboratory. As noted above the SDE method can also be used on frozen tissues.

3.1. Extraction Using SDE Buffer 1

1. Add 1 ml of SDE buffer 1, containing 1% PMSF, to the cell pellet and gently resuspend by tapping the tube (*see* **Note 8**).
2. Lay the tube lengthwise in an ice bucket and incubate on ice for 30 min with gentle mixing to ensure optimal washing of the cells.
3. Centrifuge the sample for 5 min at 4°C to pellet the cells using minimum speed necessary (510 × g for Bursal B-cells). Add the supernatant to an ice-cold microfuge tube and store at −80°C until required.
4. Gently tap the tube to loosen the pellet and then add 1 ml of SDE buffer 1. Resuspend the cell pellet and again incubate on ice, this time for 10 min.
5. Centrifuge the sample to pellet the cells, remove the supernatant to an ice-cold microfuge tube and store at −80°C until required. Repeat this procedure using 10 min incubations until proteins extracted by SDE buffer 1 are depleted (*see* **Note 9**).

3.2. Extraction Using SDE Buffer 2

1. Gently tap the microfuge tube to loosen the pellet remaining from the SDE buffer 1 extraction and add 100 μl of ice-cold SDE buffer 2 containing 1% PMSF. Gently resuspend the pellet.
2. Again, lay the tube lengthwise in the ice bucket and incubate on ice for 30 min with gentle mixing to ensure optimal washing of the cells.
3. Centrifuge the sample for 10 min at 4°C to pellet the cells (5,000 × g for Bursal B-cells).
4. Add the supernatant to an ice-cold microfuge tube and store at −80°C until required.
5. Prior to addition of more solution, tap tube gently to resuspend the pellet.

3.3. Extraction Using SDE Buffer 3

1. Gently tap the microfuge tube to loosen the pellet remaining from the SDE buffer 2 extraction and add 100 μl of ice cold SDE buffer 3 containing 1% PMSF.
2. Disrupt nucleus using five strokes of a Kontes (Fisher) disposable pellet pestle (*see* **Note10**).
3. Centrifuge the disrupted sample for 10 min, 4°C at 6,780 × g to collect the pellet.
4. Add the supernatant to an ice-cold microfuge tube and store at −80°C until required.

3.4. Extraction Using SDE Buffer 4

1. Add 1 ml of ice-cold PMSF-containing PBS.
2. Pass solution through a syringe needle several times to fragment genomic DNA (*see* **Note 11**). At this point a starting sample of viable cells may be stored at −80°C prior to further processing (*see* **Note 12**).

3. Add 10 U of DNAse I and 5 µl of 10 mg/ml RNAse A and incubate the sample for 1 hour at 37°C.
4. Centrifuge the sample at 12,000 × g for 5 min at 4°C and remove and discard the supernatant.
5. Resuspend the pellet in 200 µl SDE buffer 4 containing 1% PMSF and centrifuge at 12,000 × g for 5 min at room temperature to prevent SDS precipitating from SDE buffer 4.
6. Add the supernatant to an ice-cold microfuge tube and store at −80°C until required.
7. For some cell/tissue types, there may be a small insoluble pellet remaining. This can be stored at −80°C and directly analyzed to determine if any additional proteins can be identified.

4. Notes

1. Base buffer solutions are stable for up to 2 months when stored at 4°C in the dark *(7)*. Alternatively, we have now shown that SDE buffer 1 and 2 solutions may be stored as single-use aliquots at −80°C for 2 months (unpublished data).
2. If the PIPES does not dissolve readily, add 5 M NaOH dropwise until the solution clarifies. The final solution should be clear. We have also demonstrated (but not published) that the PIPES buffer can be replaced with HEPES (Sigma) with no loss of efficacy; SDE base buffer 1 contained a final concentration of 40 mM HEPES and SDE base buffer 2 contained 100 mM HEPES. HEPES is much easier to work with than PIPES.
3. PMSF is toxic if swallowed and contact with acid liberates very toxic gas. Use only in a chemical fume hood.
4. Digitonin is toxic by inhalation, in contact with skin and if swallowed. Use only in chemical fume hood.
5. The Triton X-100 stock solution is not buffered and will change pH very rapidly upon addition of acid or base.
6. Deoxycholate is harmful if swallowed and irritating to the respiratory system. Use only with a mechanical exhaust.
7. SDS is harmful by inhalation and if swallowed, irritating to eyes, respiratory system and skin. Use only in a chemical fume hood.
8. Viable cells were kept at 4°C and were pelleted and resuspended using minimal force to maintain cell integrity throughout the extraction procedure. Pipetting to resuspend the cell pellet was avoided.
9. We initially collected 10 SDE buffer 1 fractions. To confirm that the majority of the proteins were extracted, SDE buffer 1 samples were analysed by SDS-PAGE. Ten percent

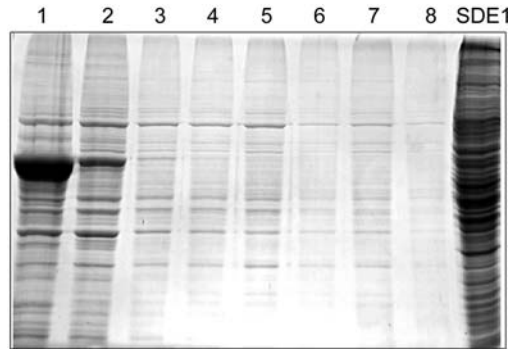

Fig. 7.1. SDE1 extractions. Depletion of the Bursal B-cell sample by SDE buffer 1 was determined by observing sequential SDE buffer 1 samples (1–8) on a 10% denaturing acrylamide gel. A 10% volume sample of each of the fractions was visualised by Coomassie blue. These samples are compared to 10% volume extracted from the same sample using SDE buffer 2.

of each fraction was separated on a 10% denaturing acrylamide gel, and the proteins visualised by Coomassie blue (**Fig. 7.1**). By doing so we were able to determine that only eight SDE buffer 1 samples were required to extract the soluble cytosolic proteins from Bursal B-cells. We recommend that the number of SDE buffer 1 extractions is optimised for each cell or tissue type.

10. Alternatively, a sonicator (Branson Sonifier 250; duty cycle constant, output control setting 3) can be used to disrupt the nucleus. Sonication should be done on ice and only in a pulsed manner.
11. A graduated series of syringe needles are used, ending with an 18-gauge needle.
12. Freezing the sample at −80°C and thawing on ice may assist in fragmentation of the genomic DNA at this step.

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Chapter 8

Enrichment of Brain Plasma Membranes by Affinity Two-Phase Partitioning

Jens Schindler and Hans Gerd Nothwang

Abstract

Plasma membranes encompass a complex and varying set of proteins essential to life. In addition, plasma membrane proteins represent the majority of all known drug targets. The characterization of plasma membrane proteomes is, therefore, of eminent importance. A current bottleneck is the lack of efficient protocols to isolate plasma membranes from tissues or entire organs. To this end, we recently established a simple and effective isolation procedure which is based on aqueous polymer two-phase systems. In this chapter, we provide a detailed protocol for the isolation of plasma membranes from brain tissue, which could easily be adapted to other sources.

Key words: Brain, plasma membrane, affinity partitioning, two-phase system, enrichment.

1. Introduction

Plasma membranes (PMs) represent the interface between a cell and its environment and thus are central to many physiological processes such as cell-cell interactions, signal transduction, and molecular transport. Their importance is stressed by the fact that PM proteins account for approximately 70% of all known drug targets *(1)*. In the brain, many PM proteins determine neuronal function, and their characterization, therefore, is essential for a better understanding of brain structure and neuronal processing. However, the analysis of this class of low abundant proteins at the high anatomical resolution required in heterogenous tissues such as the mammalian brain necessites the application of selective and efficient protocols: selectivity to reduce the contamination of the low abundant PM proteins by proteins from different

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origin; efficiency to reduce the losses of PM proteins during fractionation. One method to fulfill these criteria is affinity partitioning in aqueous two-phase systems. Aqueous two-phase systems form when solutions of two polymers are mixed above a critical concentration. Each of the resulting phases will be enriched in one of the two polymers *(2)*. In the two-phase systems, membranes differing in their subcellular origin partition preferentially in either of the two phases. This can be exploited for the efficient enrichment of a specific subcellular membrane compartment such as the PM. In a dextran/polyethylene glycol (PEG) two-phase system, for instance, PMs show the highest affinity of all subcellular membranes to partition in the top phase enriched in PEG *(2)*. In addition to the polymer composition, partitioning behavior of the membranes can be altered by salt concentrations in the two-phase system, by the polymer concentrations, and by the presence of affinity ligands such as wheat germ agglutinin (WGA) *(3–5)*. Each of these factors has to be adjusted to the tissue and subcellular compartment of interest.

The protocol we describe here is used to enrich brain plasma membranes from a crude membrane fraction by affinity two-phase partitioning *(6)*. First, microsomes containing PMs, are isolated by differential centrifugation *(7)*. In a second step, PMs are further enriched in a two-phase system of PEG and dextran. The use of the affinity ligand WGA in the following step results in the yield of highly purified PMs. In a final step, integral PM proteins are separated from peripheral PM proteins by high-salt and high-pH washes *(8, 9)*. This enables separate analysis of these two important classes of PM proteins.

2. Materials

Due to the strong influence of ions on membrane partitioning in the two-phase systems, double distilled water should be used throughout the experiments.

2.1. Preparation of Microsomes

1. Glass-Teflon homogenizer
2. Homogenization buffer: sucrose (250 mM), Tris (15 mM), complete protease inhibitor (Roche), pH 7.8

2.2. Preparation of WGA-Dextran

1. Dimethyl sulfoxide (DMSO) (*see* **Note 1**)
2. Triethylamine (*see* **Note 1**)
3. Dichloromethane (*see* **Note 1**)

2.2.1. Activation of Dextran

4. Dextran T500 (*see* **Note 2**)
5. Tresyl chloride
6. Dialysis tubes (MWCO 12,000-14,000) (Roth, Karlsruhe, Gemany)

2.2.2. Coupling of WGA

1. Coupling buffer: NaCl (500 mM), NaH_2PO_4 (100 mM), pH 7.5
2. Quenching buffer: Tris-HCl (400 mM), pH 7.5
3. Jumbosep centrifugal device (MWCO 100,000) (VWR)

2.3. Affinity of Two-Phase Partitioning

1. Dextran stock solution: Dextran T500 (20% w/w) (*see* **Note 2**)
2. PEG stock solution: PEG 3350 (40% w/w)
3. Tris-H_2SO_4: Tris (200 mM), pH 7.8 adjusted with H_2SO_4
4. Borate buffer: boric acid (200 mM), pH 7.8 adjusted with Tris
5. Li_2SO_4 stock solution: Li_2SO_4 (200 mM)
6. WGA-Dextran
7. *N*-acetyl-D-glucosamine solution: *N*-acetyl-D-glucosamine (100 mM), sucrose (250 mM), Tris (5 mM), pH 7.8

2.4. High-Salt and High-pH Washing

1. Glass-Teflon homogenizer
2. High-salt buffer: KCl (1 M), Tris (15 mM), pH 7.4
3. High-pH buffer: Na_2CO_3 (100 mM) (*see* **Note 3**)

3. Methods

3.1. Preparation of Microsomes

1. Homogenize 1.5 g of brain tissue in 4.5 mL of homogenization buffer by 20 strokes at 250 rpm in the glass-Teflon homogenizer. For smaller amounts of brain tissue, adjust the amount of homogenization buffer accordingly.
2. Centrifuge (3,000 × *g*) for 10 min at 4°C to sediment nuclei and cell debris.
3. Remove the supernatant and store on ice until further use.
4. Re-extract the pellet with a pipette in the same volume of homogenization buffer, as used for initial homogenization.
5. Centrifuge (3,000 × *g*) for 10 min at 4°C.
6. Repeat **Steps 3–5**.
7. Combine all three supernatants and centrifuge (10,000 × *g*) for 12 min at 4°C to sediment mitochondria.
8. Remove the supernatant and store on ice until further use.
9. Re-extract the pellet with a pipette in the same volume of homogenization buffer as removed before.
10. Centrifuge (10,000 × *g*) for 12 min at 4°C.
11. Repeat **Steps 8–10**.
12. Combine the three supernatants of the 10,000 × *g* centrifugation steps and centrifuge (100,000 × *g*) for 1 h at 4°C.
13. Discard the supernatant. The resulting pellet represents the microsomes that are further purified by affinity two-phase partitioning. The pellet can be stored at −80°C.

3.2. Preparation of WGA-Dextran

3.2.1. Activation of Dextran

1. Dissolve 5 g of freeze-dried Dextran T500 in 25 mL of DMSO at room temperature in a glass beaker dried in an oven at 100°C over night (see **Notes 1 and 2**). Dissolving dextran can take up to 1 h.
2. Add slowly 1 mL of triethylamine followed by 5 mL of dichloromethane within approximately 10 min under stirring to avoid precipitation of dextran (*see* **Note 1**).
3. Chill on ice while stirring (*see* **Note 4**).
4. Add slowly 0.35 g (220 μL) of tresyl chloride under vigorous stirring on ice. Stir on ice for 1 h.
5. Stir the solution at room temperature over night.
6. Add 50 mL of dichloromethane to precipitate dextran (*see* **Note 5**).
7. Wash four times with 25 mL dichloromethane while kneading the precipitate with a glass rod until firm consistency (*see* **Note 5**).
8. Dissolve the washed precipitate in 30 mL water and dialyze against distilled water until the dialysis tube contains only one clear solution.
9. Freeze-dry tresyl-dextran and store at −20°C (*see* **Notes 2 and 6**).

3.2.2. Coupling of WGA

1. Dissolve 2 g of tresyl-dextran in 10 mL coupling buffer in a glass tube and 10 mg WGA in 1 ml coupling buffer.
2. Add the WGA-solution drop wise with a pipette to the tresyl-dextran solution with vigorous vortexing (approximately 10 min). Incubate the mixture over night at 4°C with agitation.
3. Add 10 mL of quenching buffer to terminate the reaction and to inactivate unreacted tresyl groups. Incubate for 2 h at 4°C with agitation (*see* **Note 7**).
4. Add the mixture to Jumbosep centrifugal devices, fill up with distilled water to a final volume of 60 mL, and centrifuge until the volume has decreased to one third. Repeat the procedure five times (*see* **Note 8**).
5. Freeze-dry WGA-dextran (*see* **Notes 2 and 6**). Determine the coupling efficiency by Bradford assay (Pierce) using WGA as standard (*see* **Note 9**).

3.3. Affinity Two-Phase Partitioning

All steps of the affinity two-phase partitioning protocol have to be performed at 4°C. Working at room temperature prevents phase separation. The procedure is illustrated in **Fig. 8.1**. The numbers in **Fig. 8.1** correspond to the numbered two-phase systems given in **Table 8.1**, and the letters refer to the phases as indicated in the protocol given below.

1. Prepare all two-phase systems with the compositions indicated in **Table 8.1** one day prior to use. Mix them by

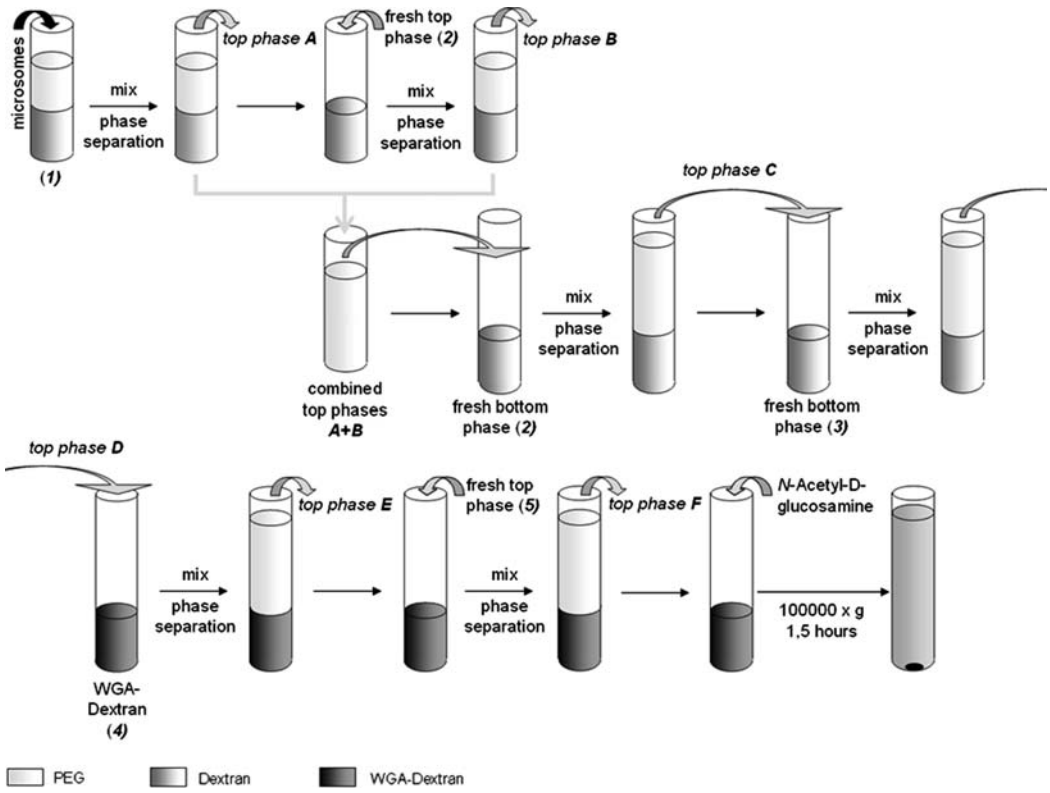

Fig. 8.1. Schematic illustration of the affinity partitioning procedure.

Table 8.1
Composition of two-phase systems

	Two-phase system				
	1	2	3	4	5
WGA-dextran	–	–	–	X[a]	–
Dextran stock solution	1.26 g	1.26 g	1.26 g	X[b]	2.52 g
PEG stock solution	0.63 g	0.63 g	0.63 g	1.26 g	1.26 g
Tris-H_2SO_4	0.3 g	0.3 g	0.3 g	–	–
Borate-buffer	–	–	–	0.6 g	0.6 g
Li_2SO_4	–	–	–	0.04 g	0.04 g
Water	1.41 g	1.81 g	1.81 g	X[c]	3.58 g

[a] $m_{WGA\text{-}dextran} = 800\,\mu g/(\text{coupling degree } [\mu g/\text{mg dextran}] \times 1{,}000)$ (*see* **Note 10**)
[b] $m_{dextran} = (0.504\,g - m_{WGA\text{-}dextran}) \times 5$ (*see* **Note 10**)
[c] $m_{water} = 8\,g - (m_{dextran} + m_{WGA-dextran} + m_{PEG} + m_{boratebuffer} + m_{Li_2SO_4})$ (*see* **Note 10**)

20 invertations, vortexing for 10 s, another 20 invertations, and store the mixtures at 4°C over night. Two-phase systems with the top phase enriched in PEG and the bottom phase enriched in dextran will form over night.

2. Add 400 µL of microsomes resuspended in homogenization buffer to the *two-phase system **1***. If less than 400 µL are used, make up to 400 µL with distilled water. Mix by 20 inversions, vortexing for 10 s, and another 20 inversions. Accelerate phase separation by centrifugation for 5 min at 150 × *g*.
3. Remove the top phase (*top phase **A***) (*see* **Note 11**) and store it at 4°C until further usage. Add a similar volume of fresh top phase from *two-phase system **2***. Mix by 20 inversions, vortexing for 10 s, and another 20 inversions. Accelerate phase separation by centrifugation for 5 min at 150 × *g*.
4. Remove the top phase (*top phase **B***) and combine it with *top phase **A***. Layer the combined *top phases **A** + **B*** onto the bottom phase of *two phase system **2***. Mix by 20 inversions, vortexing for 10 s, and another 20 inversions. Accelerate phase separation by centrifugation for 5 min at 150 × *g*.
5. Remove the resulting *top phase **C*** and mix it with fresh bottom phase from *two-phase system **3*** by 20 inversions, vortexing for 10 s and another 20 inversions. Accelerate phase separation by centrifugation for 5 min at 150 × *g*.
6. Remove the resulting *top phase **D*** and mix it with fresh bottom phase from *two-phase system **4*** by 20 inversions, vortexing for 10 s, and another 20 inversions. Accelerate phase separation by centrifugation for 5 min at 150 × *g*.
7. Discard the *top phase **E*** and mix the bottom phase with a similar volume of fresh top phase from *two-phase system **5*** with 20 inversions, vortexing for 10 s, and another 20 inversions. Accelerate phase separation by centrifugation for 5 min at 150 × *g*.
8. Remove the resulting *top phase **F***. Dilute the bottom phase with a 10-fold volume of *N*-acetyl-D-glucosamine solution and centrifuge at 100,000 × *g* for 90 min. This will release the PMs from WGA-dextran and result in their sedimentation.

3.4. High-Salt and High-pH Washing to Enrich for Integral Membrane Proteins

To enrich integral PM proteins, a high-salt and high-pH wash is recommended. Only peripheral membrane proteins will be solubilized in the washing buffers.

1. Resuspend the final pellet obtained in the affinity partitioning procedure in ice- cold high-salt buffer with a glass-Teflon homogenizer and centrifuge for 1 h at 233,000 × *g*. Repeat this step twice. Each time, the supernatant, representing peripheral PM proteins, is collected.
2. Resuspend the pellet obtained in Step 1 in ice-cold high-pH buffer with a glass-Teflon homogenizer and centrifuge for 1 h at 233,000 × *g*. Repeat this step twice. Collect each time the supernatant, which represents peripheral PM proteins.

3. Combine all six supernatants, enriched in peripheral PM proteins and store them as well as the pellet, enriched in integral membrane proteins, at −80°C.

4. Notes

1. DMSO, triethylamine and dichloromethane are dried with molecular sieves prior to use. For that purpose, dry an Erlenmeyer flask containing 0.02 g of molecular sieves (Sigma) for each milliliter solvent used next day in an oven at 100°C over night for each solvent. The next day, cool down to room temperature, and add the desired amount of DMSO, triethylamine, or dichloromethane to the molecular sieves. Close the flasks tightly with parafilm and leave them at room temperature for at least 5 h.
2. Dextran can contain up to 10% water and for that reason has to be freeze-dried. For freeze-drying, dissolve dextran in distilled water in a plastic dish with a large surface (e.g., Petri dish), freeze it at −80°C and dry it by sublimating the water under vacuum. Store the freeze-dried dextran in closed plastic tubes sealed tightly with parafilm at −20°C. Let it come to room temperature before opening in order to protect it from humidity.
3. pH has to be 11, less alkaline pH results in less elimination of peripheral membrane proteins.
4. Cooling the solution on ice usually increases the viscosity of the solution and small amounts of dextran might precipitate. If this happens, try to stir as well as possible. After half an hour, stirring should be no problem.
5. When precipitating the dextran in **Step 6**, it is clear and slimy. For washing and kneading, press this slime against the beaker with the glass rod. After washing, the product should be white and crystalline.
6. Freeze dried tresyl-dextran and WGA-dextran can be stored for several months at −20°C. Take care that tresyl-dextran and WGA-dextran do not make contact with water.
7. Tresyl groups react with the amino groups of Tris in the quenching buffer. The termination of the reaction is essential to avoid reactions with any amino group in the subsequent two-phase partitioning procedure.
8. Proper washing of WGA-coupled dextran is essential to remove uncoupled WGA and the salt of the buffers used for the coupling procedure. Salts alter the partitioning of membranes in the two-phase systems.
9. It is essential to know precisely the extent of coupling of WGA per mg dextran, as defined amounts of WGA (in the form of WGA-dextran) are used in the affinity partitioning protocol.

10. A two-phase system of 8 g with a final concentration of 6.3% of dextran and 800 μg WGA is required. As in this step, WGA is used coupled to dextran (WGA-dextran), and the amount of WGA coupled to dextran might vary between different coupling experiments, the amount of WGA-dextran, dextran, and water in the two-phase system have to be calculated.
    a) 800 μg of WGA in the form of WGA-dextran are required. With a degree of coupling of 4 μg WGA/mg dextran, 0.2 g WGA-dextran is used.
    b) A total of 0.504 g of dextran is required to get a final concentration of 6.3% in an 8 g system. As already a certain amount of dextran is present in the form of WGA-dextran, this amount has to be subtracted. This results in 0.304 g dextran in our example (0.504–0.2 g). Using a stock solution of 20%, 5 × 0.304 g (i.e., 1.52 g) of this stock solution has to be used to end up with 0.304 g dextran.
    c) The two-phase system is adjusted to 8 g using water. Hence, the amount of all other ingredients has to be subtracted to calculate how much water is missing.
11. In the two-phase systems, interphases are always assigned to the bottom phase.

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Chapter 9

Protocol to Enrich and Analyze Plasma Membrane Proteins

Jacek R. Wiśniewski

Abstract

This chapter describes a procedure for isolation and analysis of fractions enriched in plasma membranes from minute amounts of tissue. It consists of a method for extraction and fractionation of membranes and a method for enzymatic digestion of membrane proteins without use of detergents. The method for isolation of membranes comprises of a stepwise depletion of nonintegral membrane molecules from entire tissue homogenate by high-salt, carbonate, and urea washes followed by a treatment of the membranes with sublytic concentrations of a detergent and enrichment of the plasma membranes by a density gradient fractionation. Fluorometric assays for protein content and plasma membrane marker activity allow calculation of the yield and extent of plasma membrane enrichment. Reduction, carboxymethylation, and digestion with endoproteinase Lys-C are carried out on nonsolubilized membranes. The entire procedure allows processing and preparation of samples from 10–20 mg tissue, and therefore, can be extremely helpful for proteomic profiling of biopsy–size clinical samples.

Key words: Tissue proteomics, membrane proteins, plasma membrane proteins.

1. Introduction

Plasma membranes are physical barriers between cells and their external environment. Proteins integral to this membrane have important roles in cellular homeostasis including receptor function, signal transduction, cell/cell recognition, and transport of solutes and water molecules into and out of the cell. Identification, characterization, and quantification of these proteins are pivotal for studying signaling pathways, sensory mechanisms, and intercellular interactions. Disruption of cell function at the plasma membrane level is known to have strong biological repercussions in several diseases such as diabetes and cancer, just to name a few. For these reasons, characterizing and identifying differences in the

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plasma membrane protein expression in relation to clinical pathology and therapy is of significant interest. Often, however, high-quality tissue samples are only available in limited quantities, and, as such, their processing and analysis remains a fundamental challenge. For practical reasons such as shipment and storage, the vast majority of such biological material is available only in a frozen state. Unfortunately, the classical subcellular fractionation techniques, including those for purification of membrane proteins, were developed, and are suitable, only for work with fresh tissue as a starting material.

Another challenge in studying membrane proteins is their limited solubility in aqueous solutions that results in poor resolution in the commonly used two-dimensional gel electrophoresis technique, as well as weak recovery from the gels *(1, 2)*. Even though the use of strong detergents, such as SDS, allows quantitative solubilization of most membrane proteins, their use creates additional problems; efficient detergent removal, which is a prerequisite for successful mass spectrometric analysis, may be difficult or could require additional sample clean-up steps.

In the protocol described in this chapter I try to address these issues. Firstly, I describe a method for isolation of membranes consisting of a stepwise depletion of nonintegral membrane molecules from entire tissue homogenate by high-salt, carbonate, and urea washes followed by a treatment of the membranes with sublytic concentrations of digitonin and enrichment of the plasma membranes by density gradient fractionation. Secondly, assays for measuring the extent of plasma membrane enrichment are described. Finally, I describe how the integral membrane proteins can be digested without solubilization of the membrane, thus avoiding the use of detergents. In this method, proteins are directly digested on membranes under mild-denaturing conditions *(3)*. The combination of both methods followed by off-line reverse phase chromatographic separation of the released peptides and their online LC-MS/MS analysis allowed identification of 1,670 proteins from 15 mg of frozen mouse hippocampus *(4)* and the measurement of relative abundance of 967 proteins in fore- and hindbrain *(5)*. Of them 81% were known membrane proteins and 38% of the protein sequences were predicted to contain transmembrane helices *(5)*. In another study, which also employed isotope coded affinity tagging reagent HysTag *(3)*, 197 plasma membrane proteins were quantified between cortex, hippocampus, and cerebellum of mouse brain *(6)*.

2. Materials

2.1. Membrane Extraction

1. High salt buffer: 2 M NaCl, 10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA. Store at 4°C.

2. Carbonate buffer: 0.1 M Na_2CO_3, 1 mM EDTA, pH 11.3. Store at 4°C.
3. Wash buffer: 4 M urea, 100 mM NaCl, 10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA. Prepare fresh and store up to 1 day at 4°C.
4. Protease inhibitors: protease inhibitor cocktail tablets from Roche Diagnostics. Each of the above buffers should be supplemented with the recommended amount of inhibitors.

2.2. Density Gradient Centrifugation

1. Sucrose stock solution: 2 M sucrose in water. Store at 4°C.
2. Gradient buffer: 0.25 M sucrose, 100 mM NaCl, 10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA.
3. Percoll (Amersham Biosciences).
4. Digitonin stock solution: 40 mg/mL digitonin (High Purity, Calbiochem).

2.3. γ–glutamyl Transpeptidase Assay

1. Substrate stock: 2 mg/mL γ-L-Glutamic acid 7-amido-4-methylcoumarin (Glycosynth, Warrington, UK) in ethanol. Prepare fresh.
2. Assay buffer: 10 mM $MgCl_2$, 10 mM glycyl glycine, 100 mM Tris-HCl pH 9.0 (prepare fresh).
3. Standard solution: 1 mg/mL 4-methyl umbelliferone (Sigma) in ethanol.

2.4. Protein Assay

1. 100 mM SDS (20 mM final concentration).
2. 0.1 M β-mercaptoethanol.
3. 8 M urea in water.
4. Standard solution: 2 μg/mL L-tryptophanamide hydrochloride in gradient buffer.

2.5. Thiol–Alkylation and Digestion of Membrane Proteins

1. Reduction buffer 1: 0.1 M Na_2CO_3, 10 mM DTT, pH 11.3.
2. Reduction buffer 2: 0.2M NaBr, 0.2M KCl, 50 mM Tris-HCl, pH 8.0, 10 mM DTT.
3. Alkylation solution: 4 M urea, 100 mM iodoacetamide, 100 mM Tris-HCl, pH 8.
4. Carbonate buffer: 0.1 M Na_2CO_3, pH 11.3.
5. Digestion buffer: 4 M urea, 100 mM NaCl, 100 mM Tris-HCl, pH 8.0.
6. Endoproteinase Lys-C (Wako Bioproducts, Richmond, VA).

3. Methods

The following protocol describes the isolation of membranes from frozen mouse brain. This method comprises three extraction steps and a density gradient purification step. In the first extraction step, high ionic strength allows disruption of ionic bonds. The selected salt concentration is high enough to break very tight

bonds between DNA and the core histones *(7)*. In the second step, the high pH of the carbonate buffer introduces discontinuities into membrane vesicles *(8)*. The discontinuities allow efficient removal of soluble proteins from both artificial vesicles of microsomes assembled during homogenization, and from intact organelles such as mitochondria and lysosomes. Finally, the membranes are treated with 4 M urea, a concentration that is high enough for globular domains of the vast majority of proteins to melt *(9, 10)*, but that does not affect the integrity of the transmembrane helices in the lipid bilayer. In this step, nonintegral membrane proteins that were unaffected by the carbonate treatment can be washed out.

Fractionation of the total membranes is achieved by using a self-generating density gradient of Percoll. The differences in the buoyant densities are affected by addition of sublytic amounts of the nonionic detergent digitonin. This technique was initially developed for enrichment of microsomal membranes from rat liver in a sucrose gradient *(11)*. In that study, digitonin caused a selective increase of the density of microsomes, whereas organelles remained little affected. Treatment of purified membranes from brain tissues with the detergent results in a shift of all membranes, but the shift of membranes originating from the plasma membrane is less prominent, and therefore, the membrane fractions with a lower buoyant density are enriched in plasma membrane *(4)*. In the described method, the selection of fractions with the highest enrichment in plasma membranes is based on the activity measurement of γ–glutamyl transpeptidase, a plasma membrane marker enzyme, and on the determination of total protein concentration. Since the method is designed for the analysis of small amounts of biological samples, sensitive fluorometric assays are selected.

3.1. Total Membrane Extraction from Frozen Tissue

In this method all steps should be carried out in a cold room at 4°C and/or on ice.

1. Thaw 20–50 mg tissue and add 1 mL of high-salt buffer.
2. Blend the tissue using a IKA Ultra Turbax blender at maximum speed (approximately 25,000 rpm) for 30 s.
3. Ultracentrifuge the suspension in a Sorval S150AT or Beckman MLA 130 at 900,000 g for 10 min. The tubes should be balanced to a difference less than 50 mg.
4. Discard the supernatant and homogenize the pellet in 1 mL of carbonate buffer as in **Step 2**.
5. Incubate for 30 min (*see* **Note 1**).
6. Collect the nonsoluble material by centrifugation as in **Step 3** (**Steps 4–6** can be repeated 2–3 times, *see* **Note 2**).
7. Discard the supernatant and resuspend the pellet in wash buffer as described in **Step 2**.
8. Collect the crude membranes by centrifugation as in **Step 3**.

3.2. Enrichment of Plasma Membranes by Density Gradient Centrifugation

1. The following protocol describes the fractionation of membranes using a Sorvall S100AT6 (or Beckman TLA110) rotor accommodating 4.1 mL (or 4.7 mL) tubes.
2. Resuspend the crude membranes in 1 mL of gradient buffer using an IKA Ultra Turbax blender at maximum speed (approximately 25,000 rpm) for 30 s.
3. Transfer the suspension into a centrifugal vial and add:
   a. 1.44 mL (or 1.65 mL in TLA 110) of Percoll,
   b. 0.205 mL (0.27 mL) of 2 M sucrose,
   c. 1 mL (1.31 mL) of gradient buffer,
   d. 0.41 mL (0.47 mL) of digitonin solution (*see* **Note 3**).
4. Close the tubes and centrifuge at 150,000 g for 30 min. Decelerate the rotor over 5–10 min. To balance the tube another, one is prepared with the same composition of solutions 3a–d and 1 mL of gradient buffer instead of the sample suspension.
5. Remove the sample tube from the rotor and assemble the fractionation device (*see* **Note 4**).Collect fractions by the bottom gradient displacement with 2 M sucrose using a peristaltic pump at a flow rate of 0.5 mL/min. Collect at least ten 0.4 mL fractions. To remove Percoll, dilute the fractions with 0.6 ml of gradient buffer and centrifuge in a Sorval S150AT or Beckman MLA 130 at 900,000 g for 15 min. The membranes accumulate on the surface of a transparent Percoll pellet (*see* **Note 5**).
6. Resuspend each membrane pellet in 200–300 μL of gradient buffer. Aliquots of 40–50 μL are subjected to measurement of the plasma membrane marker activity and to total protein amount determination.

3.3. Biochemical Analysis of Plasma Membranes

3.3.1. γ–glutamyl Transpeptidase

1. The enzymatic activity is measured at 365 nm and 460 nm for excitation and emission of the liberated 4-methylumbelliferol, respectively, at a constant temperature of 37°C (*see* **Note 6**).
2. Mix 50 mL of assay buffer with 0.5 mL of substrate stock solution.
3. Add 3 mL of this diluted substrate to the 1 × 1 cm standard 10 mm fluorometer cuvettes and preincubate for a few minutes to reach the assay temperature of 37°C.
4. Add sample (usually 2–20 μL of the analyzed subcellular fraction obtained in **Section 3.2, Step 6**) and mix thoroughly.
5. Start recording the intensity changes at 460 nm.
6. Measure changes for 5–10 min. The slope of the recorded reaction should be straight linear because it is a zero-order reaction. If the velocity of the reaction decreases during the

recording time reduce the am ount of sample for the measurement (**Step 4**).
7. Repeat each measurement at least once.
8. Calculate for each fraction the relative specific activity and the yield of plasma membranes.
9. For absolute measurement of the released 4-methylumbelliferol, measure the intensity of fluorescence of defined amounts of the standard solution in the range of the measured activity.

3.3.2. Protein Assay

1. Mix 30 μL of the sample obtained in **Section 3.2, Step 6** with 4 μL of 100 mM SDS (final concentration of 20 mM), and 1 μL of 0.1 M β-mercaptoethanol. For measurements of protein concentrations in the initial homogenate or crude membrane fractions, dilute the samples appropriately.
2. Incubate at 96°C for 5 min and centrifuge at approximately 15,000 g for 10 min.
3. Set the spectrofluorometer for measurements at 295 nm and 350 nm for excitation and emission, respectively (*see* **Note 7**).
4. Prepare the standard curve by mixing 2 mL of 8 M urea with different amounts of tryptophanamide ranging from 1 to 20 μL of the stock solution and by measuring the emission intensity. The added amounts of tryptophanamide correspond to 2–40 ng.
5. Introduce 10 μL of the analyzed fraction prepared in **Step 1** in 2 mL of 8 M urea, and measure the emission intensity. The protein amounts can be calculated assuming tryptophan accounts for 1.3% of their total weight on average.

3.4. Digestion of Membrane Proteins

1. Combine fractions with the highest plasma membrane marker activities and collect the membranes by centrifugation at 900, 000 × g for 15 min. Remove the supernatant.
2. Resuspend the membrane pellet in 1 mL of reduction buffer and incubate on ice for 30 min.
3. Ultracentrifuge the suspension and as in **Step 1.** Discard the supernatant.
4. Resuspend the pellet in 1 mL of reduction buffer 2 and incubate on ice for 30 min. Centrifuge as in **Step 1**. Discard the supernatant.
5. Resuspend the pellet in 1 mL of alkylation solution and incubate at 25°C for 2 h.
6. Centrifuge as in **Step 1**. Discard the supernatant.
7. Resuspend the pellet in 1 mL of carbonate buffer and incubate on ice for 30 min.
8. Centrifuge as in **Step 1**. Discard the supernatant.
9. Resuspend the pellet in 100–200 μL of digestion buffer, add 1 μg of endoproteinase Lys-C per 100 μg of protein and incubate overnight at 20°C.

10. Centrifuge as in **Step 1** and collect the supernatant containing peptides.
11. The peptides can be desalted using a solid phase extraction cartridge or directly fractionated on a reverse phase column *(12)*.

4. Notes

1. Incubations should be carried out in a thermomixer (Eppendorf) with gentle mixing of the sample.
2. The introduction of discontinuities in the membrane vesicles requires a high excess of sodium carbonate. Usually at least a 50-fold (v/v) excess of the solution over membranes is required *(8)*. If the content of integral membrane proteins in the purified membranes (final product) is not at least 30–40% of total identified proteins, the sodium carbonate extraction can be repeated two or three times.
3. The concentration of digitonin is critical in the procedure. The concentration of 4 mg/mL was found to be optimal for purification of mouse brain plasma membranes. At a digitonin concentration of 8 mg/mL, lysis of membranes was observed. When working with other tissues, the amount of digitonin required for the density shift should be determined experimentally.
4. Fractionation of the density gradient is an important step; it should, therefore, be prepared in advance and performed thoroughly. Fractions can be collected manually by piercing the bottom of the tube and collecting drops or by aspirating with a pipette from the top of the gradient. In terms of reproducibility and resolution, the best results can be achieved using a fractionation device in which the gradient is displaced upwards by pumping dense sucrose from the bottom and the fractions are collected from the top.
5. Percoll can be removed by high-speed centrifugation. After centrifugation, membranes form a thin pellet over a firm and transparent pellet of the density medium. The collection of the membrane pellet is achieved by resuspending it in a small volume of buffer.
6. It is important to keep the temperature constant during the assay because both the reaction velocity and the fluorescence intensity are temperature-dependent.
7. Assaying protein concentration by measurement of the tryptophan fluorescence is a sensitive and simple method. The sensitivity of the assay depends on the type of fluorometer and on the selected setting. It typically allows reliable measurements of protein amounts as low as 10 ng.

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Chapter 10

Proteomic Analysis of the Lymphocyte Plasma Membrane Using Cell Surface Biotinylation and Solution-Phase Isoelectric Focusing

Matthew J. Peirce, Andrew P. Cope, and Robin Wait

Abstract

Plasma membrane (PM) proteins are of particular interest to cell biologists because of their role in transducing information from the external environment to the cell interior, and because of their potential as therapeutic targets. The hydrophobicity and large size of these proteins renders their analysis by conventional proteomic approaches using 2D-electrophoresis problematic, limiting our ability to evaluate alterations of cell surface architecture as a function of varying physiological, pathological, or developmental state.

In this chapter, we describe a simple method for enrichment and separation of plasma membrane proteins, prior to their identification by tandem mass spectrometry. Cell surface proteins are labeled with biotin using a reagent which does not enter the cell, purified by differential centrifugation and then affinity captured with streptavidin-agarose beads, before separation by a combination of solution-phase isoelectric focusing, and gradient gel electrophoresis, resulting in highly enriched membrane protein fractions suitable for characterization by mass spectrometry. We discuss the application of this protocol to the semiquantitative comparison of the plasma membrane proteins from resting and activated murine lymphocytes.

Key words: Lymphocyte, plasma membrane protein, solution-phase isoelectric focusing, proteomics.

1. Introduction

Proteins that reside in the plasma membrane are of particular biological interest. They sit at the interface of the cell and the extra cellular environment and are responsible for receiving and interpreting environmental cues. Thus the stimuli to which a cell can respond, and the magnitude of those responses, is

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dependent upon the profile of proteins present at the PM and their relative abundance. Moreover, transmembrane PM proteins represent uniquely "drugable" targets since they are accessible to biological fluids. This is reflected in the fact that approximately 70% of currently licensed pharmaceuticals act on transmembrane PM proteins *(1)*.

For these reasons, there has been enormous interest in applying proteomic approaches to PM proteins. However, analysis of PM proteins using classical two-dimensional electrophoresis (2DE) approaches have proved problematic *(2)*. Many PM proteins are of relatively low abundance and have regions of high hydrophobicity which can result in protein precipitation during isoelectric focusing (IEF) in immobilized pH gradient (IPG) gel strips and, consequently, under representation of PM proteins in the final gel *(3)*.

These difficulties have been obviated to some degree by "shotgun" approaches *(4)* in which peptides derived from PM proteins are resolved by 2D liquid chromatography prior to MS/MS analysis. However, these techniques are dependent upon technology (high degree of computing power and relatively sophisticated mass spectrometers) that may not be available outside specialized proteomics laboratories.

The protocols described below attempt to address some of the problems associated with proteomic analysis of PM proteins using techniques accessible to most biochemical laboratories. Using murine lymphocytes as a model system we describe a cell surface biotinylation approach using a water-soluble reagent, followed by differential centrifugation and affinity capture of the labeled proteins using an immobilized streptavidin matrix. We then describe how the recovered proteins can be resolved using solution-phase IEF, which compared to IPG-based IEF, better retains PM proteins in solution, and markedly improves protein recovery. The technique allowed identification of 75 PM proteins from murine lymphocytes and, furthermore, enabled semiquantitative comparison of PM protein profiles on resting and activated primary splenocytes *(5)*.

2. Materials

The protocol described here applies to T cell hybridomas but essentially the same protocol has been used to recover PM proteins from primary murine splenocytes and RAW 264.7 murine monocyte/macrophages (*see* **Note 1**).

2.1. Cell Surface Labeling

1. Labeling buffer: ice-cold borate buffered saline (BBS) pH 8.1; 10 mM sodium orthoborate, 2.3 mM sodium tetraborate (Borax), 115 mM NaCl (*see* **Note 2**).

2. Quenching buffer: ice-cold RPMI 1640 medium containing glutamine (*see* **Note 3**).
3. Sulpho-NHS-SS-biotin (Pierce, Perbio, Tattenhall, UK; 10 mg/ml in BBS).

2.2. Cell Homogenization and Fractionation

1. Hypotonic lysis buffer: 20 mM Tris pH 7.4, 5 mM EDTA, protease inhibitor cocktail (Sigma, Poole, UK, 1 μL/10^7 cells)
2. Dounce homogenizer with tight-fitting plunger
3. Detergent lysis buffer: hypotonic lysis buffer supplemented with; 1% v/v Triton X-100, 150 mM NaCl.

2.3. Affinity Purification

1. Immobilized streptavidin-agarose (Pierce, 1 μl/10^6 cell equivalents, *see* **Note 4**)
2. RIPA wash buffer: 50 mM Tris pH7.4, 1% v/v Triton X-100, 1% w/v deoxycholate, 0.1% SDS
3. Wash buffer 2: 1% Triton X-100
4. Elution buffer (1 ml/500 μl of added beads): 9M urea, 1% w/v DTT, 1% v/v Triton X-100, protease inhibitor cocktail (2 μl/ml).

2.4. Solution-Phase Iso-Eelectric Focusing

The protocol described here is for a pH 3-10 gradient which is adequate for the majority of samples and is advisable in the first instance. Use of other gradients (e.g. pH 3-7) requires alternative ampholytes and acid/base reservoir buffers.

1. IEF buffer: elution buffer supplemented with 1% v/v pH 3-10 ampholytes (Bio-Rad, Hemel Hempstead, UK)
2. Acid reservoir buffer: 0.1 M acetic acid
3. Base reservoir buffer: 0.1 M NaOH
4. Rotofor isoelectric focusing unit (Bio-Rad).

2.5. Sample Concentration and Clean-Up

The Rotofor fractions should be approximately 2 ml each and require concentration and removal of ampholytes, which interfere with subsequent silver staining, before separation by PAGE.

1. Centrifugal concentrators (6 ml capacity, Vivascience, Hannover, Germany)
2. SDS-PAGE clean-up kit (Amersham Bioscience, Chalfont St. Giles, UK).

3. Methods

3.1. Cell Surface Biotinylation

1. Wash cells 3× in ice-cold BBS and resuspended at 1–5 × 10^7/ml
2. Prepare a 100× solution of sulpho-NHS-SS biotin (10 mg/ml in BBS).
3. Add the biotinylating reagent to the cells to a final concentration of 0.1 mg/ml and agitate gently for 20 min at 4°C.
4. Pellet cells and wash × 2 in ice-cold glutamine-containing serum free medium (e.g. RPMI or DMEM).

3.2. Cell Homogenization and Fractionation

1. Pellet cells from quenching buffer and resuspend in ice-cold hypotonic lysis buffer at 2–5 $\times 10^7$/ml. Allow to swell and lyse for 10 min on ice.
2. Decant cells in to dounce homogenizer and subject to 30 strokes with a tight-fitting pestle (*see* **Note 5**).
3. Decant homogenate in to ultracentrifuge tubes and spin at 4,000 × g for 15 min at 4°C.
4. Recover the postnuclear supernatant (PNS) and clarify at 20,000 × g for 30 min at 4°C.
5. Aspirate as much of the supernatant of this spin (s20) as possible and recover the pellet (p20) in a small volume of detergent lysis buffer (~2 µl per million cell equivalents). Disrupt the pellet by repeated pipetting and solubilize by agitating gently for 30 min at 4°C.
6. Pellet the insoluble material (13,000 × g, 5 min at 4°C) and recover the supernatant.

3.3. Affinity Purification

1. To the detergent-soluble p20 fraction add immobilized streptavidin-agarose beads (~ 1 µl bead slurry /million starting cells).
2. Incubate overnight at 4°C with end-over-end agitation.
3. Pellet beads and remove supernatant by puncturing eppendorf and centrifuging briefly (*see* **Note 6**).
4. Wash beads (5 × 1 ml) with ice-cold RIPA buffer and then (4 × 1 ml) with wash buffer 2 to remove traces of SDS from beads. Use punctured eppendorf to remove completely supernatant of each wash.
5. Incubate over night at 4°C with 2 microlitres of elution buffer per µl of bead slurry used. Recover eluate by centrifugation of punctured eppendorf as above (*see* **Note 7**).

3.4. Solution-Phase IEF

1. Retain an aliquot (50 µl) of the eluate from **Step 3.3** and dilute the remainder in IEF buffer and supplement with ampholytes (pH 3-10) to give a final volume of 55 ml and a final ampholyte concentration of 1%.
2. With a 50 ml syringe and blunt 18-gauge needle fill the Rotofor chamber with the sample, minimizing introduction of air bubbles, and connect apparatus to a 4°C water cooler and allow sample 10 min to equilibrate.
3. Begin focusing under fixed power of 12 W monitoring current and voltage readings intermittently.
4. Allow focussing to continue for 3–4 h, or until the voltage reading is stable. Turn off the machine and under a strong vacuum harvest the fractions in to prelabeled and rinsed 6 ml centrifuge tubes (see **Notes 8**, **9** and **10**).

3.5. Sample Concentration and Clean Up

1. Pool samples as required and transfer carefully to 6 ml 10 kD cut-off centrifugal concentrator tubes (Vivascience). When

sample volume has been reduced to 50–100 μl, transfer to prerinsed eppendorf tubes.
2. Perform clean up of sample to remove ampholytes using SDS-PAGE clean up kit (Amersham Biosciences). Concentrated and cleaned samples can be used immediately or stored at −20°C.
3. Sample is then ready for SDS PAGE and visualization and analysis as required (*see* **Note 11**).

4. Notes

1. At every stage of the protocol it is essential that all tubes, instruments, and surfaces are kept scrupulously clean by washing with polished water and/or 70% EtOH to avoid contamination of the sample with environmental proteins (e.g., keratin) especially during the final concentration steps.
2. The biotinylation reaction targets primary amine groups, that is, lysine residues of proteins. It is essential that the buffer in which the reaction is performed is free of primary amine groups. For this reason, Tris-based buffers should be avoided. Either borate or phosphate buffered saline (pH 8.1) should be used. It is also important that as far as possible extraneous proteins (e.g. from serum-containing growth media) are removed before biotinylation takes place.
3. The buffer used to quench the biotinylation reaction can be any in which free primary amine groups are abundant. For example, glycine-containing buffers are often used but any glutamine-containing serum-free growth media will also make a good quenching buffer.
4. We found streptavidin-agarose beads from Pierce to yield much less background staining compared to those from Sigma under the same conditions.
5. The optimal amount of homogenization may vary between cell types. Conditions required to give complete cell lysis should be established empirically by monitoring trypan blue exclusion. The extent of cell lysis can be increased by prolonging the period of incubation in the hypotonic lysis buffer or by increasing the number of strokes with the homogenizer. Alternatively, for cells resistant to lysis (e.g., RAW 264.7), intact cells can be separated from broken cells by centrifugation (1,000 × g, 5 min, 4°C). The supernatant is retained, the intact cell pellet subjected to a second round of homogenization in fresh hypotonic lysis buffer then recombined with the supernatant of the first spin.
6. To ensure effective washing of streptavidin-agarose beads and complete recovery of the DTT eluate use a 26-gauge needle to puncture the bottom of an eppendorf tube such

that the liquid contents of the tube are recovered by placing the eppendorf inside a 6 ml plastic test tube and centrifuging (10,000 × g, 1 min). All agarose beads should be retained in the eppendorf.

7. If an IEF step is not required (i.e., if the sample is to be resolved by 1 DE only), the bead bound proteins can be eluted equally well after the washing steps by boiling (5 min) in an appropriate volume of SDS-PAGE sample buffer. Note that should you wish to visualize the biotinylated proteins using a streptavidin-HRP Western blot, reducing agent should be left out of the elution buffer in order to avoid cleavage of the biotin moiety.
8. Before harvesting, the Rotofor fractions ensure the tubing is clean by rinsing through with 0.1 M NaOH followed by two washes with Ultrapure water using a plastic transfer pipette.
9. When harvesting the Rotofor fractions, ensure that the metal teeth of the harvesting device penetrate each fraction simultaneously to avoid mixing of adjacent fractions.
10. To ensure a strong vacuum is maintained, thereby optimizing sample recovery, coat the upper edges of the plastic sample box with vacuum grease.
11. Following the clean-up step, the sample should be in a volume of approximately 100 µl. The gel system chosen for the second dimension should have sufficient capacity to accommodate the entire sample. The 20 cm Bio-Rad gel system using 1.5 mm thick combs worked well for these purposes as well as providing an extra few centimetres for greater resolution. It should also be noted that the use of gradient gels (e.g., 4–18%) gave greatly enhanced protein resolution compared to single percentage gels.

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Chapter 11

Identification of Target Membrane Proteins as Detected by Phage Antibodies

Cecile A.W. Geuijen, Arjen Q. Bakker, and John de Kruif

Abstract

The discovery of novel target antigens for antibody-based immunotherapy is still a major challenge. Antibody phage display is one of the technologies that is widely applied for the identification of novel cell surface molecules on intact eukaryotic cells and many reports describe the isolation of phage-antibodies binding to restricted cell populations such as cells in a certain pathological condition. However, the transition from cell-specific phage antibodies to the identification of the target antigens is still a major hurdle. Herein a method is described for the identification of these cell surface molecules using two complementary technologies. A genomic approach based on expression cloning can be used when cDNA libraries and antigen-negative cells are available. Otherwise, a proteomic approach based on small scale immunoprecipitation followed by large scale purification and mass-spectrometry-based identification can be applied. Correct identification of the antigens is confirmed using technologies such as recombinant expression of the target antigen followed by immunoprecipitation or cDNA transfection and FACS analysis.

Key words: Phage display, expression cloning, matrix screening, target identification, immunoprecipitation.

1. Introduction

Antibody phage display can be used to search for novel cell-specific antigens like markers selectively expressed on tumour cells *(1–6)*. In these experiments, large repertoires of antibody fragments displayed on phage are allowed to bind to a cell population of interest. Phage antibodies binding antigens that are not of interest can be depleted using 'absorber cells', such as the healthy counterparts of the tumour cells that are used in selection. Non-binding phages are washed away, followed by elution and rescue

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of the specific phages. With maturation of the phage display technology, selection of phage antibodies binding cell surfaces using these procedures has become fairly straightforward.

After selection of a panel of phage antibodies the specificity of these phages is analysed. Low affinity interactions complicate these assays *(7)*, therefore, all phage antibodies that enter the target identification process may be converted into complete IgG molecules *(8)*. The avidity effect of a bivalent IgG and the presence of an Fc tail that can be used for interaction with reagents such as protein A is an advantage over other antibody formats in both the specificity and affinity purification experiments *(9)*. Identification of the antigens recognised by these phage-derived IgG is, however, still a challenge. Classical proteomic identification methods that use a combination of affinity chromatography followed by separation on two-dimensional gels and mass spectrometry is difficult as cell surface antigens are generally hydrophobic and of a high molecular weight, both features that prevent them from being easily resolved on two-dimensional gels *(10)*. In addition, it can be very demanding to gain access to sufficient cell material for these procedures.

For the identification of phage antibody targets, we have optimised two procedures that complement each other. The first method is based on a genomic approach. Briefly, a cDNA library, made from tissue reacting with the antibody of interest, is sourced and transfected into a suitable cell line. The phage antibody is used to detect those cells that express a reactive antigen. These cells are isolated using flow cytometry where after the antigen-encoding cDNA can be recovered.

The second approach aims to purify sufficient amounts of IgG reactive antigen for mass-spectrometry based identification. Initially, small-scale immunoprecipitation experiments with biotinylated cell lysates are performed to establish whether the target antigen can be extracted while maintaining the proper conformation for antibody reactivity. This small-scale method is then optimised to establish the optimal wash and elution conditions to provide the highest purity and yields of antigen. Subsequently, these conditions are applied in large-scale affinity chromatography followed by mass-spectrometry-based identification of the purified protein. The genomic approach is less time-consuming than the proteomic approach but is critically dependent on the availability of a suitable cDNA library and a host cell line that does not express the marker of interest.

When successful, these methods result in DNA- or peptide sequences, respectively, that can be used to search databases for the target gene. If more than one candidate antigen for each phage or IgG is obtained, the correct antigen can be identified using recombinant expression of tagged versions of the antigens. Identification is confirmed when the phage or IgG

specifically detects the recombinant antigen in methods such as FACS, ELISA, or immunoprecipitation.

2. Materials

2.1. Expression Cloning

2.1.1. HEK293T/17 Culturing and Transfection

1. HEK293T/17 cells (ATCC).
2. 0.25% (w/v) Trypsin- 0.53 mM EDTA in PBS.
3. cDNA library e.g. human bone marrow cDNA library (Invitrogen). The choice of library is based on the cell types used for selection. Preferably, these cells should be tested for expression prior to the experiment.
4. Control DNA: A eukaryotic expression construct coding for membrane bound protein, for example, human CD38. A phage antibody binding this marker should be available.
5. Fugene-6 (Roche).
6. Lipofectamine (Invitrogen).
7. Trypan-blue (Sigma).
8. RPMI culture medium (Invitrogen).
9. DMEM culture medium (Invitrogen).
10. DMEM/10% FCS: DMEM culture medium supplemented with 10% heat inactivated FCS, L-glutamine and penecillin/streptomycin.
11. PBS (Invitrogen).

2.1.2. Cell Harvest and FACS Staining

1. PBS.
2. PBS/1 mM EDTA: PBS supplemented with 1 mM EDTA.
3. PBS/1% BSA: PBS supplemented with 10 g/L BSA (prepare fresh daily).
4. PBS/4% Milk: PBS supplemented with 40 g/L non-fat dry milk (prepare fresh daily).
5. Trypan blue.
6. Normal Human Serum (Bio Whittaker).
7. Antibodies recognizing the control antigen: CD38-FITC (Coulter-Immunotech), CD38-PE: (Becton Dickinson).
8. Anti-CD16-FITC, Anti-CD32-FITC, Anti-CD64-FITC (Becton Dickinson).
9. Anti-M13Biotin (Fitzgerald Ind. Int. Inc.).
10. Streptavidin-PE (Caltag).
11. 7-AAD (Becton Dickinson).
12. Phage preparation: Phage titer $> 5 \times 10^{12}$ cfu/ml.
13. Control phage preparation: Phage titer $> 5 \times 10^{12}$ cfu/ml.
14. 4 ml tube/cell strainer cap (Falcon).

2.1.3. Cell Sorting

1. PBS.
2. Hirt lysis buffer: 0.6% SDS/ (autoclaved)10 mM EDTA.
3. 5 M NaCl: NaCl 292.5 g/L, autoclaved.

2.1.4. Plasmid Rescue

1. P/C/I 25:24:1: Phenol/Chloroform/Isoamylalcohol at 25:24:1 ratio (Invitrogen).
2. Chloroform (Merck).
3. Glycogen 20 mg/ml (Roche).
4. Sodium acetetate 3 M pH 5.
5. Ethanol p.a.
6. Ethanol 75%: Ethanol p.a./Distilled water 75:25 (v/v).
7. 10 mM TRIS pH 8 (Qiagen, buffer EB).
8. ElectroMAX DH10B-T1r (Invitrogen).
9. Electroporation chamber 0.1 cm (Biorad).
10. SOC medium (Invitrogen).
11. Glass-beads 3 mm (Merck).
12. LB agar/Ampicillin: Luria Broth agar supplemented with 50 μm/ml ampicillin.
13. LB medium: Luria Broth liquid medium.
14. Plasmid isolation kits: QIAfilter Plasmid Maxi Kit or QIAprep Spin Miniprep Kit (Qiagen).

2.1.5. Plasmid Pool and Matrix Screening

1. Nitrocellulose filter.
2. 96-well deep well plate.

2.2. Affinity Purification

2.2.1. Cell Surface Labelling

1. Physiological buffer: 120 mM NaCl, 4.4 mM KH_2PO_4, 20.6 mM Na_2HPO_4, pH 7.4. Store at 4°C for no more than 2 months.
2. Quenching buffer: 100 mM glycine in physiological buffer.
3. Sulfo-NHS-LC-LC-biotin: 25 mg/ml in H_20 (Pierce).
4. Trypan blue (Sigma).

2.2.2. Cell Extraction

1. Protease inhibitor cocktail (Sigma), 1 μl for a lysate derived from 10^7 cells.
2. Triton X-100 buffer: 1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.4.
3. DOC buffer: 1% Triton X-100, 0.5% Sodium deoxycholate, 150 mM NaCl, 50 mM Tris, pH 7.4.
4. RIPA buffer: 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 7.4.

2.2.3. Immunoprecipitation

1. Protein A beads (Amersham).
2. Phage-derived IgG (*see* **Note 20**).
3. 2M Tris buffer pH 7.4.
4. NuPAGE Bis-Tris gradient gels 4–12% (Invitrogen).

2.2.4. Detection of Biotinylated Proteins

1. Block buffer: PBS supplemented with 30 g/L BSA.
2. Biotinylated streptavidin peroxidase complex (Amersham).

3. TBST buffer: 25 mM Tris-HCl pH 8.0, 125 mM NaCl, 0.1% Tween 20.
4. ECL (Amersham).

2.2.5. Establishing Wash and Elution Conditions

1. Triton X-100 buffer, DOC buffer and RIPA buffer to which NaCl is added to a final concentration of 0.2, 0.4, 0.6, 0.8 and 1.0 M.
2. Glycine buffer, pH 2.7: 1% Triton X-100, 150 mM NaCl, 0.1 M glycine, 1 mM EDTA.
3. Lysine buffer, pH 11: 1% Triton X-100, 150 mM NaCl, 0.1 M lysine, 1 mM EDTA.

2.2.6. Large-Scale Affinity Purification

1. CNBr Sepharose 4B beads (Amersham).
2. Amicon Ultracentrifugal filter with 100 kDa cut off (Millipore).
3. (Human) control IgG.
4. NuPAGE Bis-Tris gradient gels 4–12% (Invitrogen).
5. Silverquest (Invitrogen).

3. Methods

3.1. Expression Cloning

3.1.1. HEK 293T/17 Culturing and Transfection

1. Culture of HEK293T/17 cells (*see* **Note 1**) according to ATCC guidelines.
2. Remove and discard culture medium.
3. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum that inhibit the action of trypsin.
4. Add 2.0–3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5–15 min).
5. Add 6.0–8.0 ml of complete growth medium and aspirate cells by gently pipetting.
6. Add appropriate aliquots of the cell suspension to new culture vessels.
7. A sub-culture ratio of 1:4–1:8 is recommended, split every 2–3 days.
8. Incubate cultures at 37°C/10% CO_2.
9. Transfection of cDNA (sub-) library and control DNA is performed using either Lipofectamine (Invitrogen) or Fugene-6 (Roche) transfection reagent according to manufacturer's recommendations (*see* **Note 2**).
10. For pre-screening (*see* **Note 2**) of the library and bulk first round sorting use one or more 10 cm petri dishes HEK293T/17 cells transfected with Fugene-6, for second round bulk sorting use one or more 10 cm Petri dishes HEK293T/17 cells transfected with Lipofectamine

Table 11.1
Transfection set-up

Transfection size	293T/17 cells	DNA	Lipofectamine	DMEM	Fugene-6	RPMI
10 cm petridish	2.5×10^6	12 μg	72 μl	800 μl		
10 cm petridish	2.5×10^6	12 μg			15 μl	400 μl
6 well plate	0.25×10^6	2 μg			3 μl	75 μl

(*see* **Note 3**). For screening of the second round plasmid pools use 6-well plates of HEK293T/17 cells transfected with Fugene-6 (*see* **Table 11.1**).

3.1.2. Cell Harvest

1. Harvest cells 48 h after transfection (*see* **Note 4**).
2. Remove medium from transfected HEK293T/17 cells.
3. Wash cells carefully with PBS at room temperature (RT), 5 ml per 10 ml dish or 1 ml per 6-well.
4. Detach cells with PBS/1 mM EDTA, 5 ml per 10 cm dish/T75 flask or 1 ml per 6-well (*see* **Note 5**).
5. Incubate ~5 min at RT, observe cells under microscope, and when rounding up gently shake cells loose from culture dish.
6. Add an equal volume of DMEM/10% FCS to counterbalance the EDTA.
7. Flush cells gently with a pipette to make a single cell suspension.
8. Transfer cells to a 50 ml tube, put on ice (from now on keep the cells on ice to prevent clumping).
9. Pellet cells (300 × g, 5 min at 4°C), and re-suspend in ice-cold PBS/1%BSA to about $1 \sim 2 \times 10^6$/ml.
10. Count cells using trypan blue to determine total cell number, recover up to 10×10^6 cells per 10 cm petridish.
11. Top-off cells with ice-cold PBS/1%BSA to wash and pellet cells (300 × g, 5 min at 4°C).

3.1.3. Bulk FACS Staining for Cell Sorting

1. Use 10–30 × 10^6 cells transfected with (sub-) library for phage staining and cell sorting.
2. Remove all wash buffers from pelleted cells and re-suspend in adherent fluid.
3. Block cells by adding 1/10 volume of normal human serum and 1/4 volume of PBS/4% milk (*see* **Note 6**).
4. Block 500 μl test-phage preparation with 125 μl PBS/4% milk for at least 15 min on ice.
5. Mix the blocked phage with the blocked transfected cells.
6. Incubate 1 h on ice and mix gently every 10 min.
7. Wash and pellet the cells three times with 40 ml ice-cold PBS/1% BSA (300 × g, 5 min at 4°C).

8. Remove all wash buffers from cell pellet and re-suspend in adherent fluid.
9. Add anti-M13Biotin to ~1 μg/ml (1:500).
10. Incubate 30 min on ice.
11. Wash and pellet the cells once with 40 ml ice-cold PBS/1% BSA (300 × g, 5 min at 4°C).
12. Remove all wash buffers from cell pellet and re-suspend in adherent fluid.
13. Add Streptavidin-PE to 0.5 μg/ml (1:600) (*see* **Note 7**).
14. Add 10 μl each of anti-CD16-FITC, anti-CD32-FITC and anti-CD64-FITC conjugate (*see* **Note 6**).
15. Wash and pellet cells once with 40 ml ice-cold PBS/1% BSA (300 × g, 5 min at 4°C).
16. Re-suspend cells to 1–2 × 10^6 cells/ml in ice-cold PBS/1%BSA.

3.1.4. Control FACS Staining for Cell Sorting

1. Use 1 ×10^6 cells transfected with huCD38 plasmid for control stainings to set and compensate the cell sorter:
   a. Unstained control marker (CD38) positive cells
   b. Unstained control marker (CD38) positive cells plus 7-AAD (0.25 μg/ml)
   c. Control marker (CD38) positive cells plus 1 μg anti-CD38-FITC conjugate
   d. Control marker (CD38) positive cells plus 1 μg anti-CD38-PE conjugate
   e. Control marker (CD38) positive cells plus blocked anti-CD38 phage-aM13Bio/SA-PE
   f. Control marker (CD38) positive cells plus blocked test phage-aM13Bio/SA-PE (use same blocked test phage preparation as used for bulk staining)
2. Staining with fluorochrome conjugates is performed according to manufacturer's recommendation, staining with control marker specific-phage (CD38) and test-phage preparations is analogous to the bulk cell staining described above.
3. Re-suspend cells to 1–2 × 10^6 cells/ml in ice-cold PBS/1%BSA.
4. Pass all stained cell suspensions over 40 μm cell strainer filters to prevent clogging of the cell sorter.

3.1.5. Cell Sorting

1. Add 7-AAD (5 μl per 10^6 cells) to all cells except control stain A (*see* **Note 8**).
2. Sort cells at 4°C on a Vantage DIVA sorter (Becton Dickinson) or equivalent using PBS as sheath fluid.
3. Use the control stained cells to set and compensate FL1 (FITC), FL2 (PE) and FL3 (7-AAD).
4. In a FSC/SSC dot plot set gate P1 around live cells to exclude dead cells and doublets.
5. In a FSC/FL3 (7-AAD) dot plot show cells through P1 and set P2 around 7-AAD /FL3 negative cells.

6. In a FL1/FL2 dot plot show cells through P2 and set P3 just above the control transfected cells that were stained with phage-aM13Bio/SA-PE (take care to avoid any cells showing background FITC (Fc receptor) signal, *see* **Note 6**).
7. Flush the sample port tube of the sorter with FACS-Clean and -Rinse before the actual sort to prevent contamination from control-PE positive cells.
8. Sort cells in P3 using 'Yield' or 'Purity' mode (*see* **Note 9**).
9. Collect 500–10 000 cells into a 1.5 ml Eppendorf tube containing 400 µl Hirt lysis buffer.
10. After sort mix contents well in Eppendorf tube and incubate 20 min at RT.
11. Adjust NaCl to a final concentration of 1 M (add 1/4 volume of 5 M NaCl).
12. Mix well (a white precipitate forms) and incubate O/N on ice.

3.1.6. Plasmid Rescue

1. Spin the extraction in a pre-cooled microcentrifuge 20 000 × g, 10 min at 4°C.
2. Transfer supernatant to a fresh 1.5 ml Eppendorf tube.
3. Extract with an equal volume P/C/I until inter-phase is clean (Once or twice).
4. Carefully transfer upper aqueous phase to fresh tube without disturbing the inter-phase.
5. Extract once with an equal volume of chloroform to remove traces of phenol.
6. Transfer aqueous phase to fresh tube.
7. Add 2 µl glycogen and 1/10 volume 3 M NaAc pH5.
8. Add 2.5 volumes 100% Ethanol p.a.
9. Mix well and incubate > 20 min at −20°C or store in freezer until further use.
10. Spin 20,000 × g, 15 min at 4°C.
11. Carefully remove ethanol.
12. Wash with 250 µl 75% Ethanol.
13. Spin 20,000 × g, 5 min at RT.
14. Completely remove ethanol and air-dry pellet 5 min at RT.
15. Dissolve plasmid DNA/glycogen pellet in 2.5–10 µl 10 mM TRIS (Use 1 µl per 1 000 sorted cells, but at least 2.5 µl).
16. Continue with transformation procedure or store at −20°C.

3.1.7. Transformation Procedure

1. Thaw ElectroMAX DH10B-T1res electrocompetent cells on ice, mix gently (*see* **Note 10**).
2. Add 1–2,5 µl plasmid DNA to a Eppendorf tube on ice (*see* **Note 11**).
3. Add 35 µl electro-competent DH10B bacteria, mix gently by flicking the tube.
4. Transfer to a pre-chilled 0.1 cm electro-poration chamber.

5. Pulse in Biorad Gene Pulser II (or equivalent): 2.5 kV (25 kV/cm), 25 μF, 100 Ohm; resulting Tc ~ 2.5 ms.
6. Immediately after pulse, flush cells from cuvette with 965 μl SOC medium and transfer to 15 ml tube.
7. Incubate at 37°C/220 rpm for 1 h.
8. Spread appropriate aliquots of transformed bacteria on 15 cm LB-Ampicillin agar plates using 3 mm glass-beads for evenly distributed colonies (*see* **Note 12**).
9. Incubate O/N at 37°C.

3.1.8. Plasmid Pool Screening

After the second round sorting using Lipofectamine transfected cells the frequency of the target plasmid clone in the DNA pool should be about 1:1500 if the experiment is successful (*see* **Note 3**).

1. Use 1 μl rescued plasmid (corresponding to 1 000 cells) from second round sorting (Lipofectamine transfection) for DH10B electroporation as described above.
2. On 5 × 15 cm LB-Amp plates spread 20 μl transformation mix + 200 μl SOC medium: this results in approximately 300 colonies per plate (A).
3. On 5 × 15 cm LB-Amp plates spread 40 μl transformation mix + 200 μl SOC medium: this results in approximately 600 colonies per plate (B).
4. On 3 × 15 cm LB-Amp plates spread 235 μl transformation mix: this results in approximately 7 000 colonies per plate (C).
5. Incubate plates overnight at 37°C.
6. Continue with the plasmid pool screening procedure or the matrix screening procedure.

3.1.9. Plasmid Pool Screening Procedure

1. Scrape the bacteria from plates C into 10 ml LB medium and use for plasmid isolation.
2. Use the bacteria from plates A and B for replica plating.
3. Place a *dry* 15 cm nitrocellulose membrane over the bacteria and transfer to a fresh 15 cm LB-Ampicillin agar plate, the bacterial colonies facing upwards.
4. Pre-wet another 15 cm membrane on a LB-Ampicillin agar plate and then place it over the bacteria containing membrane. Carefully peel off the top membrane and place it back on the agar plate, bacteria facing upwards. Number the replica plates (A1 to A5, B1 to B5) and grow the bacteria for several hours at 37°C.
5. Store one-half of the replica plates at 4°C, use the other half for plasmid isolation.
6. Scrape the bacteria from each membrane with 5 ml LB broth and isolate a total of 10 pools of plasmid DNA, using QIAprep miniprep columns.

7. Use plasmid pools A1 to A5, B1 to B5 and C for Fugene-6 transfection of HEK293T/17 cells in six well plates, including the human CD38 positive control plasmid (12 transfections).
8. Harvest and phage-stain the cells after 2 days as was described for the sort procedure. Typically one to three pools (and at least the C pool) will stain positive (*see* **Note 12**).

3.1.10. Matrix Screening Procedure

1. Pick all colonies of the smallest pool that results in a positive signal in FACS from the stored replica plate and clonally grow in several 96-well deep well plates, containing 500 µl LB-Ampicillin, in a shaking incubator O/N at 37°C.
2. Per plate collect 200 µl from each well of row A (Plate 1, pool A, 2.4 ml).
3. Repeat for rows B to H (Plate 1, pools B–H, 2.4 ml).
4. Repeat for columns 1–12 (Plate 1, pools 1–12, 1.6 ml).
5. Collect from each pool (A–H and 1–12) 200 µl, to generate complete plate pool (P1, 4 ml).
6. Repeat for each subsequent deep well plate (up to ten).
7. Store the remaining bacterial cultures at 4°C.
8. Pool 1 ml of all pools A (Plate 1, pool A; Plate 2, pool A etc.).
9. Repeat for all other pools, B–H and 1–12.
10. Thus, generate 20 pools (A–H, 1–12) plus plate pools 1 to X (P_1 to P_x).
11. Isolate plasmid DNA or QIAprep Spin Miniprep Kit from the bacterial pools (2–4 ml) using QIAprep miniprep columns.
12. Transfect HEK293T/17 cells with the plasmid pools and harvest after 48 h.
13. FACS stain transfected cells with test phage-aM13Bio/SA-PE.
14. Possible read out: P_3 positive, pool E positive, pool 6 positive.
15. The target plasmid is in well E6, plate 3.
16. Spread some of the saved bacteria (P_3, E6) on a fresh LB-Amp agar plate and grow O/N at 37°C.
17. Pick and grow several colonies in LB-Amp medium and isolate plasmid DNA with QIAprep miniprep kit.
18. Analyse cDNA insert by DNA sequencing and confirm the target via a final transfection/phage staining experiment.

3.2. Affinity Purification

3.2.1. Cell Surface Biotinylation

1. Wash 10^8 cells 3 times by re-suspending in 20 ml ice-cold physiological buffer and spinning for 10 min at 420-× g, 4°C (*see* **Note 13**).
2. Meanwhile thaw an aliquot of solubilised EZ-link-sulfo-NHS-LC-LC-Biotin, 25 mg/ml in H_20 (*see* **Note 14**).

3. Re-suspend the cells to a concentration of 2–3 $\times$ 10^7 cells/ml in 3 ml ice-cold physiological buffer.
4. Add 80 µl biotin (25 mg/ml in H_20) per 10^8 cells.
5. Incubate 60 min in end-over-end rotor at 5 rpm at 4°C (*see* **Note 15**).
6. Block unreacted biotinylated reagent by adding 3 ml 100 mM glycine buffer.
7. Incubate 30 min in end-over-end rotor at 5 rpm at 4°C.
8. Take a 10 µl sample and check the viability of the cells with trypan blue exclusion and a microscope (*see* **Note 16**).
9. Add the biotinylated sample to a tube containing 20 ml ice-cold physiological buffer and centrifuge for 5 min at 420 $\times$ g at 4°C.
10. Re-suspend the cells in 60 ml ice-cold physiological buffer and divide in three portions of 20 ml each, centrifuge for 5 min at 420 $\times$ g at 4°C and discard the supernatant.

3.2.2. Cell Extraction

1. Re-suspend the cells to a concentration of 3×10^7 cells/ml in the three different extraction buffers, Triton X-100 buffer, DOC buffer and RIPA buffer (*see* **Note 17**).
2. Homogenise the samples by pipetting up and down in these ice-cold extraction buffers. Incubate the suspensions for 30–60 min in ice water on a shaking platform.
3. Remove insoluble material by centrifugation in an Eppendorf centrifuge for 30 min at maximum speed at 4°C.
4. Collect the supernatants, add additional protease inhibitors (1 µl per 10^7 cells) and store on ice until further use or continue with the immuno-precipitation (*see* **Note 18**).

3.2.3. Immunoprecipitation

1. Pellet 100µl protein A beads by spinning for 5 min at 700 $\times$ g at 4°C.
2. Wash the beads three times with 1 ml PBS and re-suspend in 100 µl PBS (*see* **Note 19**).
3. Pre-clear the supernatants of the different extractions with 50 µl beads by incubating them for 2 h at 4°C in an end-over-end rotor at 5 rpm.
4. Pellet the beads by spinning for 5 min at 700 $\times$ g at 4°C and transfer the supernatant using a 'Gel saver tip' (Bio plastics) to a clean Eppendorf tube.
5. Couple 4 µg of the IgG of interest, and a negative control IgG to 5 µl beads in a volume of 300 µl, in a 500 µl Eppendorf tube (*see* **Note 20**).
6. Wash the IgG coupled beads three times by spinning for 5 min at 700 $\times$ g at 4°C and re-suspend in 500 µl extraction buffer. Remove supernatant.
7. Add the pre-cleared supernatant derived from 10^7cells to the beads, re-suspend and incubate for 2 h at 4°C in an end-over-end rotor at 5 rpm.

8. Wash the beads three times by spinning for 5 min at 700 × g and re-suspend in 500 µl extraction buffer, followed by one wash in PBS. Remove supernatant.
9. Carefully remove all residual buffers using a Hamilton syringe.
10. Add 60 µl 1×SDS sample buffer and boil the sample for 10 min at 70°C. (*see* **Note 21**).
11. Load 20 µl of the samples on a NuPAGE Bis-Tris 4–12% gradient gel and run the gel according to manufacturer's instructions. Subsequently, electroblot the proteins on PVDF membrane according to manufacturer's instructions.
12. Store immuno-precipitation material that is left at this stage on ice until further use (*see* **Note 22**).

3.2.4. Immunodetection

1. Incubate PVDF membranes for 1 h at RT with block buffer (*see* **Note 23**).
2. Incubate the blots for 1 h at RT with horseradish peroxidase-conjugated streptavidin (Amersham), diluted 1:5 000 in block buffer.
3. Wash the blots three times with TBST and detect bound peroxidase by enhanced chemiluminescence according to the manufacturer's instructions (*see* **Note 24**).
4. Compare the pattern of biotinylated proteins of the antibody of interest with the pattern obtained with the negative and positive controls. In a successful immuno-precipitation the protein pattern observed can clearly be distinguished form the protein patterns observed with the controls. Expect multiple protein bands when a protein complex is precipitated (*see* **Note 25**).
5. Select the extraction buffer that results in the clearest protein pattern.

3.2.5. Establishing Wash and Elution Conditions

1. *Establish the optimal wash buffer to be used.* Perform three identical immuno-precipitations as described, in the selected extraction buffer. Before boiling the samples in SDS-PAGE buffer, perform three additional washes with one of each of the extraction buffers that contain detergents with an increasing harshness (Triton X-100-, DOC- and RIPA-buffer). Analyse the samples on Western blot for the appearance of biotinylated bands. Select the wash buffer that results in the clearest protein pattern (*see* **Note 26**).
2. *Establish the optimal NaCl concentration in the extraction buffer to be used.* Perform five identical immuno-precipitations. Before boiling the samples in SDS-PAGE buffer, wash the immuno-precipitations three times with the wash buffer (Triton X-100-, DOC- or RIPA-buffer), established in the previous point, containing an increasing

concentration of NaCl (0.2, 0.4, 0.6, 0.8 and 1 M). Analyse the samples on Western blot for the appearance of biotinylated bands. Select the high salt wash buffer that results in the clearest protein pattern (*see* **Note 26**).

3. *Establish the optimal elution conditions.* Perform two identical immuno-precipitations. Elute the bound proteins with 24 µl elution buffer of a low pH (glycine buffer, pH 2.7) or a high pH (lysine buffer, pH 11) by incubating the beads for 10 min in this buffer at room temperature. Spin down the beads, 1 min at 4 000 × g. Transfer the eluate to a clean Eppendorf and neutralise both samples by adding 24 µl 2 M Tris buffer pH 8.4. Add 12 µl 5 × SDS sample buffer to the eluate and 60 µl 1 × SDS sample buffer to the protein A beads and boil the sample for 10 min at 70°C. Analyse the samples on Western blot for the appearance of biotinylated bands. Select the buffer that results in the highest release of proteins (*see* **Note 27**).

3.2.6. Preparation of Cell Extracts for Large-Scale Affinity Purification

1. Culture a large batch of cells, $>5 \times 10^9$ cells in total (*see* **Note 28**).
2. Wash the cells twice in 200 ml ice-cold PBS per 10^9 cells.
3. Re-suspend the cells in extraction buffer at a concentration of 3×10^7 cells/ml.
4. Incubate the cells for 60 min on ice on a shaking platform.
5. Remove the insoluble material by centrifugation for 30 min at 20 000 × g on 4°C.
6. Pass the cell lysates through a 0.22 µm bottle top filter and store on ice until further use (*see* **Note 29**).

3.2.7. Large-Scale Affinity Purification

1. Purify the IgG of interest and control IgG over the 100 kDa ultra-centrifugal device to remove incomplete IgG fragments.
2. Couple the IgG of interest and control IgG each to 5 ml of CNBr-activated Sepharose CL-4B beads, according to manufacturer's recommendations.
3. Block 60 ml of CNBr-activated Sepharose CL-4B beads with 1 M ethanolamine, according to manufacturer's recommendations.
4. Divide the cell lysates in 50 ml tubes.
5. Pre-clear the lysate by adding 10 ml blocked CNBr-activated beads to each 50 ml tube and incubate at 4°C for 2 h in an end-over end rotor at 5 rpm.
6. Pellet the beads by spinning them for 30 min at 3 000 × g at 4°C and transfer the pre-cleared lysate to a clean tube.
7. Incubate the pre-cleared lysate with 5 ml CNBr-activated beads that were coupled to a negative control IgG for 2 h at 4°C in an end-over-end rotor at 5 rpm.

8. Pellet beads as before, remove pre-cleared lysate and filter the pre-cleared lysate through a 0.22 μM filter bottle top filter on ice.
9. Load the CNBr-activated Sepharose CL-4B beads coupled to the IgG of interest in a XK25 26/10 column (*see* **Note 30**) and connect the column to an AKTA FPLC 900, or similar piece of equipment.
10. Equilibrate the column with 30 ml of the optimal extraction buffer at 2 ml/min.
11. Apply the pre-cleared lysate at 1 ml/min.
12. Following loading the column, wash the column with five column volumes of extraction buffer and collect the flow-through.
13. Wash the column with five column volumes of the optimal wash buffer and collect the flow-through.
14. Wash the column with five column volumes of the optimal high salt wash buffer and collect the flow-through.
15. Wash the column with five column volumes of TX-100 buffer and collect the flow-through (*see* **Note 31**).
16. Elute the column with five column volumes of the optimal elution buffer.
17. Collect 0.5 ml fractions in tubes containing 1 μl protease inhibitor cocktail and 100 μl 1 M Tris-HCl Tris Base pH 8.1 (when elution is performed in glycine buffer pH 2.7) or 50 μl 1 M citric acid (when elution is performed in lysine buffer pH 11).
18. Put each 0.5 ml each fraction immediately on ice.
19. Analyse 15 μl volumes of the fractions and of the flow-through of different steps on a SDS-PAGE 4–12% gradient NuPage gel.
20. Stain the gel with a silver stain compatible with MS analysis such as Silverquest (Invitrogen), according to manufacturer's instructions.
21. Select the eluted fraction showing the expected protein profile as determined in the optimisation experiments.
22. Excise the purified protein bands from the gel.
23. Analyse the bands by mass spectrometry.
24. Compare the resulting peptide tag with databases, for example, http://www.ncbi.nlm.nih.gov/BLAST/
25. Confirm the identified antigen by expressing the cognate cDNA fused to a tag sequence and subsequent immunodetection (*see* **Note 32**).

4. Notes

1. The selected host cell line to be used should stain negative for the target protein. We selected the HEK293T/17 cell line for it's high transfection efficiency characteristic. The

presence of the SV40 large-T antigen was of no effect in our experiments since the used library vector (pSPORT) did not contain a SV40 ori. The use of this cell line with a SV40 ori containing library vector or the use of a cell line other than HEK293T/17 will most probably affect the parameters described in this protocol.

2. Select any eukaryotic full-length cDNA library isolated from tissue expected to express the target molecule. A pre-screen staining of library-transfected (Fugene-6) versus untransfected target cells can be performed to assess the presence of the target plasmid in the cDNA expression library. We observed signals ranging from 0.05% up to 10% (CD38) using our phage panel in a pre-screen experiment. In case a pre-screen staining of 1% or more is found, one might consider proceeding directly to a 'second round' Lipofectamine transfected sort, followed by the plasmid pools screening procedure.
3. During our experiments, we have found a difference in plasmid copy number per cell after transfection with either Fugene-6 or Lipofectamine reagent. Approximately 5,000 copies were present per cell after Fugene-6 transfection and around 1,500 copies per cell when using the Lipofectamine method. As a consequence of this, each positive cell contains only one copy of the target plasmid within a pool of, respectively, 5,000 or 1,500 irrelevant plasmid molecules. To enlarge the total coverage of the cDNA library during the pre-screening procedure and first round cell sorting we prefer to use the Fugene-6 transfection method. For second round sorting we choose to use the Lipofectamine reagent, thereby increasing the frequency of the target plasmid to 1:1 500. For the screening of second round plasmid pools, we prefer the more convenient Fugene-6 protocol.
4. A 48-h incubation of the transfected cells is chosen to obtain the maximal expression level of the target protein. Longer incubation times may result in pseudo-transfected cells, that is, cells still expressing the protein on the surface whilst having lost the target plasmid after cell division.
5. The proteolytic activity of trypsin might result in a decrease or even loss of the target epitope. To avoid any of these problems we choose to use EDTA to detach the transfected cells from the culture surface. The EDTA treatment of HEK293T/17 cells that were transfected with the Lipofectamine reagent is difficult, for this reason we split the cells 24 h after transfection using trypsin to detach and culture the cells in two T75 culture flasks for another 24 h.
6. The human bone marrow cDNA library contains the coding genes for Fc-receptors (CD16, CD32, CD64), which will bind the antibodies used for detection, resulting in false

positive PE signals. To block these receptors, human serum is added. In addition, FITC conjugated anti-CD16, CD32 and CD64 are used to pull the Fc-receptor expressing cells into the FL1 channel, which will prevent isolation of these cells and genes.

7. Avoid the presence of culture media in staining procedures that use streptavidin since most media contain biotin that will interfere with the staining protocol.
8. We often observe up to about 50% dead cells, probably due to the EDTA detachment method and/or the presence of toxic protein products expressed from the cDNA library.
9. Use 'Yield' mode (allowing contaminating cells in the 'sort envelope') for first round cell sorting showing < 0.1% phage positive cells. If a higher percentage of positive cells is observed use the 'Purity' mode (which will discard 'sort envelopes' with contaminating cells). Be aware that any contaminating cell will contribute to 1,500–5,000 extra irrelevant plasmid molecules (**Note 2**).
10. The use of DH10B bacteria is crucial because its genotype is permissive for eukaryotic methylated DNA.
11. Use the complete plasmid preparation for first round cloning, use as many aliquots of competent bacteria as required.
12. Expect to recover 15 (Lipofectamine) to 50 (Fugene-6) colonies per sorted cell. Since we estimated that transfected cells contain 1,500–5,000 plasmid copies per cell (*see* **Note 3**), the plasmid rescue efficiency appears to be about 1%. The latter observation implies that from 1,000 sorted cells only 10 target plasmids will be retrieved.
13. The cell line used in this method should express a high density of the target antigen. Therefore, several cell lines should be tested for their reactivity with the antibodies in FACS. For the final selection, a cell line that grows in suspension is preferred, since these cells can be more conveniently grown to larger volumes.
14. The EZ-link-sulfo-NHS-LC-LC cannot cross the cell membrane and contains a long spacer arm of 30 angstrom that will reduce the probability of steric hindrance. Dissolve biotin in water to an end-concentration of 25 mg/ml and store in aliquots at −20°C.
15. With this procedure, only surface exposed proteins are labelled and fragile cells can survive. Biotinylation at higher temperatures up to room temperature with robust cells, results in an increased biotinylation of the membrane proteins, partly due to endocytic turnover.
16. When >20% of the cells are dead the contribution of biotinylated intracellular proteins becomes significant in the immuno-precipitation data.

17. The choice of extraction buffers is extremely important in the immuno-precipitation experiments. The buffer must not only lyse the cells, but should also effectively solubilize the antigen of interest without denaturing or altering its immuno-reactivity. Therefore, three different buffers are tested with increasing stringency that will increase the probability of solubilizing the antigen of interest. However, this will also increase the probability of denaturing the antigen.
18. Freeze thawing of biotinylated lysates will result in less reactivity. During storage on ice, precipitates may form and these have to be removed before continuing with the next step of the protocol.
19. The pellet of beads is very loose, remove the first 800 µl with a 1 ml tip and the rest of the fluid with Gel saver Tips (Bio plastic).
20. For successful immuno-precipitations using these procedures, the antibodies should have an affinity of at least 10^{-8} M. Immuno-precipitations with low affinity antibodies might succeed by increasing the avidity of the beads. Therefore, coupling 20–100 µg of IgG should increase the concentration of antibody molecules on the beads.
21. Start analysing the immuno-precipitations on a non-reduced gel. If reactive bands appear, analyse the same sample under reducing conditions.
22. Do not store material under freezing conditions, since freeze thawing effects the reactivity of the lysates.
23. Do not use milk powder-based blocking buffers as these contain biotin.
24. Black or white bands can appear at the size of 150 kDa. When these bands also appear in the negative control lane this is the result of IgG coming off the beads.
25. The use of different extraction buffers can result in the appearance of different protein profiles, these can vary between one prominent single protein band and multiple protein bands.
26. Wash buffers that contain detergents or NaCl concentration with increasing harshness, wash away impurities that can disrupt heteromeric protein complexes. As a result, fewer proteins can appear in the immuno-precipitation and the target protein becomes more distinct.
27. Expect not all protein to be released from the protein A beads.
28. Preparation of a large cell lysate is required to obtain enough material and increase the success of the procedure. If it is not possible to culture cells in these quantities at once, cells can also be cultured and extracted in portions that are combined to 5×10^9 cells prior to use.

29. Filter the lysates in portions of 100 ml or less, since lysates will block the 0.22 μM filters.
30. To prevent clogging of the column a diameter of > 25 mm is required.
31. DOC and SDS precipitate at a low pH, therefore, it is essential to remove all traces of SDS and DOC from the column prior to elution with glycine buffer.
32. To confirm the identification of the antibody target, immuno-detection of recombinant expressed protein should be performed. Depending on the antigen identified, FACS staining or immuno-precipitation can be used. In immuno-precipitations, cells transfected with a tagged version of the identified target will be required.

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Chapter 12

Membrane Protease Degradomics: Proteomic Identification and Quantification of Cell Surface Protease Substrates

Georgina S. Butler, Richard A. Dean, Derek Smith, and Christopher M. Overall

Abstract

The modification of cell surface proteins by plasma membrane and soluble proteases is important for physiological and pathological processes. Methods to identify shed and soluble substrates are crucial to further define the substrate repertoire, termed the substrate degradome, of individual proteases. Identifying protease substrates is essential to elucidate protease function and involvement in different homeostatic and disease pathways. This characterisation is also crucial for drug target identification and validation, which would then allow the rational design of specific targeted inhibitors for therapeutic intervention. We describe two methods for identifying and quantifying shed cell surface protease targets in cultured cells utilising Isotope-Coded Affinity Tags (ICAT®) and Isobaric Tags for Relative and Absolute Quantification (iTRAQ™). As a model system to develop these techniques, we chose a cell-membrane expressed matrix metalloproteinase, MMP-14, but the concepts can be applied to proteases of other classes. By over-expression, or conversely inhibition, of a particular protease with careful selection of control conditions (e.g. vector or inactive protease) and differential labelling, shed proteins can be identified and quantified by mass spectrometry (MS), MS/MS fragmentation and database searching.

Key words: Protease, membrane protease, matrix metalloproteinase, MMP, degradome, ICAT, iTRAQ, shedding, protease substrate identification, quantitative mass spectrometry.

1. Introduction

The matrix metalloproteinases (MMPs) are a family of 23 extracellular zinc-dependent endopeptidases implicated in a number of physiological and pathological processes such as cancer and inflammation *(1)*. Classically, degradation of extracellular matrix proteins has been the sole function attributed to MMPs, but in

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recent years it has become apparent that these enzymes process a wide-range of bioactive molecules, both soluble (e.g. chemokines and growth factor binding proteins) *(1)* and cell surface molecules (such as receptors and cell-adhesion molecules) *(2)*. Four members of the MMP family are type-I membrane proteins, and type two are GPI-anchored, so implicating them in shedding cell surface and pericellular target proteins. However, the full substrate repertoire, or degradome, of individual members of the MMP family remains largely unknown. This is a confounding factor for the treatment of diseases such as cancer with MMP inhibitor drugs, since blockade of the processing of undetermined substrates can have unpredictable outcomes, including toxicity and side-effects.

Proteomics offers the opportunity to screen for protease substrates in complex biological systems *(3)*. We describe two labelling protocols, Isotope-Coded Affinity Tag (ICAT) and Isobaric Tags for Relative and Absolute Quantification (iTRAQ) that are available in kit form (Applied Biosystems Inc.). These reagents allow the identification and quantification of up to 1,000 proteins in two or more complex samples, without the need for 2D-gel electrophoresis, where the isoelectric focussing dimension is not suitable for very hydrophobic membrane proteins. The workflows for ICAT and iTRAQ are compared in **Fig. 12.1**.

The ICAT reagent consists of an acid-cleavable biotin tag, a linker containing nine carbon-12 (light reagent) or nine carbon-13 (heavy reagent) atoms and a thiol reactive group *(4, 5)*. The latter covalently reacts with cysteine residues. Isotopically heavy and light-labelled protein samples are combined and digested to peptides with trypsin. Excess reagents, trypsin in particular, are removed by cation exchange, then cysteine-labelled biotin-tagged peptides are isolated by avidin-affinity chromatography and the biotin-tag is released by acid-cleavage. These chromatography steps reduce the complexity of the sample, which improves coverage of the proteome and therefore, experimental reproducibility. Purified labelled cysteine-containing peptides are separated by microcapillary high-performance liquid chromatography (HPLC) and subjected to MS. Each peptide labelled with heavy or light reagent is chemically identical, but the 9 Da mass difference is resolved by MS and the ratio of signal intensity of heavy: light labelled peptide provides relative quantification of each peptide in the 2 samples. Each peptide undergoes collision-induced dissociation (CID), that is, the peptide is fragmented, during tandem mass spectrometry (MS/MS). The fragment ion spectrum allows identification of the amino acid sequence, whereupon the protein from which the peptide originated can be identified by database searching.

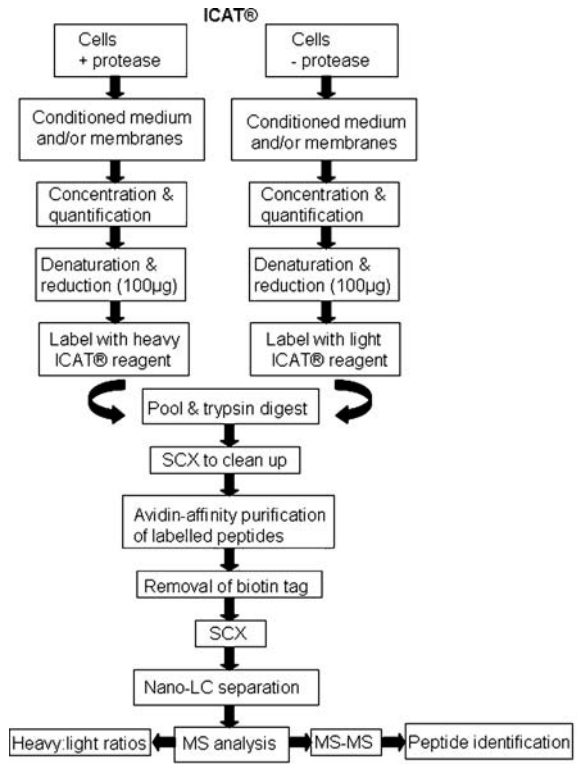

Fig. 12.1. Schematic representation of protocol for analysis of membrane protease substrates by ICAT.

In contrast to ICAT, iTRAQ labels the NH_2-terminus (and lysine side chains) of *all* tryptic peptides prior to combining the samples *(6)*. The iTRAQ tag consists of an amine-specific peptide reactive group (NHS) linked to an isobaric tag that consists of a balance group and a reporter group. The reporter group varies between the different tags – currently there are eight different reagents, allowing the simultaneous quantification of peptides from up to eight different samples – masses of the reporter are 114.1, 115.1, 116.1, 117.1 (4-plex), plus 113.1, 118.1, 119.1 and 121.1 (8-plex), and the balance varies accordingly to maintain the mass of the tag at 145.1 Da, thus ensuring identical behaviour of tagged peptides during chromatography and in single MS mode. Protein samples are reduced, alkylated and digested with trypsin. All tryptic peptides are labelled with iTRAQ reagent (a different reagent for each sample), then pooled and fractionated by strong cation-exchange HPLC. A labelled peptide present in two or more samples appears as a single precursor ion by MS, due to the consistent mass of the iTRAQ tag. Quantification is achieved following MS/MS when the reporter ion fragments along with the peptide, thus, allowing relative quantification and amino acid sequencing of the peptide from each

sample, which can then be matched to its parent protein by database searching.

As a model system, we have used ICAT to identify substrates of MMP-14 that were shed from the cell membrane or pericellular environment to the conditioned medium of cultured human breast cancer cells transfected with MMP-14 compared with vector or an inactive MMP-14 mutant *(7)*. Similarly we have identified shed substrates of MMP-2 in conditioned media from cultured *Mmp2–/–* murine fibroblasts transfected with low levels of active MMP-2 compared with a catalytically inactive mutant by ICAT *(8)* and at different time points by iTRAQ *(9)*. We have also utilised a MMP inhibitor drug to block shedding activity and have identified shed substrates that decrease in conditioned medium and increase in membrane preparations *(10)*.

2. Materials

Warnings are given for hazardous materials, but Material Safety Data Sheets (MSDS) should be read prior to starting the protocol. MSDS information can be obtained at:
http://www.piercenet.com/resources/browse.cfm?RequestTimeOut=180&fldID=449 and https://docs.appliedbiosystems.com/msdssearch.html.

2.1. Cell Culture

1. Culture medium, serum, supplements and selection reagents appropriate for cell-type used.
2. Phosphate buffered saline (PBS).
3. Phenol red free, serum free DMEM (Gibco BRL).

2.2. Sample Collection and Preparation

2.2.1. Protease Inhibitors

1. Protease Inhibitor Stocks
2. 50 mM phenylmethylsulfonyl fluoride (PMSF)
3. 100 mM EDTA
4. 1 mM leupeptin
5. 10 mM pepstatin A

2.2.2. Concentration of Conditioned Medium

1. 0.2 μm pore size filtration unit
2. Concentrators with 3 or 5 kDa cut-off membrane (Centriprep and Microcon, Amicon) OR
3. C4 and C18 SPE cartridges, 3 ml (Grace VYDAC)
4. Acetonitrile (ACN)
5. 100% trifluoroacetic acid (TFA)

6. 50 mM Hepes, pH 8.0
7. 10% (w/v) sodium dodecyl sulfate (SDS)
8. 8 M deionised urea

2.2.3. Preparation of Membrane-Enriched Fraction

1. Versine:140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4. $7H_2O$, 1.8 mM NaH_2PO_4, 0.5 mM EDTA, 11 mM glucose, pH 7.4
2. Membrane buffer: 50 mM Tris, pH 6.8, 200 mM NaCl, 10 mM $CaCl_2$
3. Cell disruption bomb (Parr Instrument Co., Moline, Illinois)
4. Nitrogen gas supply
5. Membrane resuspension buffer: 200 mM Tris, pH 8.8, 5 mM EDTA, 6 M urea, 0.05% SDS
6. Homogeniser (Polytron)

2.3. Protein Quantification

1. Bicinchoninic Acid (BCA) protein assay kit (Pierce)

2.4. Isotope-Coded Affinity Tag (ICAT) Labelling

MSDS information on constituents of the Applied Biosystems ICAT kit can be obtained at https://docs.appliedbiosystems.com/msdssearch.html.

2.4.1. Protein Denaturation, Reduction, Labelling and Trypsin Digestion

1. Acetone
2. Acetonitrile (ACN)
3. Cleavable ICAT reagent assay kit (Applied Biosystems) containing:
4. Denaturing Buffer, pH 8.5 (contains 50 mM Tris and 0.1% SDS)
5. Reducing reagent (contains 50 mM TCEP)
6. Heavy and light cleavable ICAT reagents
7. Trypsin (TPCK-treated)

2.4.2. Sample Clean Up by Cation Exchange

1. Cation-exchange loading buffer: 10 mM KH_2PO_4, 25% ACN, pH 3.0
2. Cation-exchange elution buffer: 10 mM KH_2PO_4, 25% ACN, pH 3.0, 0.35 M KCl
3. Cation-exchange cleaning buffer: 10 mM KH_2PO_4, 25% ACN, pH 3.0, 1 M KCl
4. Cation-exchange storage buffer: 10 mM KH_2PO_4, 25% ACN, pH 3.0 + 0.02% NaN_3
5. Cation-exchange cartridge (POROS®50, 50 μM particle size, 4.0 × 15 mm)

2.4.3. Purification of Labelled Peptides and Biotin Tag Removal

1. Affinity elution buffer: 0.4% TFA, 30% ACN
2. Affinity loading buffer: 2X PBS, pH 7.2
3. Affinity wash buffer 1: 1X PBS, pH 7.2
4. Affinity wash buffer 2: 50 mM ammonium bicarbonate, 20% methanol, pH 8.3
5. Affinity storage buffer: 2X PBS, pH 7.2, 0.02%NaN_3

6. Avidin-affinity cartridge (4.0 × 15 mm)
7. Cleaving-reagents A and B

2.5. Isobaric Tags for Relative and Absolute Quantification (iTRAQ) Labelling

MSDS information on constituents of the Applied Biosystems iTRAQ kit can be obtained at https://docs.appliedbiosystems.com/msdssearch.html.

2.5.1. Protein Denaturation, Reduction, Trypsin Digestion and iTRAQ Labelling

1. Acetone
2. iTRAQ labelling kit (Applied Biosystems) containing dissolution buffer, denaturant, Tris (2-carboxyethyl) phosphine-hydrochloride (TCEP) (reducing agent), methyl methanethiosulfonate (MMTS) (cysteine modification), iTRAQ reagents
3. Sequencing grade modified trypsin (Promega)
4. 0.5 M triethylammonium bicarbonate (supplied as a liquid)
5. Ethanol

2.6. Multi-Dimensional Liquid Chromatography for Fractionation of Labelled Peptides

2.6.1. Strong Cation Exchange (SCX)

1. SCX loading buffer: 10 mM KH_2PO_4, pH 2.7, 25% ACN
2. SCX elution buffer: 10 mM KH_2PO_4, pH 2.7, 25% ACN, 0.5 M KCl
3. Polysulfoethyl A, 100 mm × 4.6 mm, 5 μm-300 Å strong cation exchange column (Poly LC Inc.)
4. HPLC instrument

2.6.2. Nano-LC Separation

1. Solvent A (2% ACN, 0.1% formic acid)
2. Solvent B (98% ACN, 0.1% formic acid)
3. PepMap^{TM} C18 nano-trapping column, 300 μm ID × 5 mm (Dionex)
4. PepMap^{TM} C18 nano-separation column, 75 μm ID × 15 cm (Dionex)
5. HPLC instrument capable of nano (<1 μl/min) flow rates
6. Nanospray ionisation source (New Objective Inc.)

2.7. MS Analysis of Labelled Peptides

1. Electrospray Qstar Pulsar i Quadrupole Time-of-Flight Mass Spectometer (Applied Biosystems/Sciex) or similar tandem mass spectrometer capable of analysis to 100 m/z, with HPLC connected inline to permit analysis of peptides directly from the column
2. Analyst QS software (Applied Biosystems/MDS Sciex)

3. ProteinPilot™ software (Applied Biosystems)
4. MASCOT software (Matrix Science)

3. Methods

3.1. Cell Culture

1. Grow cells (*see* **Note 1**) in culture medium in roller bottles (*see* **Note 2**) or T175 tissue culture flasks so that cells will be 80–90% confluent at the time of medium collection (*see* **Note 3**).
2. Wash cells three times with PBS and grow overnight in serum-free culture medium (*see* **Note 4**).
3. Repeat extensive washing of cells with PBS and culture in serum-free, phenol-red free culture medium (*see* **Note 5**) for the desired time before collection of conditioned medium and cell membranes.

3.2. Sample Collection and Preparation

3.2.1. Conditioned Medium

1. Harvest conditioned medium and immediately add protease inhibitors (final concentrations 0.5 mM PMSF, 1 mM EDTA, 10 μM leupeptin, 10 μM pepstatin A) (*see* **Note 6**). Keep the conditioned medium cool (4°C) or on ice from this point on.
2. Clarify conditioned medium by centrifugation to remove any cells (500 × g, 5 min) and filter through 0.2 μm pore size to remove particulate matter (*see* **Note 7**).

3.2.2. Concentration of Conditioned Medium

This protocol uses C4 and C18 hydrophobic resins that allow fast and simple concentration of large samples of conditioned medium (*see* **Note 8**). Aim for a final protein concentration of 1–2 mg/ml.

1. Using gravity flow, condition C4 and C18 cartridges with 2 ml of 100% ACN and equilibrate with 5 ml of 0.1% TFA in ddH_2O (*see* **Note 9**).
2. Adjust conditioned medium samples to 0.1% TFA.
3. Connect C4 and C18 columns in tandem and load 30 ml of sample onto the C4 column with the flow through passing onto the C18 column.
4. After 30 ml of sample has been loaded, disconnect C4 and C18 columns and wash separately with 4 ml of 0.5% ACN/0.1% TFA in ddH_2O (*see* **Note 10**).
5. Re-equilibriate both columns separately with 4 ml of 0.1% TFA in ddH_2O and repeat Steps 3–5 until all sample has been loaded.

6. Wash columns separately with 4 ml of 0.5% ACN/0.1% TFA in ddH_2O.
7. Elute proteins from each column with 1 ml 75% ACN, 0.1% TFA/ddH_2O into 1.5 ml microcentrifuge tubes (*see* **Note 11**).
8. Combine C4 and C18 eluates and reduce to ~50 µl by centrifugation under vacuum (e.g. in a Speedvac).
9. Dilute to 500 µl with 50 mM Hepes pH 8.0 (*see* **Note 12**). If any precipitate is observed, add SDS to a final concentration of 0.2% and sonicate briefly in a sonicating bath. If precipitate does not resolubilize, add an equal volume of deionised 8 M urea (*see* **Note 13**).

3.2.3. Preparation of Membrane-Enriched Fraction

1. Pre-chill N_2 bomb with ice.
2. Following PBS washes, detach cells using Versene (*see* **Note 14**).
3. Centrifuge cells (500 × g, 5 min) in a 50 ml Falcon tube, resuspend in 20 ml cold membrane buffer and centrifuge again.
4. Resuspend cells in 20 ml membrane buffer containing protease inhibitors as above (**Section 3.2.1**).
5. Fill cell disruption bomb with fresh ice and place Falcon tube with cells inside, assemble the N_2 bomb with lid making sure the outlet tube extends into the Falcon tube.
6. Pressurise bomb to 500 psi using N_2. Shut off valves, disconnect tubing, and incubate cells for 30 min, making sure pressure is maintained.
7. Open outlet valve gently and release lysed cells into a tube or small tissue culture flask (*see* **Note 15**).
8. Centrifuge the cell lysate (1,100 × g, 10 min) at 4°C.
9. Transfer supernatant to a suitable tube and centrifuge the supernatant (48,000 × g, 60 min) at 4°C (*see* **Note 16**).
10. Resuspend the pellet in freshly prepared membrane resuspension buffer (1–3 ml) and homogenise (Polytron 11,500 rpm, 5 s) (*see* **Note 17**).

3.3. Protein Quantification

1. Determine the protein concentration of concentrated conditioned medium or membrane preparations using the microscale protocol supplied with the bicinchoninic acid (BCA) protein assay kit.

3.4. Isotope-Coded Affinity Tag (ICAT) Analysis

A comprehensive protocol is supplied by Applied Biosystems with the Cleavable ICAT Reagent kit. It is recommended to run through the procedure with a test protein prior to embarking upon complex samples. The procedure is described below for two samples that are labelled with heavy $[^{13}C]_9$ or light $[^{13}C]_0$ cleavable ICAT reagents.

3.4.1. Acetone Precipitation

1. Add 10 volumes pre-chilled (−20°C) acetone to 100 μg of sample on ice, mix gently and incubate overnight at −20°C or −70°C to precipitate proteins.
2. Centrifuge (16,000 × g, 15 min) at 4°C (a small pellet should be visible). Carefully pour off acetone and allow pellet to air dry until just moist but not powder-dry (*see* **Note 18**).

3.4.2. Protein Denaturation and Reduction

The proteins are denatured and reduced to expose the cysteine residues for labelling.

1. Resuspend pellet in 80 μl denaturing buffer.
2. Add 2 μl reducing reagent, vortex and spin briefly.
3. Incubate in a boiling water bath for 10 min, vortex and spin briefly (*see* **Note 19**).

3.4.3. ICAT Labelling

1. Resuspend each vial of cleavable ICAT reagent in 20 μl ACN.
2. Add test and control sample to either heavy or light ICAT reagent, vortex and spin briefly.
3. Incubate 2 h at 37°C, vortex and spin.

3.4.4. Trypsin Digestion

1. Resuspend a vial of TPCK-treated trypsin (supplied in kit) in 200 μl ddH_2O.
2. Combine the labelled samples in one of the sample vials.
3. Rinse the empty sample vial with the trypsin and transfer all to tube containing the combined samples, vortex and spin.
4. Incubate for 18 h at 37°C, vortex and spin.

3.4.5. Sample Clean Up by Cation Exchange

To remove the trypsin, TCEP, SDS and unincorporated ICAT label from the peptides, the sample is cleaned on a cation-exchange column (*see* **Note 20**).

1. Equilibrate the cation-exchange cartridge (supplied with the kit) with 2 ml cation-exchange loading buffer.
2. Dilute the labeled samples with cation-exchange loading buffer (2 ml). Ensure pH is between 2.5 and 3.3 using pH paper, if not, add more loading buffer.
3. Load the sample slowly (1 drop/s).
4. Wash with 1 ml loading buffer.
5. Slowly elute peptides as a single fraction with 500 μl cation-exchange elution buffer.
6. Clean the cartridge with cation-exchange cleaning buffer and store in cation-exchange storage buffer at 4°C.

3.4.6. Purification of Labeled Peptides by Avidin Affinity Chromatography

Labeled peptides are captured and separated from unlabelled peptides by avidin-affinity chromatography via the biotin tag. Following elution of labeled peptides, the biotin tag is removed using acid.

1. Inject 2 ml affinity elution buffer onto avidin cartridge (supplied with kit) to liberate low-affinity binding sites.
2. Equilibrate the cartridge with 2 ml affinity loading buffer.
3. Neutralise cation-exchange fraction(s) with 500 µl affinity loading buffer.
4. Inject neutralised fraction slowly (1 drop/s) onto avidin cartridge.
5. Wash cartridge with 500 µl affinity loading buffer.
6. Wash cartridge with 1 ml affinity wash buffer 1 (reduces salt concentration)
7. Wash cartridge with 1 ml affinity wash buffer 2 (removes non-specifically bound peptides)
8. Wash cartridge with 1 ml ddH_2O.
9. Inject 50 µl affinity elution buffer (discard eluate).
10. Inject 750 µl affinity elution buffer and collect eluate (contains labeled peptides).
11. Dry peptides by centrifugation under vacuum.
12. Repeat Steps 1–11 for subsequent fractions (*see* **Note 21**).
13. Clean the cartridge with 2 ml affinity elution buffer and store in affinity storage buffer at 4°C.

3.4.7. Removal of the Biotin Tag

1. Combine 95 µl cleaving-reagent A with 5 µl cleaving-reagent B, vortex and spin.
2. Use the freshly prepared cleaving-reagent to resuspend the labeled peptides, vortex and spin.
3. Incubate 2 h 37°C.
4. Spin and evaporate to dryness by centrifugation under vacuum.
5. Redissolve each dried fraction in 5 ml SCX loading buffer.
6. Proceed to **Section 3.6** Multi-dimensional Liquid Chromatography of labeled peptides.

3.5. Isobaric Tags for Relative and Absolute Quantification (iTRAQ) Labelling and Analysis

Up to eight samples can be labelled with eight different iTRAQ tags according to the manufacturer's instructions. The protocol supplied by Applied Biosystems is summarised below with optimisation for labelling of proteins from conditioned medium.

3.5.1. iTRAQ Labelling of Sample

1. Acetone precipitate 100 µg of each sample as described in **Section 3.4.1**.
2. Resuspend pellet in 30 µl iTRAQ Dissolution Buffer using 1–2 µl iTRAQ Denaturant, to completely dissolve the precipitate.
3. Reduce proteins with 3.5 mM TCEP at 60°C for 1 h (*see* **Note 22**) and rapidly spin sample to bottom of tube.
4. Block cysteine residues with 6.7 mM MMTS at room temperature for 10 min.

5. Digest the proteins with sequencing grade modified trypsin (1:10 w/w, i.e. 10 μg trypsin for 100 μg protein) in 0.5 M triethylammonium bicarbonate overnight at 37°C.
6. Dry digests by centrifugation under vacuum and resuspend in 30 μl 0.5 M triethylammonium bicarbonate.
7. Dissolve each vial of iTRAQ label in 70 μl ethanol.
8. Add sample to iTRAQ label and incubate at 25°C for 1 h.
9. Pool equal amounts of 2–8 differently labelled samples.
10. Dilute labelled samples to 5 ml with SCX loading buffer (*see* **Note 23**).
11. Proceed to **Section 3.6** Multi-dimensional Liquid Chromatography of labeled peptides.

3.6. Multi-Dimensional Liquid Chromatography of ICAT or iTRAQ Labelled Peptides

HPLC is used to fractionate the labelled peptides to simplify the proteome before analysis thus improving coverage.

3.6.1. Strong Cation Exchange

1. Equilibrate the Polysulfoethyl A SCX column for 20 min in loading buffer at 0.5 ml/min (*see* **Note 24**).
2. Load sample and wash with loading buffer at 0.5 ml/min.
3. Elute the tryptic peptides using a 30-min gradient of 0–35% SCX elution buffer at 0.5 ml/min collecting one-minute fractions.
4. Select 10–15 fractions containing the greatest concentration of tryptic peptides according to the $A_{214\,nm}$ chromatogram (*see* **Note 25**).
5. Dry fractions by centrifugation under vacuum.

3.6.2. Nano-LC Separation

1. Redissolve and acidify each fraction just prior to analysis using 100 μl solvent A.
2. Inject onto a C18 trapping column connected to a nanospray mass spectrometer.
3. Equilibrate sample on the column with 95% solvent A, 5% solvent B for 15 min to wash away salts and contaminants (*see* **Note 26**).
4. Switch inline with the mass spectrometer and run a 40 min linear gradient from 95% to 40% solvent A (*see* **Note 27**).
5. Increase gradient to 80% solvent B over 5 min to remove unbound peptides.
6. Re-equilibrate the column for 15 min in 95% solvent A, 5% solvent B before injecting the next sample.

3.7. MS Data Acquisition

Analysis of the separated peptides is performed using a QStar Pulsar i Quadrupole Time-of-Flight Mass Spectrometer (Applied

Biosystems/Sciex) or similar instrument. For ICAT, quantification of the light and heavy-labelled peptides is carried out in MS mode, and peptide sequencing is carried out in MS/MS mode. For iTRAQ, the quantification and sequencing of labeled peptides are performed in MS/MS mode. MS data can be acquired automatically from the QSTAR LC/MS/MS system using the latest version of Analyst QS or similar software. Parameters are instrument-specific and should be adjusted daily to acquire maximum signal.

1. Execute an information-dependent acquisition method consisting of a 1 s TOF MS survey scan of mass range 400–1,200 amu and two 2.5 s product ion scans of mass range 100–1,500 amu (*see* **Note 28**). Select the two most intense peaks over 20 counts, with a charge state 2–5 for MS/MS fragmentation.
2. Use a 6 amu window to prevent peaks from the same isotopic cluster from being fragmented again. Add the ions selected for fragmentation to an exclusion list for 180 s to avoid repeat fragmentations of the same abundant ions, thus increasing the number of unique ions sequenced. Set the curtain gas to 20 and use nitrogen as the collision gas, with an ionization tip voltage of 2,700 V.

3.8. Data Analysis

3.8.1. ICAT Data Analysis

1. Determine ICAT ratios of isotopically heavy $[^{13}C]_9$- to light $[^{13}C]_0$-labeled tryptic peptides using Protein Pilot software and average for multiple peptides derived from a single parent protein (*see* **Note 29**).
2. Identify proteins from peptide sequences using MASCOT. Select a sequence database to be searched such as the MS protein sequence DataBase (MSDB; Imperial College, London, UK), National Centre for Biotechnology Information (NCBI) non-redundant database or SwissProt.

3.8.2. iTRAQ Data Analysis

1. Calculate the ratios of the 113.1, 114.1, 115.1, 116.1, 117.1, 118.1, 119.1 and 121.1 amu signature mass tags generated upon MS/MS fragmentation from the eight iTRAQ tags using ProteinPilot^{TM} or similar software (*see* **Note 30**).
2. Within the MASCOT search software, set the MS and MS/MS tolerances used in the iTRAQ analysis, such as 0.2 Da (*see* **Note 31**). Select a sequence database to be searched such as the MS protein sequence DataBase (MSDB; Imperial College, London, UK), National Centre for Biotechnology Information (NCBI) non-redundant database or SwissProt (*see* **Note 32**).

3. Set 'iTRAQ (N-term)', 'iTRAQ (Lys)' and 'MMTS (C)' (methyl methanethiosulphonate modification of cysteines) as fixed modifications, and 'iTRAQ (Y)' and 'oxidation (M)' as variable modifications. Select 'trypsin' as the enzyme, allow a maximum of one missed cleavage, select '2+' and '3+' for peptide charge, and select 'monoisotopic'.

3.9. Validation

The amount of data generated by both ICAT and iTRAQ analyses can be overwhelming. Some suggestions for tackling the large amount of data to extract useful information follow.

3.9.1. Deciding Which 'Hits' are Significant

1. Limit peptides to those identified with high confidence (≥ 99% confidence).
2. For iTRAQ, include only those proteins identified by two or more peptides. This is not practical for ICAT as there far fewer cysteine-containing peptides identified per protein.
3. Identify all known substrates for the protease under study and set 'cut-offs' for test:control ratios based on these. This will be more biologically relevant for the system under study than setting arbitrary numerical 'cut-offs', for example, > 2 and < 0.5 change.
4. Select proteins with ratios above the highest cut-off and below the lowest cut-off.
5. Choose those proteins that may be biological substrates of the protease under study (based on location, function etc.) for biochemical validation. For example, select proteins that are known substrates of other protease family members, or family members of known substrates.
6. If there is any doubt as to the assignment of a peptide to a spectrum, manually validate all MS/MS spectra with Protein Pilot software.

3.9.2. Validation of Substrates

ICAT and iTRAQ provide a degradomic screen for protease substrates. Candidate substrates require further *validation*.

1. Validate cleavage *in vitro* using purified protease and putative substrate.
2. Follow up with cell-based and *in vivo* models.

4. Notes

1. Whether using ICAT for duplex experiments, or iTRAQ where 2–8 samples are compared, test and control samples need to be carefully selected to obtain the desired information *(3)*. When dealing with protease substrate identification, the most obvious choice is expression of the protease of interest compared with empty vector on

a protease null background. In this case, an increase in shedding of substrates by the protease would result in a label ratio protease:control of > 1 in conditioned medium. Catalytically inactive mutants can also be used (e.g. mutation of catalytic glutamic acid to alanine in MMPs), but these can also have a dominant negative effect where levels of binding proteins as well as substrates can change due to binding at the active site without cleavage and release, as well as at exosites (regions distinct from the active site which localise or orientate substrates for cleavage, e.g. MMP hemopexin domain). Alternatively, recombinant protease could be added exogenously to cell cultures. The multiplex nature of iTRAQ allows for time or drug/growth factor dose-dependent monitoring of shed substrates. A complementary approach is to look for the disappearance of shed substrates from the conditioned medium upon protease inhibition, or a build-up of uncleaved substrate, preferably using a specific inhibitor with a vehicle control, or siRNA knockdown of the protease gene with a scrambled siRNA control – in these cases the label ratio would be active:inhibited < 1 in conditioned media and > 1 in membrane preparations as the uncleaved substrates build up.

2. Roller bottles may be useful for experiments involving conditioned medium as they allow a small amount of medium (50 ml) to bathe several fold more cells than in a flask. It is, however, not so easy to remove cells from roller bottles for membrane preparations, and some cells do not adhere well.
3. Do not let cells reach 100% confluency as uncharacterised signalling events may occur, such as receptor internalization.
4. These steps remove abundant serum proteins (e.g. BSA) to increase specific labeling of cell-derived proteins and remove serum protease inhibitors that could block trypsin digestion.
5. Phenol-red free medium is used to avoid carry-over of phenol red through the labelling protocol.
6. Protease inhibitors are required to 'freeze' the profile of shed proteins upon termination of the experiment and to prevent further degradation, for example, by protease originating from dead cells. A specific inhibitor of the protease of interest could also be added at this point. Care should be taken that inhibitors of trypsin are removed prior to trypsin digestion, such as in the C4/C18 concentration step.
7. Samples can be stored at 4°C for 24 h or −80°C for 1 week, however, precipitation of some proteins, such as fibronectin, may occur. For best results, the samples should be concentrated directly after clarification.
8. There are various options for concentration of conditioned medium. C4 and C18 hydrophobic resins allow quick and easy concentration of large samples: C4 columns bind pep-

tides and proteins greater than 10,000 Da and C18 columns bind peptides and proteins below 10,000 Da. Connecting the columns in tandem increases throughput and reduces protein loss. We have also used 3 or 5 kDa cut-off concentrators (Centriprep and Microcon, Amicon), but there are losses of protein both in adsorption to the filter and by passage of small proteins into the filtrate and concentration of large volumes of multiple samples is relatively time-consuming. Protein precipitation techniques have varying degrees of success: TCA precipitation is possible, although the pellet is often difficult to resolubilize. Ammonium sulphate precipitation may give variable recovery depending on protein concentration. Acetone precipitation should not be used as it precipitates amino acid additives from the culture medium that interferes with the determination of protein concentration by BCA assay.

9. Use good quality deionised H_2O throughout such as Milli-Q® or Barnstead Nanopure.
10. Washing after loading 30 ml sample removes bound riboflavin, a yellow culture medium additive. It is recommended to prepare fresh wash buffer each time as ACN and TFA evaporation reduces their working concentrations.
11. Mass spectrometry laboratories routinely use Eppendorf® microcentrifuge tubes. Do not store the eluates containing ACN in microcentrifuge tubes that are specially coated to reduce protein binding as the coating can be solubilized and will lead to polymer contamination and interference with MS.
12. Use Hepes and never Tris or other amine buffers which react with the amine-targeted iTRAQ label.
13. The Applied Biosystems iTRAQ protocol states that urea must be below 1 M. Further steps of acetone precipitation and dilution for trypsin digestion will reduce the urea concentration to below this limit. Use of fresh deionised urea and minimising heat will avoid the carbamylation of lysine residues by urea breakdown products.
14. Avoid trypsin when detaching cells from culture flasks as this will digest the cell surface proteome! Use chelating agents such as EDTA (Versine) which interfere with adhesion, for example, integrin function, and cause cells to detach without damaging the cell. For MDA-MB-231 cells an extra 5 mM EDTA was added to the versine before the cells would detach.
15. Take care, the lysate can splatter and be lost! Parafilm loosely wrapped around the outlet tube and neck of the tube or flask can help.
16. We have used a relatively crude membrane preparation; however, these methods are equally applicable to purified plasma

membrane prepared by ultracentrifugation and by means other than using a cell disruption bomb.

17. We used buffer containing 6 M urea, 0.05% (w/v) SDS, which is compatible with ICAT-labelling to solubilize the membrane-enriched fraction. Using this, we identified many type-I and multi-pass membrane proteins *(10)*. It is possible to increase the urea concentration to 8 M and SDS up to 4% (w/v) to maximise the solubilization and labelling of hydrophobic multi-pass membrane proteins *(11)*. However, the increased concentration of SDS and urea then inhibits the trypsin digest. An alternative method, such as SDS-PAGE and in-gel tryptic digests of gel slices, followed by avidin-affinity chromatography was used by these authors *(11)*. However, acetone precipitation could also be used prior to trypsin digestion (*see* **Note 18**).
18. Acetone precipitation is useful for removing substances that interfere with the labelling protocols, such as thiols, high levels of detergents and denaturants, and primary amines (iTRAQ). For membrane proteins that require urea and SDS for solubility iTRAQ - acetone precipitate after reduction and cysteine blocking, prior to tryptic digest and labelling; ICAT - acetone precipitate after labelling, but prior to trypsin digestion. Do not use acetone precipitation after trypsin digestion as the peptides do not precipitate well and sample will be lost. For membrane preparations containing high concentrations of urea, or if the conditioned media proteins were resolubilized with 8 M urea in 3.2.2.9, then repeat the precipitation step in 10 volumes −20°C acetone and precipitate for 1 h at −20°C to further reduce the concentration of urea in the sample, as at concentrations > 6 M, urea tends to be precipitated with the proteins.
19. To minimise carbamylation, carry out reduction at 37°C for 1 h if samples contain urea.
20. For less complex samples, use the ICAT Cation Exchange Buffer Pack and Cartridge (POROS®50, 50 μM particle size (4×15 mm) that are supplied with the kit. This column is reusable up to 50 times. If the sample is highly complex, better resolution may be achieved on a high-resolution cation-exchange column such as Polysulfoethyl A 200 mm × 2.1 mm, 5 μm-300 Å SCX column (Poly C Inc), using 10 mM KH_2PO_4, pH 3, 25% ACN as the starting buffer and eluting the sample into fractions using a gradient 0–100% 10 mM KH_2PO_4, 350 mM KCl pH 3, 25% ACN over 60 min at a flow rate of 0.2 ml/min, collecting 2 min fractions. The column can be cleaned using 10 mM KH_2PO_4, 1 M KCl pH 3, 25% ACN.
21. The avidin-affinity cartridge is reusable up to 50 times.

22. Using a temperature block with a heated lid will avoid condensation.
23. The combination of phosphate buffer and potassium salts gives good SCX resolution, but if contamination of the MS with potassium and phosphate is a concern, ammonium and acetate salts can be substituted. However, acetate absorbs at 214 nm, confounding the $A_{214\,nm}$ chromatogram.
24. The use of an SCX column with 300 Å pore size is recommended to give high-resolution fractionation of large tryptic peptides.
25. Most peptides will be eluted from the HPLC in the initial 15–20 min period. To decrease MS time and expense, generally only the fractions containing the majority of tryptic peptides are processed. There are at least three major peaks observed in the $A_{214\,nm}$ chromatogram of every iTRAQ experiment that are not due to peptides but are artefacts of the iTRAQ labelling chemistry. These peaks can be monitored as quality control and experiments lacking these peaks can be stopped before they consume expensive MS time.
26. If potassium and phosphate salts were used in the SCX procedure, thorough washing of peptides bound to the C18 trapping column is important.
27. By using the UV absorbance of the SCX fractions to estimate the total peptide concentration, the reverse phase HPLC gradient duration can be lengthened to compensate for a higher peptide concentration in a particular fraction to avoid detection saturation.
28. Analyst QS is software for data acquisition and processing using the QSTAR LC/MS/MS system. A 1 s TOF MS survey scan of mass range up to 3,000 m/z is possible although in practice peptides above 1,500 m/z are rare and tend to fragment poorly. A survey scan of 800 m/z is optimal to limit the duty cycle time and thus lower under-sampling.
29. Protein Pilot® software locates and determines heavy: light peak ratios and performs database searches using data extracted for cysteine-containing peptides. MASCOT is a search engine that matches MS data to protein sequence databases, thus, identifying parent proteins from peptides.
30. ProteinPilot™ uses the Paragon algorithm to identify proteins and will report both the protein name and the iTRAQ ratio. Mascot (*see* **Note 29**) can be linked through ProteinPilot™ or used alone to confirm the identity of proteins determined through the Paragon algorithm.
31. The Paragon algorithm will automatically select the MS and MS/MS tolerances based upon the accuracy of the instrument used to acquire the data. Normally, a MS tolerance of 50–100 ppm and a MS/MS tolerance of 0.15 Da are

sufficient. The MS/MS tolerance can be set lower to identify more peptides, however, more false positives are likely.

32. MSDB is a non-identical protein sequence database specifically designed for mass spectrometry analysis, whereas NCBI and SwissProt sequences are non-redundant, rather than non-identical, so fewer matches may be obtained for a MS/MS search.

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Chapter 13

Purification of Basolateral Integral Membrane Proteins by Cationic Colloidal Silica-Based Apical Membrane Subtraction

Robert J.A. Goode and Richard J. Simpson

Abstract

Epithelial cell polarity mediates many essential biological functions and perturbation of the apical/basolateral divide is a hallmark of epithelial to mesenchymal transition in carcinoma. Therefore, correct targeting of proteins to the apical and basolateral surfaces is essential to proper epithelial cell function. However, proteomic characterisation of apical/basolateral sorting has been largely ignored, due to ineffectual separation techniques and contamination of plasma-membrane preparations with housekeeping proteins. Here we describe a method that strips the apical membrane from the adherent cells and releases the intracellular contents, thereby leaving the basolateral membrane available for stringent washes and collection. Analysis of the basolateral membrane of an adherent colon adenocarcinoma cell line resulted in 66% of identified proteins being integral membrane proteins, which possessed either a transmembrane domain or lipid modification, including 35 CD antigens. Based on the abundance of peptides from basolateral marker proteins, this method efficiently captures basolateral integral membrane proteins, with minimal contamination from other membranes and basic proteins.

Key words: Membrane, cationic colloidal silica, basolateral, integral membrane proteins, plasma membrane, epithelial cell polarity.

Abbreviations: MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FCS, fetal calf serum; CCS, cationic colloidal silica; DMEM, Dulbecco's Modified Eagle's Medium; T-V, trypsin versene; PMCB, plasma-membrane coating buffer; PAA, polyacrylic acid.

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1. Introduction

Epithelial cells line the surface of every organ in the body and perform various critical functions, including directional secretion and absorption, transcellular transport, selective permeability and protection from infection. This is achieved through polaristion, into apical and basolateral membranes divided by tight junctions. Loss of tight junctions and polarity is characteristic of epithelial to mesenchymal transition, which is a hallmark of carcinoma.

It has been noted that a major challenge in proteomics is characterisation of integral membrane proteins *(1)* and, in particular, cell-surface proteins *(2)*. Many techniques to purify cell-surface proteins have been applied to characterise the plasma-membrane proteome *(3)*; however, the majority of methods identify many abundant intracellular, mitochondrial, nuclear and endoplasmic reticulum proteins *(4–8)*. Furthermore, apical/basolateral partitioning has largely been ignored in proteomic characterisation of the plasma membrane thus far. In the past, radioactive and biotin labeling of membranes from differentiated epithelial layers grown on permeable membranes have commonly been used for determination of apical/basolateral separation *(9)*; however, mass spectrometry-based proteomic characterisation requires higher yields than these methods can easily or safely generate in a cost-effective manner, and membrane permeability (and hence cell-surface specificity) of some common biotinylation reagents can be an issue *(10)*.

The method described in this chapter selectively purifies integral membrane proteins from basolateral membranes of confluent, adherent epithelial cell lines and is easily scalable. It is based on the cationic colloidal silica (CCS) method described by Chaney and Jacobson in the early 1980s *(11)*. The original method is based on positively charged silica beads binding to the negative phospholipids in the exposed membrane to form a dense pellicle which can be isolated by density cushion centrifugation. Originally, the method was applied to isolate plasma membranes from suspension cells, but many variations of the original method have been described for purifying luminal membranes in the endothelium *(12–14)*, caveolae *(14–16)*, and basolateral membranes from adherent cell lines *(17)*. The last method is described here in detail and uses the principle that coating of the apical membrane with a silica pellicle allows its selective removal upon lysis, leaving the basolateral membrane attached to the culture vessel and available for subsequent washing and collection (**Fig. 13.1**). By applying basic and ionic washes peripheral membrane proteins are removed *(18, 19)*, thereby purifying the basolateral integral membrane proteins.

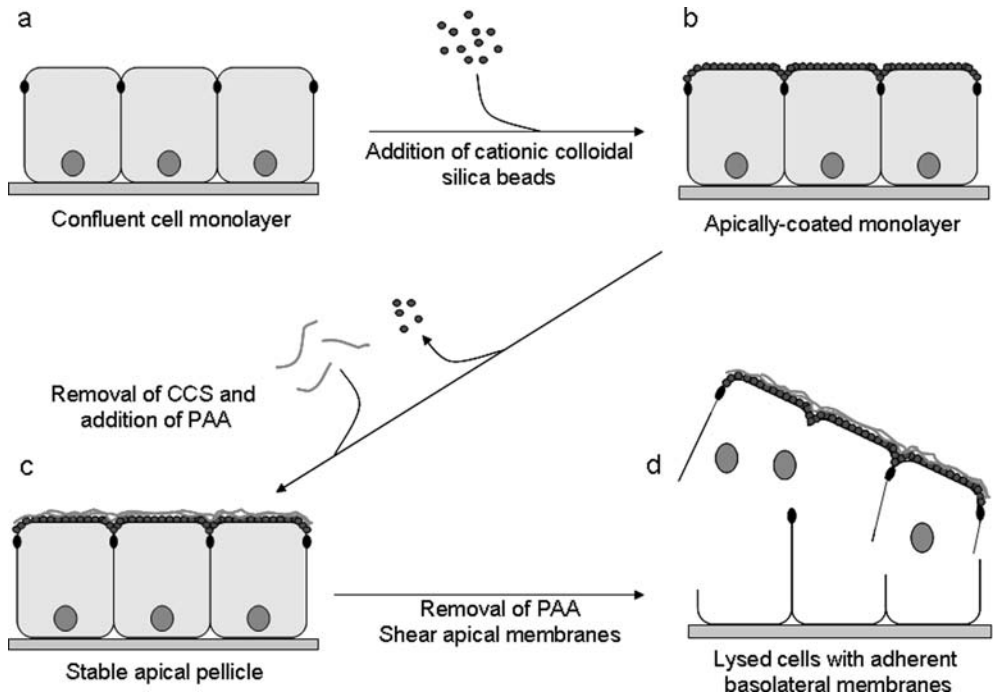

Fig. 13.1. Schematic of cationic colloidal silica-based apical membrane subtraction. A confluent monolayer culture is grown until polarised (**a**) The apical membrane coated with cationic colloidal silica beads (**b**). The beads are overcoated with negatively charged PAA to form a stable pellicle (**c**). Finally the pellicle and attached apical membrane are sheared from the underlying cells, causing cell lysis and release of intracellular organelles, while leaving the basolateral membranes attached to the tissue culture vessel for subsequent collection and characterisation (**d**).

Using this purification strategy coupled to a SDS-PAGE/LC/MS/MS strategy *(5)* we have characterised the basolateral integral membrane proteome from the polarised HCA7 colon adenocarcinoma cell line (Goode et al, manuscript in preparation). Using stringent manual validation, 194 proteins were identified of which 96 were annotated with transmembrane domains in SwissProt *(20)* and an additional five were predicted to be membrane-spanning proteins according to TMHMM *(21)*. In addition, 28 of the non-transmembrane proteins were annotated in SwissProt with lipid or glycosylphosphatidylinositol modifications. This is one of the highest proportions (129 proteins, 66.5%) of integral membrane proteins reported for a proteomic characterisation of plasma membranes. Furthermore, 35 CD antigens were identified and the most abundant proteins in the preparation were common basolateral marker proteins, such as various integrins *(22)* and Na+/K+ ATPase *(23)*, as well as desmoglein, a transmembrane protein found in intercellular junctions on the lateral membrane *(24)*.

2. Materials

Throughout the protocol, MilliQ quality water should be used for making all aqueous solutions and the pH of solutions should be adjusted using either 5 M HCl or 1 N NaOH, where appropriate. Unless noted, chemicals were of analytical grade from Sigma.

2.1. Preparation of 30% w/v CCS Stock Solution

1. Waring blender
2. Aluminium chlorohydroxide complex (CHLORHYDROL®, Reheis Inc)
3. Nalco 1060 colloidal silica beads, 500 Å nominal diameter (Ondeo Nalco Co) (*see* **Note 1**)
4. Water bath

2.2. Cell Culture

1. Well differentiated epithelial cell line, such as HCA7 colon adenocarcinoma cells (HCA7 colony 29, ECACC) (*see* **Note 2**)
2. Phosphate-buffered saline (PBS): 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, and adjust to pH 7.4 with HCl
3. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Invitrogen)
4. Fetal calf serum (FCS) (ThermoTrace, Melbourne, Australia)
5. Trypsin-Versene (T-V) (GIBCO, Invitrogen)
6. 150 mm tissue culture dishes (BD Falcon)

2.3. Membrane Coating (see Note 3)

1. PBS + salts (PBS+S): supplement PBS with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (*see* **Note 4**). Prepare 500 mL and store at 4°C.
2. Plasma-membrane coating buffer (PMCB): 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM MES, 135 mM NaCl, pH 5.3. Prepare 1 L and store at 4°C.
3. 5% w/v CCS solution: dilute 16.6 mL of 30% w/v derivatised silica stock solution to 100 mL with cold PMCB and then centrifuge at 800×g for 5 min to remove aggregated beads. Prepare on the day of experiment and store at 4°C.
4. 0.5% w/v polyacrylic acid working solution (0.5% PAA): dilute 2 mL of 25% w/v PAA solution (Aldrich) to 90 mL with cold PMCB (*see* **Note 5**). Adjust to pH 6–6.5 using pH paper to measure pH, and make up to 100 mL with PMCB. Prepare on the day of experiment and store at 4°C.
5. Lysis buffer: 2.5 mM imidazole, pH 7 (*see* **Note 6**). Prepare 500 mL and store at 4°C. On the day of the experiment, dissolve four protease inhibitor tablets (Complete EDTA-free, Roche) in 200 mL Lysis buffer and store at 4°C.
6. Basic wash solution: 100 mM sodium carbonate, pH 11.4. Prepare 700 mL and store at room temperature.

7. Ionic wash solution: 5 M sodium chloride. Prepare 200 mL and store at room temperature.
8. Large trays. These will hold ice and must fit ten 15 cm cell culture dishes arranged as a single layer.
9. Liquid waste receptacle, such as a 1 L beaker.
10. 10 mL plastic disposable syringe.
11. 18 gauge needle. Modify the tip of the needle according to **Note 7**.
12. Cell scraper.
13. Ultracentrifuge (Beckman Coulter) with SW28 rotor and polyallomer 25 × 89 mm centrifuge tubes (Beckman).
14. Benchtop microfuge.
15. Rocker platform.

3. Methods

This is an adaptation of the method of Spector et al. *(17)* for adherent cell lines, which derives from the original cationic colloidal silica method of Chaney and Jacobsen *(11)*. It uses the principle that coating of the apical membrane with a silica pellicle allows its selective removal upon lysis, leaving the basolateral membrane attached to the culture vessel and available for subsequent washing and collection (**Fig. 13.1**). The use of the pellicle serves two main purposes: it improves the efficiency of cell lysis and therefore reduces intracellular protein contamination of the harvested basolateral membranes and it allows the separation of the sheared apical membranes from intracellular membranes. However, in our experience the apical membranes are heavily contaminated with basic non-membrane proteins, which is presumably due to interaction with the negatively charged PAA coating. Therefore, the recovery of apical membranes is not discussed in this chapter, but is covered in Spector et al. *(17)*.

This method is only applicable to adherent cells, and is best performed on polarised, confluent monolayer cultures. Use of sub-confluent cultures may lead to suboptimal apical/basal fractionation and reduced yields. For preparation of plasma membranes from suspension or loosely adherent cultures the original method of Chaney and Jacobsen may be used *(11)*, however significant contamination with basic proteins and intracellular proteins is often observed.

3.1. Preparation of 30% w/v Cationic Colloidal Silica Stock Solution

1. To a Waring blender with 300 mL water, add 35 g of aluminium chlorohydroxide complex.
2. Mix 450 g colloidal silica in 90 mL of water (*see* **Note 1**).
3. Turn on the blender to moderate speed and add the colloidal silica solution. Blend at maximum speed for 2 min.

4. Transfer the solution to a 1 L flask and cover with a 150 mL beaker. Place flask in a water bath at 80–90°C for 30 min with occasional stirring.
5. Remove from water bath, allow to cool and incubate overnight (16 h) at room temperature.
6. Return the solution to the blender and while on moderate speed adjust the pH to 5.0 with 1 N NaOH, using pH paper to monitor.
7. Leave to incubate for 24 h at room temperature.
8. Readjust the pH if necessary, and store at room temperature (*see* **Note 8**). This stock suspension is (nominally) 30% w/v cationic colloidal silica.

3.2. Cell Culture (see Note 2)

1. Grow HCA7 cells in a 175 cm^2 tissue culture flask in DMEM with 10% v/v FCS (DMEM+FCS) at 37°C with 10% CO_2.
2. Upon reaching ~80% confluence, remove medium and wash the cells briefly with PBS. Add 2 mL T-V, rock the flask to ensure coverage of cells and return to incubator for 10–15 min.
3. Add 18 mL DMEM+FCS to trypsinised cells and add 2 mL of cell suspension to each of ten 150 mm tissue culture dishes containing 25 mL DMEM+FCS. Culture the cells in a humidified 37°C incubator with 10% CO_2. Media should be renewed at least twice a week until the cells are confluent and polarised.

3.3. Membrane Coating

1. Prepare trays and ice boxes filled with ice. Put PMCB, CCS, PAA and lysis buffer in iceboxes.
2. Wash cells with cold PBS+S (*see* **Note 4**), and then add ~10 mL cold PBS+S per dish. Transfer tissue culture dishes to ice trays.
3. Decant PBS+S into the waste receptacle and pour on 10–20 mL PMCB per dish.
4. Decant PMCB and add ~5 mL 5% CCS per dish. Swirl to cover entire tissue culture dish. Proceed quickly to ensure incubation is ~1 min per dish (*see* **Note 9**).
5. Swirl 5% CCS solution and decant. Add ~10 mL PMCB to wash cells.
6. Swirl PMCB, decant and add ~5 mL 0.5% PAA. Swirl to cover. Again incubate for ~1 min.
7. Swirl and decant 0.5% PAA. Wash the cells by adding ~10 mL PMCB.
8. Remove PMCB and briefly rinse with lysis buffer. Remove rinse and add 10 mL lysis buffer with protease inhibitors. Incubate at 4°C or on ice for 30 min with occasional swirling to ensure coverage of the dishes.

9. Incubate dishes at room temperature for 5–10 min.
10. Using a 10 mL syringe and modified needle assembly, take up lysis buffer from the dish and spray at a downward angle of ~45° onto the plate using a waving motion to maximise surface coverage and sufficient force to tear the apical membrane away, while leaving the basal membrane ghosts attached to the dish (*see* **Note 10**). Repeat until the entire plate is lysed. Normally, duplicate coverage of each plate is sufficient and can be achieved using 10–12 syringe volumes. Use one last syringe volume with the dish tilted at a 10° angle and spray from the top to bottom to wash the sheared colloidal silica coated membranes to one side of the plate. Remove the sheared apical membrane and released cytosolic components, maximising removal of any visible particulates (apical membranes) (*see* **Note 11**). Add 10 mL lysis buffer with protease inhibitors to the dish, swirl to cover and incubate at 4°C until all plates are sprayed.
11. Rock the dishes at room temperature for 5 min on a rocking platform, ensuring complete coverage of the membranes, then decant lysis buffer. Add 5–10 mL ionic wash solution and return to rocking platform for an additional 5 min.
12. If desired, the salt solution may be collected for analysis and stored on ice (*see* **Note 12**), or discarded. Then, rinse dishes briefly with ionic wash solution and discard. Rinse membranes briefly with ~5 mL basic wash buffer and discard then add ~5 mL basic wash buffer to membranes and incubate on the rocking platform for 5 min at room temperature.
13. Completely remove the basic wash solution (*see* **Note 12**) and add 3.5 mL basic wash solution to each culture dish (*see* **Note 13**). Using a cell scraper, firmly scrape the bottom of the dish to displace the basal membranes. This is best achieved by holding the dish at an angle and scraping towards the bottom, ensuring total coverage of the plate. Carefully transfer all the scraped membranes into a single centrifuge tube.
14. Harvest basolateral membranes by centrifugation at 14,000 × g for 30 min at 4°C in an ultracentrifuge using a SW28 rotor.
15. The pellet appears as a thin translucent layer (often with cracks) across the bottom of the centrifuge tube. Carefully discard the supernatant without disturbing the pellet.
16. Resuspend the precipitate in ~500 μL basic wash buffer and transfer it to a 1.5 mL microfuge tube. Wash the centrifuge tube with an additional 500 μL basic wash buffer and transfer the washes to the microfuge tube.
17. Centrifuge the resuspended membranes in a benchtop centrifuge at maximum speed (16,000 × g) for 20 min at 4°C.

18. Remove the supernatant and resuspend the pellet in water. Centrifuge the membranes in a benchtop centrifuge for 20 min and remove the supernatant.
19. The pellet can be solubilized for further analysis, such as SDS-PAGE, western analysis and proteomic characterization (*see* **Note 14**). If not proceeding directly with analysis, freeze the pellet using a dry ice-ethanol bath and store at −70°C; however, protein degradation is observed after storage of membranes.

4. Notes

1. Small particles, such as the silica beads, pose a health hazard if inhaled. Therefore, when working with powdered particles use appropriate precautions, at a minimum, work in a laminar flow cabinet and wear a protective mask.
2. Any adherent polarising epithelial cell line can be used with this protocol, subject to it remaining adherent during the coating and wash steps. Additionally, sub-confluent cultures and non-polarising cell lines may be used with this protocol to purify the adherent plasma membrane, which are normally rich in integral plasma-membrane proteins, especially cell adhesion molecules (unpublished observations).
3. The volumes of buffer provided are appropriate for a preparation using ten 150 mm culture dishes. Buffers should be prepared within a week of the experiment, unless noted.
4. Some cell lines spontaneously lift in PBS. By supplementing PBS with 1 mM MgCl2 and 1 mM CaCl2 this can often be prevented from occurring. If the cell line in use does not lift in PBS alone, supplementation with salts is unnecessary.
5. Use a 5 mL pipette to dispense PAA, as it is extremely viscous. Allow time for uptake of the PAA by the pipette and gentle movement of the pipette may aid uptake. Use a wheel rotator in a cold room to ensure the PAA is homogeneous before adjusting pH. Also, PAA may damage the probe of pH meters. To check the pH of the PAA solution, pipette a small volume (1–2 μL) onto pH paper, such as Neutralit pH 5–10 (Merck).
6. Imidazole is corrosive and harmful if inhaled or absorbed through skin. Handle with care, at a minimum use gloves and work in a fume hood.
7. Modification of the needle increases the effective area sprayed and, though not necessary, reduces the time and increases the coverage of the plates during the cell lysis step compared with using a normal needle. The aim of the modification is to deflect the liquid flow at the needle exit to generate a broad spray. Using wire cutters and a gentle

twisting motion, clip off the tip of the angled needle opening. This should bend the resulting straight edge slightly across the flow path and still leave a large opening to prevent the needle blocking.

8. The silica derivatisation can be confirmed by precipitation of the silica beads at increasing pH. This can be measured by 50-fold dilution of silica stock with PMCB of varying pH and measurement of $OD_{500\,nm}$. A dramatic increase in OD should occur when diluting 30% CCS stock with PMCB of pH 7.
9. Cell viability is extremely time dependant during coating (steps 4-7); therefore, the entire process should be completed in as short a time as possible. Normally, 10 dishes can be coated with CCS and PAA (steps 3–7) in less than 10 min. To expedite the coating and prevent drying of the cells, buffers are decanted into a waste receptacle, replaced with the next buffer and the dish replaced on ice before proceeding to the next dish. Addition of buffers to dishes should be done by pouring the appropriate buffer onto the sidewall of the dish with the dish held at about a 30–45°C angle. This minimises the mechanical forces applied to the cells and therefore the potential for cells lifting.
10. The basolateral membrane ghosts/footprints should be viewed by phase contrast microscopy to ensure complete cell lysis and removal of sheared apical membranes. They appear as extremely transparent outlines of cells, and therefore are more easily viewed during the washes after removal of sheared apical membranes.
11. Sheared apical membranes may be retained on ice and purified by discontinuous gradient centrifugation according to the method of Chaney and Jacobsen *(11)*, however, significant contamination with basic proteins, such as histones and ribosomal proteins, has been observed in our experience. This problem of basic protein contamination of plasma membranes also occurs when membranes are prepared by classical isolation methods *(5)*; however, it may be exacerbated by their affinity for the negatively charged PAA coating. This is easily detected by three intense histone bands in SDS-PAGE around 20 k.
12. Washes may also be collected for analysis of basolateral membrane associated proteins; however, these are often dominated by cytoskeletal proteins. Buffer exchange is also required prior to SDS-PAGE analysis due to the ionic concentration in the wash buffers. This can be achieved by numerous methods such as precipitation or chromatographic buffer exchange, and is left to the researcher's preference.
13. Additional washes, such as a chelator wash with aqueous 10 mM ethyldiamine tetraacetic acid (EDTA), may be

undertaken to further remove peripheral membrane proteins. However, due to the metal dependence of attachment in some cell lines, EDTA washing may release the basolateral membranes from the culture vessel resulting in their loss. Therefore, EDTA washes are optional and should only be performed after testing of the metal dependence of membrane attachment by phase contrast microscopy.

14. Solubilization of membranes may be achieved using a variety of reagents. For routine SDS-PAGE shotgun proteomic characterisation *(5)* the pellet can be directly solubilized in SDS sample buffer; however, incomplete solubilization by SDS has been reported for lipid rafts *(25)*. Alternative solubilization strategies include organic solvents (such as hexafluoro-2-propanol), organic acids (such as trifluoroacetic acid or formic acid) or chaotropes with alternate detergents (such as urea/thiourea in combination with ASB-14).

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Chapter 14

Moving Closer to the Lipid Raft Proteome Using Quantitative Proteomics

Leonard J. Foster

Abstract

Lipid rafts are small subdomains of the plasma membrane enriched in cholesterol, sphingolipids, saturated phospholipids and specific proteins. They are thought to act as coordination centres for signal transduction pathways so their protein composition is of particular biological interest. Rafts are refractory to solubilization in non-ionic detergents so they can be biochemically enriched by floatation on a sucrose density gradient but several other membranes co-migrate with rafts in such a procedure. Qualitative proteomic analysis of such detergent-resistant membranes fails to distinguish true raft proteins from co-migrating contaminants. This chapter describes a quantitative proteomic application of Stable Isotope Labelling by Amino acids in Cell culture (SILAC) for distinguishing true raft proteins in detergent-resistant preparations.

Key words: SILAC, cyclodextrin, cholesterol, detergent extraction, MSQuant, Triton X-100, detergent-resistant membranes.

1. Introduction

The lipid raft theory *(1, 2)* was first put forward to explain the partitioning behaviour of cholesterol and sphingolipids in membranes. Glycosphingolipids, in particular, cluster laterally in membranes via weak hydrogen bonding between the carbohydrate head groups, and then cholesterol intercalates into any voids between the saturated hydrocarbon chains. Certain protein families also appeared to be targeted to rafts, including doubly acylated tyrosine kinases, glycosylphosphatidylinositol (GPI)-anchored proteins and certain transmembrane domain proteins *(3–6)*. Lipid-anchored proteins probably partition into

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rafts themselves while other proteins may bind raft-specific lectins to become raft-associated. The concentration in rafts of various groups of proteins led to speculation that rafts are involved in several fundamental processes, including endocytosis, bacterial invasion, polarization of epithelial cells and T-cell receptor (TCR) signalling.

As mentioned, rafts are typically enriched biochemically by taking advantage of their resistance to solubilization in non-ionic

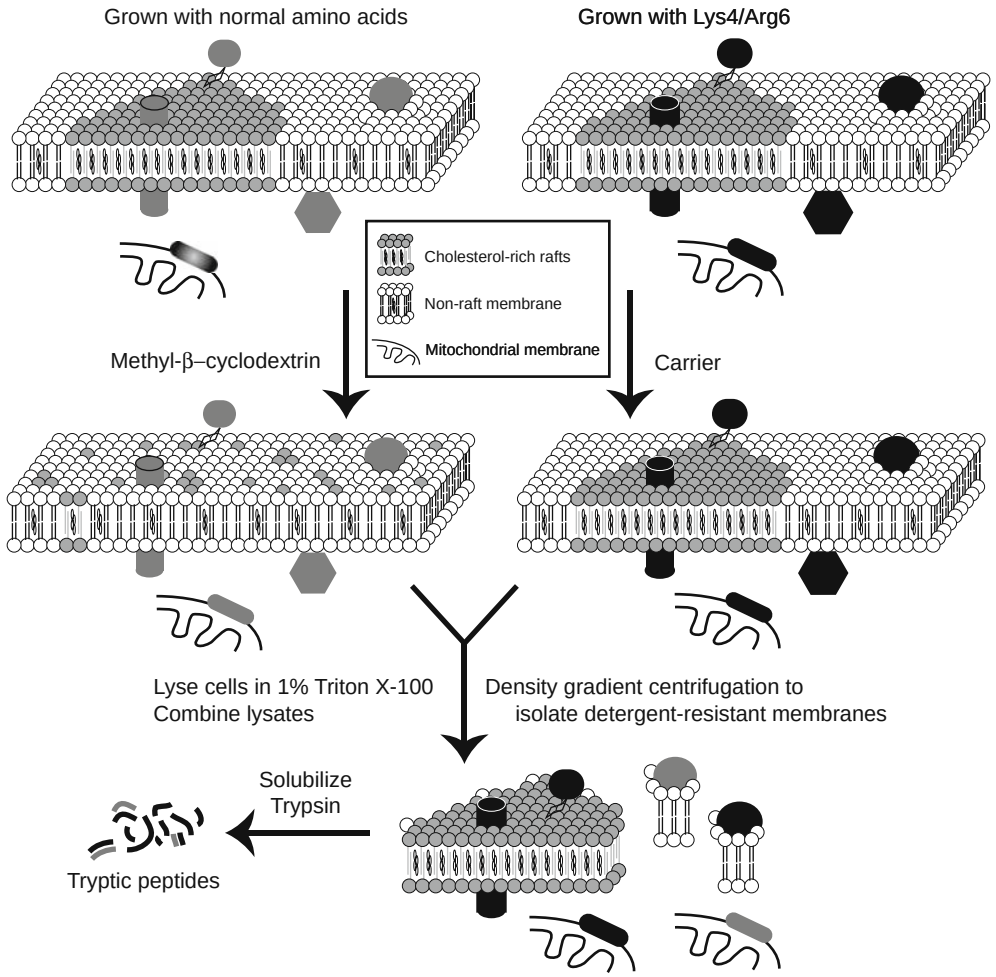

Fig. 14.1. Use of SILAC to distinguish true raft proteins. Two sets of HeLa cells are raised, one in Lys0 and Arg0 (light cells, *grey* proteins) and the other with Lys4 and Arg6 (heavy cells, *black* proteins). Cholesterol is then depleted from the light cells with methyl β-cyclodextrin (MβCD) while the heavy cells were treated with carrier. Both cell populations are lysed and combined prior to isolating detergent-resistant membranes by floatation in a discontinuous sucrose gradient. MβCD treatment causes the rafts in the light cells to lose their structural integrity so that they would no longer be detergent-resistant, unlike rafts originating from the heavy cells. Conversely, co-purifying non-raft proteins from both cell populations will still enrich equally well from heavy and light cells since they are not affected by the MβCD treatment. Heavy:light ratios for raft components as measured by the relative ion intensities of tryptic peptides should be high, while ratios for co-purifying proteins grouped around 1 (Reprinted from *(9)*).

detergents. There are now almost two dozen proteomic studies of detergent-resistant membrane (DRM) fractions focused on ascertaining the protein composition of lipid rafts, which we have reviewed extensively elsewhere *(7, 8)*. The standard approach has been simply to identify as many proteins as possible in biochemically prepared DRMs but this approach overlooks the cholesterol-dependence test that has been the minimum standard required in cell biological studies for considering a protein a raft component. To overcome this, we have previously employed quantitative proteomics to define the cholesterol-dependence of proteins in the detergent resistant fraction prior to MS analysis. By applying this more stringent criterion (**Fig. 14.1**) we were able to show that as many as 40% of proteins found in DRMs are simply co-purifying with rafts and not actually components of rafts themselves *(9)*. This chapter describes in detail the methods that were developed for that original study and that are currently in use in our laboratory.

2. Materials

The description here includes heavy isotope forms of arginine and lysine instead of the triply-deuterated leucine that was used in the original study. Arginine and lysine are the amino acids of choice now because virtually every tryptic peptide should contain at least one of these residues so most peptides can be quantified.

2.1. Cell Culture and SILAC

1. Dulbecco's modified Eagle's Medium lacking L-arginine and L-lysine (Caisson Labs, Rexburg, ID) supplemented with penicillin/streptomycin (HyClone, Ogden, UT) and 10% dialyzed fetal bovine serum (Invitrogen/Gibco, Bethesda, MD).
2. Trypsin solution (0.25%) with ethylenediamine tetraacetic acid (EDTA, 1 m*M*) (HyClone).
3. Phosphate-buffered saline (PBS).
4. Normal isotopic abundance L-arginine (Arg0) (Sigma-Aldrich, St. Louis, MO) stock solution at 84 mg/mL in PBS.
5. Normal isotopic abundance L-lysine (Lys0) (Sigma-Aldrich) stock solution at 146 mg/mL in PBS.
6. $^{13}C_6$ L-arginine (Arg6) (Cambridge Isotope Laboratories, Cambridge, MA) stock solution at 90 mg/mL in PBS.
7. D_4 L-lysine (Lys4) (Cambridge Isotope Laboratories) stock solution at 150 mg/mL in PBS.
8. Light SILAC media: Arg and Lys-free media supplemented with Arg0 and Lys0 stock solutions diluted 1:4000.
9. Heavy SILAC media: Arg and Lys-free media supplemented with Arg6 and Lys4 stock solutions diluted 1:4000.

2.2. DRM Isolation

1. DMEM with penicillin/streptomycin and Arg0/Lys0 or Arg6/Lys4 at 1:4000 dilutions of above stock solutions. Do not add serum.
2. PBS.
3. Methyl β-cyclodextrin (Sigma-Aldrich) stock solution in water.
4. Lysis buffer containing 1% Triton X-100 (Sigma-Aldrich) in 25 m*M* 2-morpholinoethanesulphonic acid (MES, Sigma-Aldrich) at pH 6.5. Protease Inhibitor Cocktail (Roche Applied Science, Laval, QC) added fresh. Solution MUST be kept ice cold.
5. Coomassie Plus reagent (Pierce, Rockford, IL).
6. 90% sucrose in MES-buffered saline (MBS) (25 m*M* MES – pH 6.5, 150 m*M* NaCl) (*see* **Note 1**).
7. Surespin 630 swinging-bucket ultracentrifuge rotor (Sorvall, Guelph, ON).
8. 36 mL thin-walled ultracentrifuge tubes (Seton Scientific, Los Gatos, CA).
9. 35% sucrose in MBS.
10. 5% sucrose in MBS.
11. 3 mL syringe with 25G needle (Becton Dickinson, Oakville, ON).
12. MBS.
13. Rubber cell scrapers (Sarstedt, Montreal, QC).
14. Semimicrovolume Disposable Polystyrene Cuvettes (Bio-Rad, Hercules, CA).
15. 17 mL thick-walled ultracentrifuge tubes (Seton Scientific).

2.3. Proteolytic Digestion

1. 100% Ethanol.
2. 2.5 *M* sodium acetate, pH 5.0.
3. Glycogen, 20 μg/μL (Molecular Biology Grade, Roche).
4. 6 *M* urea/2 *M* thiourea in 25 m*M* Tris, pH 8.0 (*see* **Notes 2** and **3**).
5. Digestion buffer: 50 m*M* ammonium bicarbonate (Sigma-Aldrich). 40 mg ammonium bicarbonate in 10 mL of water gives a 50 m*M* solution very close to pH 8.0 so no additional pH adjustment is necessary.
6. 0.5 μg/μL Sequencing grade, modified porcine trypsin (Promega, Nepean, ON) in Digestion Buffer.
7. 0.5 μg/μL dithiothreitol (DTT) (Sigma-Aldrich) in digestion buffer.
8. 2.5 μg/μL iodoacetamide (IAA) (Sigma-Aldrich) in digestion buffer.
9. RapiGest: 0.5% RapiGest (Waters, Milford, MA) in digestion buffer.

2.4. LC/MS/MS

1. Buffer A: 0.5% acetic acid in distilled, deionized water with total organic content less than 1 part-per-billion.
2. Buffer B: 0.5% acetic acid, 80% acetonitrile (HPLC grade, Fisher) in distilled, deionized water with total organic content less than 1 part-per-billion
3. 96-well, full skirt PCR plates with Teflon sealing mats (Axygen Scientific, Union City, CA).
4. C_{18} reversed phase (3 μm Reprosil, Dr. Maisch, Germany) 15 cm analytical column packed with a self-assembling frit in a 20 cm long, 360 μm outer diameter, 75 μm inner diameter, 8 μm tip opening fused silica emitter (New Objectives, Ringoes, NJ).
5. C_{18} Stop And Go Extraction (STAGE) tips. Using a 16G flat needle (Hamilton, Reno, NV) punch out small disks of C_{18}-embedded Empore disks (3M, London, ON, USA) and tap them into the end of a 200 μL disposable pipette tip.
6. Sample Buffer: 3% acetonitrile, 1% trifluoroacetic acid, 0.5% acetic acid
7. HPLC-grade methanol.

3. Methods

This procedure describes the isolation and analysis of lipid rafts from HeLa *(9)* cells but any cell type that can be grown in defined media can be substituted here, although certain cell types do not respond well to SILAC media. The MS described is also for our particular system but any LC/MS/MS system could be used, although the mass accuracy of the Orbitrap makes it very desirable.

3.1. Cell Culture and SILAC

One confluent 10 cm plate of HeLa cells is passaged with trypsin/EDTA and one fifth of the cells are seeded into a 10 cm plate with 8 mL of light SILAC media and one fifth into a 10 cm plate with 8 mL of heavy SILAC media (a 1:5 dilution). Once the two plates of SILAC cells near confluence they are each split 1:5 into two more 10 cm plates, allowed to grow to confluence again and then split into six 14 cm plates. The total increase in cell population by this method is approximately 150-fold, or more than 2^7.

3.2. DRM Isolation

1. Once the six heavy plates and six light plates are confluent remove the media, wash them once with 14 mL PBS and add serum-free DMEM with the appropriate heavy or light Arg and Lys. Replace plates in the incubator and serum-starve the cells for 18 h.

2. Add MβCD directly to the light cells (final concentration: 5 m*M*) for 1 h and at the same time add an equal volume of carrier (ethanol) to the heavy cells (*see* **Note 4**).
3. Wash cells three times with ice-cold PBS and place on ice immediately. Allow excess liquid to drain to one side of each plate by resting them at an angle but be sure to have the entire bottom surface of each plate in contact with the ice. Once the liquid has drained aspirate the last little bit.
4. Add 1 mL of ice-cold lysis buffer to each plate and scrape cells off with a rubber cell scraper. Use the scrapper to collect as much liquid as possible on one side of the plate and transfer the lysates into a 15 mL plastic tube, keeping the heavy and light lysates in separate, well-marked tubes (*see* **Note 5**).
5. Using 2 mL lysis buffer, wash all six plates of each condition to obtain as much material as possible. Use the rubber cell scrapper to remove all liquid from each plate.
6. Rotate the heavy and light lysates in an end-over-end rotator for 1 h. IMPORTANT: keep the rotator at 4°C.
7. Clarify each lysate by centrifuging the heavy and light tubes for 10 min at 600*g* in a centrifuge cooled to 4°C.
8. Measure the relative protein concentrations of each clarified lysate using the Coomassie Plus kit. Put 500 μL distilled, deionized water in each of three semimicrovolume cuvettes. Add 3 μL heavy lysate in one and 3 μL light lysate in the other. Add 500 μL Coomassie Plus reagent to each cuvette and mix well. Read the absorbance of the heavy and light samples at 595 nm after blanking the spectrophotometer with the cuvette containing no lysate.
9. Before isolating DRMs, equal amounts of protein from the heavy and light lysates must be mixed. For the lysate with the lower 595 nm absorbance transfer the supernatant from the clarification (Step 7) into a 50 mL plastic tube, carefully noting the volume transferred. To calculate the volume of the other lysate required, take the volume noted above and multiply by the quotient of the lower to the higher absorbance reading. Transfer this volume of the second lysate into the same tube with the first lysate (*see* **Note 6**).
10. To this tube, also add a volume of 90% sucrose in MBS equal to the combined volume of the two mixed lysates. Mix well until no schlieren (lines of mixing) are visible and then transfer to a clean 36 mL thin-walled ultracentrifuge tube.
11. On top of the lysate solution carefully layer 5 mL of 35% sucrose in MBS and then fill the remaining space in the tube with 5% sucrose in MBS. Place a small mark at the 5/35% interface.
12. Centrifuge the sucrose density gradient for 18 h at 166,000*g* at 4°C in a swinging-bucket rotor. A light-scattering, fuzzy

white band should be visible near the mark made in Step 11.

13. Insert a 25G needle connected to a 3 mL syringe through the side of the centrifuge tube approximately 2 mm below the bottom of the light-scattering band (DRMs) and, keeping the needle perpendicular to the length of the tube and the beveled side facing upwards, apply suction with the syringe (*see* **Note 7**). Stop when all of the light-scattering material has entered the syringe.
14. Eject the extracted DRMs into a 15 mL tube and dilute to 15 mL with ice-cold MBS. Mix well and transfer to a clean 17 mL thick-walled ultracentrifuge tube.
15. Pellet DRMs by centrifuging for 2 h at 166,000*g* at 4°C.
16. Remove supernatant and wash pellet gently with MBS.

3.3. Proteolytic Digestion

1. Solubilize DRM pellet in 100 μL urea/thiourea. Pipette up and down vigorously to break up pieces of membrane. Transfer solution to a clean 1.5 mL microfuge tube.
2. Add 1 mL absolute ethanol, 40 μL 2.5 *M* sodium acetate and 20 μg glycogen to the DRM/urea/thiourea suspension, mix well and allow to stand for 90 min at room temperature (*see* **Note 8**).
3. Pellet proteins by centrifuging at 16,000*g* for 10 min at room temperature. Discard supernatant and note size of pellet. A pellet with a diameter of 2–3 mm corresponds to approximately 25 μg of protein. Use this as a very rough guide to gauge how much protein is present for subsequent digestion steps. Unless the scale of the preparation is huge there will not be enough protein to measure it directly.
4. Re-solubilize membranes and proteins in a minimal volume of RapiGest (<50 μL if possible) and heat sample to 99°C for 10 min.
5. Add DTT at a 1:50 ratio by mass of DTT:protein (estimated in Step 3) and incubate for 30 min at 37°C.
6. Add IAA at a 1:50 ratio by mass of IAA:protein and incubate for 30 min at 37°C.
7. Add trypsin at a 1:50 ratio by mass of enzyme:protein and incubate overnight at 37°C.

3.4. LC/MS/MS

1. Acidify digested proteins by adding 2/3 the volume of sample buffer.
2. Decompose the RapiGest by incubating acidified digest for 30 min at 37°C.
3. Clarify RapiGest breakdown products by centrifuging for 10 min at 16,000*g*.
4. Prepare a STAGE tip by forcing 20 μL of methanol through it followed by 20 μL buffer A.

5. Load a volume of the digest corresponding to approximately 2 μg protein onto the STAGE tip and force it through slowly (20 μL/min).
6. Wash excess salts from the STAGE tip with 20 μL buffer A.
7. Elute peptides off of STAGE tip directly into a well of a PCR plate using 10 μL buffer B. The bed volume of the STAGE tip is less than 1 μL so 10 μL represents at least 10 column volumes and should result in complete elution.
8. Evaporate the acetonitrile from the eluted peptides in a vacuum centrifuge and re-dissolve peptides in 3.2 μL sample buffer.
9. Analyze peptides by LC/MS/MS – we use an 1100 Series nanoflow HPLC (Agilent, Palo Alto, CA) coupled through a 20 μm inner diameter fused silica capillary to an analytical column/emitter (*see* **Section 2.4**) using a liquid-electrode T-junction (Proxeon Biosystems A/S, Odense, Denmark). The autosampler is set to pick up and inject 3.0 μL and the HPLC pump loads it at 600 nL/min under 6% buffer B. The flow rate is then dropped to 200 nL/min and a linear gradient is developed from 6% B to 30% B over 60 min before jumping to 80% B to flush the system (*see* **Note 9**). This separation system is coupled online to an LTQ-Orbitrap (Thermo Electron, Bremen, Germany) set to operate in data-dependent mode. A survey scan (60,000 resolution, full-width, half-max (FWHM)) is acquired in the Orbitrap while the five most abundant multiply charged ions are fragmented in the LTQ *(10)*.
10. Extract fragment spectra from the resulting .RAW file using Extract_MSN.exe, part of the BioWorks package, and search against the desired protein library (*see* **Note 9**) using Mascot and/or X!Tandem. If the Orbitrap is well tuned the peptide mass tolerance for the database search should be no more than 5 parts-per-million while the fragment mass tolerance should be set to 0.8 Da. Allow no more than one missed cleavage and allow heavy arginine and lysine as variable modifications (162.12124 and 132.1201 Da, respectively). The Mascot search results are then saved in the 'Peptide Summary' format (*see* **Note 10**).
11. Associate the database search result file with the corresponding .RAW data file in MSQuant (http://msquant.sourceforge.net/) *(11)* and under the Options menu set the quantitation type to the corresponding stable isotope-labelled amino acids used in **Section 3.1**.
12. Open the search file by double-clicking, select all the parsed proteins and start the quantitation under the Automation menu.
13. When MSQuant has extracted all the quantitative information double-check those proteins highlighted in orange

to be sure that the LC peak assignments have been made correctly then export all protein and peptide information to Microsoft Excel for easier manipulation. Those proteins with a high heavy:light ratio are sensitive to cholesterol disruption and are considered real components of lipid rafts while those proteins with a ratio near 1.0 are insensitive to cholesterol disruption and are likely to be co-purifying contaminants *(9)*.

4. Notes

1. A solution of 90% sucrose is best prepared by adding one third of the sucrose at a time to the required volume of water, allowing it to fully dissolve before adding the next third and then the final third. Keep the container covered as this process take some time and dust will settle in the beaker otherwise. If necessary, some gentle heating can be applied to aid the solubilization.
2. Urea and thiourea should first be solubilized in water and incubated overnight with a mixed bed ion exchange resin to remove cyanate ions that can carbamylate peptides then the Tris can be added and the solution brought to the proper pH. Solution should then be aliquoted and stored at −80°C.
3. A solution of urea will form a carbamate ion over time that can and will modify amino acid side chains. This reaction is very dependent on heat so it is crucial to perform all digestion steps in urea at room temperature rather than 37°C. Some people prefer to recrystallise urea to remove the carbamate. Our approach is to dissolve the appropriate mass of urea and thiourea to make 250 mL of a 6/2 *M* solution in about 220 mL of water and then add a mixed bead ion exchange resin with stirring for several hours or overnight. Then the resin is pelleted by centrifugation, the appropriate buffer added and the solution adjusted to the correct pH before bringing the volume to 250 mL. This solution is then aliquoted into single-use aliquots and stored at −80°C.
4. Successful SILAC analysis depends on complete incorporation of the labelled amino acids into the sample. Hypothetically, if no proteins were degraded during replication and cells were allowed to divide through six cycles after adding SILAC media then the amount of original, unlabelled protein remaining in the cells would be 2^{-6} or about 1.5%, which is most likely less than the isotopic impurity level in the labelled amino acids being used. We try to grow all cells in SILAC media for at least six population doublings before doing an experiment but even so some cell types are able to metabolise certain amino acids so it is important to check for

complete labelling in each cell type under each set of growing conditions.

5. When dealing with several plates of cells at once it may be necessary to complete the lysis of one condition before moving on to the second set. The main reason for this is the timing; dealing with several plates can take a long time and will introduce an additional variable to the experiment. It is also important that the cells get to 4°C as quickly as possible.
6. This preparation will only yield perhaps 50 ug of DRM protein so when mixing the heavy and light lysates it is more important to maximise yield rather than aim for mixing a specific amount of protein each time an experiment is repeated. Therefore, we typically only measure the relative protein concentrations of the two lysates and use as much as possible.
7. When extracting a band from an ultracentrifuge tube it is important to keep the needle in a steady position relative to the tube, otherwise, the level of the extraction will jump around and reduce the resolution of the gradient.
8. Glycogen is added to the protein precipitation solution as it helps achieve more quantitative precipitation and because it makes visualising the pellet much easier when small masses of protein are involved. As glycogen is purely a carbohydrate it does not get digested in downstream steps and will be removed at the STAGE tip steps.
9. The elution gradient should be adjusted to give LC peak widths of approximately 20 s at half the maximal height. The reason for this is sampling: the mass spectrometer needs enough time to fully sample each ion over its elution curve in order for the downstream quantitation to be accurate.
10. The Mascot results must be saved in the Peptide Summary view chosen from the main search results window in order to be compatible with MSQuant.

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Chapter 15

Use of Sequential Chemical Extractions to Purify Nuclear Membrane Proteins for Proteomics Identification

Nadia Korfali, Elizabeth A. L. Fairley, Selene K. Swanson, Laurence Florens, and Eric C. Schirmer

Abstract

The nuclear envelope (NE) is a double membrane system that is both a part of the endoplasmic reticulum and part of the nucleus. As its constituent proteins tend to be highly complexed with nuclear and cytoplasmic components, it is notoriously difficult to purify. Two methods can reduce this difficulty for the identification of nuclear membrane proteins: comparison to contaminating membranes and chemical extractions to enrich for certain groups of proteins. The purification of nuclear envelopes and contaminating microsomal membranes is described here along with procedures for chemical extraction using salt and detergent, chaotropes, or alkaline solutions. Each extraction method enriches for different combinations of nuclear envelope proteins. Finally, we describe the analysis of these fractions with MudPIT, a proteomics methodology that avoids gel extraction of bands to facilitate identification of minor proteins and membrane proteins that do not resolve well on gels. Together these three approaches can significantly increase the output of proteomics studies aimed at identifying the protein complement of subcellular membrane systems.

Key words: Nuclear envelope (NE), endoplasmic reticulum (ER), microsomal membrane (MM), integral membrane protein, inner nuclear membrane (INM), outer nuclear membrane (ONM), detergent, chaotrope, alkaline, multidimensional protein identification technology (MudPIT).

1. Introduction

At the core of the nuclear envelope (NE) is a double membrane system. One half of this system, the inner nuclear membrane (INM) faces the nucleus and contains many integral membrane proteins *(1–3)*. Most of these appear to be type II integral proteins and several have been shown to bind chromatin *(4, 5)*

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and/or the intermediate filament lamin polymer that lines the INM *(6)*. The lamin polymer can also interact directly with the membrane via a farnesyl group at the C-terminus of B-type lamins *(7)* and a GNAERG group at the N-terminus of lamin C2 *(8)*. The other half of this membrane system, the outer nuclear membrane (ONM), faces the cytoplasm and, studded with ribosomes, is clearly a subcompartment of the endoplasmic reticulum (ER) *(9, 10)*. It contains some unique proteins that appear to connect the NE to cytoplasmic filament systems *(11)*. These two lipid bilayers connect at the pore membrane (PoM) where they flow around the nuclear pore complexes (NPC), large macromolecular assemblies that regulate the directional trafficking of soluble molecules between the nucleus and cytoplasm *(12, 13)*. Recent work suggests that the NPCs actively regulate trafficking of transmembrane proteins as well *(14)*. Between the ONM and INM is the lumen of the NE, which may also contain unique proteins or functions: for example, most of the mass of the integral NPC protein gp210 resides in the lumen *(15)*. Together the ONM and associated transmembrane proteins, PoM and associated transmembrane proteins, lumen and associated proteins, INM and associated transmembrane proteins, and the connected lamin polymer constitute the NE.

Because of its inherent inner complexity and outer connectivity the NE cannot be purified to homogeneity, complicating efforts to identify the full complement of NE proteins. We developed a subtractive proteomics approach specifically to identify membrane proteins unique to the NE. As there are no expected contaminating membrane structures within the nucleus, the major expected contaminant would be ER membranes: indeed single membrane vesicles likely to have been derived from vesiculated fractionated ER are observed in NE preparations by electron microscopy *(16)*. Therefore, proteins appearing in both NEs and a separately analyzed ER fraction are discarded from the dataset *(16)*. In contrast, contamination from soluble proteins could come from the ER, cytoplasmic filaments, or the nucleoplasm; thus it is not possible to choose a single contaminating fraction for subtraction. The ER-rich fraction was obtained by using standard protocols for purification of microsomal membranes that are made completely free of nuclear membrane because the large intact nuclei are readily pelleted before membranes are floated on sucrose cushions *(17, 18)*. NEs are prepared by first isolating nuclei *(19)*, then douncing to vesiculate other membranes that are removed by floating on sucrose cushions, and finally digesting chromatin to remove nucleoplasmic contents *(20, 21)*.

To enrich for transmembrane proteins, NE and microsome fractions are extracted with either alkaline solutions *(16)* or chaotropes *(22)*, which should solubilize cytoskeletal and chromatin components/contaminants while leaving transmembrane

proteins embedded in the insoluble membrane. To enrich for proteins associated with the intermediate filament lamin polymer NE fractions were extracted with salt and detergent, as the lamina (lamins and interacting transmembrane proteins) is defined biochemically by its general insolubility at NaCl and Triton X-100 concentrations of up to 1 M and 2%, respectively. Detergents solubilize membrane proteins by mimicking the lipid-bilayer environment. At low concentrations they integrate into the lipid bilayer, but as the bilayer becomes saturated the membrane disassembles to form mixed micelles. There is no general rule for which type of detergent is optimal to extract a particular membrane protein or type of membrane protein; thus one detergent may extract a subset of both contaminants and NE proteins while another may extract a different subset of both. Indeed, extraction characteristics differ even among lamin subtypes *(23)*. The variability in what each chemical extracts is highlighted by the minimal overlap between the proteins identified using alkali extraction and those identified using salt/detergent extraction, yet proteins in both datasets were strongly predicted to contain membrane-spanning segments by computer prediction and concentrated at the NE when tested by transient transfection of tagged versions *(16)*.

To minimise loss of membrane proteins that often do not resolve well on gels *(24)*, proteins were identified using Multi-Dimensional Protein Identification Technology *(25–27)* in which the extracted NE fraction is directly digested without prior separation of proteins. The complex peptide mixture is then resolved on combined reverse phase and cation exchange microcapillary columns (LC/LC), eluting directly into ion trap tandem mass (MS/MS) spectrometers. MS/MS datasets are searched for peptide sequence information using SEQUEST against a database combining mammalian protein sequences *(28)*. DTASelect is used to compile SEQUEST outputs into protein level information, and filter spectrum/protein matches based on SEQUEST-defined parameters *(29)*. Multiple runs are compared using CONTRAST *(29)*. To identify transmembrane proteins within the dataset, additional computational algorithms are employed.

2. Materials

2.1. Preparation of Blood Lymphocytes

2.1.1. Hardware

1. Buffy coats from local blood bank.
2. Tissue-culture laminar flow hood.
3. Standard supplies (centrifuge tubes, gloves, pipettes, scissors, tissue-culture flasks, etc.).

2.1.2. Solutions

1. Phosphate buffered saline (PBS): 4.3 mM sodium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, 1.4 mM potassium phosphate.
2. Ficoll-Hypaque 1077 density gradient medium.
3. RPMI medium: RPMI 1640 (e.g. Cambrex 09-774), 10% fetal bovine serum, 100 U potassium penicillin, 100 μg/ml streptomycin sulfate, 100 μg/ml gentamicin sulfate.

2.2. Preparation of Nuclear Envelopes

2.2.1. Hardware

1. Loose fitting (Wheaton type B pestle) 15 ml glass Dounce homogeniser with clearance of between ~0.1 and 0.15 mm (*see* **Note 1**).
2. Swinging-bucket rotor (e.g. Beckman Coulter SW28 rotor with Beckman Coulter 344058 Ultra-Clear 25 × 89 mm centrifuge tubes).
3. Local standard light microscope, glass slides and coverslips.
4. Large bore luer lock stainless steel needles (14 gauge or larger) of greater length than centrifuge tubes and luer lock syringes.

2.2.2. Solutions

Sucrose solution names are defined by the initials for the primary components: S for sucrose, H for HEPES, K for KCl, and M for $MgCl_2$ (*see* **Note 2**).

1. Phosphate buffered saline (PBS): 4.3 mM sodium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, 1.4 mM potassium phosphate.
2. DNase resuspended at 10 U/μl in H_2O.
3. RNase resuspended in H_2O at 10 mg/ml and boiled for 20 min (*see* **Note 3**).
4. Protease inhibitors (*see* **Note 4**): all solutions require freshly added 1 mM AEBSF [4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride] from a 1 M solution in H_2O (*see* **Note 5**), 1 μg/ml aprotinin (from a 1 mg/ml stock in H_2O), 1 μM pepstatin A [from a 1 mM stock in DMSO (dimethyl sulfoxide)], 10 μM leupeptin hemisulfate (from a 10 mM stock in H_2O) and 10 μM 3,4-dichloro isocoumarin (e.g. Sigma D7910, from a 10 mM stock in DMSO) (*see* **Note 6**).
5. Hypotonic lysis buffer: 10 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl and freshly added 2 mM DTT (dithiothreitol; from a 1 M solution in H_2O) and protease inhibitors.
6. 0.25 M SHKM: 50 mM HEPES pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose and freshly added 1 mM DTT and protease inhibitors.
7. 2.2 M SHKM: 2.2 M Sucrose, 50 mM HEPES pH 7.4, 25 mM KCl, 5 mM $MgCl_2$ and freshly added 1 mM DTT and protease inhibitors (*see* **Note 7**).

8. 30% SHKM: 0.9 M Sucrose, 50 mM HEPES pH 7.4, 25 mM KCl, 5 mM $MgCl_2$ and freshly added 2 mM DTT and protease inhibitors. This can also be prepared by mixing 67 ml 0.25 M SHKM with 33 ml 2.2 M SHKM.
9. 30% SHM buffer: 0.9 M sucrose, 10 mM HEPES pH7.4, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$ and freshly added 2 mM DTT and protease inhibitors.
10. 10% SHM buffer: 0.3 M sucrose, 10 mM HEPES pH7.4, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$ and freshly added 2 mM DTT and protease inhibitors.

2.3. Preparation of Microsomal Membranes

2.3.1. Hardware

1. The same hardware is required as for *Preparation of Nuclear Envelopes*.
2. A type 45 Ti fixed-angle rotor or equivalent that can provide 150,000*g* and matching tubes (e.g. Beckman-Coulter 348176).

2.3.2. Solutions

The same sucrose solutions used for *Preparation of Nuclear Envelopes* can be used in preparing microsomes. In particular the 2.2 M SHKM, 0.25 M SHKM and a mixture of the two to 1.86 M sucrose will be required.

2.4. Chemical Extractions

2.4.1. Hardware

1. TLA100.3 rotor for table-top ultracentrifuge or equivalent and corresponding tubes (e.g. Beckman-Coulter 343778 polycarbonate 11 × 34 mm tubes).

2.4.2. Solutions

1. Salt wash: 50 mM HEPES pH 8.0, 500 mM KCl, 5 mM $MgCl_2$ with freshly added 2 mM DTT and protease inhibitors.
2. Alkaline extraction: 0.1 N NaOH, 1 mM DTT in H_2O.
3. Salt/Detergent extraction (i) Triton X-100 resuspended at 1.0% in a solution containing 25 mM HEPES pH 7.5, 400 mM NaCl. (ii) Octyl ß-D-glucopyranoside (also called n-Octyl glucoside) resuspended at 1.0% in a solution containing 25 mM HEPES pH 7.5, 400 mM NaCl. (iii) Empigen BB resuspended at 0.3% in a solution containing 25 mM HEPES pH 7.5, 400 mM NaCl.
4. Chaotrope extraction: 0.1 M Na_2CO_3, 4 M urea.

2.5. Digestion of Proteins

2.5.1. Hardware

1. Eppendorf Thermo Mixer R and Thermo mixer R block for 1.5 ml tubes.
2. pH Indicator Strips, 7.5–14.

2.5.2. Solutions

1. 90% Formic Acid.
2. Cyanogen Bromide at 500 mg/ml in 90% Formic Acid.

3. Ammonium Hydroxide solution, NH_4OH, in water at 0.9 g/ml density.
4. Urea, solid.
5. HPLC grade water.
6. Tris(2-Carboxylethyl)-Phosphine Hydrochloride, TCEP (e.g. Pierce, 20490), as a 1 M stock in HPLC grade water, stored at −20°C.
7. Iodoacetamide, IAM, made fresh weekly as a 500 mM stock in HLPC grade water, and stored at −20°C.
8. Hydrochloric acid, HCl
9. Tris base, as a 1 M solution in HPLC grade water, pH adjusted to 8.5 with HCl, stored at 4°C.
10. Endoproteinase LysC, sequencing grade (Roche, 11047825001), as a 1 μg/μl stock in in HPLC grade water, stored at −20°C.
11. Calcium chloride as a 500 mM stock in HPLC grade water, stored at room temperature.
12. Trypsin, modified sequencing grade, as a 0.1 μg/μl stock in HPLC grade water, stored at −20°C.

2.6. Microcapillary Column Preparation

2.6.1. Hardware

1. Laser Puller (e.g. Model P-2000 Sutter Instrument Co).
2. Polyimide coated fused silica, 50 μm i.d. × 365 μm o.d. (e.g. Polymicro Technologies, TSP 050375).
3. Polyimide coated fused silica, 100 μm i.d. × 365 μm o.d. (e.g. Polymicro Technologies, TSP 100375).
4. Polyimide coated fused silica, 250 μm i.d. × 365 μm o.d. (e.g. Polymicro Technologies, TSP250350).
5. Column Scribe (e.g. Chromatography Research Supplies, 205312).
6. M-520 In-line Micro Filter Assembly (e.g. UpChurch Scientific, M-520).
7. 0.5 μm PEEK Filter end fitting (e.g. UpChurch Scientific, M-120X).
8. Microtight 395 μm Sleeves (e.g. UpChurch Scientific, F-185X).
9. Pressurisation Device (Brechbuehler, Inc., Houston, TX, or MTA for blueprints available by request from John Yates, Scripps Research Institute, La Jolla, CA).
10. Agilent 1100 series G1379A degasser, G1311A quaternary pump, G1329A autosampler, G1330B autosampler thermostat, and G1323B controller (Agilent Technologies, Palo Alto, CA).

2.6.2. Solutions

1. HPLC grade Methanol
2. HPLC grade Acetonitrile
3. 90% Formic Acid
4. HPLC grade water

5. Ammonium Acetate
6. C_{18} Aqua Reversed Phase, 5 μm (e.g. Phenomenex, bulk material, 04A-4299) (*see* **Note 8**).
7. Partisphere Strong Cation Exchange, 5 μm (e.g. Whatman, WC4621-1507; *see* **Note 9**).
8. Buffer A: 5% Acetonitrile, 0.1% formic acid, in HPLC grade water.

2.7. Multidimensional Chromatography Coupled to Tandem Mass Spectrometry

2.7.1. Hardware

1. LCQ DECA-XP^{plus} tandem mass spectrometer (Thermo Electron, San Jose, CA).
2. Nano electrospray stage (e.g. Thermo Electron Nanospray II ion source or PicoView Source from New Objective).
3. MicroTee Assemblies (e.g. UpChurch Scientific, P-775).
4. Micro Ferrule for 360 μm OD tubing (e.g. UpChurch Scientific, F-152).
5. Gold wire 0.025 in diameter (e.g. Scientific Instrument Services, W352).

2.7.2. Solutions

1. Buffer A: 5% acetonitrile, 0.1% formic acid, in HPLC grade water.
2. Buffer B: 80% acetonitrile, 0.1% formic acid, in HPLC grade water.
3. Buffer C: 500 mM ammonium acetate, 0.5% acetonitrile, 0.1% formic acid, made with HPLC grade water and filtered.

2.8. Analysis of MS/MS Dataset

Linux Computer cluster (over 100 nodes) dedicated to SEQUEST analysis.

2.9. Determination of Transmembrane Proteins

Many computer algorithms that can be used to predict transmembrane proteins are freely available online and discussed elsewhere in this volume. Those we typically use are listed:

1. TMHMM http://www.cbs.dtu.dk/services/TMHMM-2.0/
2. TMPred www.ch.embnet.org/software/TMPRED_form.html

3. Methods

The first step in NE enrichment is the isolation of nuclei from other cellular proteins and membranes. Factors critical to this process are the size ratio of nucleus to cytoplasm, the concentration of cytoplasmic filament systems and nuclear density, which all vary among different tissues. The protocol described here is one we have optimized for purifying NEs from human blood leukocytes (generally 60–80% lymphocytes; *see* **Note 10**). We generally recover ~65 million NEs from one buffy coat (~100 million leukocytes; *see* **Note 11**).

3.1. Preparation of Blood Lymphocytes

1. Buffy coats are obtained from a blood bank according to local permissions and protocols. If dealing with more than one buffy coat at a time it is important to keep them separate until **Section 3.2.7**, otherwise the mixed lymphocyte activation response can rapidly alter expression profiles and cells may aggregate, forming rosettes.
2. Open six 50 ml conical tubes (e.g. Falcon^{TM} Conical Centrifuge Tubes, 50 ml capacity) in a laminar flow cabinet/tissue culture hood (*see* **Note 12**).
3. Wipe scissors and blood bag with 70% EtOH. Cut one of the strands of tubing coming from the bag while holding upright. Tilt end of tube over first 50 ml conical tube and pour 12.5 ml into each tube using tilt angle to regulate gravity flow.
4. Dilute blood with 4 volumes PBS (50 ml total per tube that has a full 12.5 ml of blood). Calculate the total volume and divide by 15: this gives the number of tubes required for Step 5.
5. Pour 15 ml of Ficoll-Hypaque 1077 media into fresh tubes.
6. Gently overlay with 15 ml of diluted blood (*see* **Note 13**).
7. Centrifuge at 400*g* for 30 min at room temperature.
8. Three layers will be obtained after centrifugation: the middle layer containing principally lymphocytes should appear cloudy white (*see* **Note 14**). Carefully dispense of the upper layer with a pipette. Collect all the intermediate phase (on average 4–6 ml) into a new tube, taking care to disrupt any clumps.
9. Dilute the material with four volumes of PBS and centrifuge at 250*g* for 10 min at room temperature(*see* **Note 15**).
10. Resuspend each pellet in 25 ml PBS, combine (two tubes into one), and centrifuge at 250*g* for 10 min at room temperature (*see* **Note 16**).
11. Resuspend each pellet in RPMI medium and transfer into 75 cm^2 tissue-culture flasks (*see* **Note 17**).
12. One buffy coat will generally yield ~120 million leukocytes and can be seeded at between one and ten million cells per ml.

3.2. Preparation of Nuclear Envelopes

1. Count cell number and pellet (in 50 ml tubes) at 250*g* for 10 min at room temperature (*see* **Note 18**).
2. Carefully and completely decant supernatant and resuspend the pellet from each blood pack from Step 1 in 0.5 ml ice-cold PBS by gentle agitation (*see* **Note 19**). Accumulate so each tube has ~25 million cells and add additional PBS to 30 ml (*see* **Note 20**).
3. Withdraw a 10 µl sample for microscopic analysis, then pellet the rest at 250*g* for 10 min at 4°C.

4. Pour off buffer carefully by inversion, carefully re-right the tube, and let PBS settle. Remove the remaining PBS with a pipette tip. Place pellets on ice and resuspend the first pellet in 7 ml ice cold hypotonic lysis buffer with freshly added DTT and protease inhibitors (*see* **Note 21**). Let incubate on ice for 10 min, following an aliquot under the microscope throughout (*see* **Note 22**). Good swelling is depicted in **Fig. 15.1**: compare panel A (before swelling) with panel B (swelling). When such a state is reached, the Dounce step *(6)* should be performed.
5. Five minutes after resuspension of the first pellet, similarly resuspend the second pellet (*see* **Note 23**).
6. When the 10 min incubation is complete, cells should be moved to the 15 ml loose Dounce homogenizer and lysed by 10 vigorous strokes. Immediately add 1/10 volume of 2.2 M SHKM and 1/10 volume of 1 M KCl (*see* **Note 24**).
7. Remove to centrifuge tubes and underlay with a cushion of 1/10th volume 30% SHKM using a 14 gauge needle and syringe. Pellet nuclei at 2,000*g* in a swinging- bucket rotor (e.g. 4,000 rpm in a Beckman Coulter J6-MC floor model centrifuge) for 20 min at 4°C.
8. Decant the supernatant and resuspend pellets in 11 ml 0.25 M SHKM. If they appear at all aggregated, give a few sharp strokes in the Dounce homogeniser with the loose pestle to uniformly resuspend them. Add 2.2 M SHKM to a final concentration of 1.9 M sucrose (39 ml) (*see* **Note 25**).
9. Dispense 25 ml into each SW28 ultracentrifuge tube and underlay with 5 ml of 2.3 M SHKM using a 14 gauge needle in a luer lock syringe (**Fig. 15.2**).
10. Balance the tubes by exchange between the upper nuclei-enriched lysate phase and spin in the SW28 rotor for 2 h at 82,000*g* (25,000 rpm).

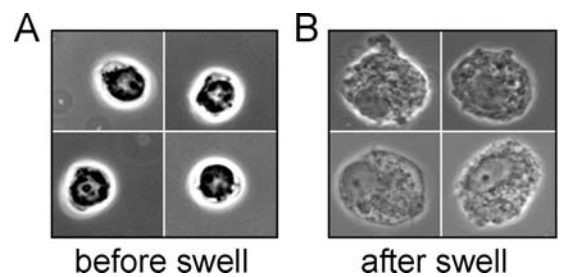

Fig. 15.1. Swelling of lymphoctyes for hypotonic lysis. (**A**) Cells prior to treatment. Nuclei make the greater part of cell mass. (**B**) The same cells after swelling in hypotonic lysis buffer. Note that some cells have burst even without Dounce homogenisation. It is critical to Dounce the cells quickly at this stage and make the solution isotonic or the nuclei will also swell and burst.

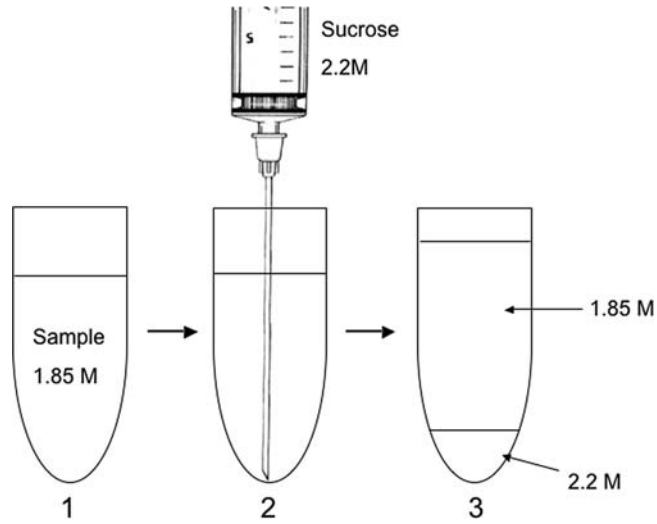

Fig. 15.2. Schematic detailing the addition of sucrose cushions. After distributing the nuclei-enriched lysate into SW28 ultracentrifuge tubes, a long large bore needle is inserted to the base of the tube to underlay with high sucrose buffer. Because of the high viscosity of the 2.2 M sucrose solution, it is important to use a needle of at least 14 gauge: it takes several minutes to underlay each tube if an 18 gauge needle is used compared to 30 s with a 14 gauge. It is important to use a luer lock syringe because the viscosity of the solution can produce high pressure on the connection.

11. Move to a cold room and remove any floating white material with a spatula, then pour off the rest of the supernatant by rapid inversion. Keep the tubes upside down in the cold for 10 min to drain them. Then gently wipe out the inside walls of tubes with a folded kimwipe (or equivalent towel), being very careful not to touch the pellet (*see* **Note 26**).
12. Resuspend each pellet in a small volume (~2 ml) of 0.25 M SHKM with freshly added 2 mM DTT and protease inhibitors, being careful to avoid touching the walls.
13. Dounce to break the aggregates of nuclei and transfer into an ice-cooled 15 ml conical tube. Wash the homogeniser with 1 ml 0.25 M SHKM and add to the resuspended nuclei. Take a small aliquot for counting nuclei (step 14) and another to place on a slide for step 16.
14. Count nuclei using a hemocytometer and calculate the total number from the volume currently spinning in the centrifuge. The nuclei should be clean of contaminating cell fragments and appear as in **Fig. 15.3A**.
15. Resuspend in 10% SHM with freshly added 2 mM DTT and protease inhibitors at 1.5 million nuclei/ml. Add 4 U/ml DNase and 1 μg/ml RNase and incubate at room temperature for 15 min. Transfer into ice-cooled tubes and centrifuge at 6,000*g* (~5,000 rpm) in a floor model intermediate speed centrifuge with a swinging-bucket rotor (e.g. Beckman-Coulter J6-MC) for 10 min at 4°C.

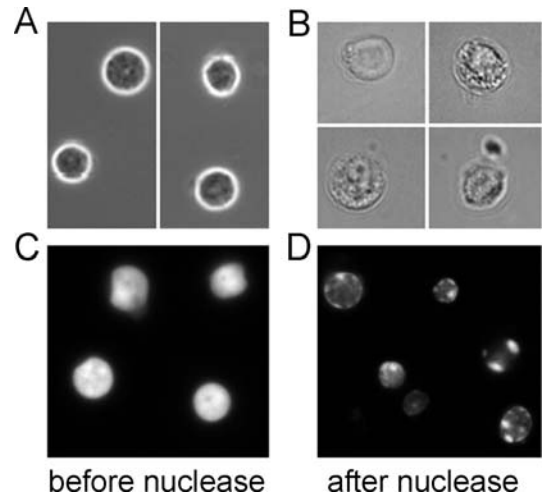

Fig. 15.3. Removal of nucleoplasmic contents to make NEs. (**A**) Isolated nuclei before nuclease treatment. (**B**) Nuclei after chromatin digestion. Note the change from phase dense to phase lucent. When the cells have reached this stage, the digestion is complete and pelleting yields the final NE fraction. (**C** and **D**) DNA staining. Isolated nuclei (**C**) and NEs (**D**) are also depicted by fluorescence microscopy after staining with Hoechst 33342. Most of the DNA staining has disappeared from the chromatin-digested NEs.

16. Resuspend the pellet in 10% SHM with freshly added 2 mM DTT and protease inhibitors at 3 million nuclei/ml. Dounce if necessary, transfer into a 15 ml conical tube. Add 8 U/ml DNase and 2 μg/ml RNase and incubate at room temperature for 15 min. Observe digestion on the microscope in parallel. The phase grey of the nuclei should diminish (**Fig. 15.3**, compare **A** and **B**).
17. When 90% of nuclei are no longer phase-grey, underlay the solution with 30% SHM with freshly added DTT and protease inhibitors. Spin for 30 min at 6,000*g* using a swinging-bucket rotor (e.g. 5,000 rpm in a Beckman-Coulter floor model J6-MC centrifuge; *see* **Note 27**).
18. Carefully aspirate off the supernatant (do not decant by pouring) as the pellet will be very soft (*see* **Note 28**).
19. Resuspend the pellet in the same volume of 10% SHM. Withdraw a 10 μl sample and count NEs using a hemocytometer to calculate the yield of the prep:

    percentage yield is calculated as (final number of NEs/total number initial cells) × 100

20. Although there is no time during the digestion to stain nuclei with DAPI or Hoechst dyes to visualise more directly the extent of chromatin digestion, if this is desired samples can be taken at this point and fixed for later staining. The loss of chromatin observed by phase contrast light microscopy (**Fig. 15.3A** and **B**) can be more clearly observed by DNA staining (**Fig. 15.3C** and **D**).

21. Aliquot the NEs to centrifuge tubes (chosen for desired storage method and concentration) and spin at 6,000*g* (5,000 rpm) for 10 min (no cushion) at 4°C (*see* **Note 29**).
22. Carefully aspirate the supernatants and immediately flash-freeze in liquid nitrogen and store at −80°C.

3.3. Microsomal Membrane Purification

Because lymphocytes have very little cytoplasm to produce microsomes, we instead used the HL-60 human cell line that can be induced into different blood cell lineages. Treatment with phorbol esters induces differentiation into megakaryocytes with associated attachment and spreading of the cells. We predicted that this would produce microsomes in sufficient quantity while being largely similar in protein composition to the lymphocyte ER.

1. Seed 1×10^7 HL-60 cells per 10 cm tissue- culture dish.
2. Add PMA (Phorbol 12-myristate acetate; Calbiochem 524400) from a 5 mg/ml stock in acetone to a final concentration of 1 μg/ml.
3. When the cells have adhered and spread (2–3 days), collect by scraping with a teflon cell scraper, transfer into a centrifuge tube and pellet at 250*g* for 10 min.
4. Wash once in PBS and collect again by centrifugation.
5. Follow Steps 4–7 in *Preparation of Nuclear Envelopes* (**Section 3.2**).
6. Recover the supernatant from *Preparation of Nuclear Envelopes* Step 7 and add EDTA to 0.5 mM to inhibit metalloproteinases. Centrifuge at 10,000*g* 11,500 rpm, Type 45 Ti Beckman for 15 min to remove mitochondria (*see* **Note 30**).
7. Mix the post-mitochondrial supernatant with 5 volumes (e.g. 1 ml + 5 ml) of 2.2 M SHKM (with 0.5 mM EDTA, 2 mM DTT, and protease inhibitors) to achieve a final sucrose concentration of roughly 2 M.
8. Float the microsomes by pouring 20 ml of the diluted membranes in each SW28 rotor tube and overlay with 6 ml of 1.86 M SHKM with 0.5 mM EDTA, 2 mM DTT and protease inhibitors. Finally, overlay this with 2.3 ml of 0.25 M SHKM with 0.5 mM EDTA, 2 mM DTT and protease inhibitors. Centrifuge at 57,000*g* (21,000 rpm) in type 45 Ti rotor for 4 h (*see* **Note 31**).
9. The microsomes will be in the interface between the 1.86 M and 0.25 M sucrose layers and should appear largely white and translucent. There will be material in and above the 0.25 M layer, which should be discarded; thus it is best to remove material by tube puncture from the side though a syringe inserted between the layers from the top.
10. Dilute the membranes with four volumes of 0.25 M SHKM (with 0.5 mM EDTA, freshly added DTT and protease inhibitors) and pellet at 152,000*g* (44,000 rpm in a type

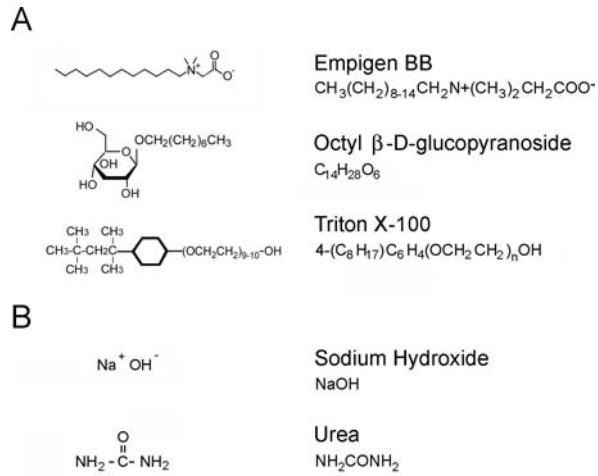

Fig. 15.4. Structures of chemicals used to extract NEs. (**A**) The three detergents used have very different structures. Empigen BB is a zwitterionic detergent that was used in a recent proteomics study, where it was reported to preferentially solubilize nuclear pore complex core proteins *(33)*. As a Zwitterionic detergent it has both properties of non-ionic and ionic detergents. Both octyl ß-D-glucopyranoside (also called octyl glucoside) and Triton X-100 are non-ionic detergents. Octyl glucoside has a glycosidic head group, while Triton X-100 has a polyoxyethylene head group. The non-ionic detergents are generally thought to be better suited to break lipid-protein interactions than protein-protein interactions. They also have the advantage of being unaffected by the concentration of salt, which enables further removal of chromatin contaminants from the NEs. (**B**) Alkaline and chaotrope stuctures for NaOH and urea respectively.

45 Ti, 48,000 rpm in a type 50 Ti, or 60,000 rpm in a TLA100.3 rotor) for 75 min (*see* **Note 32**). There should be a translucent pellet with the appearance of a fat droplet in the corner of the tube. Decant supernatant and store at −80°C.

3.4. Chemical Extractions

It is wise to check an aliquot of the NE preparation by Coomassie blue stained SDS-PAGE as 'Purified' NEs often contain a prominant histone band indicating a large degree of chromatin contamination. If this is the case, they may be salt washed prior to further chemical extraction. Formulas for the chemicals used to extract are depicted in **Fig. 15.4**.

3.4.1. Salt Wash

1. To salt wash, resuspend the NE pellet in the salt wash solution at ~5 million NEs/ml and let stand on ice 15 min with occasional mixing.
2. Pellet by centrifugation at 20,000*g* (~14,000 rpm) in a cooled microcentrifuge for 30 min and decant supernatant.

3.4.2. Alkali (NaOH) Extraction

1. Resuspend NEs (no more than 10 million/ml or an equivalent amount of microsomes, *see* **Note 33**) in 0.1 M NaOH on ice.

2. Transfer immediately to TLA100.3 ultracentrifuge tubes and pellet insoluble material at 104,000*g* (50,000 rpm) for 35 min at 4°C (*see* **Note 34**).
3. Wash the pellet quickly with double distilled H_2O (*see* **Note 35**).
4. Either freeze at −80°C or directly process for MS.

3.4.3. Extraction with Salt and Detergent

1. Resuspend NE pellet in any salt/detergent buffer at no more than 10 million NEs/ml and incubate on ice for 15 min with occasional mixing.
2. Pellet by centrifugation at 20,000*g* (~14,000 rpm) in a cooled microcentrifuge for 30 min and decant supernatant.
3. Wash the pellet with the same buffer minus detergent, re-pellet and either freeze at −80°C or directly process to digest for MS.

3.4.4. Chaotrope Extraction

1. Resuspend the pellet in chaotrope buffer and incubate on ice for 15 min.
2. Pellet the insoluble material in a TLA100.3 table top ultracentrifuge rotor or equivalent at 104,000*g* (50,000 rpm) for 35 min at 4°C.
3. Wash the pellet with double distilled H_2O and either freeze at −80°C or directly process to digest for MS.

Relative recovery of representative nuclear or nuclear membrane proteins using the methods described here is depicted in **Fig. 15.5**.

3.5. Digestion of Proteins

As described in *(26)*, the membrane pellets were first partially solubilized in formic acid and cyanogen bromide to chemically cleave large portions of proteins, before digesting these larger peptides with endoproteinase Lys-C and trypsin.

1. On day one, resuspend the dried membrane pellets in 100 µl of CNBr at 500 mg/ml in 90% formic acid, mix by pipetting and leave under a fume hood overnight in the dark (e.g. in a cardboard freezer box).
2. On day two, transfer 100 µl of partially solubilized membranes to a 15 ml conical tube on ice (*see* **Note 36**), and add NH_4OH drop by drop to neutralise formic acid until there is no more bubbling and the pH is ~8.5 (check after every 100 µl added with 1 µl using pH indicator strips). Once the appropriate pH is reached, the sample can be transferred back to a 1.5 ml tube. The final volume should be around 500 µl, that is, a 3- to 5-fold dilution.
3. Add solid urea to 8 M (taking into account the fact that adding urea will cause the volume to increase to about 800 µl) and then add TCEP to a final concentration of 5 mM. Allow the reduction to proceed at room temperature

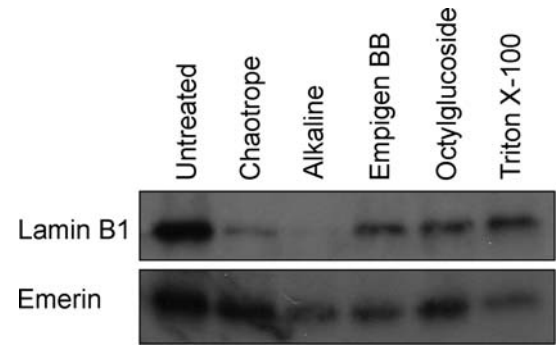

Fig. 15.5. Comparison of leukocyte/lymphocyte NEs extracted with Na_2CO_3/urea (chaotrope), NaOH (alkaline), Empigen BB, ß-octyl glucoside, or Triton X-100. Extracted proteins were resolved by SDS-PAGE (loading 0.5 million starting NEs per lane on Bio-Rad 15-well mini-gels), transferred to PVDF membranes, and reacted with antibodies to either lamin B1 or the integral membrane proteins of the inner nuclear membrane emerin. The chaotrope and alkaline treatments removed most lamins, while detergents had little effect. In contrast, chaotrope and alkaline treatments had little effect on emerin, yet each detergent extracted this integral protein to a different degree with the most protein remaining after extraction with octylglucoside. More emerin was removed by Triton X-100 extraction than by any other treatment. This parallels results from the first proteomic analysis of NETs in which emerin was not identified in a Triton-extracted fraction, but was in the chaotrope-extracted fraction *(22)*. In that same study, another integral inner nuclear membrane protein (LBR) was lost from the chaotrope-extracted fraction and retained in the Triton-extracted fraction.

for 30 min, then add IAM to 20 mM, and let the carboxiamidomethylation of free cysteines proceed for 30 min at room temperature in the dark.

4. Re-check the pH at this step using pH indicator strips and adjust with 1 M Tris-HCl pH 8.5 if necessary before adding endoproteinase Lys-C at 1 µg/µl for an estimated enzyme to protein ratio of 1:100 (w/w). Let the digestion proceed at 37°C, for at least 6 h.
5. Dilute the digestion mix to 2 M urea by adding 0.1 M Tris-HCl, pH 8.5 (*see* **Note 37**). Add $CaCl_2$ to 2 mM, then trypsin at 0.1 µg/µl for an estimated enzyme to protein ratio of 1:100 (w/w). Let the reaction proceed overnight at 37°C.
6. On day three, quench the reaction by adding formic acid to 5%. Either store the peptide mixtures at −20°C or load directly onto microcapillary columns.

3.6. Microcapillary Column Preparation

The large final sample volume (3 × 1,150 µl) would take a very long time to load onto traditional 100 µm columns, so we use the 'split' column approach (**Fig. 15.6**), in which the sample is loaded onto larger diameter open-ended columns packed with reverse phase and strong cation exchange resins, before being connected to a resolving 100 µm column packed with reverse phase (*see (30)*) for a detailed description of the following steps).

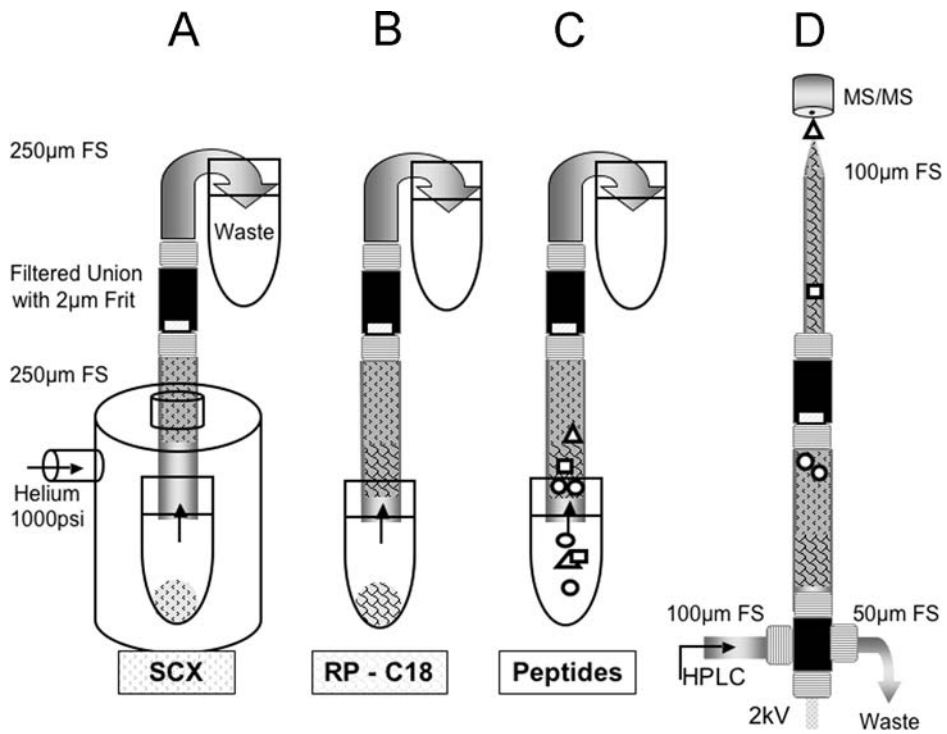

Fig. 15.6. Split-3-phase microcapillary column: Packing, Loading and Setup in LC/LC/MSfMS Mode. (**A**) Two 250 µm fused silica tubing are connected via a filtered union. One end of the set up (on the frit side) is inserted into a high-pressure deviced and packed using Helium pressure with SCX material in a slurry. (**B**) The column is loaded with a slurry of Aqua C-18 RP, then washed with Methanol and Buffer A. (**C**) The complex peptide mixture is pressure-loaded onto the 250 µm column. (**D**) A pulled 100 µm single-phase column is connected to the loaded and washed 2-phase column, and installed in-line with a quaternary HPLC pump and a tandem MS.

3.6.1. Single-Phase Fused-Silica 100 µm Microcapillary Column

1. Place 40 cm of 100 µm i.d. × 365 µm o.d. fused silica into P-2000 laser puller and pull tip to about a 5 µm opening.
2. Make a slurry of 5 µm C_{18} Reverse Phase (Aqua Phenomenex), at 15 mg/ml in 500 µl methanol.
3. Pack fused silica column with 8–9 cm of 5 µm C_{18} RP using high-pressure loading device.
4. Wash with methanol for at least 10 min.
5. Equilibrate in Buffer A for at least 30 min.

3.6.2. Double-Phase Fused-Silica 250 µm Microcapillary Column

1. Connect two 250 µm i.d. × 365 µm o.d. fused silica capillaries (about 30 cm) with a 2 µm filtered union.
2. Make slurries of 5 µm C_{18} Reverse Phase (Aqua Phenomenex) and of 5 µm strong cation-exchange material (Partisphere SCX, Whatman), both at 15 mg/ml in 500 µl methanol.
3. Pack first with 3–4 cm of Partisphere SCX, followed by 2–3 cm of Aqua RP.
4. Wash with methanol for at least 10 min.
5. Equilibrate in Buffer A for at least 30 min.

3.6.3. Off-Line Loading and Desalting

1. Spin samples down at 14,000 rpm for 30 min and transfer to a new tube.
2. Load sample to 250 μm column.
3. Wash with Buffer A (1.5 ml).
4. Connect 250 μm i.d. column to equilibrated 100 μm double-phase column.

3.7. Multidimensional Chromatography Coupled to Tandem Mass Spectrometry

3.7.1. Chromatography

1. Install the loaded and washed split-3-phase microcapillary column on the nanoelectrospray stage (**Fig. 15.6D**), connecting it with the quaternary HPLC pump using a microtee.
2. Cut the overflow of 50 μm fused silica capillary to the appropriate length (about 40 cm) to have a flow rate at the tip of the column of about 200–300 nl/min (i.e. back pressure of ~40 bars), while the HPLC flow rate is kept constant at 0.1 ml/min throughout the chromatography.
3. Engage a 12-step chromatography run (24 h) on samples with the gradient parameters described in **Table 15.1**. In such sequences of chromatographic events, peptides are sequentially eluted from the SCX resin to the RP resin by increasing salt steps (increase in Buffer C concentration), followed by organic gradients (increase in Buffer B concentration). For the last chromatography step, wash in high salt with 100% Buffer C, followed by the acetonitrile gradient (repeated twice).
4. Apply a 2.4 kV voltage to the eluting peptides via a gold wire connecting the microtee and the mass spectrometer.

3.7.2. Tandem Mass Spectrometry

Chromatographic gradients and MS/MS data acquisition are controlled by the Xcalibur™ data system. For runs performed on LCQ-Deca tandem mass spectrometers, the acquisition scheme is a cycle of one full MS scan (from 400 to 1,600 m/z), followed by three MS/MS events at 35% collision energy on the top three most intense ions. Dynamic exclusion is enabled for 5 min, allowing ions of lesser intensities to be analyzed. This cycle is repeated continuously throughout the chromatography. One RAW file is generated for each chromatographic step and needs to be converted into a dat file using the XCalibur file converter function.

3.8. Analysis of MS/MS Dataset

1. Convert each dat file into a ms2 file *(31)* using extract-ms followed by 2–3 *(32)* to remove spectra of poor quality and assign a charge state to the precursor ion when possible.
2. Use SEQUEST *(28)* to search the MS/MS datasets against a database of sequences downloaded from NCBI (National Center for Biotechnology Information). We combined 27,960 human, 26,180 mouse, 21,205 rat, 21,909 chimp, 5,373 orangutan, 903 gorilla and 2,777 macaque protein sequences on 2005-02-17, and complemented with

Table 15.1
Gradient profiles for a 12-step MudPIT chromatography

Step #	Time (min)	Buffer A (%)	Buffer B (%)	Buffer C (%)
1	0	100	0	0
1	16	60	40	0
1	17	0	100	0
1	20	0	100	0
2 through 10	0	100	0	0
2 through 10	3	100	0	0
2 through 10	3.1	98	0	X*
2 through 10	5	98	0	X*
2 through 10	5.1	100	0	0
2 through 10	10	100	0	0
2 through 10	10.1	100	0	0
2 through 10	25	85	15	0
2 through 10	117	55	45	0
11 and 12	0	100	0	0
11 and 12	2	100	0	0
11 and 12	2.1	0	0	100
11 and 12	22	0	0	100
11 and 12	22.1	100	0	0
11 and 12	27	100	0	0
11 and 12	37	80	20	0
11 and 12	85	30	70	0
11 and 12	90	0	10	0
11 and 12	90.1	0	10	0
11 and 12	95	0	10	0
11 and 12	95.1	100	0	0
11 and 12	97	100	0	0

*X is equal to 5, 10, 15, 20, 30, 40, 50, 60 and 80% C is Steps 2 through 10, respectively.

172 sequences from usual contaminants (human keratins, IgGs. . .) (*see* **Note 38**.)

3. Bring together the peptide information contained in the SEQUEST output files and organise these into protein level

information using DTASelect *(29)*. DTASelect is also used to select and sort peptide/spectrum matches passing the criteria defined in **Table 15.2**. In particular, the validity of peptide/spectrum matches is assessed using the SEQUEST-defined parameters, cross-correlation score (XCorr) and normalised difference in cross-correlation scores (DeltCn). Spectra/peptide matches are only retained if they have a DeltCn of at least 0.08 and minimum XCorr of 1.8 for singly-, 2.5 for doubly- and 3.5 for triply charged spectra. In addition, the peptides have to be at least 7 amino acids long and the preceding residue in the protein sequence has to be a methionine, an arginine or a lysine (to account for the combined cyanogen bromide/trypsin digestion protocol).

Table 15.2
Filtering criteria applied to spectrum/peptide matches and proteins

Parameter	Value
Minimum +1 XCorr	1.8
Minimum +2 XCorr	2.5
Minimum +3 XCorr	3.5
Minimum DeltCN	0.08
Minimum charge state	1
Maximum charge state	3
Maximum Sp rank	10
Tryptic status requirement	Any
Multiple, ambiguous IDs allowed	FALSE
Preceding residue must be one of	KRM
Minimum sequence length	7
Maximum sequence length	100
Purge duplicate peptides by protein	XCorr
Include only loci with unique peptide	FALSE
Remove subset proteins	FALSE
Exclude protein names matching	Contaminant
Exclude protein descriptions matching	KERATIN
Minimum redundancy for low coverage loci	10
Minimum peptides per locus	1

4. Compare protein lists with multiple runs using CONTRAST *(29)*. Although some argue that at least two peptides should be recovered for higher confidence, we allow single peptide identifications in the final dataset because some well-characterised NETs were also detected by single peptides in previous studies, likely indicating their lower abundance *(16)*.
5. Additional utilities of DTASelect/CONTRAST are used to create subset databases in Fasta format containing only the proteins in the final list and to generate tab-delimited text files that can be consolidated into relational databases using MSAccess.

3.9. Determination of Transmembrane Proteins

Use Fasta format protein sequence files to search for predicted transmembrane segments using various algorithms. These are detailed elsewhere in this volume (*see* **Note 39**).

4. Notes

1. In **Section 3.2.6** the number of leukocytes aliquoted for each Dounce step should be correspondingly altered if a smaller or larger Dounce homogeniser is used.
2. $MgCl_2$ concentration in the original procedure was 5 mM throughout; however, if NEs are being prepared for viewing by electron microscopy, dropping the concentration through most of the procedure to 0.1 mM will yield better structure. If this is done, the $MgCl_2$ must be increased back to 5 mM, or to 2 mM with 0.5 mM $CaCl_2$ as we use, during DNase and RNase treatment for the enzymes to function properly.
3. This may seem counter-intuitive as the goal of the procedure is to degrade both RNA and DNA, but these enzymes are commonly prepared from bovine pancreas, and therefore, may have contaminating proteases unless recombinant proteins are used.
4. The optimal protease inhibitors will vary according to the tissue being investigated; so it is important to search the literature to determine what proteases are present at high concentrations in the tissue of choice. The choice for blood covers a wide general range (inhibiting serine, trypsin, cysteine, and aspartic proteases), but also includes coumarin which inhibits granzyme B, which is particularly abundant in blood cells.
5. We had traditionally used phenylmethylsulfonyl fluoride (PMSF) because it is much less expensive and large volumes are needed for the procedure. However, we have found that some batches tend to precipitate and form crystals when added to the solutions and when this happens we have observed nuclei/NEs aggregating on these crystals under the microscope. Thus, if using PMSF crystal formation

should be tested for each batch prior to its use in the procedure. When used, it can be added from a 100 mM stock in EtOH.

6. If general protease cocktails are used, it is important to make certain that they do not contain EDTA as this has been reported to negatively affect NE preparation.
7. The solution can be prepared by adding 220 ml of a 2.5 M (85%) sucrose stock to 12.5 ml 1 M HEPES, 6.25 ml 1 M KCl, 1.25 ml 1 M $MgCl_2$ and 10 ml H_2O.
8. Phenomenex now recommends the use of 'Synergi Hydro-RP as an improved alternative to Aqua 125Å'.
9. Bulk material is not available. The resin is extracted from the HPLC column (cut in half with hacksaw), washed with methanol, dried and stored as a powder.
10. Chapters detailing modifications of the NE protocol for rodent liver and muscle are being prepared for other volumes in the *Methods in Molecular Biology* series.
11. As with most protocols there is an optimal middle ground with too little or too much starting material resulting in lower yields. Because of the timing of centrifuge steps and layering gradients, one person can only easily manage two buffy coats at a time; however, four can easily be processed in a day in two sets. It is useful to increase the total number of blood packs used for a NE preparation as it is difficult to see the nuclear pellets after pelleting through the sucrose cushions, but the use of too much starting material can saturate the sucrose gradients. The maximum capacity of the SW28 rotor used in this procedure would be approximately eight buffy coats.
12. This is an excess to ensure that a sufficient number of tubes are open to accommodate the volume in the bag (which is variable); only four are usually required.
13. It is helpful when layering to tilt the tube at a 45° angle and direct the pipette tip at a right angle to the side of the tube so that the force of the flow is distributed over a wide area of the wall of the tube.
14. Sometimes the cells will be clumped and sometimes also have a red tinge. Neither invalidates the preparation. However, if cells are in large clumps extra care should be made to disrupt them when resuspending, or rosette formation may occur in the next pelleting step and reduce yields. The red tinge is due to erythrocyte contamination: if it is critical to remove all erythrocytes, then another ficoll gradient may be engaged after washing the cells with PBS and pelleting.
15. The supernatant will appear cloudy: this is because it is dense with platelets and does not reflect a loss of leukocytes/lymphoctyes.
16. If the supernatant still appears cloudy, this step can be repeated.

17. The pellet resuspends with less clumping when using a small volume (i.e. 5–7 ml) before transferring cells into 75 cm^2 tissue-culture flasks.
18. If processing bloods from multiple donors, it is important to keep each blood separate until after the hypotonic lysis.
19. Care must be taken to remove as much supernatant as possible in order to reduce highly concentrated proteins from serum that could block swelling in Step 4.
20. The cells are distributed thus, so each tube can be successively resuspended in hypotonic lysis buffer in Step 4 to maintain an identical swell time for each douncing.
21. As many protease inhibitors are short-lived, it is important to add them fresh to buffers shortly before use throughout the procedure.
22. We have noted that each blood takes a different amount of time for the cells to swell. Thus, 10 min is a guideline, but not an absolute.
23. It is important to stagger the pellets because protracted incubation of the cells in hypotonic buffer will also lyse nuclei.
24. This serves to stabilise the nuclei as, otherwise, they also will swell and lyse during the subsequent steps, particularly the long incubation in the sucrose gradients.
25. The 50 ml volume assumes that cells from 1–2 buffy coats are being processed. If more are being processed, the pellets should be resuspended so that (based on initial cell counts and assuming full recovery thus far) no less than 50 million and no more than 200 million nuclei are loaded per SW28 tube (25 ml).
26. Contaminating ER and other membranes that are rich in proteases have floated in the sucrose away from the nuclear pellet and these now line the walls of the tube. Therefore, it is important to avoid contact between the protease rich walls and the pellet. It is also important to keep the tubes inverted in the cold room after pouring off the supernatant until the sides can be wiped in order to prevent the remaining film collecting by gravity in the bottom of the tube.
27. It is very important to use a swinging-bucket rotor when spinning through the sucrose cushion at this point in order to float any chromatin that is released away from the NEs. In a fixed-angle rotor the cushion will be distributed thinly and NEs will have more chromatin contamination.
28. The supernatant may appear slightly cloudy, but this is mostly chromatin that has been ejected and should give a dark, worm-like appearance under the microscope quite distinct from NEs.
29. Faster speeds with shorter times may be used in microcentrifuge tubes provided that the NEs are not intended for ultrastructural analysis.

30. The pellet here will typically be much larger than the nuclear pellet.
31. Although the membranes are floated better using a swinging-bucket rotor, a fixed- angle rotor can also be used (e.g. type 45 Ti). In this case, volumes should be increased to 35 ml of the diluted membranes, 9.5 ml of the 1.86 M SHKM, and 3 ml of the 0.25 M SHKM. This final volume fills the tubes sufficiently to minimise the chance of tube collapse, while minimising the chance of leakage into the rotor. Centrifuge at 57,000*g* (~27,000 rpm) for 5 h.
32. The type-45 Ti rotor tubes must be filled to close to the top or they can collapse.
33. Microsome protein levels comprise a less predictable fraction of the starting material as compared to NEs. Therefore, amounts are determined by either Coomassie staining on SDS-PAGE or by an alternative protein quantitation assay.
34. It is critical to start the centrifugation step immediately since, loss of membrane proteins was observed even after just 10 min of additional incubation on ice.
35. The pellet should be very hard, so there is no need to recentrifuge after adding the H_2O.
36. Tansfering from an 1.5 ml eppendorf tube to a larger volume tube is to avoid losing sample during the bubbling that occurs because of the neutralization process.
37. At this step, because of the large volume (~850 μl), the sample will have to be split into three aliqots of ~1,150 μl in 1.5 ml eppendorf tubes.
38. This makes for a pretty redundant list of proteins, but not all protein sequences have been predicted in the annotated human genome and sometimes missing proteins will appear in another related organism.
39. We have used TMHMM since the second version has become available, but used TMPred earlier because the first version of TMHMM failed correctly to predict several of the well-characterised NETs. It is wise to compare the results from different algorithms and to search for multimers of beta barrels as well as transmembrane helices.

Acknowledgments

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Chapter 16

Isolation of Extracellular Membranous Vesicles for Proteomic Analysis

Rommel A. Mathias, Justin W. Lim, Hong Ji, and Richard J. Simpson

Abstract

Membranous vesicles are constitutively released by a multitude of cell types. Following fusion of multivesicular bodies with the plasma membrane, endocytic vesicles, 30–90 nm in size termed exosomes are released extracellularly. Whilst several groups have reported the presence of exosomes in cell-culture conditioned medium, their biological and physiological functions still remain unclear. In addition, exosomes have been detected in body fluids associated with disease, further demonstrating their potential as diagnostic biomarkers. This protocol employs size filtration followed by ultracentrifugation to isolate and purify exosomes from the colon carcinoma cell line LIM 1215. Morphological visualisation and characterisation is based on electron microscopy and western blotting, whilst protein identification is achieved using a combination of 1D SDS-PAGE and LC-MS/MS.

Key words: Membranous vesicles, conditioned medium, exosomes, LIM 1215, proteomics.

Abbreviations: Multivesicular Bodies (MVB); Intraluminal Vesicles (ILV); Plasma Membrane (PM); Membranous Vesicles (MV); Conditioned Medium (CM); Electron Microscopy (EM); Fetal Calf Serum (FCS); Insulin-Transferrin-Selenium (ITS); One Dimensional Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (1D SDS-PAGE); Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS); Phosphate Buffered Saline (PBS); Tris-Buffered Saline with Tween-20 (TBST 20); Dithiothreitol (DTT); Iodoacetic Acid (IAA); Room Temperature (RT).

1. Introduction

Intracellular sacs filled with small, uniform, membrane-enclosed structures were first described following endocytosis and recycling of the transferrin receptor in reticulocytes *(1, 2)*. Inward budding of the limiting membrane in multivesicular bodies (MVBs) is believed to generate small intraluminal vesicles (ILVs) contained

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within MVBs *(3)*. MVBs not targeted for lysosomal degradation, fuse with the plasma membrane (PM) causing the ILVs to be released extracellularly *(3)*. These extracellular membranous vesicles (MVs) were initially purified from cell-culture conditioned medium (CM) by centrifugation, and visualised with electron microscopy (EM) *(4, 5)*. The term exosome has been given to MVs with endocytic origin, approximately 30–90 nm in diameter *(6)* (*see* **Note 1**).

Exosomes have been purified from the CM of haematopoietic cells *(7–10)*, epithelial cells *(11, 12)*, tumour cells *(13–15)* and clinical samples such as urine *(16)*. Initially, exosomes were thought mechanistically to remove proteins from the cell PM *(5)*. Since then exosomes have been implicated in extracellular communication *(3)*, and are believed to be involved in antigen presentation due to the presence of Major Histocompatibility Complex (MHC) class I/II molecules *(17)*. Tumour cells release MVs into their extracellular environment *(18,19)*, and this is believed to aid tumour progression and metastasis *(20, 21)*. Since the presence of MVs may associate with disease, and MVs have been identified in easily accessible body fluids such as urine *(22)*, they have the potential to be used as biomarkers to predict the early onset of disease. Therefore, analysing MVs released from LIM 1215 cells may allow the identification of candidate protein biomarkers for the early detection of colon cancer.

Whilst several exosome purification approaches currently exist, most procedures collect the CM of viable cell-cultures and isolate exosomes by differential centrifugation *(7–13)*. However, increased purity can be achieved using sucrose density gradients *(23)*. In addition, flow cytometry has been used recently to purify exosomes by targeting a cell-specific surface antigen *(24)*. In the protocol described here, we present a serum-free method to isolate and purify exosomes from the CM of the colon carcinoma cell line LIM 1215. Since routine cell-culture requires the use of Fetal Calf Serum (FCS) that contains bovine MVs, a stringent washing procedure has been implemented. Cells are cultured for 24 h in serum-free medium supplemented with Insulin-Transferrin-Selenium (ITS). Harvested CM is filtered through a 0.1 μm membrane to eliminate larger constituents released from the PM, concentrated, and the exosomes isolated by ultracentrifugation.

LIM 1215 exosomes are visualised with EM, characterised morphologically with immunogold staining EM, and characterised biochemically with Western blot analysis that targets exosome-associated markers such as the tetraspanin protein CD9, and the intestinal epithelial cell-specific A33 antigen *(11, 12)*. In addition, exosome proteins are identified using 1D SDS-PAGE in conjunction with LC-MS/MS (*see* **Fig. 16.1**).

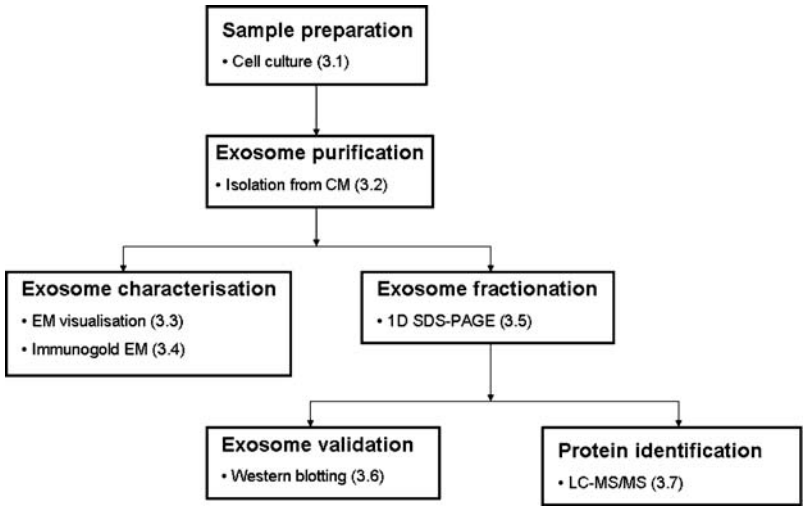

Fig. 16.1. Experimental overview for membranous vesicle isolation, characterisation, and identification. After sample preparation, CM is harvested for exosome purification. Isolated exosomes can be characterised morphologically via EM, or fractionated via 1D SDS-PAGE. Fractionated exosomes are probed via western blot analysis with exosome-specific antibodies, or subjected to LC-MS/MS for protein identification. The sections in this chapter describing each method are indicated in parenthesis.

2. Materials

2.1. Cell Culture and Sample Preparation

1. LIM 1215 cells (ATCC, Manassas, VA, USA)
2. RPMI medium (GIBCO, Invitrogen, Carslbad, CA, USA)
3. Fetal Calf Serum (FCS) (ThermoTrace, Victoria, Australia)
4. T-175 tissue culture flasks (BD Falcon, Franklin Lakes, NJ, USA)
5. Trypsin/EDTA (GIBCO, Invitrogen)
6. 150-mm tissue culture dishes (BD Falcon)
7. RPMI (phenol red-free) medium (GIBCO, Invitrogen)
8. Insulin-Transferrin-Selenium-A (ITS) (GIBCO, Invitrogen). 1% ITS collection medium; dilute 100 μL ITS with 9.9 mL RPMI (phenol red-free).

2.2. Exosome Isolation and Purification

1. Complete EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Dissolve 1 tablet per 50 mL of solution.
2. VacuCap 60 filter unit (0.1 μm) (Pall, Cornwall, UK)
3. Amicon ultra centrifugal filter device (5 kDa MWCO) (Millipore, Billerica, MA, USA)
4. Phosphate Buffered Saline (PBS): 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, and adjust to pH 7.4 with HCl. Wash buffer: dissolve 1 protease inhibitor tablet in 50 mL of PBS.

2.3. Exosome Visualisation with EM

1. PBS (*see* **Section 2.2**)
2. 0.1 M Sodium cacodylate (ProSciTech, Queensland, Australia): mix 2.14 g sodium cacodylate with 100 mL water, and adjust to pH 7.3 with HCl.
3. 2.5% Glutaraldehyde stock solution (Merck, Darmstadt, Germany): mix 10 μL of 25% Glutaraldehyde stock solution with 90 μL of Cacodylate buffer.
4. Nescofilm (AZWELL, Osaka, Japan)
5. Formvar film/200 mesh copper grids (ProSciTech)
6. Dumostar HP matt finish tweezers (ProSciTech)
7. Uranyl acetate (ProSciTech): mix 2.5 g of uranyl acetate with 50 mL of water. Cover with foil and stir overnight. Store solution at 4°C.
8. 3 MM chromatography paper (Whatman, Maidstone, England)
9. Gelatine capsules (ProSciTech)

2.4. Immunogold Labelling of Exosomes for EM Visualisation

1. PBS (*see* **Section 2.2**)
2. 2% (w/v) Paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA): mix 1 g of paraformaldehyde with 45 mL of PBS. Heat to 60°C and shake until dissolved. Add PBS to a final volume of 50 mL and adjust to pH 7.4 with NaOH. Store aliquots at −20°C.
3. 2.5% (v/v) Glutaraldehyde solution, Nescofilm, Dumostar HP matt finish tweezers, Uranyl acetate staining solution, 3 MM chromatography paper and Gelatine capsules (refer to **Section 2.3**)
4. Formvar film/200 mesh nickel grids (ProSciTech)
5. 0.05 M Glycine (Bio-Rad Laboratories, Hercules, CA, USA): mix 0.375 g of glycine with 100 mL of PBS.
6. 0.2% Triton X-100 (Roche Diagnostics): mix 5 mL of Triton X-100 with 45 mL of PBS. Mix 2 mL of this solution with 98 mL of PBS.
7. BSA, cold-water fish skin gelatin, and goat serum (ProSciTech). Blocking buffer: mix 5 g of BSA, 0.2 g of cold-water fish skin gelatin and 5 mL goat serum with 95 mL of PBS.
8. Incubation buffer: mix 0.2 g of BSA with 100 mL of PBS. Store at −20°C.
9. Mouse monoclonal anti-human A33 antibody (Ludwig Institute for Cancer Research-Austin, Victoria, Australia): dilute the primary antibody in Incubation buffer to a final concentration of 5 μg/mL.
10. Ultra small gold-conjugated goat anti-mouse antibody (ProSciTech): dilute the secondary antibody in incubation buffer according to the manufacturer's instructions (1:50).
11. Immunogold silver staining kit (ProSciTech). Store at 4°C.

2.5. 1D SDS-PAGE

1. Sample buffer: 9 M urea, 4% (w/v) CHAPS; dissolve 5.4 g urea in 8 mL water. Add 0.4 g CHAPS and add water to a final volume of 10 mL. Store aliquots at −20°C.
2. 2X SDS-PAGE Sample loading buffer: 125 mM aqueous Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.005% (w/v) bromophenol blue , 50 mM dithiothreitol (DTT).
3. NuPAGE 4–12% Bis-Tris Gels (Invitrogen Corporation)
4. 20X NuPAGE MES Running buffer (Invitrogen Corporation): 1 M MES, 1 M Tris-base, 69.3 mM SDS and 20.5 mM EDTA. Mix 25 mL of 20X MES Running buffer with 475 mL water.
5. SeeBlue plus 2 (Invitrogen Corporation): pre-stained molecular weight protein marker.
6. NuPAGE Antioxidant solution (Invitrogen Corporation)

2.6. Western Blot Analysis

1. 10X Tris-buffered saline with Tween-20 (TBST): aqueous 1.5 M NaCl, 0.2 M Tris-HCl and 1% Tween-20. Prepare 1X TBST buffer by mixing 100 mL of TBST with 900 mL water.
2. Blocking buffer: mix 1 g of skimmed milk powder with 20 mL of 1X TBST.
3. Mouse monoclonal anti-human A33 antibody (Ludwig Institute for Cancer Research-Austin, Victoria, Australia): dilute the antibody with 1X TBST buffer to a final concentration of 2 μg/mL.
4. Mouse anti-human CD9 antibody (SantaCruz Biotechnology, SantaCruz, CA, USA): dilute the antibody with 1X TBST buffer to a final concentration of 0.2 μg/mL.
5. Goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Bio-Rad): dilute the antibody with 1X TBST buffer according to the manufacturer's instructions (1:5,000).
6. ECL plus western blotting detection kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).
7. Super Rx X-ray film (Fuji photo film, Tokyo, Japan)

2.7. LC-MS/MS and Protein Identification

1. 96-well Microplate (Greiner bio-one, Frickenhausen, Germany)
2. Ammonium hydrogen carbonate (100 mM stock): dissolve 0.79 g AnalaR grade ammonium bicarbonate (BDH, West Chester, PA, USA) in 100 mL of water.
3. DTT (10 mM stock): dissolve 15.4 mg DTT (Calbiochem, San Diego, CA, USA) in 10 mL of 100 mM ammonium hydrogen carbonate.
4. Iodoacetic acid (IAA, 55 mM stock): dissolve 90 mg IAA (Sigma-Aldrich) in 1 mL of 1 M NaOH. Add 9 mL of 100 mM ammonium hydrogen carbonate.

5. Sequencing-grade modified Trypsin (Worthington, Indianapolis, USA). Dissolve in 50 mM acetic acid, and dilute to 6 ng/μL with 100 mM ammonium hydrogen carbonate.
6. Autosampler microvials (Agilent, Santa Clara, CA, USA)
7. AnalaR-grade Formic acid (BDH). Buffer A: dilute 1 mL formic acid with 999 mL of water.
8. CHromAR-grade Acetonitrile (Mallinckrodt, Phillipsburg, NJ, USA). Buffer B: add 600 mL of ACN to 400 mL of water, and add 935 μL of formic acid.
9. Reverse-phase capillary column (Vydac, USA). Column specifications: C4MS, 150 mm length × 0.15 mm inner diameter, butyl-silica 300 Å pore size, 5 μm particle diameter.

3. Methods

3.1. Cell Culture and Sample Preparation

1. Culture LIM 1215 cells in RPMI medium containing 5% (v/v) FCS, at 37°C in 10% CO_2.
2. Grow cells in a T-175 culture flask, and passage upon reaching 80% confluence with Trypsin/EDTA.
3. Seed cells into ten 150-mm tissue culture dishes and grow to approximately 70% confluence.
4. Gently wash each dish with 10 mL RPMI medium (*see* **Note 2**).
5. Repeat Step 4.
6. Gently wash each dish with 10 mL RPMI (phenol red-free) medium.
7. Repeat Step 6.
8. Add 10 mL of 1% ITS collection medium to each dish, and culture cells for 24 h (*see* **Notes 3 and 4**).

3.2. Exosome Isolation and Purification

1. Harvest the 100 mL of CM (*see* **Note 5**).
2. Add protease inhibitor tablets to the CM.
3. Centrifuge the CM at 1,900 × *g* at 4°C for 10 min to remove cell debris and transfer the supernatant to a new tube.
4. Filter the CM through a 0.1 μm membrane filter and collect the filtrate in a new tube.
5. Concentrate the filtrate to a final volume of 1 mL using centrifugal filtering devices (*see* **Note 6**).
6. Centrifuge the retentate at 100,000 × *g* at 4°C for 1 h to pellet the LIM 1215 exosomes (*see* **Note 7**).
7. Without disturbing the pellet, carefully remove and discard the supernatant (*see* **Note 8**).
8. Carefully resuspend the pellet in 1 mL of Wash Buffer to wash the exosomes.

9. Centrifuge the exosome suspension at 100,000 × *g* at 4°C for 1 h to re-pellet the washed exosomes.
10. Repeat Steps 7–9 to wash the exosomes again (*see* **Note 9**).
11. Snap freeze the purified exosome pellet and store at −80°C, or assay for protein concentration, or proceed with EM analysis (*see* **Note 10**).

3.3. Exosome Visualisation with EM

1. Resuspend 10 μg of exosome pellet in 2 μL of PBS, and vortex briefly to loosen the pellet (*see* **Note 11**).
2. Add 15 μL of 2.5% Glutaraldehyde solution and incubate at room temperature (RT) for 30 min to fix the exosomes (*see* **Note 12**).
3. Set-up a working area in a chemical fume hood (*see* **Note 13**).
4. Pick up a copper grid with tweezers and lay it on the Nescofilm with the formvar-coated side facing upwards (*see* **Note 14**).
5. Pipette 3–6 μL (2–4 μg) of fixed exosome suspension onto the grid and leave to dry completely (*see* **Note 15**).
6. Pipette 100 μL of 0.1 M Cacodylate buffer onto the Nescofilm that should form a droplet due to surface tension.
7. Place the grid on top of the droplet, ensuring the sample-containing side is facing the droplet.
8. Stir the droplet with a pipette tip, gently washing the grid for 10 min.
9. Repeat Steps 6–8 twice.
10. Pipette 150 μL of water onto the Nescofilm.
11. Transfer the grid to this droplet and gently wash for 5 min.
12. Repeat Steps 10–11.
13. Pipette 50 μL of Uranyl acetate staining solution onto the Nescofilm (*see* **Note 16**).
14. Transfer the grid to this droplet and incubate for a minimum of 10 min to stain the exosomes.
15. Gently touch the corner of the grid with chromatography paper to remove excess Uranyl acetate. Do not disturb the sample.
16. Once the grid is fully dry, store it in a gelatine capsule until imaging can be performed on an electron microscope. **Figure 16.2** shows exosomes derived from LIM 1215 cells.

3.4. Immunogold Labelling of Exosomes for EM Visualisation

1. Resuspend 20 μg of exosome pellet in 20 μL PBS and vortex briefly to loosen the pellet (*see* **Note 11**).
2. Add 20 μL of 2% Paraformaldehyde solution and incubate at RT for 1 h.
3. Set-up a working area in a chemical fume hood (*see* **Note 13**).

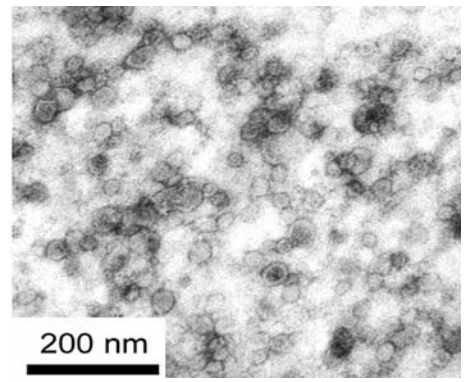

Fig. 16.2. Visualization of LIM 1215 exosome morphology. Electron micrograph showing the morphology of exosomes isolated from LIM 1215 cells. Imaging was carried out using a Philips 10 electron microscope at 25,000× magnification.

4. Pick up a nickel grid with tweezers and lay it on the Nescofilm with the formvar-coated side facing upwards (*see* **Notes 14 and 17**).
5. Pipette 3–6 μL (2–4 μg) of fixed exosome suspension onto the grid and leave to dry completely (*see* **Note 15**).
6. Pipette 100 μL of PBS onto the Nescofilm that should form a droplet due to surface tension.
7. Place the grid on top of the PBS droplet; ensuring sure the sample-containing side is facing the droplet.
8. Stir the droplet with a pipette tip, gently washing the grid for 10 min.
9. Repeat Steps 6–8 twice.
10. Pipette 50 μL of 0.05 M glycine solution onto the Nescofilm.
11. Transfer the grid to this droplet and incubate for 20 min to quench any free aldehyde.
12. Pipette 50 μL of 0.2% Triton X-100 solution onto the Nescofilm.
13. Transfer the grid to this droplet and incubate for 5 min to permeabilise the exosome membrane (*see* **Note 18**).
14. Repeat Steps 6–8 twice.
15. Pipette 50 μL of blocking buffer onto the Nescofilm.
16. Transfer the grid to this droplet and incubate for 30 min to block any non-specific antibody binding.
17. Pipette 100 μL of Incubation buffer onto the Nescofilm.
18. Transfer the grid to this droplet and incubate for 5 min. Occasionally, stir the droplet with a pipette.
19. Repeat Steps 17 and 18.
20. Pipette 30 μL of primary antibody solution onto the Nescofilm (*see* **Note 19**).
21. Transfer the grid to this droplet and incubate for a minimum of 30 min (can be left at 4°C overnight) (*see* **Note 20**).

22. Repeat Steps 17 and 18 six times.
23. Pipette 30 μL of secondary antibody solution onto the Nescofilm (*see* **Note 21**).
24. Transfer the grid to this droplet and incubate for a minimum of 4 h (can be left at 4°C overnight).
25. Repeat Steps 17 and 18 six times.
26. Repeat Steps 6–8 twice.
27. Pipette 50 μL of 2.5% Glutaraldehyde solution onto the Nescofilm.
28. Transfer the grid to this droplet and incubate for 10 min to post-fix the exosomes.
29. Pipette 150 μL of water onto the Nescofilm.
30. Stir the droplet with a pipette tip, gently washing the grid for 5 min.
31. Repeat Steps 29 and 30 twice.
32. Remove the developer and enhancer from the Immunogold silver staining kit and allow the reagents to reach RT.
33. Mix both reagents according to the manufacturer's instructions.
34. Incubate the grid in the enhancement mixture at RT for 5–25 min (*see* **Note 22**).
35. Dip the grid in a tube of water 20 times.
36. Repeat Step 35 three times, using a new tube of water each time.
37. Pipette 50 μL of Uranyl acetate staining solution onto the Nescofilm (*see* **Note 16**).
38. Transfer the grid to this droplet and incubate for a minimum of 10 min to stain the exosomes.
39. Gently touch the corner of the grid with chromatography paper to remove excess Uranyl acetate. Do not disturb the sample.
40. Once the grid is fully dry, store it in a gelatine capsule until imaging can be performed on an electron microscope.

3.5. 1D SDS-PAGE

1. Resuspend the exosome pellet in an appropriate volume of Sample buffer (*see* **Note 23**).
2. Add the exosome suspension to 2X SDS-PAGE Sample loading buffer in a 1:1 (v/v) ratio (*see* **Note 24**).
3. Vortex to mix, then centrifuge briefly to bring all liquid to the bottom of the tube (*see* **Note 25**).
4. Set-up a 1D gel in an appropriate electrophoretic gel apparatus (*see* **Note 26**).
5. Add 200 mL of 1X Running buffer to the inner chamber and 300 mL to the outer chamber of the gel tank.
6. Using gel-loading pipette tips, load each sample into a well. Also, load 5 μL of SeeBlue plus 2 molecular weight protein marker to a separate well.

7. Add 0.5 mL antioxidant solution to the 1X Running buffer in the upper chamber of the gel tank, to ensure that electrophoresis is carried out under reducing conditions.
8. Connect the electrodes to a power supply and run at 150 V at room temperature until the bromophenol blue dye front reaches the bottom of the gel (~1 h.).
9. Remove the gel from its cassette and continue with western blotting or LC-MS/MS analysis (*see* **Note 27**).

3.6. Western Blot Analysis

Western blotting for proteins of interest is performed using standard protocols (*see* **Note 28**).

1. After transferring, place the membrane in 20 mL of blocking buffer and incubate on a rocking platform at room temperature for 1 h (can be left at 4°C overnight).
2. Discard the blocking buffer and rinse the membrane twice with 1X TBST buffer.
3. Place the membrane in 5 mL of primary antibody solution (A33 or CD9) and incubate on a rocking platform at RT for 1 h.
4. Remove the primary antibody solution, and wash the membrane with 25 mL of 1X TBST buffer for 10 min (*see* **Note 29**).
5. Repeat Step 4 twice.
6. Place the membrane in 5 mL of secondary antibody solution and incubate on a rocking platform at room temperature for 1 h.
7. Discard the secondary antibody solution, and wash the membrane with 25 mL of 1X TBST buffer for 10 min.
8. Repeat Step 7 twice.
9. Prepare 2 mL of ECL plus reagent according to the manufacturer's instructions.
10. Cover the membrane with the chemiluminescent substrate and gently agitate for 3 min.
11. Develop the image onto film in a darkroom under safe light conditions. **Figure 16.3** shows purified LIM 1215 exosome proteins subjected to 1D SDS-PAGE and blotted with A33 and CD9 antibodies.

3.7. LC-MS/MS and Protein Identification

Protein identification is obtained via trypsin digestion, followed by nano-liquid chromatography coupled on-line to an electrospray ion trap mass spectrometer *(25, 26)*.

1. After staining, excise bands of interest from the 1D gel and place into a 96-well plate for trypsin digestion (*see* **Note 30**).
2. Wash each gel slice extensively with deionised water, reduce with DTT at 37°C for 30 mins, and then alkylate with IAA at room temperature for 30 min (*see* **Note 31**).

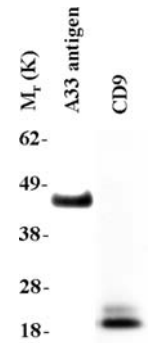

Fig. 16.3. Biochemical characterisation of LIM 1215 exosomes. Western blot analysis of LIM1215 exosomes. Purified exosomes were subjected to 1D SDS-PAGE and blotted with antibodies against the tetraspanin protein CD9, and the intestinal epithelial cell-specific A33 antigen.

3. Digest with trypsin at 37°C for a minimum of 4 hours (*see* **Note 31**).
4. Concentrate tryptic digests to 10 μL and transfer to glass autosampler vials for LC-MS/MS (*see* **Note 32**).
5. Ensure the HPLC system is coupled on-line to the mass spectrometer for automated MS/MS analysis of individually isolated peptide ions.
6. Fractionate 8 μL of sample by reverse-phase HPLC using a linear 60 min gradient from 0–100% Buffer B, with a flow rate of 0.8 μL/min.
7. Operate the mass spectrometer in data-dependent mode (triple play) to automatically switch between MS, Zoom MS (automated charge state determination) and MS/MS acquisition, selecting the most intense precursor on for CID fragmentation. Employ dynamic exclusion for 3 min when three consecutive precursor ions of the same mass are observed (*see* **Note 33**).
8. Generate peak lists from the CID spectra for database searching. The following parameters are suggested for extract-msn: minimum mass 700; maximum mass 5,000; grouping tolerance 1.4; intermediate scan 1; minimum group count 1; automated calculation of charge state – 1+, 2+, or higher; 30 peaks minimum per spectrum (*see* **Note 34**).
9. Database search the .mgf files to assign protein identity. The following parameters are suggested when searching: carboxymethylation of cysteine as a fixed modification, NH_2-terminal acetylation and oxidation of methionine as variable modifications, peptide mass tolerance ±3 Da, MS/MS fragment mass tolerance ±0.8 Da and allow up to two missed tryptic cleavages (*see* **Note 35**).

4. Notes

1. Several terms can be used to describe extracellular vesicles depending on definition. In this protocol, we have adopted the term exosomes to describe MVs ~30–90 nm in size, collected and isolated from the conditioned medium of LIM 1215 cells.
2. Ensure that medium used in washing steps is pre-warmed to 37°C. The addition of medium should take place along the dish wall to minimise the disruption of adherent cells. Washing is done by gently swirling medium around the dish several times. This stringent washing procedure is employed to remove the FCS present during normal culturing conditions.
3. The concentration of ITS used for collection should be optimised (normally 0.1% to 1%) for cell viability using an assay such as the Trypan blue dye exclusion assay *(27)* and for cell proliferation using an assay such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay *(28)*.
4. FCS depleted of bovine MVs can be used during collection as an alternative to ITS. Depleted FCS refers to FCS that has been subjected to ultracentrifugation at 100,000 × *g* at 4°C for 16 h. Depleted FCS is added to RPMI (phenol red-free) for LIM 1215 exosome collection.
5. The CM should be kept on ice throughout the duration of exosome isolation.
6. The time it takes to concentrate the CM is cell type and density dependant.
7. The ultracentrifuge used in this step is the Optima MAX (Beckman Coulter) with a TLA 100.2 SN 569 rotor.
8. The exosome pellet that forms following centrifugation may not be readily visible. Furthermore, extreme care must be taken when removing the supernatant so the pellet is not disturbed.
9. The second wash further increases exosome purity. After this final wash, ensure that all traces of PBS are removed from the pellet/tube.
10. Protein concentration can be determined using any standard protein assay such as the BCA assay *(29)*. The protein yield of exosomes derived from LIM 1215 cells is approximately 0.16 μg per million cells over 24 h. Typical protein yield from other tumour cell exosomes is 0.3–0.5 μg per million cells over 48 h *(30)*.
11. Exosomes that have been freshly purified should be used to obtain good quality electron micrographs (i.e. proceed directly from **Section 3.2 to 3.3** without storing at −80°C).

12. Many protocols commonly use 2.5% (v/v) Glutaraldehyde for fixation. We have found that using a reduced amount of Glutaraldehyde (0.6–1%) is sufficient to produce good quality electron micrographs.
13. Cover the floor of the hood with clean bench coat. Cut a small section of Nescofilm and lay it on top of the bench coat. The Nescofilm will be the working platform for the following steps.
14. It is imperative to determine which side of the grid is formvar-coated for correct sample loading. Visually, it appears as the darker matt-finished side (as opposed to the shiny copper/nickel side).
15. The amount of exosome sample loaded on each grid should never exceed 4 μg; otherwise, the formvar coating on the grid can collapse during imaging.
16. Uranyl acetate must be filtered with a 0.2 μm syringe filter every time before usage as it precipitates during storage. Uranyl acetate contains the isotope ^{238}U of uranium. The specific activity of ^{238}U in laboratory grade uranyl acetate does not exceed 10,000 Bq per gram. ^{238}U is an alpha emitter, and there are beta and gamma emitting decay products. Handling of uranyl acetate and disposal of uranyl acetate related wastes should follow relevant guidelines.
17. Always include a negative control grid. Diluted goat serum should be used instead of the primary antibody in step 20.
18. Omit Steps 12 and 13 if the target antigen is on the exosome surface.
19. For double labelling, two primary antibodies are mixed and applied simultaneously.
20. Incubation with primary and secondary antibodies should be performed in a tightly sealed plastic box containing pieces of wet tissue. This will limit evaporation and maintain the antibody concentration over a long incubation period. Overnight incubation is recommended for the primary antibody in step 21.
21. For double labelling, two different sizes of conventional immunogold conjugates (e.g. 6 nm and 15 nm gold particles) are used and applied simultaneously.
22. A series of experiments should be set up to optimise the period of the incubation of the grid in the silver enhancement mixture.
23. The volume of sample buffer used depends on exosome protein yield. Try and resuspend the pellet to achieve a concentration of 1 μg/uL.
24. The amount of exosomes run on 1D is usually 10 ug for western blotting and 50 ug for protein identification via LC-MS/MS.

25. Do not heat the sample prior to electrophoresis as the sample buffer contains urea and heating will cause the protein to be carbamylated.
26. The gel apparatus used is the Xcell SureLock mini-cell (Invitrogen). Exosome samples can be run on Bis-Tris gels with different gradients, as well as on 4–20% Tris-Glycine gels (Invitrogen).
27. Prior to protein identification via LC-MS/MS, gels are stained and proteins visualized with dyes such as Coomassie blue R250 (BioRad), or Imperial Protein Satin (Pierce).
28. Alternatively, proteins can be transferred rapidly from gel to membrane using the iBlot Dry Blotting System (Invitrogen).
29. The primary antibody solution can be reused by adding sodium azide (exercise caution as azide is highly toxic) to a final concentration of 0.02% (v/v) and stored at 4°C.
30. A 1D gel cutter (The Gel Company) is used to divide a single gel lane into 1 mm fractions for trypsin digestion. The gel stamp normally produces around 70 individual gel slices per lane.
31. The robotic liquid handling station (Waters-Micromass) is used to perform automated reduction, alkylation and trypsin digestion. Digested samples can be stored at this stage in 96-well plates at −20°C.
32. Digests are concentrated by centrifugal lyophilisation using the SpeedVac System (Savant).
33. These operational parameters are used when MS-based peptide sequencing is performed using an LCQ-DECA mass spectrometer (Thermo-Finnigan).
34. Peak lists were extracted using extract-msn (Thermo), and in-house software used to generate .mgf files for database searching.
35. Mascot Daemon (Matrix Science) was used to search .mgf files against the LudwigNR_subset database for protein identification. Three hundred and thirty two proteins were identified in total of which 24% were either PM or membrane associated. Sixteen percent of the 332 proteins contained at least one putative transmembrane domain.

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Part III

Separation of Membrane Proteins

Chapter 17

Enrichment of Human Platelet Membranes for Proteomic Analysis

David W. Greening, Kristen M. Glenister, Rosemary L. Sparrow, and Richard J. Simpson

Abstract

Platelets (thrombocytes) are the smallest human blood cells and are pivotal in processes of hemostasis and thrombosis. Central to their function, the activation of platelets includes a complex interplay of adhesion and signalling molecules mediated via the plasma and inner membrane. Because platelets are enucleated, the analysis of the proteome is the best way to approach their biology. Here, we employ mass spectrometry (MS)-based proteomics to characterise membrane proteins derived from non-stimulated human platelets. This protocol details the extraction and purification of platelet membrane proteins from whole blood using SDS-PAGE in conjunction with LC-MS/MS. This method allowed the identification, and characterization of 207 platelet membrane proteins (PMP) from approximately 9.95×10^9 platelets *(16)*.

Key words: Platelet, thrombocyte, platelet membrane, platelet proteomics, mass spectrometry.

Abbreviations: Mass spectrometry (MS); tandem mass spectrometry (MS/MS); liquid chromatography (LC); 1/2D-gel, one/two-dimensional gel electrophoresis; sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); buffy-coat (BC); platelet rich plasma (PRP); platelet membrane protein (PMP); phosphate-buffered saline (PBS); fetal calf serum (FCS); red blood cell (RBC); white blood cell (WBC).

1. Introduction

Human platelets (thrombocytes) are specialised enucleated blood cells derived from megakaryocytes, approximately 2–4 by 0.5 μm in size containing a mean volume of 7–11 fl *(1)*. In blood, they are the second most numerous corpuscle, normally circulating between $150–450 \times 10^9$/L *(1)*. They are multifunctional

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and involved in many pathophysiological processes including hemostasis and thrombosis, clot retraction, vessel constriction and repair, inflammation including promotion of atherosclerosis, host defence and even tumour growth/metastasis *(2)*. Their shape and small size enables them to access the edge of blood vessels, thereby enabling them constantly to survey vasculature integrity. Upon vessel wall damage, platelets undergo highly regulated functional responses including membrane adhesion, spreading, release reactions, aggregation, pro-coagulant activity, microparticle formation and clot retraction *(3)*.

Platelets do not lend themselves to analysis by traditional molecular biology techniques due to the fact that they are produced in the bone marrow from megakaryocytes as cytoplasmic fragments without genomic DNA *(4)*. Whilst valuable information may be gathered from studies of messenger RNA *(5)*, the rapid signalling and regulatory events in platelets are not governed by, or dependent on, alterations in gene expression *(6)*. In contrast, proteomics, or analytical protein chemistry, has the potential to define platelet biology as it offers the opportunity to comprehensively describe proteins involved in specific elements of platelet function and stages of activation in normal and disease biophysiology.

Due to their importance in platelet function, platelet membrane proteins (PMPs) and cytoskeletal proteins are an important focus for proteomic analysis *(7, 8)*. Membrane proteins throughout platelets perform important functions, including cell signalling regulation through surface receptors, cell–cell interactions and mediating intracellular signalling via the open canalicular and dense tubular systems (*see* **Note 1**) *(9, 10)*. Despite this importance, only a limited number of platelet proteomic studies have been undertaken *(11–14)*. Past studies have had issues with the preparation of platelet source (e.g. platelet-rich plasma (PRP), buffy-coat (BC) derived, platelet-pheresis: *see* **Notes 2–3**), in addition to the technical challenge of analysing complex protein mixtures (dynamic range of protein abundances). To circumvent the latter, current proteomic analyses are directed towards extensive sample prefractionation (e.g. 2D-gel analysis, multidimensional chromatography separation) and/or enrichment of sub-proteome(s) of interest *(15)*.

In this protocol, we describe a strategy for the isolation and purification of platelet concentrates from whole blood. As a first step towards a detailed platelet proteome analysis, we have focused on the platelet membrane proteome due to the functional importance of platelet membrane biology. We detail a method for isolating platelet membranes from intact, quiescent (resting) human platelets obtained from the BC layer. Using SDS-PAGE, PMPs from $9.95 \pm 5 \times 10^9$ platelets were fractionated for subsequent LC-MS/MS analysis. This protocol allowed the

identification, and characterisation of 203 PMP of which 28% were plasma/integral membrane, membrane associated,or GPI-bound proteins and 12.5% of total proteins contained at least one putative transmembrane domain *(16)*.

2. Materials

Throughout the protocol, Milli-Q deionized water (HPLC grade, ≥18 MΩ) should be used for making up all aqueous solutions (A10-Synthesis water polishing system; Millipore, Australia). All washing, lysis and HPLC buffers should be prepared using clean glassware on the day analysis is to be performed. All chemicals purchased from BDH Chemicals, Merck, Victoria, Australia, unless specified. The pH of solutions should be adjusted using either 5 M HCl or 1 M NaOH, where appropriate.

2.1. Blood Collection

1. Standard PL146 Optipac blood packs (Baxter, La Châtre, France) containing citrate phosphate dextrose (CPD) anticoagulant.
2. Centrifugation unit, programmable temperature setting (Hettich Roto Silenta RS, Hettich Zentrifugen, Tuttlingen, Germany).

2.2. Platelet Concentrate Preparation

1. Plasma storage bags (with platelet additive solution; T-sol) (Baxter, La Châtre, France). Platelet additive solution (T-sol; 116 mM NaCl, 30 mM sodium acetate trihydrate, 10 mM sodium citrate dihydrate, pH 7.0–7.4 at 22°C).
2. Standard PL2410 platelet concentrate storage bags (Baxter, La Châtre, France).
3. Centrifugation unit, programmable temperature setting (Hettich Roto Silenta RS, Hettich Zentrifugen, Tuttlingen, Germany).
4. OptiPure PC PLX-5 Leukofilter device (Baxter, La Châtre, France).
5. Fenwal automated Optipress II device (Baxter, La Châtre, France).
6. Linear Platelet Reciprocator (Melco Engineering Corp., Glendale, CA) with storage at 22°C.

2.3. Platelet Sample Analysis

1. Cell-Dyn 3200 haematology analyser (Abbott Diagnostics/Laboratories, Il).
2. Phosphate-buffered saline solution (PBS); 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4.

This mixture also is supplemented with 3% (v/v) fetal calf serum (FCS) or 0.5% (w/v) BSA.

3. Flow cytometry analyser: FACScan using Cellquest software (Becton Dickinson, Palo Alto, CA).
7. Fluoroscein isothiocyanate (FITC) conjugated anti-CD41 (BD Biosciences, Bio-Scientific, Australia). Store at 4°C.
8. Phycoerthyrin (PE) conjugated anti-glycophorin A (BD Biosciences, Bio-Scientific, Australia). Store at 4°C.

2.4. Preparation of Platelet Membranes

1. 15 mL polypropylene centrifuge tubes (BD Biosciences, Bio-Scientific, Australia).
2. Platelet wash buffer (PWB); 6.95 mM Na_2HPO_4, 9 mM Na_2EDTA, 140.3 mM NaCl, pH 7.0 at 22°C.
3. Platelet lysis buffer (PLB); 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT (dithiothreitol) (Bio-Rad Laboratories, Australia), 0.5 mM PMSF (Phenylmethylsulphonylfluoride), pH 8.0 at 22°C.
4. 2D Quant kit, as per manufacturer's instructions (Amersham Pharmacia Biotech, Australia).
5. Bovine serum albumin (BSA) (Sigma, Australia), prepared (1 mg/mL) and store at −20°C.
6. NuPAGE® LDS sample buffer (Invitrogen), store at 4°C.
7. SDS reducing sample buffer; 0.35 M Tris-HCl (pH 6.8 at 25°C), 10% (w/v) SDS, 30% (w/v) glycerol, 9.3% (w/v) DTT.

2.5. Protein Separation

1. Mark 12 protein standard mix (Invitrogen, Australia), store at 4°C.
2. 20X NuPAGE® MES SDS running buffer (MES 50 mM, pH 7.2, Tris Base 50 mM, SDS 0.1%, EDTA 1 mM, pH 7.3), (Invitrogen, Australia). Prepare 1 L of 1X running buffer with 50 mL 20X running buffer and 950 mL water.
3. Precast SDS polyacrylamide 12-well gel (4–20% Bis-Tris precast gel, Invitrogen, Australia).
4. Gel apparatus (XCell *Surelock*™ Electrophoresis Mini-Cell gel tank (Invitrogen, Australia)).
5. Colloidal Coomassie protein stain (Bio-rad Laboratories, Australia).

2.6. In-gel Digestion and Peptide Extraction

1. 96-well flat-bottom tissue culture plate.
2. Reduction/alkylation buffers (*see* **Table 17.1**).
3. Sequencing grade modified trypsin (Worthington), prepared in 50 mM acetic acid, diluted to 6 ng/μL in 100 mM ammonium bicarbonate, stored covered at −20°C.
4. SpeedVac, centrifugal lyophilisation (Savant AES1010, Savant, U.S.A).

Table 17.1
Reagents for reduction/alkylation buffers

Buffer	100 mM ammonium bicarbonate
Dehydration	100% acetonitrile
Reduction	10 mM DTT
Alkylation	55 mM (Sigma #I 1149) iodoacetamide
Digestion	Sequencing grade modified trypsin (Worthington), made up in 50 mM acetic acid, diluted to 6 ng/μL in 100 mM ammonium bicarbonate, stored covered at −20°C.
Extraction	1% (v/v) formic acid/2% (v/v) acetonitrile in water (HPLC/Spectro grade; Pierce, Australia)

2.7. Nano-LC Analysis

1. HPLC solvents. Solvent A; aqueous 0.1% (v/v) formic acid (HPLC/Spectro grade; Pierce, Australia). Solvent B; 60% (v/v) aqueous acetonitrile (ChromAR grade; Mallinkrodt) in water containing 0.09% formic acid (v/v; HPLC/Spectro grade; Pierce, Australia).

2.8. On-line MS/MS Analysis

1. Mass spectrometry program; Extract-MSN; Bioworks 3.1 (Thermo Finnigan, USA).

3. Methods

3.1. Blood Collection (see Note 4)

1. Whole blood (450 mL ± 10%) is collected with informed consent from random, voluntary donors, according to routine procedure (Australian Red Cross Blood Service, Melbourne). Blood is collected into conventional quadruple (bottom-top)-bag systems containing 63 mL of citrate phosphate dextrose anticoagulant. For platelet preparation, a maximum collection time of 12 min is allowed.
2. The whole-blood units are stored at 22°C.
3. For each donation, component preparation is performed within 24 h of collection.

3.2. Platelet Concentrate Preparation (17)

1. The initial separation of blood is performed by centrifugation. Whole-blood unit bags are centrifuged stepwise at 22°C to separate blood into plasma, BC and red blood- cell (RBC) components (*see* **Note 2**).

2. Centrifugation is initially performed at 4200 × *g* for 10 min at 22°C.
3. The specific separation of whole blood into plasma, buffy coat and red cells is done using automated equipment (*see* **Note 5**). Each whole-blood unit is separated at room temperature using an automated optical component preparation device (Fenwal Optipress II; Baxter).
4. Two empty plasma storage bags are connected both on the top and bottom outlets for collection.
5. Both plasma and RBCs are physically pressed off the top and bottom outlets, respectively, into the separate connected bags (rate 40 mL/min).
6. The pressing device is halted and outlet tubes automatically clamped and sealed when the cell layer reaches the installed vertical optical sensor.
7. The bag containing the remaining BC is subsequently sealed (approximately 50 mL).
8. Blood-type matched BCs from five donor units are then pooled by connecting them in series via sterile docking to a plasma storage bag containing platelet additive solution (T-sol).
9. The pooled platelet concentrate is rested for 1 h at ambient temperature (22°C) before further purification.
10. The pooled platelet concentrate is centrifuged for 7.5 min at 445 × *g* at 22°C.
11. This platelet-rich supernatant is subsequently leukocyte reduced by filtration (*see* **Note 6**) into a separate platelet storage bag, again using the automated programmed equipment (OptiPure PC PLX-5). The device is programmed to terminate expression when red cells are detected. The content of the filter is drained by gravity into the platelet storage bag.
12. This storage bag is automatically clamped and sealed, and contents gently mixed for approximately 2 min.
13. A sample (25 mL) is removed aseptically from the filtered, mixed platelet concentrate for quality control (pH, cell counts, and bacterial contamination) (*see* **Section 3.3**).
14. Platelet concentrates are stored on a platform agitator at 22°C under gentle agitation (50–70 oscillations/min; Linear platelet reciprocator) until sampling (24 h) (*see* **Note 7**).

3.3. Platelet Sample Analysis (see Note 8)

1. The level of contaminating red cells in the platelet samples is estimated by flow cytometry (FACScan, with Cellquest software) *(17)*.

2. Platelet counts are determined using a haematology analyser (CellDyn 3200, Abbott). Residual white blood cells (WBCs) are determined using an absolute counting assay using Tru-COUNT tubes. Propidium iodide (PI) is used to detect nucleated cells (WBCs) under standard conditions.
3. For RBC detection, isolated pooled platelets (1×10^9) are labelled with 5 μL anti-CD41/Fluoroscein isothiocyanate (FITC)-conjugated monoclonal antibody and 5 μL anti-glycophorin A/Phycoerthyrin (PE)-conjugated monoclonal antibody for 30 min at 4°C in the dark in standard PBS containing 3% (v/v) FCS 0.5% (w/v) BSA.
4. Platelets are subsequently washed once (1500 × *g*, 5 min), and the pellet resuspended in 300 μL PBS.
5. FACScan flow-cytometer is used for analysis. Logarithmic mode is used to acquire fluorescence and forward scatter data (*see* **Note 8**). A minimum of 10,000 events should be acquired. For immunofluorescence analysis, gating boundaries should be pre-defined to exclude cell fragments or background noise. All data analysed with Cellquest software.
6. Quality assurance (recommended) specifications for pooled leukocyte-filtered platelets: platelet count greater than 300×10^9 per pooled platelet unit, white cell count less that 1×10^6 per pooled platelet unit, red cell count less that 6×10^9 per pooled platelet unit, volume greater than 200 mL.

3.4. Preparation of Platelet Membranes

1. After incubation for 24 h, samples of the platelet concentrate (10 mL) are removed and centrifuged in polypropylene centrifuge tubes at 500 × *g* for 1 min at 22°C to sediment any agglutinates (i.e. excess RBC and WBC presence).
2. Supernatant suspensions are carefully transferred to fresh polypropylene tubes and platelets subsequently pelleted at 1100 × *g* for 15 min.
3. The sedimented platelets are washed five times with platelet wash buffer (PWB) and pelleted each time at 1100 × *g* for 15 min at 22°C.
4. The washed platelet pellet is re-suspended in gentle platelet lysis buffer (PLB) and incubated on ice for 10 min.
5. The lysate is centrifuged at 3000 × *g* for 10 min at 22°C to separate soluble (supernatant) and insoluble (pellet) fractions.
6. The insoluble fraction (representing the crude membrane preparation) is re-suspended in 200 μL of SDS reducing sample buffer.
7. Total protein content of the insoluble membrane fraction and soluble lysate is estimated using the 2D Quant kit, as per the manufacturer's instructions. Serial dilutions of BSA are used to obtain a standard calibration curve.
8. Both insoluble and soluble fractions are stored at −20°C.

3.5. Protein Separation

1. A platelet membrane protein sample (50 μg) is mixed with NuPAGE® LDS sample buffer (in the ratio sample: buffer; 2:1).
2. The sample mixture is heated for 10 min on a heat block at 70°C, and cooled prior to sample loading.
3. Separation is performed using a precast 12-well SDS polyacrylamide gel (4–20% Bis-Tris precast gel, Invitrogen) (*see* **Fig. 17.1**).
4. 500 mL of 1 × MES SDS running buffer (Invitrogen) is prepared – approximately 200 mL in the upper (inner) buffer compartment and 300 mL in the lower (outer) buffer compartment.
5. Samples are loaded into defined gel lanes. Mark 12 protein standards (Invitrogen) are used for molecular weight comparison.
6. Gel apparatus is setup and protein separation is performed at 150 V (constant voltage) until tracking dye reaches the bottom of the gel (approx 90 min).
7. Immediately following electrophoresis the gel should be washed with water and stained with colloidal Coomassie blue, as previously described *(19)*.

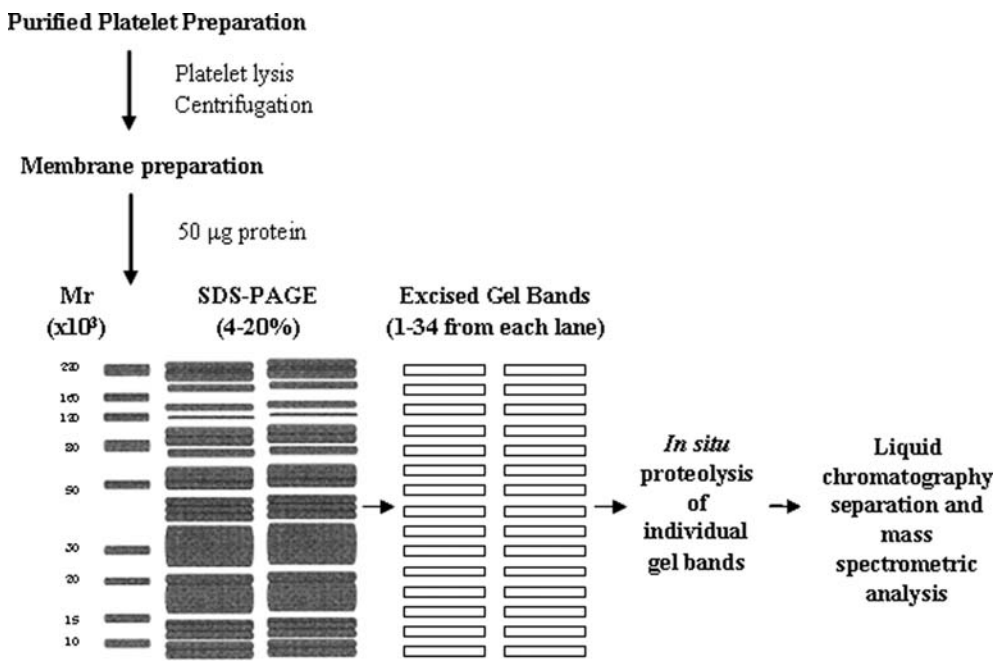

Fig. 17.1. Schematic representation of the strategy used for the proteomic analysis of enriched membrane preparation from purified buffy-coat human platelets. Both lanes of the 1D-gel were cut into 1.0–1.5 mm sections, each section digested *in situ* with trypsin, and LC-MS analysis performed on each of the 2 × 34 peptide extracts (Adapted from *(46)*).

3.6. In-gel Digestion and Peptide Extraction

1. After staining, gel sections are excised (1.0–1.5 mm slices) from a single lane using a scalpel (or gel cutter), extensively washed each time in deionized water (*see* **Fig. 17.1**).
2. The excised gel sections (approximately 30–40 sections) are placed in a 96-well flat-bottom tissue culture plate and digested with trypsin (0.05 μg), as described according to the protocol of Mortiz et al. *(20)* (*see* **Table 17.1**).
3. Samples washed with 100 mM ammonium hydrogen carbonate buffer.
4. Proteins are subsequently reduced with 10 mM DTT for 30 min and alkylated by incubation with 55 mM iodoacetamide in 100 mM ammonium hydrogen carbonate buffer at room temperature for 30 min.
5. Gel pieces are dehydrated in acetonitrile and rehydrated with 6 ng/μL of trypsin in 50 mM ammonium hydrogen carbonate buffer.
6. Digestion is performed by incubation at 37°C for a minimum of 4 h.
7. Peptides are extracted by application of 30 μL of 1% aqueous (v/v) formic acid in 2% (v/v) acetonitrile, followed by two subsequent 30 μL extractions of 50% acetonitrile.
8. Tryptic digests are concentrated to 10 μL by centrifugal lyophilisation ready for liquid chromatography-mass spectrometry (LC-MS).
9. Protein digests are transferred into 100 μL glass autosampler vials ready for injection.

3.7. Nano-LC Analysis

1. Peptide fractionation is achieved by capillary reversed-phase (RP)-HPLC (Agilent Model 1100 capillary HPLC) using a butyl-silica (C4) 150 × 0.15 mm I.D. RP-capillary column (Vydac^{TM}-C4MS, 5 μm, 300 Å Vydac, U.S.A.) developed with a linear 60 min gradient from 0–100% B with a flow rate of 0.8 μL/min. Samples (~7 μL) are loaded onto the column via the autosampler.
2. Buffer conditions: Solvent A; 0.1% (v/v) aqueous formic acid and Solvent B; 0.1% aqueous formic acid/60% (v/v) acetonitrile.
3. The column temperature is maintained at 45°C and eluent monitored for UV absorption at 215 and 280 nm.
4. The capillary HPLC is coupled on-line to the ESI-IT mass spectrometer for automated MS/MS analysis of individually isolated peptide ions *(20)*.

3.8. On-line MS/MS Analysis

1. Raw spectra are extracted using Extract-MSN as part of Bioworks 3.1 (Finnigan, San Jose, U.S.A.).
2. The parameters that are *recommended* to create peak lists are as follows: minimum mass 400; maximum mass 5000; grouping tolerance 1.5; intermediate scans 1; minimum group

count 1; automated calculation of charge state; 30 peaks minimum per spectrum; peptide charge states 1+, 2+ or higher; ±2 Da peptide mass tolerance; ±0.5 Da MS/MS fragment mass tolerance.

3. Parent ion masses are determined based on the isotope cluster spacing in the zoom scan spectrum.
4. Individual spectra files (.dta file extension) are subsequently generated prior to database searching

4. Notes

1. Platelets have two discrete membrane systems: (a) the surface-connected open canalicular system (OCS) derived from the plasma membrane of the megakaryocyte *(21)* and (b) the dense tubular system (DTS) derived from the smooth endoplasmic reticulum of the mega-karyocyte and the site of calcium sequestration *(22)*. The OCS is continuous with the plasma membrane in platelets and is thought to be the channel through which internal platelet granules release their contents during stages of activation. This extensive cytoplasmic counterpart of the membrane results in extensive cytoplasmic presence in platelet purification strategies.
2. Referred to as random donor platelets, platelet concentrates (PCs) are prepared by centrifugation of standard units of whole blood. As discussed, there are two methods for doing this: (1) the BC method (*see* **Section 3.2**) and (2) the PRP method (an example provided, *see* **Note 9**). The PRP method is used in the United States Blood Bank Service, whereas the BC method is in common use in Blood Banks across Australia and Europe. Both preparations differ principally in centrifugation procedures due to density differences associated with platelets and WBCs. In the PRP method, an initial soft spin produces PRP, which is separated from red cells, and subsequently platelets pelleted at a higher *g* force. As a consequence, the resulting platelet concentrates obtained from the PRP method contain 10^8 to 10^9 WBCs or ~50% or more of the leukocytes from the original unit of whole blood *(23)*. In contrast, BCs are pooled and centrifuged at low *g* force to suspend the platelets in the supernatant, consequently limiting platelet pelleting and reducing the level of cellular contaminants *(24, 25)*.
3. Typically referred to as platelet-pheresis, apheresis involves the use of a single-donor, where blood removed from one vein passes through a blood-cell separator centrifugation system with removal of the platelets or other cellular components and return of the red cells, leukocytes and most of the plasma, in addition to saline replacement, to the donor *(26)*. This procedure involves processing 4–5 L of donor's blood, resulting

in a platelet-pheresis product that contains platelet numbers equivalent to 6–9 units of PCs prepared from whole blood.

4. All biological specimens and materials coming in contact with them are considered biohazards. Universal precautions and OSHA (Occupational Safety and Health Administration) and institutional requirements (http://www.osha.gov/SLTC/biologicalagents/index.html) should be followed, including gloves, eye protection or working in a biosafety cabinet for blood processing. Handle as if capable of transmitting infection and dispose of with proper precautions in accordance with federal, state and local regulations. All equipment (storage, shipping, and centrifuge) must be labelled as biohazard. Never pipette by mouth. Wear suitable protective clothing and gloves. All centrifugation and preparation temperatures must be adhered to. Temperatures below room temperature can result in cold-induced platelet activation *(27,28)*.
5. Worldwide, plasma and RBCs are separated from units of whole blood by the use of automated expressor methods. Although one type of instrument is used in this example, many different automated devices are sufficient. The automatic separation units are programmed to deliver BCs of approximately 50 mL, with a hematocrit (complete blood count) of ~40%. The resultant BCs are subsequently pooled with a small quantity of plasma for the preparation of platelet concentrates. For transfusion purposes, most patients require a dose of platelets larger than can be provided by platelets from one unit of whole blood, therefore, several platelet concentrates are often pooled.
6. A 170–240 μm mesh filter has been used since the early days of blood transfusion to avoid the entry of debris and clots into the recipient's bloodstream. Presently filters have a non-woven web of mostly synthetic fibres, arranged with a proximal porous layer designed to retain gels that form from degenerating leukocytes in packed red cells during storage *(29)*. Performing leukodepletion soon after collection and component preparation has the advantage that the release of intracellular substances such as elastase and histamine *(30)*, in addition to other cellular components from disintegrating leukocytes, can be avoided.
7. Platelets are stored at 20–24°C using continuous gentle horizontal agitation in storage bags specifically designed to permit O_2 and CO_2 exchange to optimise platelet quality *(31–33)*. This combination of storage container, agitation, preservative solution, temperature and the use of approximately 50 mL of plasma, permits satisfactory preservation of platelets for up to 7 days *(34)*. However, several instances of bacterial contamination of PCs stored for this period have been reported *(35, 36)*, and storage time from collection to transfusion is now limited to 5 days *(37)*. An increased pH in the storage medium (>7.3), has been suggested as a cause of storage damage *(38)*.

When pH decreases below 6.2, platelets irreversibly lose viability *(39)*. Methods for the *in vitro* evaluation of platelet posttransfusion viability and function have been searched for. No ideal method is presently available, although visual checks of swirling and osmotic shock response indicate the maintenance of discoid shape and viability, respectively *(40)*.

8. Acquisition and analysis can be performed on scatter gating or fluorescence gating. Scatter gating (gating on forward scatter [FSC] and side scatter [SSC]) can be difficult when the platelet count is low or when there is aggregation in the sample. In both normal and disease states, and especially when activated, platelets and debris can have overlapping light-scatter signatures. For scatter gating, debris and background noise is excluded by setting the appropriate FSC threshold. Fluorescence gating can be done on the activation independent platelet marker, and then the light-scatter profile of the positive population independently analysed. Venous blood typically demonstrates three subpopulations of particles; the majority of the particles consist of single intact platelets. A second population, typically representing 5% of all particles, exhibits greater light scatter than single platelets and represents platelets associated with large WBCs. A third population, representing 5–15% of the particles whose light scatter is lower than single intact platelets, includes platelet-derived micro particles with an average diameter of 0.1 μm. For fluorescence gating, exclude debris and background noise by setting the appropriate threshold. See references for further descriptions *(18, 41, 42)*
9. Platelet rich plasma preparation
    1. Whole blood collected following informed consent, by venipuncture of human volunteers who had denied taking medication known to interfere with platelet function within the previous 2 weeks.
    2. Blood (8.5 mL) is collected aseptically using a 21-gauge needle by venipuncture into a standard vacutainer tube containing acid citrate dextrose (ACD) anticoagulant *(43–45)*. Preparation of PRP should be immediately performed.
    3. The PRP is obtained by centrifugation (110 × *g*, 15 min, 22°C) of the whole-blood.
    4. The upper 25% (PRP sample) is removed for further analysis to limit contamination by white cells and red cells.

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Chapter 18

Detergents and Chaotropes for Protein Solubilization Before Two-Dimensional Electrophoresis

Thierry Rabilloud

Abstract

Because of the outstanding ability of two-dimensional electrophoresis to separate complex mixtures of intact proteins, it would be advantageous to apply it to all types of proteins, including hydrophobic and membrane proteins. Unfortunately, poor solubility hampers the analysis of these molecules. As these problems arise mainly in the extraction and isoelectric focusing steps, the solution is to improve protein solubility under the conditions prevailing during isoelectric focusing. This chapter describes the use of chaotropes and novel detergents to enhance protein solubility during sample extraction and isoelectric focussing, and discusses the contribution of these compounds to improving proteomic analysis of membrane proteins.

Key words: Hydrophobic protein, membrane proteins, protein solubilization, isoelectric focusing, chaotropes, detergents, zwitterionic detergents.

1. Introduction

The solubilization of proteins for 2D electrophoresis-based proteomics is a difficult task. Two-dimensional electrophoresis with isoelectric focussing (IEF) as the first dimension is one of the worst possible circumstances for protein solubilization. Proteins must reach their pI, at which solubility is at its minimum, but still remain in solution. Moreover, since mobility decreases when proteins approach their pI, IEF has to be performed using strong electric fields (200 V/cm compared to the 10–20 V/cm normal In SDS-PAGE). This means, in turn, that salts and ionic compounds in general are incompatible with IEF. Moreover, any solubilizing agent used prior to IEF must not alter the original pI of

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the proteins, which precludes the use of strong ionic detergents (e.g. SDS). However, low amounts (up to 0.03% w/v) of ionic detergents can be used, provided that conditions favouring the exchange of SDS for other, non-ionic detergents are employed *(1–3)*. This ensures removal of bound SDS from the proteins, but means that the benefits of SDS are lost for the IEF dimension. However, the use of SDS has been often recommended as a way to ensure a complete initial solubilization before IEF.

Apart from these factors intrinsic to the protein world, other common problems are attributable to non-proteinaceous compounds present in many biological samples. For example, nucleic acids act as mobile ion exchangers at the low-ionic strength required by IEF, and can completely blur the 2D-electrophoresis pattern when present at too high a concentration *(4)*. Other classes of compounds (lipids, salts etc.) encountered in many samples also create their own artefacts.

There are, thus, different problems depending on the starting material. This chapter will, however, focus on the intrinsic solubilization of proteins, and will mainly deal with the chaotropes and detergents used for initial solubilization and for IEF.

There is an interplay between chaotropes and detergents for protein solubilization before IEF and 2D electrophoresis, though both are necessary, since systems running with either detergents or chaotropes alone show very poor efficiency, in contrast to the effectiveness of SDS in SDS-PAGE. From this example, it is clear that non-ionic detergents are unable by themselves to denature and solubilize proteins as SDS can. This is because IEF-compatible detergents do not bear any electrical charge, whereas this feature is essential for the efficiency of both anionic detergents such as SDS, and also for cationic detergents including CTAB or BAC16 that are used in other forms of 2D electrophoresis (*see* chapters 6 and 19 of this volume). Because of the low efficiency of electrically neutral detergents, it is necessary to add chaotropes to improve denaturation and solubilization of proteins.

The role of chaotropes in the solubilization process is to break the inter- and intra-molecular non-covalent interactions in the sample (e.g. hydrogen bonds, dipole-dipole interactions, and hydrophobic interactions), and thereby facilitate protein unfolding. Although ionic bonds are not directly affected by non-ionic chaotropes such as urea and thiourea, the influence of these chaotropes on the dielectric constant of water also alters the strength of the ionic bonds. However, chaotropes alone are not efficient for complex membrane samples. For example, they are unable to solubilize the lipid bilayer of membranes, which is required for membrane protein solubilization. Furthermore, by unfolding proteins, chaotropes expose additional hydrophobic residues to the solvent, and thus increase the likelihood of aggregation. The addition of detergents, which are specialised

molecules for handling hydrophobic effects in aqueous solvents, considerably reduces this aggregation problem.

As mentioned, the constraints imposed by IEF preclude the use of substances which are ionized within the pH limits of the IEF dimension, that is, only nonionic or zwitterionic compounds can be used. This narrows the choice of chaotropes to members of the amide and urea families, as guanidines and amidines are charged below pH 12. Among these, urea is long established. More recently, the addition of thiourea to urea has shown interesting features for protein solubilization *(5)* and also limits protease action *(6)*.

Among the range of commercially available uncharged detergents, two subfamilies can be distinguished. Nonionic detergents do not have any charges on the molecule, whereas zwitterionic detergents have an equal number of balancing negative and positive charges. Depending on the pK of the ionisable groups, some detergents can be ionic in one pH range (where at least one group is titrated) but zwitterionic in another, while others may be zwitterionic over the complete pH range. For example, classical betaines (which have a quaternary ammonium and a carboxylic group) are positively charged at low pH, because the carboxylic group is partially protonated. More than 2 pH units above the pK, however, the carboxylic group is fully deprotonated, and they behave as a zwitterions. Conversely, sulfobetaines, bearing a quaternary ammonium and a sulfonic group, are zwitterionic over the entire 0–14 pH range, as both groups are ionised. Of course, only detergents completely zwitterionic over the pH range of interest can be used for IEF.

The detergents historically used for 2D electrophoresis are Triton X100 (or NP-40) (nonionic), and CHAPS (zwitterionic). These have been used extensively in combination with urea, but have not proved very efficient with sparingly soluble membrane proteins *(7)*. However, recent work has shown that either specially designed zwitterionic detergents *(8–10)*, or carefully selected non ionic detergents *(11)* can solubilize membrane proteins. It is interesting to note that Triton X100 is poor when used with urea alone but is much more efficient in urea-thiourea *(11)*. This is also true of linear alkyl oligo ethylene glycol compounds *(11)* such as the Brij® detergents. However, the most efficient nonionic detergents are glycosides such as octyl glucoside and dodecylmaltoside. The latter seems to be efficient both in urea *(12)* and in urea-thiourea *(11, 13)*. An example of the variations in protein solubilization induced by the choice of detergent can be seen in **Fig. 18.1**. The multiple variables playing a role in the solubilization process have also been investigated in *(14)*. However, it should not be concluded from the preceding that dodecyl maltoside is the absolute best choice for protein solubilization for 2D electrophoresis, since the optimal detergent will also depend

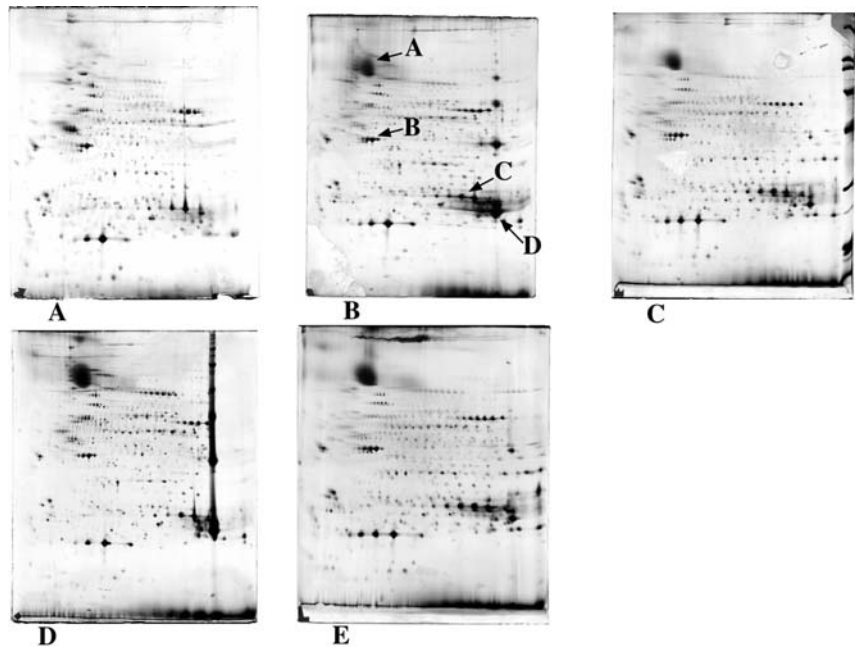

Fig. 18.1. 2D electrophoretic separation of human red blood cell membrane proteins. Red blood cell plasma membrane proteins (150 μg) were loaded on the 2D gels. The first dimension is a 4–8 linear pH gradient, and the second dimension a 10% acrylamide gel. The identities of several proteins are indicated: A: band 3 (12 transmembrane domains); B: actin; C: stomatin (1 transmembrane domain); D: globin dimer. The proteins were extracted and focused in a solution containing 7 M urea, 2 M thiourea, 20 mM DTT, 0.4% carrier ampholytes and (**A**) 4% CHAPS, (**B**) 2% Triton X-100, (**C**) 2% Brij56, (**D**) 2% dodecyl maltoside, (**E**) 2% ASB14.

on the sample. However, previous work has established some kind of a shortlist, so the best candidates for protein solubilization, at least as a first screen, should be chosen from dodecyl maltoside, ASB14, C7BzO, Brij56 and C13E10.

2. Materials

2.1. Biological Material

Human red blood cell membranes, which are a very good control for membrane protein solubilization tests, are prepared as follows (all steps are carried out between 0° and 4°C).

Ten ml of freshly drawn human blood, collected on EDTA or citrate, are diluted with 10 ml of PBS supplemented with 1 mM EDTA. The diluted blood is loaded on a 10 ml cushion of Ficoll/sodium diatrizoate solution (d: 1.077, trade names Ficoll Hypaque or equivalent) and centrifuged at 2000*g* for 20 min. The supernatant (plasma and white blood cells) is discarded, and the red blood cells are re-suspended in 10 ml of PBS-EDTA and centrifuged at 2000*g* for 20 min. The pellet is re-suspended in 20 times its volume of 2 mM EDTA pH 8, resulting in hemolysis.

The hemolysate is centrifuged at 50,000*g* for 20 min. The dark red supernatant is discarded, leaving a double-layered pellet, with a tight, light red pellet at the bottom and a bluish-white, fluffy pellet on top. This white layer is the membrane pellet, the red layer being composed of protease-rich granules. The white layer is re-suspended in 30 ml PBS-EDTA, centrifuged at 50,000*g* for 20 min, and the supernatant is removed. After a final wash with 2 mM EDTA, the final pellet is re-suspended in a minimal volume of 10 mM Tris-HCl pH 7.5, 0.2 M sucrose, 1 mM EDTA, then aliquoted and stored at −80°C (preferably) or −20°C (for short term storage). The protein concentration is estimated by a standard protein assay.

2.2. Equipment

1. A tabletop ultracentrifuge, used for membrane preparation and to remove insoluble material.
2. Immobilised pH gradient strips (IPG, linear and non-linear pH 3–10 gradient, 18 cm length, (GE Healthcare)).
3. IPGphor apparatus (GE Healthcare) for isoelectric focusing of proteins.
4. Tube gels electrophoresis equipment (Bio-Rad), for first dimension gel electrophoresis.
5. Protean II (Bio-Rad) for SDS-PAGE electrophoresis.

2.3. Reagents

1. Dodecyl maltoside, Triton X100 and CHAPS are best used from 20% (w/v) stock solutions in water. These solutions should be stored at 4°C and have limited stability (a few weeks).
2. C13E10, Brij 56, ASB14 are best used from 20% (w/v) stock solutions in ethanol/water (50/50 v/v). These solutions are stable for several months at room temperature.
3. Urea stock solution for IEF. It is difficult to go beyond 9 M urea at room temperature, which is the concentration used when urea is the sole chaotrope. This means that urea is best added as a solid (*see* **Note 1**).
4. Urea-thiourea stock solution. The final chaotrope concentrations are 7 M urea and 2 M thiourea. This means that a 1.25× concentrated solution can be prepared, which is simpler than weighing small amounts of solid urea and thiourea for each sample. For 10 ml of this concentrated solution, weigh 5.25 g of urea and 1.9 g of thiourea. Detergents (e.g. CHAPS or Triton X-100) which are fully compatible with urea, can be added at this stage. Those detergents which are less compatible with urea (e.g. ASB14) must be added during dilution to the final concentration. A total volume of 4.2 ml of liquid must be added to the urea and thiourea to give 10 ml (*see* **Notes 2** and **3**). This solution is stable for several months at −20°C.

5. Tributylphosphine is a liquid (4 M when pure). A 40-fold dilution in dimethyl formamide is made just prior to use. This solution is further diluted 50-fold in the sample solution.
6. Tris carboxyethyl phosphine is a solid. A 1 M stock solution in water is made, and is stable for several months at −20°C.

3. Methods (*see* Note 4)

3.1. Solubilization in Urea for IEF

3.1.1. Solubilization from a Solid Sample, for example, Tissue or Cell Pellet

In this case, the contribution of the sample to the final solubilization volume can often be neglected. A solution containing 9–9.5 M urea, (*see* **Note 1**), the detergent (selected from those listed in **Section 2.3**) at 2–4% w/v concentration, carrier ampholytes (0.4% w/v for IPG, 2% w/v for CA-IEF) and a reducing agent (50 mM DTT or 5 mM TBP or 5 mM TCEP) is added to the solid sample, resulting in a liquid extract. Protein extraction is helped by sonication in an ultrasonic bath for about 30 min. Insoluble material is removed by ultracentrifugation at 200,000*g* for 30 min at room temperature.

3.1.2. Solubilization from a Suspension or Solution

In this case, the volume of the sample must usually be included for calculation of the final concentrations. As a rule of thumb, the sample solution can represent up to 35% of the final volume. Solid urea, water and stock solutions of the detergent, ampholytes and reducing agents are added to the liquid sample to give the extraction solution.

3.2. Solubilization in Urea–Thiourea for IEF

The use of sample application by in-gel re-hydration of IPG strips *(15)*, means greater sample volumes can be used. This is especially true if home made strips are used, as these can be made wider than commercial IPG strips and so accommodate volumes up to 1 ml. It is thus often possible to dilute liquid samples, or solid samples re-suspended in a minimal volume of water, with the concentrated chaotrope solution. If the detergent can be predissolved in the concentrated chaotrope solution, which may also contain the reducing agent, then the sample volume can represent up to 20% of the total extraction volume. If the detergent has to be added last, with a urea concentration not exceeding 8 M, then it is more convenient to use one volume of sample, one volume of detergent stock solution, and to add eight volumes of concentrated chaotrope solution. If this results in to too high a final volume, two alternative approaches can be considered:

(1) Place a volume of a stock detergent solution equal to the sample volume in a sample tube and evaporate in a Speed-Vac. Then add the sample and four volumes of concentrated chaotrope solution.

(2) Assume that the sample volume will constitute 40% of the final volume. Weigh the corresponding quantities of urea, thiourea and solid detergent. Dissolve in the sample solution using an ultrasonic bath.

In all cases, 30–60 min extraction at room temperature is optimal prior to centrifugation (200,000*g*, 30 min, room temperature) to remove insoluble material.

3.3. Running IEF Gels with Membrane Proteins

The detergents historically used for IEF and 2D electrophoresis of soluble proteins (CHAPS or Triton X100) are fully compatible with urea (*see* **Note 5**). However, temperatures greater than 15°C are needed to maintain urea and thiourea in solution.

Some of the detergents used for improved solubilization of membrane proteins, especially those with a linear alkyl tail (e.g. ASB 14, ASB 16, and Brij56) show a urea tolerance that is strongly temperature dependent (*see* **Note 5**). Since detergent precipitation ruins electrophoretic separations, the detergent concentration and running temperature must be carefully controlled. For all detergents mentioned here, using 2% detergent in a 7 M urea/2 M thiourea solution and running the gels at 22°C (thermostated) ensures complete solubility of the chaotrope-detergent mixture and optimal solubilization in the sense that no additional proteins appear on the 2D gels if the detergent concentration is increased.

The downstream steps of 2D electrophoresis (IEF gel equilibration and SDS-PAGE) are performed exactly as for soluble proteins and no adjustment of the standard protocols is necessary.

4. Notes

1. The partial specific volume of urea is useful to know in order to make concentrated urea solutions. One gram of urea occupies 0.75 ml in solution. For most detergents (and thiourea), 1 g occupies 1 ml. As an example, 1 ml of aqueous extract added to 900 mg urea gives 1.675 ml of a 9 M urea solution. Similarly 1 g urea added to 1 ml aqueous extract, results in 1.75 ml of a 9.5 M urea solution. Urea must also never be warmed above 37°C, or protein carbamylation may occur.
2. Most of these extraction solutions contain less than 50% water. This means that dissolution of solids is difficult, particularly as temperatures above 37°C cannot be used (*see* **Note 1**). The use of an ultrasonic bath (sold for cleaning objects and glassware) is of great help for these difficult solubilizations.
3. Many detergents are not fully compatible with urea. Depending on the detergent structure, the urea concentration and the temperature, insoluble detergent-urea complexes can form. Detergents with linear alkyl chains (e.g. ASB 14, Brij 56) are

especially prone to this problem. (Commercial linear sulfobetaines are effectively unusable, as they do not tolerate urea concentrations above 4 M).

4. Many detergents and reducing agents used in sample preparation interfere with standard protein assay methods. In the worst case, one can end with a solubilization cocktail which is incompatible with any protein assay method. It may, therefore, be advisable to determine the protein concentration in the initial sample, prior to extraction, especially when dealing with suspensions. This means though, that it will be impossible to assess the efficiency of the solubilization process.
5. In concentrated aqueous solutions, urea forms channel-like structures, which are able to accommodate organic compounds, especially those with a linear, saturated alkyl chain, forming so-called inclusion compounds which are less soluble than either component separately. This is the molecular basis for the urea-driven precipitation of many detergents. This phenomenon is highly temperature dependent, as is the solubility of the detergent/urea inclusion compounds.

This problem can be alleviated by subtle modifications to the structure of the detergents, for example by inclusion of an amide bridge or introducing mixed alkyl and aryl hydrophobic domains, as aryl structures are too big to insert into the urea channels.

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Chapter 19

Two-Dimensional Separation of Membrane Proteins by 16-BAC-SDS-PAGE

Hans Gerd Nothwang and Jens Schindler

Abstract

Defining membrane proteomes is fundamental to our understanding of many physiological and pathophysiological processes. Their separation and identification is hence a key issue in basic and biomedical research. Due to their hydrophobic character, few high-resolution techniques for separation are available for qualitative and quantitative approaches. For gel-based methods, the two-dimensional 16-BAC/SDS-PAGE is the method of choice. This technique separates proteins in the first dimension using an acidic buffer system and the cationic detergent benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) and in the second dimension by SDS-PAGE. Here, we describe a detailed protocol for the separation of proteins by 16-BAC/SDS-PAGE.

Key words: 16-BAC, membrane protein, two-dimensional gel electrophoresis, protein separation.

1. Introduction

Often, gel-based high-resolution separation of membrane proteins is desirable. Examples include the characterization of subcellular proteomes and their subsequent identification by mass spectrometry, the quantitative comparison of membrane compartments in different developmental stages or in wildtype versus disease conditions, or the identification of different isoforms of a given membrane protein of interest. For many complex protein mixtures, separation at high-resolution can routinely be performed by conventional two-dimensional gel electrophroesis using isoelectric focusing (IEF) in the first dimension and

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SDS-PAGE in the second dimension *(1–3)*. However, the separation of hydrophobic membrane proteins, particularly those with more than one transmembrane domain, by isoelectric point and molecular weight has been poor *(4, 5)*. This is due to low recovery of these proteins *(6, 7)* which is caused by several factors. First, membrane proteins do not solubilize well in nonionic detergents and the low ionic strength of the media required for IEF *(4, 8)*. Second, membrane proteins often precipitate at pH values close to their isoelectric point and will not transfer to the second dimension *(8)*. Third, glycosylation of membrane proteins introduce charge heterogeneity which leads to streaking in the first dimension *(8)*. Finally, remaining lipids within the membrane preparation might interfere with the IEF process *(4)*.

For membrane proteins, IEF as first dimension was, therefore, replaced by a modified benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) gel system *(8)*. In this system, which was pioneered by Macfarlane *(9, 10)*, proteins are resolved using the cationic detergent 16-BAC, a stacking gel of pH 4.1, and a separation gel at pH 2.1. Similar to SDS-PAGE, proteins are separated according to their molecular mass. The combination of the 16-BAC gel with SDS-PAGE, therefore, resolves the proteins along the diagonal of the second dimension (**Fig. 19.1**). However, the properties of the acidic 16-BAC system are sufficiently different from the alkaline SDS system to allow for substantial resolution in the second dimension.

Successful applications of the 16-BAC-SDS-PAGE include the analysis of the membrane proteins of synaptic vesicles *(8)*,

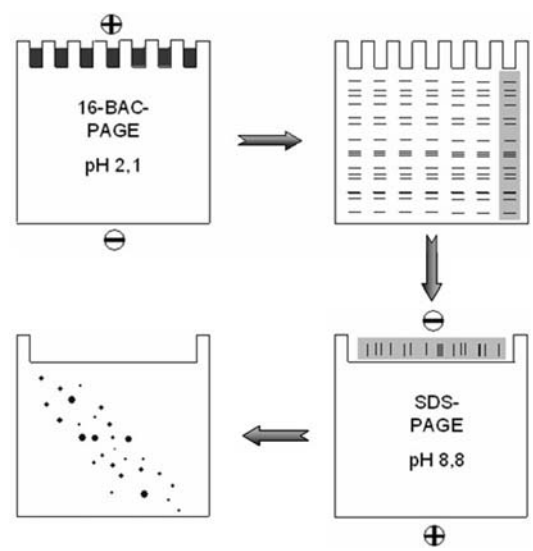

Fig. 19.1. Schema of 16-BAC-SDS-PAGE. Note the switch in polarity of the electrodes between the 16-BAC-PAGE and the SDS-PAGE.

the proteome of the nuclear envelope *(11)*, Golgi-associated proteins *(12)*, and the proteins of peroxisomes *(13)*. Furthermore, this system can be applied to proteins with very acidic or alkaline isoelectric points such as histones which are not resolved by conventional IEF *(14, 15)*.

2. Materials

The protocol described here applies to the often used standard sized gel of 20 × 20 cm with a thickness of 1 mm in the first dimension and a thickness of 1.5 mm in the second dimension. For other gel sizes, the volumes have to be adjusted accordingly.

2.1. 1st Dimension 16-BAC-PAGE

1. 16-BAC (250 mM) (*see* **Note 1**)
2. Stock acrylamide solution: acrylamide (30%), bisacrylamide (0.8%) (*see* **Note 2**)
3. Ascorbic acid (80 mM) (*see* **Note 3**)
4. Bisacrylamide (2%) (*see* **Note 2**)
5. Cationic electrophoresis buffer: 16-BAC (2.5 mM), glycine (150 mM), phosphoric acid (50 mM) (*see* **Note 4**)
6. Destaining solution: methanol (45%), acetic acid (10%) (*see* **Note 5**)
7. $FeSO_4$ (5 mM) (*see* **Note 6**)
8. H_2O_2 (30%)
9. Reequilibration buffer: Tris-HCl (100 mM), pH 6.8
10. Sample buffer (2X): urea (7.5 M), 16-BAC (10%), glycine (10%), DTT (75 mM), Pyronin Y (0.05%) (*see* **Note 7**)
11. Separating gel buffer: KH_2PO_4 (300 mM), pH 2.1 with HCl
12. Stacking gel buffer: KH_2PO_4 (500 mM), pH 4.1 with HCl
13. Staining solution: Coomassie Brillant Blue R-250 (0.25%), methanol (45%), acetic acid (10%)
14. Urea

2.2. 2nd Dimension (SDS-PAGE)

1. Ammoniumperoxodisulfate (10%)
2. Anionic electrophoresis buffer: Tris (25 mM), glycine (192 mM), SDS (0.1%)
3. Isopropanol (75%)
4. *N,N,N′,N′*-Tetramethylethylenediamine (TEMED)
5. Reducing buffer: Tris (75 mM), glycine (576 mM), SDS (0.3%), β-mercaptoethanol (5%)
6. SDS (10%) (*see* **Note 8**)

7. Sealing solution: agarose (0.1%), traces of bromophenol blue
8. Separating gel buffer: Tris-HCl (1.5 M), pH 8.8
9. Stacking gel buffer: Tris-HCl (500 mM), pH 6.8
10. Stock acrylamide solution: acrylamide (30%), bisacrylamide (0.8%) (*see* **Note 1**)

3. Methods

3.1. Gel Casting of 16-BAC Gel (1st Dimension)

Cast the gel one day prior to use to ensure complete polymerization and keep at room temperature. Gels should be used within 5 days.

1. Assemble a sandwich of two clean glass plates with 1 mm spacers.
2. Prepare the following separating gel solution in a 50 mL plastic tube (*see* **Notes 9** and **10**).

	Final concentration	**50 mL**
Urea	3 M	9.1 g
Acrylamide/bisacrylamide	7.5%	12.5 mL
Separating gel buffer	75 mM	12.5 mL
Ascorbic acid	4 mM	2.5 mL
$FeSO_4$	8 μM	80 μL
16-BAC	2.5 mM	500 μL
Water		12.5 mL
H_2O_2	0.002%	3.3 μL

3. Pour the separating gel solution into the gel cassette, until it reaches a position of 1 cm from the bottom of the comb that will form the loading wells. Once this is completed, you will find excess gel solution remaining in your flask. After polymerization, dispose this in an appropriate waste container. Do **not** pour it down the sink.
4. To ensure that the gel sets with a smooth surface carefully cover the separating gel with KH_2PO_4, pH 2.1 (300 mM). When set, a very clear refractive index change can be seen between the polymerized gel and the overlaying solution.
5. When the separating gel has set, pour off the overlaying solution. Prepare the following stacking gel solution in a 12 mL plastic tube.

	Final concentration	10 mL
Urea	1.67 M	1 g
Acrylamide/bisacrylamide	5%	1.67 mL
Bisacrylamide	0.2%	1.173 mL
Stacking gel buffer	125 mM	2.5 mL
Ascorbic acid	4 mM	500 μL
$FeSO_4$	4 μM	8.5 μL
16-BAC	1.75 mM	70 μL
Water		3 mL
H_2O_2	0.0025%	0.8 μL

6. Pour the stacking gel solution into the gel cassette until the solution reaches the cutaway edge of the gel plate. Place the well-forming comb into this solution, and leave it to set over night at room temperature. Mark the positions of the wells on the taller glass plate (which becomes the front glass plate of the sandwich during the electrophoretic separation) to facilitate loading of the samples.

3.2. Gel Casting of SDS-Polyacryamide Gel (2nd Dimension)

Cast the gel one day prior to use to ensure complete polymerization.

1. Assemble a sandwich of two clean glass plates with 1.5 mm spacers (*see* **Note 11**)
2. Prepare the following separating gel solution in a 200 mL flask (*see* **Note 9**)

	Final concentration	100 mL
Acrylamide/bisacrylamide	10%	33.4 mL
Separating gel buffer	375 mM	25 mL
SDS	0.1%	1 mL
TEMED	0.04%	40 μL
Ammoniumperoxodisulfate	0.1%	1 mL
Water		39.54 mL

3. Pour the separating gel solution into the gel cassette. Stop, when the solution reaches a position which is 1 cm plus the width of 1 lane of the 16-BAC gel below the cutaway edge of the short glass plate. Once this is completed, you will find

excess gel solution remaining in your flask. After polymerization, dispose this in an appropriate waste container. Do **not** pour it down the sink.

4. To ensure that the gel sets with a smooth surface carefully cover the separating gel with isopropanol. When set, a very clear refractive index change can be seen between the polymerized gel and the overlaying solution.
5. When the separating gel has set, pour off the overlaying solution. Prepare the following stacking gel solution in a 12 mL plastic tube.

	Final concentration	6 mL
Acrylamide/bisacrylamide	5%	1 mL
Stacking gel buffer	375 mM	1.5 mL
TEMED	0.04%	7.5 μL
Ammoniumperoxodisulfate	0.75%	45 μL
Water		3.5 mL

6. Pour the stacking gel solution into the gel cassette until the solution is 1 cm above the separating gel. A comb with a continuous large well and one additional small well for the protein marker (normally used for preparative gels) may be placed into the cassette, but this is not absolutely necessary. Let the gel set overnight at room temperature.

3.3. 16-BAC-SDS PAGE

1. Mount the gel cassette into the electrophoresis chamber filled with cationic electrode buffer.
2. Mix equal volumes of protein sample with 2X sample buffer and warm up to 60°C for 5 min. After washing the wells with cationic electrode buffer, load the 16-BAC gel with the samples (*see* **Notes 12** and **13**).
3. Electrophoretic separation in the 16-BAC gel is carried out from anode to cathode (as opposed to SDS-PAGE) at 4°C with 15 mA over night until the dye runs out of the gel.
4. After electrophoresis in the first dimension, remove the gel from the gel cassette by gently separating the two plates of the cassette.
5. Stain the gel for 20 min with staining solution followed by destaining of the gel with destaining solution until the lanes are visible on the gel (*see* **Note 5**).
6. Wash the gel four times for 10 minutes with reequilibration buffer (*see* **Note 14**).
7. Excise the desired lane from the 16-BAC gel using a 15–18 cm long Gel releaser. Transfer the lane on the Gel releaser to the gel cassette and layer it on top of the SDS gel without

any space between the two gels. Place the gel strip between the glass plates close to one edge. To improve the sliding of the lane into the gel cassette, moisten the glass plates.

8. Cover the gel for 5 min with reducing buffer. Remove reducing buffer with a 20-gauge needle fixed to a 5 mL syringe.
9. If a preparative comb was used, add protein marker to the small well. If no well is available, pipette carefully 5–10 μL of marker proteins on a 10 × 5 mm Whatmann No. 1 filter paper and slide the filter piece carefully along the free edge into the glass cassette, stopping just above the stacking gel (*see* **Note 15**).
10. Remove the reducing buffer and fix the gel with sealing solution melted at 95°C.
11. Electrophoresis is carried out from cathode to anode at 4°C with a fixed current of 15 mA in the stacking gel and 25 mA in the separating gel until the dye runs out of the gel.
12. Remove the gel from the cassette by gently separating the two plates of the cassette and stain (*see* **Notes 16** and **17**).

4. Notes

1. The stock solution of 16-BAC can precipitate even at room temperature. If this happens, warm to 60°C to solubilize. Store at room temperature.
2. Acrylamide is a potential neurotoxin and should be handled with great care. Prepare stock solution in an extractor hood and wear gloves.
3. Ascorbic acid should be used within 2 weeks.
4. Phosphoric acid is a 85% solution which should be regarded as a 8.67 M solution.
5. Destaining solution can be reused but evaporation of methanol increases destaining time. You might add some methanol to keep destaining time short.
6. $FeSO_4$ should be prepared as a fresh solution prior to use, as it oxidizes in aqueous solutions to Fe^{3+}.
7. Solubilize and store at 60°C in a water bath, as it precipitates otherwise.
8. SDS stock solution can precipitate even at room temperature. If this happens, warm up to solubilize.
9. Polymerization of the 16-BAC gel is initated by the homolytic dissociation of H_2O_2 into OH radicals, and in case of the SDS gel by dissociation of ammoniumperoxodisulfate into SO_4^-. radicals.
10. Work rapidly, as the gel polymerizes within minutes. Therefore, do not degas.

11. The gel of the second dimension is 50% thicker to ensure easier layering of the first dimension onto the second dimension.
12. We use between 300 and 400 μg membrane proteins for a 20 × 20 cm gel, when the second dimension is stained with colloidal Coomassie blue. Adjust protein for other gel sizes and staining methods.
13. Fill nonused lanes with the same volume of sample buffer as used for the sample preparation. This will reduce smiling effects.
14. According to the literature, cut lanes from the first dimension can be stored for few days in destaining solution. However, we observed diffusion of proteins during storage, which results in notably lower quality in total separation of the proteins after the second dimension.
15. The filter paper should not be in contact with the stacking gel, as the protein markers might diffuse laterally.
16. Staining procedure depends on intended use of the gel. For simple protein pattern recognition, all stains ranging from different silver stainings to coomassie blue stainings or fluorescent dyes can be applied. If identification of proteins by mass spectromety is planned, use a stain comptabile with mass spectrometry such as colloidal Coomassie blue staining.
17. In our hands, molecular weights of the identified proteins as judged by their migration in relation to known marker proteins deviate to a larger extent than in conventional two-dimensional gel electrophoresis. This is due to the different biochemical properties of membrane proteins compared to the hydrophilic proteins most commonly used as marker proteins.

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Part IV

Identification and Quantification of Membrane Proteins

Chapter 20

MudPIT Analysis: Application to Human Heart Tissue

Kelli G. Kline and Christine C. Wu

Abstract

Although two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used as the standard proteomic approach for separating proteins in a complex mixture, this technique has many drawbacks. These include a limited molecular mass range, poor separation of highly acidic or basic proteins, and exclusion of the majority of membrane proteins from analysis. Considering the important functions of many membrane proteins, such as receptors, ion transporters, signal transducers, and cell adhesion proteins, it is increasingly important that these proteins are not excluded during the global proteomic analysis of cellular systems. *Mu*lti*d*imensional *P*rotein *I*dentification *T*echnology (MudPIT) offers a gel-free alternative to 2D-PAGE for the analysis of both membrane and soluble proteins.

The goal of this chapter is to provide detailed methods for using MudPIT to profile both membrane and soluble proteins in complex unfractionated samples. Methods discussed will include tissue homogenization, sample preparation, MudPIT, data analysis, and an application for the analysis of unfractionated total tissue homogenate from human heart.

Key words: MudPIT, 2D-PAGE, proteomics, membrane proteins, human heart explants.

1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with mass spectrometry has typically been used to identify proteins within complex mixtures. This technique utilizes isoelectric focusing (IEF) to separate proteins in the first dimension according to their charge (pI, isoelectric point) and then in a second dimension using polyacrylamide gel electrophoresis (PAGE), which separates based on the protein's molecular weight (**Fig. 20.1**) *(1)*.

This method can be used to separate over 1,000 proteins; however, membrane proteins are under-represented using this

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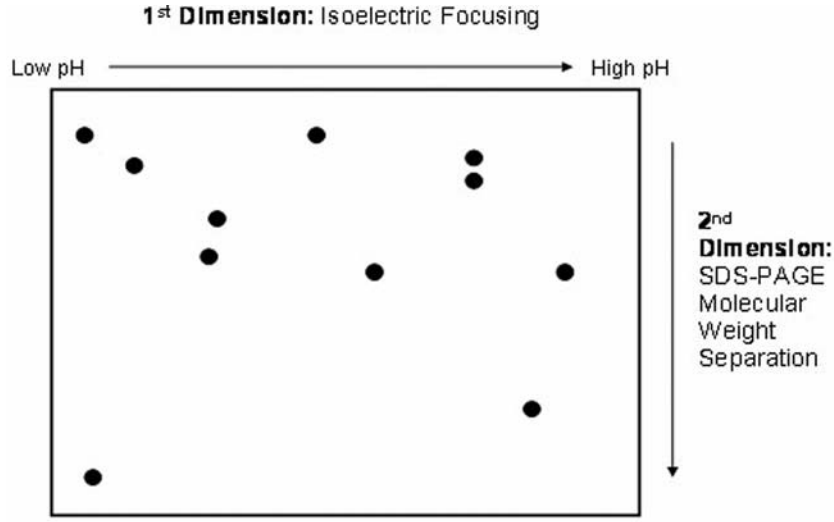

Fig. 20.1. Two-dimensional polyacrylamide gel electrophoresis.

platform due to their poor solubility in the sample buffer and resolution in 2D-PAGE *(2)*. Membrane proteins are typically insoluble in the nonionic or zwitterionic detergents utilized in IEF, or they precipitate out of the IEF solution at pH values close to their pI *(3)*. In general, 2D-PAGE techniques can effectively separate membrane proteins that have one or two transmembrane segments, but not those with three or more, which means that approximately 1% of integral membrane proteins are actually resolved using current 2D-PAGE *(4,5)*. Gel-based approaches are also generally biased toward detection of highly abundant proteins and housekeeping enzymes and yield decreased detection of those proteins with extremes in isoelectric point and molecular weight, a limitation that is exacerbated by the fact that up to 7% of proteins have pI $\geq$10 *(6–8)*. An additional limitation of 2D gel approaches is the complexity of the data analysis. There has not been a program developed that is automatic in initial spot recognition and in the subsequent matching of spots between gels when presented with complex 2D gel images that contain thousands of protein spots. Most of the programs conventionally used require a significant amount of user hands-on time to edit images before they can be entered into the database. However, many of these limitations are currently being overcome. For example, immobilized pH gradients that can resolve proteins with extreme acidic or basic pI's are currently being utilized, and these advancements will allow gel-based techniques to be more widely used in the future for complex samples *(9)*.

The 2D-gel electrophoresis (2D-GE) approach has been used extensively in the study of many complex diseases, including human heart failure. Heart failure is a complex, multifactorial

disease that is characterized by the inability of the heart to maintain cardiac output that is sufficient for the body's needs. In one study of human heart disease using 2D-GE 1600 gel spots were identified, with an unlisted number of proteins identified from these spots *(10)*. However, the study was limited mainly to the examination of soluble proteins and those highly abundant myofilament proteins such as myosin and desmin. In another study using 2D-GE and human heart tissue, 110 proteins were identified from 374 excised gel spots *(11)*. The fraction of membrane proteins in each of these samples were not reported.

As an alternative to 2D gel approaches, several multidimensional liquid phase based separation techniques have been investigated. Among these techniques is the *Mu*ltidimensional *P*rotein *I*dentification *T*echnology (MudPIT) developed by the Yates group *(12)*. This technique uses biphasic capillary columns packed with both strong cation exchange material (SCX) and reverse phase material (RP) to separate whole protein mixtures that have been digested and loaded directly onto the column. The solid phase column packing material allows for the separation of peptides based on both their charge and hydrophobicity. The column is then placed in-line with a tandem mass spectrometer to facilitate electrospray ionization, fragmentation, and detection of the ions as they are eluted off the multidimensional separation column (**Fig. 20.2**).

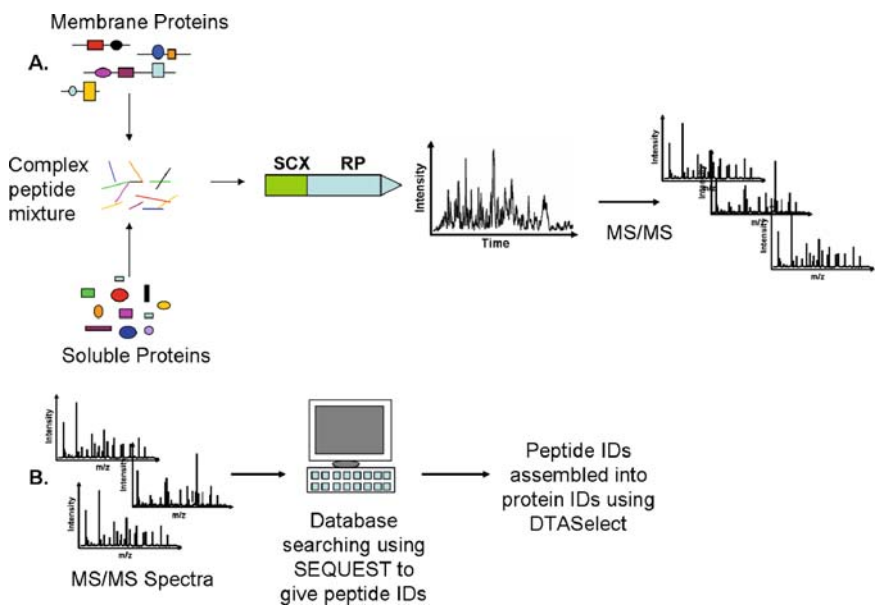

Fig. 20.2. Overview of multidimensional protein identification technology.

An advantage of the MudPIT approach is that it provides a more unbiased proteomic analysis. It facilitates the identification of both high- and low-abundance proteins, and hydrophobic proteins that are unresolved by 2D gels with sensitivity in the low femtomole range *(13)*. This technique can be used to circumvent many of the current 2D gel limitations. For example, this system has the high resolving power of multidimensional high-pressure liquid chromatography (HPLC), the sensitive peptide detection capabilities of tandem mass spectrometry, the analytical power of highly developed database searching software, and the possibility of creating automated data collection systems *(14, 15)*. Most importantly for our discussion of membrane proteins, MudPIT is compatible with sample preparations that optimize for the analysis of membrane proteins, which are generally excluded from 2D gel analysis. Washburn and colleagues developed a method in which membranes are solubilized with 90% formic acid, hydrolyzed with CNBr, and digested with endoproteinase-LysC and trypsin, leading to increased analysis of peptides from hydrophobic proteins *(16)*. Similarly, an acid labile detergent, *Rapi*Gest, developed by the Waters Corporation can be used with the protease trypsin to partially solubilize both cytosolic and membrane proteins for MudPIT analysis (similar detergents are now available through Protein Discovery, Inc.) *(17,18)*. It has been predicted that ~30% of all open reading frames in the human genome encode membrane proteins, and Washburn et. al., showed that using the 90% formic acid solubilization method they were able to identify 19% of the total membrane proteins predicted from the genome *(19,20)*. Even more recently, a high pH method has been used to create intact membrane "sheets" from nonsolubilized membrane samples. Proteinase K, a nonspecific protease, can then be used to cleave the solvent accessible domains of membrane embedded proteins, which ultimately can provide information about membrane protein topology *(21, 22)*. With the continuing advances in the MudPIT technique it is clear that it will continue to be advantageous for the analysis of membrane proteins, which are generally excluded from traditional 2D gel based analyses.

The MudPIT approach has also been used in the study of heart disease as an alternative to the conventional gel-based studies *(23)*. This shotgun proteomics platform for studying heart disease may result in a more comprehensive analysis than to those conducted in gel-based systems because it provides a global analysis that is inclusive of membrane proteins and utilizes a dynamic range capable of detecting both high-abundance proteins, such as actin and myosin, and low-abundance proteins, such as cell-urface receptors. Furthermore, MudPIT can be used with unfractionated protein samples in conjunction with RNA microarray studies utilizing the same tissue specimen. While microarray studies allow for the unbiased profiling of global changes in mRNA

levels, the MudPIT analysis of total cellular proteins allows for the global analysis of myocardial protein expression that originated from the same initial subject. Simultaneous proteome and transcriptome profiling has been shown in plant species using 2D-gel methods; however, the sequential extraction of protein and mRNA from human tissue for profiling has yet to be fully explored *(24,25)*. Studies of this type are being performed by our group at the University of Colorado Health Sciences Center using TRIzol protein precipitate from RNA-extracted human ventricular tissue from patients with nonfailing hearts and those diagnosed with dilated cardiomyopathy. The advantage of this approach is that by examining unfractionated protein samples both genomics and proteomics can be applied to create a comprehensive global profile of myocardial gene/protein expression from a single heart sample.

The methods described in this chapter detail (1) tissue homogenization, (2) sample preparation to facilitate parallel microarray and proteomic analysis, (3) MudPIT, and (4) proteomic data analysis.

2. Materials

2.1. Tissue Homogenization and Protein Precipitation

1. Tissue homogenizer, 4.5 mm probe diameter (Biospec Products, Bartlesville, OK)
2. Table top centrifuge, eppendorf 5417R (eppendorf, Westbury, NY)
3. 0.4 M KH_2PO_4/K_2HPO_4 buffer, pH 6.7 or TRIzol (Invitrogen, Carlsbad, CA)
4. Methanol
5. Chloroform

2.2. Protein Digestion I – RapiGest/Trypsin Method

1. *Rapi*Gest SF powder (Waters Corporation, Milford, MA)
2. 50 mM Ammonium Bicarbonate buffer, pH 7.8
3. 500 mM dithiothreitol (DTT)
4. 500 mM iodoacetamide (IAA, light sensitive)
5. 500 mM HCl
6. 250 ng/μl Trypsin, modified, sequencing grade (Promega, Madison, WI)
7. 100 mM $CaCl_2$
8. Thermomixer (eppendorf, Westbury, NY)

2.3. Protein Digestion II – Formic Acid/CnBr/Trypsin Method

1. 90% Formic acid
2. CNBr
3. 30% Ammonium Hydroxide
4. Saturated Ammonium Bicarbonate in water
5. Solid Urea

6. 500 mM DTT
7. 500 mM iodoacetamide
8. EndoLys-C protease (Roche, Indianapolis, IN)
9. 100 mM Tris-HCl, pH 8.5
10. 100 mM $CaCl_2$
11. 250 ng/μl Trypsin, modified, sequencing grade
12. pH paper

2.4. Preparation of Samples for Column Loading

2.4.1. Rapigest/Trypsin Method

1. HCl
2. Microcentrifuge

2.4.2. Formic acid/CnBr/Trypsin Method

1. 90% Formic Acid

2.5. Column Packing and Sample Loading

1. P-2000 laser puller (Sutter Instruments; Novato, CA)
2. HPLC packing materials: SCX (Whatman, Florham Park, NJ) and AQUA C18 RP (Phenomenex, Torrance, CA)
3. Stainless steel pressure bomb
4. HPLC pump, Agilent 1100 (Agilent, Palo Alto, CA)

2.6. Chromatography

1. Buffer A:5% acetonitrile, 0.1% formic acid.
2. Buffer B: 95% acetonitrile, 0.1% formic acid.
3. Salt solutions, ammonium acetate:100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, 900 mM, 1 M, 5 M.
4. HPLC pump, Agilent 1100 (Agilent, Palo Alto, CA)
5. Microelectrospray ionization source (built in-house)
6. Temperature controlled autosampler (Agilent, Palo Alto, CA)
7. Ion trap mass spectrometer, Finnigan LTQ linear ion trap (ThermoElectron Corp., Waltham, MA)
8. Xcalibur interface software (ThermoElectron Corp., Waltham, MA)

2.7. Sample Analysis

1. SEQUEST (see http://www.sequest.org)
2. DTASelect (see http://fields.scripps.edu/DTASelect/)

3. Methods

3.1. Tissue Homogenization and Protein Precipitation

1. Tissue homogenization (1–10 mg) is done in 800 μL cold phosphate buffer pH 6.7 (or using TRIzol solution to extract intact RNA concurrently, *see* **Note 1**), using a Tissue Tearor

or other tissue homogenizer. For samples from cell culture, a Dounce homogenizer can be used with either cold phosphate buffer or TRIzol.
2. Pellet out nuclei and unbroken cells using a low speed spin (1500 rpm) for 5 min on table top centrifuge.
3. Take postnuclear supernatant and perform a high speed spin (14,000 rpm) for 30 min using microcentrifuge to pellet out total cellular membranes (*see* **Note 1**).
4. Move total protein pellet or membrane pellet to new microcentrifuge tube and begin to precipitate membrane proteins by adding 4 volumes of MeOH and 1 volume of $CHCl_3$.
5. Vortex.
6. Add 3 volumes of water
7. Vortex and spin down at 14,000 rpm for 2 min and then remove the bottom $CHCl_3$ organic layer.
8. Add fresh $CHCl_3$ (1 volume) and spin down at 14,000 rpm for 2 min and again remove the bottom $CHCl_3$ layer. Also remove the water layer leaving only the interphase layer which contains your protein precipitate.
9. Add fresh $CHCl_3$ (1 volume) and MeOH (3 volumes) to the protein precipitate, vortex, and spin down at 14,000 rpm for 2 min. Remove all the supernatant and leave the protein pellet to air dry for approximately 2 min.

3.2. Protein Digestion I – RapiGest/Trypsin Method

1. Make 0.1% *Rapi*Gest diluted in 50 mM ammonium bicarbonate buffer, pH 7.8 (1 mg *Rapi*Gest per 1 ml 50 mM ammonium bicarbonate)
2. Using locking lid microcentrifuge tubes add 50 µl 0.1% *Rapi*Gest solution to membrane protein pellet. Work the pellet back into solution by alternating pipetting and vortexing (*see* **Note 2**).
3. Once the membrane protein pellet is fully solubilized the sample should be heated to 100°C for 2 min and then left to cool until it reaches room temperature.
4. Reduce the solution with DTT (final concentration of 5 mM) for 30 min at 60°C and then cool the sample to room temperature
5. Alkylate the solution with iodoacetamide (final concentration 15 mM) in the dark for 30 min at room temperature (*see* **Note 3**)
6. Add modified trypsin for a final concentration of 1:100, enzyme:protein. Incubate in a thermomixer overnight at 37°C with shaking.

3.3. Protein Digestion II – Formic Acid/CNBr/Trypsin Method

1. Begin with a membrane pellet that contains ~200 µg protein and add 50 µl CNBr solution (500 mg/ml CNBr in 90% formic acid) (*see* **Notes 4** and **5**).

2. Resuspend the pellet in the CNBr/formic acid solution by alternating vortexing and pipetting. If you cannot achieve a homogenous solution, then add another 10 µl CNBr/formic acid solution. However, do not exceed 100 µl in a single microcentrifuge tube because you will run out of room in subsequent neutralization steps.
3. Incubate the homogenous solution in the dark overnight at room temperature.
4. The next day, neutralize your highly acidic sample by SLOWLY adding 2 volumes of 30% ammonium hydroxide (1 volume = volume of CNBR/formic acid solution initially added).
5. Neutralize the sample further by SLOWLY adding 1–2 volumes of saturated ammonium bicarbonate solution. Check the pH by spotting a drop of the sample onto pH paper and continue neutralization until the sample has reached a pH of 8.0–8.5 (*see* **Note 6**).
6. Add solid urea to the sample to a final concentration of 8.0 M and vortex until completely solubilized.
7. To reduce the proteins add DTT to a final concentration of 25 mM and incubate at 60°C for 30 min.
8. Follow by alkylating the proteins using iodoacetamide (final concentration 100 mM) and incubate at room temperature in the dark for 30 min.
9. The EndoLys-C protease should be added to your sample (1:100, enzyme:protein). Incubate in a thermomixer overnight at 37°C with gentle shaking.
10. The next day, dilute the initial solution volume by a factor of two with 100 mM Tris, pH 8.5, and add $CaCl_2$ (final concentration 1 mM).
11. Add modified trypsin (1:100, enzyme:protein) and incubate at 37°C in a thermomixer overnight with shaking.

3.4. Preparation of Samples for Column Loading

3.4.1. RapiGest/Trypsin Method

1. Add HCl (final concentration of 100 mM) to the trypsin digested sample and incubate at 37°C for 45 min.
2. Spin sample at 14,000 rpm (4°C) for 10 min. A cloudy pellet should appear.
3. Separate the supernatant from the pellet and place the supernatant into a fresh eppendorf tube (spin again if needed to make sure all cloudy material has been removed).

3.4.2. CNBr/Formic acid/Trypsin Method

1. Microfuge sample (14,000 rpm) for 10 min to pellet any insoluble debris. Move supernatant to a new tube and adjust it to 5% formic acid for loading onto the chromatography column.

3.5. Column Packing and Sample Loading

1. Laser puller is used to pull a 5 μm tip from a fused silica tube (~17 cm long) of 100 μm inner diameter (i.d.) and 365 μm outer diameter (o.d.).
2. The column is then packed with 8 cm 5 μm C18 RP material followed by 4 cm of 5 μm SCX material using a pressure bomb that uses helium *(26)* (*see* **Notes 7** and **8**).
3. Equilibrate column on HPLC with Buffer A for 30 min.
4. Load sample onto column using pressure bomb and follow by washing the column for 10 min with buffer A (*see* **Notes 9** and **10**).

3.6. Chromatography Conditions

1. Chromatography setup is controlled through the Xcalibur interface on the tandem mass spectrometer (*see* **Notes 11** and **12**)
2. Following the 10 min wash with Buffer A (Step 4 of 3.5; see above), Step 1 of the MudPIT run is initiated by applying a short pulse of water onto the column (50 μl).
3. The initial water pulse is followed by a long shallow increase in buffer B until 80% acetonitrile is reached.
4. Buffer A (100%) is used to re-equilibrate the column for 10–15 min.
5. Step 2 of the 12 Step MudPIT is initiated by addition of 100 mM ammonium acetate (50 μl) directly onto the column by means of the autosampler. This is followed by the shallow gradient of buffer B to 80% acetonitrile and reequilibration with 100% Buffer A for 10 min.
6. Steps 3–11 in the 12-step MudPIT are done similar to Step 2 described above with increasing concentrations of ammonium acetate salt loaded on to the column (50 μl) followed by the shallow gradient of buffer B to 80% acetonitrile and reequilibration with 100% buffer A for 10 min. The salt concentrations for Steps 3–11 are 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, 900 mM, and 1mM, respectively.
7. Step 12 is done using 5 M ammonium acetate salt solution (100 μL) loaded onto the column, followed by the shallow gradient of buffer B to 80% acetonitrile and reequilibration with 100% buffer A for 10 min.

Representative base peak chromatogram from one 12-step MudPIT run containing total cellular protein from a nonfailing human heart sample is shown in **Fig. 20.3**.

3.7. Sample Analysis

1. Acquired tandem mass spectra can be analyzed using correlation software such as SEQUEST, which correlates the experimental tandem mass spectra with theoretical mass spectra from a protein sequence database in order to identify the peptide sequences detected.

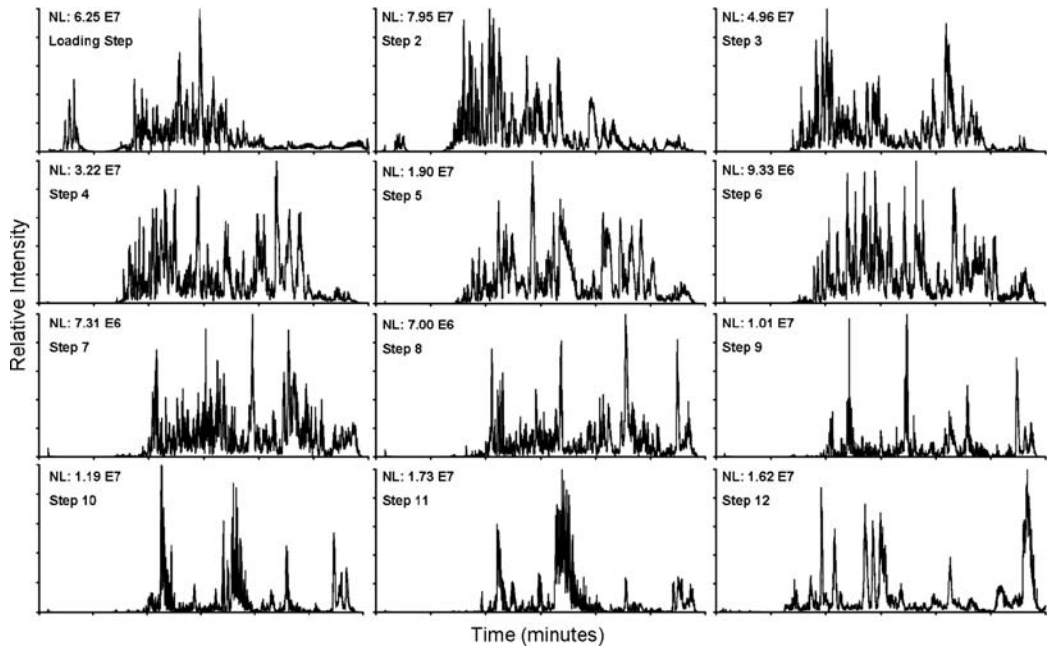

Fig. 20.3. Chromatography profile for a human heart sample prepared using the *Rapi*Gest/Trypsin digestion protocol. In a typical 12-step MudPIT analysis >1500 unique proteins are identified with an FDR < 5%, approximately 13.5% of which are predicted by the TMHMM prediction algorithm to be membrane proteins.

2. The identified peptide sequences can then be assembled back into proteins using software such as DTASelect *(27)* (*see* **Note 13**).
3. The Contrast program is used for the comparison and filtering of data sets generated by multiple MudPIT runs to determine the protein content of multiple related samples.

4. Notes

1. To look at total protein content (soluble and membrane) perform the methanol/chloroform protein precipitation on the postnuclear supernatant following the low-speed spin in Step 2. In addition, if microarray and proteomic analyses are to be done concurrently on the same tissue sample, a TRIzol extraction of the tissue is done to yield RNA, DNA, and total protein fractions *(28)*. A methanol/chloroform protein precipitation can then be done on the total protein fraction. For the experiments described here, a TRIzol extraction was done on human heart tissue followed by protein precipitation to yield total cellular protein components.

2. The membrane protein pellet can be difficult to get back into solution. To facilitate its solubilization an insulin syringe can be used to pull the solution in and out in order to break up the pellet.
3. To reduce the amount of chymotryptic activity the trypsin enzyme is capable of add $CaCl_2$ (final concentration 1 mM) to the solution following reduction with iodoacetamide.
4. CNBr is a toxic reagent and all solutions containing CNBr should be handled in a hooded chemical area and disposed of properly.
5. If you have no idea how much protein is in your pellet, a rough approximation of 200 μg protein would be a pellet the size of an "o" at 12 point font.
6. Be sure to add the basic solutions drop by drop, otherwise, the highly acidic sample will bubble over and some of your sample may be lost. In addition, it is encouraged that following neutralization you let the sample sit for 15 min and then recheck the pH before adding the solid urea to be sure the pH was adjusted correctly.
7. C18 RP and SCX material should be placed in HPLC grade methanol forming a slurry to be utilized for column packing.
8. There is a large variety of C18 RP material commercially available. Aqua C18 (Phenomenex, Torrance, CA), Luna C18 (Phenomenex, Torrance, CA), Prodigy C18 (Phenomenex, Torrance, CA), Zorbax C18 (Agilent, Palo Alto, CA), and Resolve C18 (Waters Corporation, Milford, MA) are examples. Each RP material will yield slightly different retention times depending on the sample composition *(29)*. For the experiment shown here, Aqua C18 was used.
9. Samples need to be free of particles/debris before loading onto the column since the small inner diameter of the column makes it prone to clogging. If problems with clogging occur, consider using a 3 cm C18 RP packed in a 250 μm i.d. column and connect to the biphasic column using an in-line filter assembly (Upchurch, Oak Harbor, WA). This facilitates the loading and desalting of viscous samples and/or large volumes.
10. Loading of formic acid/CNBr/trypsin samples can take up to five times longer than *Rapi*Gest/Trypsin samples.
11. Using a column heater at ~40°C during all chromatography steps can improve peak resolution.
12. The chromatography conditions listed here are for a typical 12-step (24 h) MudPIT run. The longer acetonitrile gradients (80–110 min) may allow for more peptides to be eluted from the column onto the mass spectrometer and detected and analyzed. The number of salt pulses and the length of acetonitrile gradients can be modified depending on the

complexity of the sample to achieve the best resolution and number of peptides identified.

13. Other peptide identification software is commercially available. SEQUEST is available through ThermoElectron Corporation and Mascot is available through Matrix Science Inc *(30)*.

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Chapter 21

Liquid Chromatography MALDI MS/MS for Membrane Proteome Analysis

Nan Wang, J. Bryce Young and Liang Li

Abstract

Liquid chromatography (LC) can be combined with matrix-assisted laser-desorption ionization (MALDI) mass-spectrometry (MS) by using automated off-line fraction collection of eluates onto a MALDI plate. Commercial tandem MS systems are available for generating product ion spectra of MALDI-produced peptide ions. The MALDI MS/MS spectra can be searched against a proteome database for protein identification. In this chapter, a protocol of sequential solubilization and digestion of membrane proteins involving methanol- and SDS-assisted trypsin digestion and microwave-assisted acid hydrolysis is presented. The process of LC eluate deposition onto a MALDI plate along with practical considerations for achieving optimal performance of eluate deposition is described. Issues related to MALDI MS and MS/MS spectral acquisition are discussed. Database searching and manual inspection of MS/MS spectra of singly charged MALDI-produced peptide ions for positive protein identification are also addressed.

Key words: Trypsin digestion, microwave-assisted acid hydrolysis, LC-MALDI MS and MS/MS.

1. Introduction

The current bottom-up proteomic approach in which proteins are first converted into peptides followed by mass spectrometric sequencing of peptides for protein identification is mainly based on the use of liquid chromatography (LC) combined with electrospray ionization (ESI) tandem mass-spectrometry (MS/MS). Matrix-assisted laser-desorption ionization (MALDI) is a complementary ionization technique to ESI. For a given protein digest, some peptides are readily ionized by ESI while others may be more amenable to MALDI. Recent advances in both LC-MALDI interfacing technology and MALDI MS/MS

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instrumentation make LC-MALDI MS/MS an attractive technological platform for bottom-up proteome analysis *(1–15)*. Despite considerable effort in attempting to interface LC to MALDI in an on-line fashion *(16–24)*, no viable interfaces for on-line LC-MALDI are currently available. LC-MALDI is currently best done using an off-line on-plate deposition approach (i.e. LC eluates are deposited onto a MALDI sample plate as discrete spots or a continuous track). Several interfacing devices, including commercial designs, are available to combine capillary LC with MALDI *(25)*. A heated droplet interface, while not presently available commercially, can be used to combine microbore LC with MALDI *(26)*. LC-MALDI MS and MS/MS experiments can be carried out using a variety of tandem mass spectrometric instruments. In many cases, spectral acquisition can be done automatically.

Applying LC-MALDI MS/MS to membrane proteome analysis presents a special challenge, particularly in the areas of protein solubilization and digestion. We have reported a sequential protein solubilization method to fractionate a complex proteome into several groups of proteins based on their solubility differences in various solvent systems *(27)*. In addition, we have reported a microwave-assisted acid hydrolysis (MAAH) method for degrading proteins into peptides for MS/MS sequencing *(28, 29)*. Because this method does not require solubilization of proteins, it is particularly useful in handling insoluble proteins, such as highly denatured or very hydrophobic membrane proteins.

In this chapter, practical aspects of running LC-MALDI MS/MS for bottom-up proteome analysis are discussed. A detailed protocol of combining sequential protein solubilization with trypsin digestion and MAAH for handling membrane proteome samples is reported.

2. Materials

The protocol described is for the analysis of the membrane proteome of zebrafish liver tissue extracts. The protocol may also be applied to other samples, such as cell extracts.

2.1. Sequential Protein Digestion

1. Zebrafish were taken from the Zebrafish Breeding Facility at the University of Alberta Biosciences Aquatics Facility (courtesy of Dr. Greg Goss and Andrew Waskiewicz), where they were kept at 28°C. Strain A/B zebrafish were used in this study. All animals were treated according to established approved animal care protocols.

2. Membrane protein extraction buffer: compartmental protein extraction buffer (Kit K3013010 Biochain Institute, Inc.).
3. Serine protease inhibitor: 2 μM phenylmethylsulfonyl fluoride (PMSF).
4. Protein quantification reagents: BCA™ protein assay kit (Pierce Biotechnology, Inc).
5. Disulfide bond reducing reagents: 90 mM dithiolthreitol (DTT, 6.94 mg in 500 μL H_2O).
6. Alkylation reagent: 200 mM iodoacetamide (IAA, 18.5 mg in 500 μL H_2O) (*see* **Note 1**).
7. Protein precipitation solvent: acetone, precooled in a −80°C freezer.
8. Protein solubilization solutions: 50 mM ammonium bicarbonate (NH_4HCO_3), 60% (v/v) methanol/water, 1% (w/v) sodium dodecyl sulfate (SDS) in deionized water.
9. Protein digestion (proteolysis) reagent: sequencing grade modified trypsin (Promega).
10. Microwave-assisted acid hydrolysis reagent: 25% (v/v) trifluoroacetic acid (TFA) in deionized water (*see* **Note 2**).
11. Solvents for strong cation-exchange (SCX) separation: A: 5 mM KH_2PO_4, pH 3.0 + 5–25% ACN; B: 5 mM KH_2PO_4, pH 3.0 + 5–25% ACN + 500 mM KCl.
12. For grades of all the reagents (*see* **Note 3**).
13. Microwave oven for MAAH experiment: Panasonic 1 200 W (2450 MHz) domestic microwave oven.
14. Filter for LC solvents: LCR Membrane, PTFE, 0.45 μm, 47 mm (Millipore).

2.2. LC-MALDI

1. Agilent 1 100 HPLC with binary pump.
2. C18 columns with various internal diameters and lengths (*see* **Note 4**).
3. LC-MALDI Interface: A homebuilt impulse-driven heated droplet deposition device was used for this work *(30)*. However, whether a commercial or homebuilt LC-MALDI apparatus is used, all share common features: LC eluate flows out of exit tubing prior to deposition to a MALDI plate, LC eluate is collected as sequential droplets in an array on a MALDI plate, and matrix chemical is added to each droplet either before or after the eluate droplets are deposited. Nano- and capillary-flow LC-MALDI interfaces are available commercially from Dionex/LC Packings (Sunnyvale, CA), Applied Biosystems/Sciex (Foster City, CA), Shimadzu (Tokyo, Japan), Proxeon (Odense, Denmark), Agilent (Santa Clara, CA) and others. Please refer to a recent product survey for a comprehensive overview of the available LC-MALDI technologies *(25)*.

4. MALDI MS: Bruker Reflex III MALDI TOF MS was used for generating MS spectra and ABI QqTOF tandem Mass Spectrometers were used for generating both MS and MS/MS spectra (*see* **Note 5**).

3. Methods

3.1. Sample Processing

The following steps were used to extract membrane proteins from zebrafish liver tissue extracts.

3.1.1. Membrane Protein Extraction

1. Excise the zebrafish livers and place them in a 1.5 mL flat-bottomed eppendorf (Flex-Tube, eppendorf) containing lysis buffer (buffer + protease inhibitor cocktail from compartmental extraction kit + 2 μM PMSF). Place on ice.
2. Follow the instructions of the protein extraction kit to extract the membrane components (*see* **Note 6**).
3. Quantify the protein in the membrane fraction using BCA^{TM} protein assay kit.
4. Aliquot the extracts and store at −70°C for later analysis.

3.1.2. Reduction, Alkylation and Acetone Precipitation

1. Check the pH of the protein solution by spotting 1 μL on pH paper. Adjust the pH to ∼8 using 1 M or 100 mM NH_4HCO_3 if necessary.
2. Add the reducing reagent to the protein extracts to a final concentration of 5–10 mM. Incubate the solution for 60 min at 37°C (*see* **Note 7**).
3. Cool the sample to room temperature. To alkylate the free thiol groups, add an equal volume of the alkylation reagent (200 mM IAA) as that of DTT used in the previous step. Incubate the sample at room temperature in the dark for 60 min.
4. Add the precooled acetone gradually (with intermittent vortexing) *(31)* to the protein extract to a final concentration of 80% (v/v) (*see* **Note 8**).
5. Keep the protein solution at −80°C for 10 min and then at −20°C overnight.
6. Centrifuge the solution for 10–12 min at 4°C at 20 000 g.
7. Decant the supernatant. Wash the pellet using cold acetone 1–3 times. Air-dry the pellets at room temperature.

3.1.3. Sequential Solubilization and Trypsin Digestion of the Protein Pellet

1. Redissolve the pellet by adding 50–100 μL of 50 mM ammonium bicarbonate each time. Apply 20 min intermittent vortexing to facilitate protein solubilization (*see* **Note 9**).
2. Centrifuge the solution at 4°C for 10 min at 20 000 g. Estimate the protein concentration with the BCA assay.
3. Add trypsin in a 1:40 enzyme:protein ratio (w:w) to the supernatant and incubate overnight at 37°C (*see* **Note 10**).

4. Acidify the digest solution using 10% TFA and store at −80°C.
5. Use 60% MeOH to resuspend and redissolve the remaining pellet from step 2 with sufficient vortexing. Produce a final protein concentration of 0.5 mg/mL *(32–34)* (*see* **Note 11**).
6. Add trypsin at an enzyme:protein ratio of 1:30. Incubate the solution at 37°C for 5 h.
7. Centrifuge the solution at 4°C for 10 min at 20 000 g. Save the pellet for the next redissolving step. Acidify the supernatant and evaporate the MeOH using a vacuum centrifuge.
8. Redissolve the pellet using 10–20 μL of 1% SDS each time. Apply 20 min intermittent vortexing *(26, 35)* (*see* **Note 12**).
9. Dilute the solution 20 times. Estimate the protein concentration with the BCA assay again.
10. Add trypsin to achieve a final enzyme:protein ratio (w:w) of 1:30. Incubate for 2 days at 37°C to complete the digestion.
11. Centrifuge the solution at 4°C for 10 min at 20 000 g. Save the pellet, if any, for the next redissolving step. Acidify the supernatants and store at −80°C.

3.1.4. Microwave-Assisted Acid Hydrolysis (MAAH) of the Protein Pellet

The intrinsic character of membrane proteins makes them difficult to dissolve, even after the organic-assisted and detergent-assisted solubilization procedures have been applied. The application of limited acid hydrolysis to the remaining pellet facilitates the production of specific peptides for tandem MS analysis. The acid hydrolysis process can be dramatically accelerated to the scale of seconds or minutes by the use of a domestic microwave. The following steps are taken to achieve MAAH of the insoluble protein pellet.

1. Resuspend the protein pellet in 100 μL 25% TFA *(28)* (*see* **Note 13**).
2. Place 20 μL of the protein suspension in a 1.5 mL polypropylene centrifuge tube. Cap the tube and seal with Teflon tape (*see* **Note 14**).
3. Place the tube in a domestic microwave oven. Also place 100 mL water in a loosely covered container beside the sample vial to absorb extra microwave energy (*see* **Note 15**).
4. Microwave the sample for 10 min (*see* **Note 16**).
5. Cool the hydrolysate and dry it thoroughly using a vacuum centrifuge to remove the TFA.
6. Reconstitute the peptides in 100 μL 0.1% TFA aqueous solution.
7. Centrifuge at 20 000 g for 5 min. If there is still pellet left, repeat steps 1–6. Pool all the supernatants and store them at −80°C until further analysis.

3.1.5. Strong Cation-Exchange (SCX) Separation of the Peptide Mixtures

Tryptic digests, obtained from **Section 3.1.3**, can be either combined or analyzed separately during the LC run, depending on

the sample amount and the complexity of the proteome. However, due to the differences of peptides generated by MAAH from those of trypsin digests (they may or may not contain K or R) the hydrolysates are preferably analyzed separately from the tryptic peptides. In any case, the digests obtained sequentially are usually still very complex. Thus, two-dimensional LC separations are required to reduce this complexity and facilitate the MALDI MS/MS analysis. SCX is typically applied as the first dimension of separation. In this step the peptides are fractionated according to their net charge under acidic conditions.

1. Prepare two mobile phases: A: 5 mM KH_2PO_4, pH 3.0 + 5–25% ACN; B: 5 mM KH_2PO_4, pH 3.0 + 5–25% ACN + 500 mM KCl (*see* **Note 17**).
2. Condition the SCX column (e.g., 2.1 × 250 mm PolySULFOETHYL Aspartamide™ Strong Cation Exchange Column, 5 μm, 300 Å) using mobile phase A (*see* **Note 18**).
3. Adjust the pH of the peptide sample to lower than three so that all the peptides are positively charged. Centrifuge the peptide solution at 20 000 g for 5 min to remove any particles in the solution (*see* **Note 19**).
4. Load the peptide solution on to the column and apply a linear salt gradient. For example, a 40 min salt gradient can be designed as: 0% buffer B for 10 min to wash off the nonionic and anionic components, 0–8% buffer B for 2 min, 8–40% buffer B over 25 min, 40–90% buffer B over 2 min and back to 0% buffer B in 1 min) at 0.2–0.3 mL/min for a narrow bore SCX column.
5. Record the UV absorbance of the eluate at 210 nm and collect 1 min fractions with a fraction collector.
6. Reconcentrate the SCX fractions to ~10 μL. It may be necessary to combine adjacent fractions if they have low UV absorbance. Reconstitute the peptides in 0.1% TFA solution.

3.2. LC-MALDI MS

3.2.1. RP-LC Separation

1. Prepare two mobile phases: A – 0.1% TFA (v/v) in 4% (v/v) acetonitrile in deionized water; B – 0.1% TFA (v/v) in acetonitrile (*see* **Note 20**).
2. Equilibrate the RP column at 100% A for 10–20 column volumes.
3. Load samples via manual injection or an autosampler.
4. Initiate gradient program according to guidelines set in **Note 21**.

3.2.2. Eluate Deposition

This section assumes the use of a commercial LC-MALDI fraction collector, or a homebuilt apparatus that produces discrete fractions on a MALDI plate (*see* **Note 22**).

1. Prepare LC-MALDI fraction collection apparatus: load a clean MALDI plate, and load the matrix solution delivery reservoir if using on-line matrix addition (*see* **Section 3.2.3**).
2. Choose the time over which fraction collection should occur, that is, when peptides will elute from the column. Peptides are typically eluted from the column by eluant that is between 7 and 45% organic solvent.
3. Enter the fraction period into the LC-MALDI fraction collector software (the time 'window' over which the eluate is collected for each individual fraction) (*see* **Note 23**).
4. Begin fraction collection at the designated start time; begin matrix solution infusion if performing on-line matrix addition.
5. Ensure that droplets remain confined to their discrete fractions to avoid crossover contamination between fractions.

3.2.3. Matrix Addition (On-Line and Off-Line)

Common matrices for peptide samples are alpha-cyano-4-hydroxy-cinnaminic acid (CHCA) and 2,5-dihydroxy-benzoic acid (DHB). CHCA is considered a 'hot' matrix, and may cause more in-source fragmentation than a 'cool' matrix, such as DHB. DHB is water- soluble and is easier to use with LC-MALDI. Matrix addition can be done either on-line or off-line (*see* **Note 24**).

3.2.3.1. On-Line Matrix Addition

In this mode of operation, matrix compound is premixed with the post-column LC eluate via a mixing T prior to fraction collection.

1. Ensure that the matrix infusion device is primed and ready, and that there is no obstruction to proper flow (matrix chemical can very quickly and easily cause problems in the fluid delivery system).
2. Prepare matrix solution: 100 mg/mL DHB in methanol (acidify to 0.5% formic acid for better MALDI sensitivity).
3. Load matrix solution into the matrix delivery system, typically a syringe pump.
4. Prime fluid delivery system to purge air bubbles.
5. When ready to begin collecting LC-MALDI fractions infuse at a flow rate equal to the LC flow rate.
6. Monitor crystallization process to ensure best results (*see* **Note 25**).

3.2.3.2. Off-Line Matrix Addition

In this mode of operation, matrix compound is added after the completion of the fraction collection process.

1. Prepare matrix solution: 60 mg/mL DHB in 40% methanol (acidify to 0.5% formic acid for better MALDI sensitivity).
2. Deposit matrix solution directly over the deposited fractions and allow to dry. The volume of matrix solution to be

deposited depends on the diameter of the desired MALDI spot. For a 2 mm spot, a maximum of 2 μL should be deposited.

3.2.4. MS Spectral Acquisition

The available approaches for the mass spectrometric analysis of a proteome are varied and diverse. For example, newer instruments allow the collection of MS and MS/MS spectra from LC fractions in a fully automated fashion with dynamic exclusion capability, much like LC-ESI MS and MS/MS. However, in LC-MALDI where the LC fractions are collected on a MALDI plate, the solid phase nature of the matrix crystals give LC-MALDI analyses distinct and attractive attributes. Highly sensitive and complete MS survey scans can be carried out without switching immediately to MS/MS mode. When the survey scan of all fractions is complete the MS data can be inspected either manually or automatically to generate a peak list. This peak list is then used to direct the MS/MS data acquisition. The MS/MS data quality can be inspected, and further MS/MS experiments can be performed if necessary. The solid phase nature of matrix crystals allows multiple analyses of the same fraction until the analytes are consumed. Multiple analyses on LC-MALDI fractions allow detailed characterisation to be carried out, such as the determination of modification sites of a peptide of interest. To summarize, a two-stage data acquisition process, that is, MS survey scan followed by MS/MS of selected peptide ions, involves the following steps:

1. Load prepared LC-MALDI plate into a mass spectrometer.
2. Acquire MS survey scan of all collected fractions. Ensure that the survey mass range is sufficient for your sample, that is, 500–3 000 m/z for tryptic peptides.
3. Survey the MS data and choose candidate peaks for further MS/MS analysis.
4. Acquire MS/MS data for each candidate peak. Attempt to acquire a balanced product ion spectrum so that all product ions are well represented and have sufficient signal: noise or until the modification sites can be revealed from the MS/MS spectral data. Reacquire MS/MS data if necessary to obtain spectra of adequate quality.
5. (Optional) Store LC-MALDI plate in cool/dry/dark conditions for future analysis.

3.3. Data Processing and Database Searching

An on-line search dialogue is available at www.matrixscience.com, as are comprehensive tutorials for the proper use and interpretation of database search results. The following is a brief overview of the steps involved in performing a database search using raw LC-MALDI MS/MS data:

1. Raw MS/MS data is parsed by a software algorithm to produce a text format peak list.

2. This peak list is uploaded to either the public Matrix Science Website or a dedicated in-house server.
3. The user must specify a protein database (Note: choice of the database depends on the proteome of interest. General databases are available for common organisms whose genomes have been sequenced. For special applications, such as certain bacteria or organisms, it may be necessary to produce a custom database).
4. Key details must be supplied, such as the type of digestion enzyme used, the types of chemical modifications expected either through post-translational processes or chemical derivatisation, and the estimated mass tolerance of the mass spectrometer.
5. The number of modifications to be searched at a time has a great effect on the computational 'cost'; while it may be tempting to choose as many modifications as possible to maximize the number of identified proteins, this approach may lead to insignificant results and highly inflated computational times.

Manual MS/MS data interpretation may also be used, and is good practice in order to verify the search results. Manual inspection of the search results is an important aspect of this type of analysis, since false positives (and negatives) are an unavoidable aspect of any statistical technique. Manual inspection can reduce the incidence of false positives and false negatives. In brief, manual MS/MS interpretation involves identifying product ion masses and elucidating peptide sequence information from the mass difference between product ions. Collision-induced dissociation (CID) processes produce predominantly b- and y-ions. A peptide sequence can be elucidated from the C-terminal end by piecing together the y-ions in the product ion spectrum (and from the N-terminal end with the b-ions). Manual interpretation is complicated by the presence of both b- and y-ions in the same product ion spectrum since the ion masses will overlap, making it difficult to differentiate b-ion from y-ion. Further complicating matters are losses of ammonia and water from the peptide.

These rules were based on our accumulated experience over the past several years:

1. y-ions are often of higher intensity than b-ions. However, if the C-terminus of the parent peptide is lysine (K) instead of arginine (R) and the N-terminus is a basic amino acid, such as histidine (H), we expect to see higher intensity with b-ion series than y-ions.
2. If the C-terminus of the peptide is lysine (K) instead of arginine (R), y_1 may not be visible.
3. Fragmentations on the C-terminal side of aspartic acid (D) and N-terminal side of proline (P) are mostly favoured in MALDI QTOF MS/MS. They usually generate the most

dominant fragment ions (not only y-ions or b-ions, but also possible internal fragments) in the MS/MS spectrum.
4. Fragmentations on the C-terminal side of glutamic acid (E), asparagine (N), glutamine (Q), alanine (A), glycine (G), valine (V), leucine (L) and isoleucine (I) are preferred to some extent over other amino acids. The resulting fragment intensity increases are not as significant as that in the D or P cases.
5. Fragments containing basic amino acids, such as arginine (R), lysine (K) or histidine (H) yield relatively higher peak intensity.

4. Notes

1. Iodoacetamide stock should be freshly prepared before use in an amber vial.
2. TFA solution must be prepared in the fume hood with caution.
3. It is suggested that all chemicals must be ACS grade or higher. Solvents should be HPLC grade or higher.
4. Most LC-MALDI interfaces are limited to capillary-bore flow rates, so it follows to use a capillary-bore column. Length is dependent on the complexity of the sample, but 10–15 cm is typical. Columns that are packed with high-quality silica and are end-capped are preferable. Chromatographic performance is superior with these columns and peak artifacts, such as peak tailing, are avoided.
5. The most popular MALDI instruments for proteomics studies include TOF, quadrupole-TOF (QTOF), and TOF-TOF spectrometers. Choice of instrument will depend on the experimental requirements, such as whether product ion spectra are needed, and performance, such as speed of data collection, spectral quality and sensitivity. MALDI-TOF instruments rely on a technique known as post-source decay (PSD) to produce product ion spectra. The quality of PSD spectra generated in reflectron TOF is not as good as those from CID. CID methods are available using MALDI QTOF and TOF-TOF instruments. Of the two, QTOF instruments produce better quality product ion spectra than TOF-TOF instruments, but the latter can acquire data far more quickly.
6. A variety of lysis buffers for membrane protein extraction can be used. These buffers usually contain a certain amount of detergent (e.g. NP 40, SDS, Triton X-100) so that membrane proteins can be released. Commercial lysis buffers from Pierce and Sigma are also good choices if a suitable extraction protocol is not available for a given sample.

7. Reduction can also be performed at 50°C for 30 min or 95°C for 5 min if a fast reaction is needed. More concentrated DTT stock solution (up to 900 mM) can be used to avoid over-dilution of the protein solution.
8. Reduction and alkylation steps are performed before acetone precipitation of the proteins for the purpose of facilitating the MAAH method to hydrolyze the leftover protein pellets. The power of both the reduction and alkylation reagents is limited under the strong acidic condition. This procedure also helps the cleanup of the unreacted reagents in the protein solution.
9. Keep adding the NH_4HCO_3buffer until no pellet can be dissolved. We find that usually 10–20% of the membrane protein pellet can be dissolved during this step. The final concentration of the supernatant is around 0.1–1 μg/μL.
10. Trypsin is the typical enzyme of choice due to its high-cleavage specificity. The enzyme:protein ratio can be raised if completion of digestion is a concern.
11. The volume of 60% methanol needed to dissolve the pellet varies for different kinds of membrane protein samples.
12. At least 10 μL of 1% SDS is necessary to dissolve a 200 μg protein sample. 2% SDS can also be used which, however, will lead to a larger volume of the final protein solution because subsequent 40 times dilution will be necessary. For 1% SDS-dissolved protein solution, 20 times dilution before the enzyme digestion is essential to reduce the detergent interference on the enzyme activity.
13. It is essential to choose an appropriate acid to achieve the optimal performance for hydrolyzing complex membrane protein samples. An appropriate acid produces little side reactions on proteins and can be easily removed after the hydrolysis. Inorganic acids like HNO_3 and H_2SO_4 are unsuitable due to their oxidative property. The non-volatile acid, H_3PO_4, is not a good choice either. Organic acids like formic acid and acetic acid have the potential of introducing modification to the N-terminus of peptides which will further complicate the downstream MS analysis. Both HCl and TFA are good choices in terms of suitability for MAAH analysis. The acid concentration is critical for the performance of MAAH analysis. Low-acid concentrations (e.g., 0.1 M HCl or 0.3 M TFA) result in fragments containing the C- and/or N-terminus. At a higher concentration (e.g. 1.5 M HCl or TFA), HCl is found to produce more nonspecific cleavages and a lower signal-to-noise ratio. This leads to less efficient and less sensitive downstream LC-MS/MS analysis for comprehensive membrane proteome profiling. However, TFA shows a glycine cleavage specificity (cleavages on both sides of glycine in peptides are always abundant), which can

aid protein identification. Hydrolysis using 3 M TFA (25% v/v) for complex protein pellets generates optimal results in terms of the number of peptides generated and sequence information.

14. The volume of the acid in each tube is limited so that the sample tube can tolerate the vapour pressure produced during the microwave irradiation.
15. Make sure to fill the container with the same volume of water for each microwave experiment. Variations in water volume can cause irreproducible acid hydrolysis performance.
16. Microwave irradiation time is also very critical to ensure the performance of the acid hydrolysis process. 10 min microwave radiation using the highest power level is the optimal time for 25% TFA acid hydrolysis for membrane protein mixtures. Prolong the microwave radiation time if a microwave oven with lower power level (e.g. 900 W) is used. Use protein standards like BSA or cytochrome C to test the optimal time for a particular microwave oven (29). An optimized hydrolysis should produce peptides in the molecular mass region between 500 and 2 500 Da. If the microwave irradiation time is too long, mainly low mass peptides (<1 000 Da) are generated. If too short, high mass peptides (>2 500 Da) are dominant and difficult to sequence by conventional tandem MS/MS.
17. Mobile phases should be filtered before use to avoid particulate plugging of the inlet frit. A proper percentage of the organic modifier (i.e. 20% ACN (v:v)) can enhance the resolving power of the SCX fractionation. 500 mM KCl is normally sufficient to elute most of the peptides. However, higher concentrations of KCl can be applied to elute the most basic peptides.
18. Proper selection of the solid phase for SCX is essential. Silica-based material with a bonded coating of an anionic polymer is commonly used for peptide separation in SCX mode. Wide-pore (300 Å) silica material is recommended to supply reasonable permeability and resolving power. Make sure to use columns of appropriate scale (i.e. analytical or narrow bore) according to the amount of peptides to be loaded. An upper load limit of 1 mg for an analytical column is also recommended.
19. Peptide samples in a high concentration of salt do not bind with the ion exchanger and elute with the void volume. In order to minimize this behavior the samples must be diluted to bring down the salt concentration. A large volume, dilute peptide sample with a low concentration of salt can be effectively separated by SCX chromatography because there is less salt to interfere with peptides binding the ion exchanger, and

provided that the quantity of peptide sample is below the binding capacity of the resin.

20. Mobile phases should be filtered through 0.45 μm filters that are resistant to chemical attack and do not contain extractables (polyfluoro polymer filters are a good choice). Unlike LC-ESI, with LC-MALDI it is possible to use TFA for separation because solvents are evaporated during the deposition process and MALDI is relatively tolerant to interferences such as ion pairing reagents.
21. In most proteomic samples, salt, buffer and even detergents may be present as contaminants. The solvent gradient program will depend on the complexity and cleanliness of the proteomic sample, but will typically feature: (1) a desalting portion at 0–10% organic followed by (2) a slow rise from 10% to 50% organic at a rate of 0.25–1.0%/min, (3) a fast rise from 50% to 80% organic at a rate of 1–2%/min, and (4) a return to original starting conditions. The slow rise portion of the gradient is the most crucial part of the program, as the slope has enormous bearing on the resolution of components in the separation. Shallow slopes are better able to resolve components at the expense of greater time spent during the gradient; steep slopes are quicker but sacrifice sample separation. Column conditioning and equilibration is necessary to ensure best performance. LC practitioners will run a gradient program followed by an isocratic program at the starting conditions prior to running their analytical gradient. After the analytical gradient, it is prudent to run a gradient program to clean the column followed by isocratic equilibration at the starting conditions for the analytical gradient. If the sample is particularly complex or hydrophobic it may be necessary to perform additional gradient steps to ensure that the column is flushed. The amount of column equilibration necessary depends on the solvent conditions, column dimensions and the packing material, but is generally held to be 10–20 column volumes at the solvent composition with which the analytical gradient begins.
22. LC-MALDI is typically a fraction collection process where the LC eluate is collected as a series of discrete fractions on a MALDI plate. While most often carried out with nano- or capillary-bore LC separations, higher flow rate separations are possible but require special apparatus to accommodate the increased eluate volume. For standard LC-MALDI, limit collected fraction volume to no more than 2 μL for a 2 mm diameter MALDI spot; for smaller diameter spots, volume must be further limited.
23. Ensure that plate density is great enough to collect fractions of the LC eluate of interest. The fraction collector

can be set to produce fractions every 1–60 s depending on sample complexity (the LC separation of complex samples should be collected in more fractions to minimize ion suppression during mass-spectrometry). For very complex samples and for chromatography with very narrow peak widths the number of peptides that can be identified by MS analysis is increased by collecting narrow fractions, such as 5 s. This phenomenon is due to suppression of minor ions by major ions. Setting fractions to very short durations, that is, less than the average chromatographic peak width, causes splitting of any given component over two or more fractions. This practice is useful if it is necessary to preserve the chromatographic integrity of the separation. However, this ultimately limits sensitivity, especially in MS/MS mode since the sample is essentially spread over several spots. The user must ascertain the ultimate goal of the experiment and choose the best LC-MALDI approach to suit the experiment. MS/MS quality is greatly dependent on the quantity of sample available for analysis. The sensitivity of mass spectrometers in MS/MS mode is typically much less than 1% of MS mode sensitivity, and thus MS/MS quality hinges on the ability to maximise the signal strength of precursor ions. It is imperative then that ion suppression is minimised by producing fractions only as wide as average chromatographic peak widths and by developing chromatographic methods that resolve as much as possible the components of the sample.

24. MALDI spot preparation is not an exact science and a certain degree of skill is required for best results. It is important to experiment with the spotting process to become familiar with the behaviour of the matrix chemical during crystallization.
25. Some commercial MALDI plates have hydrophobic/hydrophilic surface patterns that attempt to concentrate drying droplets into smaller spots. Beware of the surface condition of these plates as these technologies are hindered by poor solvent choice and can be rendered useless if the surface pattern is disrupted.

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Chapter 22

Cysteinyl-Tagging of Integral Membrane Proteins for Proteomic Analysis Using Liquid Chromatography-Tandem Mass Spectrometry

Srijeet K. Mitra and Michael B. Goshe

Abstract

Membrane proteomic analysis is of considerable interest due to the role of receptors, ion channels, and membrane-associated proteins that are critical components in cellular control and differentiation. Consequently, proteomic investigations of membrane proteins under a variety of conditions and stimuli are being conducted. Although abundant and biologically significant, large-scale proteomic analysis of highly hydrophobic integral membrane proteins containing multiple transmembrane domains (TMDs) is more difficult and requires alternative methods than those routinely used for whole-cell proteomic studies. This chapter contains a method for extraction, solubilization, cysteinyl-labeling, proteolysis, and identification of hydrophobic integral membrane proteins for large-scale proteomic analysis using liquid chromatography-tandem mass spectrometry (LC/MS/MS). Application of this method enables proteome-wide identification of integral membrane proteins from both bacterial and plant tissues. The method is also amenable to quantifying integral membrane protein expression and posttranslational modifications using isotopically enriched media or various stable isotope-labeling and/or affinity isolation reagents such as iTRAQ and cICAT. Since the protocol can easily be extended to various cell and tissue types, we anticipate that the method will be of interest to those who are trying to characterize the membrane proteome and gain some insight regarding the role of receptors, ion channels, and other membrane proteins involved in signal transduction and cellular differentiation pathways.

Key words: Membrane proteins, mass spectrometry, liquid chromatography, labeling, biotinylation, enrichment, proteomics, bacteria, plant, Arabidopsis.

1. Introduction

Integral membrane proteins are critical components of the cellular membrane. Based on genomic analysis, it has been estimated that 20–30% of all open reading frames of the genome

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encode integral membrane proteins *(1)*. Although highly abundant and biologically significant, large-scale proteomic analysis of the highly hydrophobic integral membrane proteins containing multiple transmembrane domains (TMDs) is analytically challenging. Techniques using two-dimensional polyacrylamide gel electrophoresis mass spectrometry (2-D-PAGE-MS) often result in under-representation of these water insoluble proteins. Consequently, much effort has been devoted to membrane proteomic strategies relying on liquid chromatography-tandem mass spectrometry (LC/MS/MS) to detect and identify constituent peptides of enzymatically digested proteins obtained from various organisms and cell types.

Sample preparation methods for isolating membrane proteins routinely involve the use of detergents or chaotropes. These reagents can be difficult to remove and residual amounts often interfere with chromatographic separation and suppress peptide ionization as well as downstream MS detection by electrospray ionization (ESI). To increase the identification of hydrophobic integral membrane proteins by LC-ESI-MS/MS analysis, a sample preparation method using methanol was first developed on bacterial systems to extract, solubilize, and digest integral membrane proteins for large-scale proteomic analysis *(2)* and has proven to be useful for a variety of membrane protein systems including *Escherichia coli (3)*, red blood cells *(4)*, *Corynebacterium glutamicum (5)*, lipid rafts *(6)*, mammalian tissues *(7)*, and plant tissues *(8, 9)*. In addition, specific and quantitative biotinylation of cysteinyl residues of integral membrane proteins using the methanol method for affinity isolation and identification of low abundance integral membrane proteins was also developed *(10, 11)*. This tagging process reduced sample complexity and provided a cysteinyl-constraint to aid in peptide identification. The large number of proteins identified within the membrane subproteome of *Pseudomonas aeruginosa* demonstrates the efficacy of the developed method to solubilize and label hydrophobic integral membrane proteins and provides a reliable and reproducible route for large-scale membrane proteomic analysis using isotope-coding that is not possible with traditional surfactant-based 2-D-PAGE-MS technology.

The biotin-tagging method described in this chapter is presented in essentially three distinctive parts. First, we address how to generate microsomal fractions highly enriched in membrane proteins from bacterial cells and plant tissues. Second, we describe the use of methanol to extract and solubilize membrane proteins from the lipid component that permits quantitative biotinylation of cysteinyl residues. Third, we describe the effective tryptic digestion of the labeled proteins and subsequent affinity isolation of

the biotinylated-peptides using immobilized avidin that is directly amenable to LC/MS/MS analysis. On the basis of its design, the method also provides the ability to quantify integral membrane protein expression and posttranslational modifications using isotopically enriched media or various stable isotope-labeling and/or affinity isolation reagents such as cICAT and iTRAQ (Applied Biosystems) to enable a variety of expressional and functional proteomic studies.

2. Materials

The protocols described pertain to those used previously to obtain membrane proteins from bacteria *(2, 10)* and plant tissues *(8)* as outlined in **Fig. 22.1**. For those interested in applying this enrichment and affinity labeling method to different cell or tissue types, please refer to **Note 1**.

2.1. Enriching Integral Membrane Proteins from Bacteria

1. *Deinococcus radiodurans* strain R1 cells from American Type Culture Collection N0.13939 (Manassas, VA).
2. Lysis buffer: 50 mM Tris, pH 7.3.
3. Centrifuge capable of 3,200 × *g* at 4°C.
4. French press (Amnico Rochester, NY).
5. Bicinchoninic acid (BCA) assay reagents from Pierce (Thermo Fisher Scientific).
6. 100 mM Sodium carbonate, pH 11 (Thermo Fisher Scientific).
7. Shaker/rotisserie (Labquake, Conroe, TX).
8. Ultracentrifuge capable of 115,000 × *g* at 4°C.
9. Water (18 MΩ) was purified using a Barnstead Nanopure Infinity water purification system (Dubuque, IA).

2.2. Solubilizing Membrane Proteins from Bacteria for Labeling and Proteolysis

1. 50 mM Ammonium bicarbonate, pH 8.
2. Sonicating bath (Bronson Model 1510, Danbury CT).
3. Siliconized polypropylene Eppendorf tubes.
4. Methanol (HPLC grade).

2.3. Bacterial Membrane Protein Labeling and Proteolysis

1. Tributylphosphine (97% Aldrich; make a 0.1 M stock solution in methanol).
2. (+)-Biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine (iodoacetyl-PEO-biotin) from Pierce (Thermo Fisher Scientific).

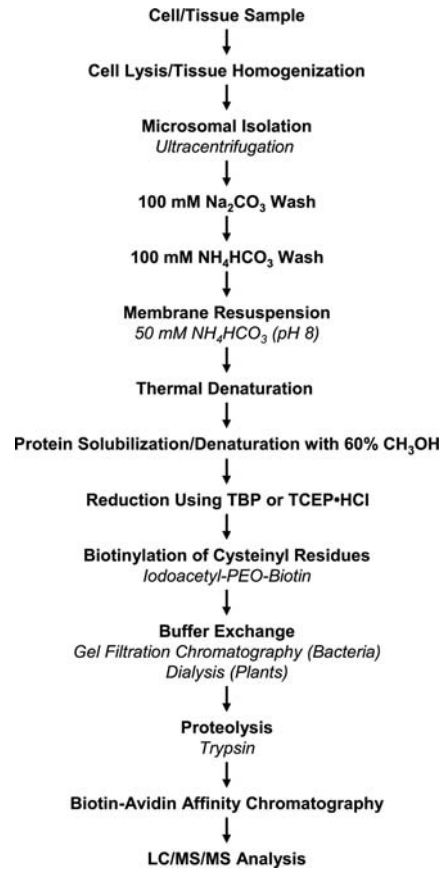

Fig. 22.1. Flow-diagram of the protocol for cysteinyl-tagging of integral membrane proteins.

3. D-Salt Excellulose gel filtration columns (5 ml) from Pierce (Thermo Fisher Scientific).
4. Sequencing grade-modified trypsin from Promega (Madison, WI).

2.4. Enriching Integral Membrane Proteins from Plant Tissue

1. *Arabidopsis thaliana* ecotype Columbia-0 seeds were obtained from Arabidopsis Stock Center at The Ohio State University, Columbus, OH.
2. Lysis buffer: 50 mM Tris pH 7.5 5 mm EDTA, 0.2% casein hydrolysate, 0.6% polyvinylpyrrolidone (PVP) (Sigma), 5 mM ascorbate, 5 mM DTT, and protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN).
3. Knife Blender (Oster mini-blender, Model Galaxie).
4. Centrifuge capable of 6,000 × *g* at 4°C.
5. 100 mM Sodium carbonate, pH 11 (Thermo Fisher Scientific).
6. Shaker/rotisserie (Labquake, Conroe, TX).

7. Ultracentrifuge capable of 115,000 × *g* at 4°C.
8. Water (18 MΩ) was purified using a Barnstead Nanopure Infinity water purification system (Dubuque, IA).

2.5. Solubilizing Membrane Proteins from Plant Tissue for Labeling and Proteolysis

1. 50 mM Ammonium bicarbonate, pH 8 (BioChemika Ultra ≥ 99.5%, Fluka).
2. Sonicating probe (60 Sonic dismembrator Model F60, Thermo Fisher Scientific).
3. Siliconized polypropylene Eppendorf tubes.
4. Methanol (HPLC grade).
5. BCA Assay Kit from Pierce (Thermo Fisher Scientific).

2.6. Plant Membrane Protein Labeling and Proteolysis

1. Tris 2-carboxyethyl phosphine hydrochloride (TCEP·HCl) from Pierce (Thermo Fisher Scientific).
2. (+)-Biotinyl-iodoacetamidyl-3, 6-dioxaoctanediamine (iodoacetyl-PEO-biotin) from Pierce (Thermo Fisher Scientific).
3. Dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA).
4. Sequencing grade-modified trypsin (Promega, Madison, WI).

2.7. Affinity Purification of Biotinylated Peptides

1. Phenylmethanesulfonyl fluoride (PMSF) (make a 0.1 M stock solution in methanol).
2. Ultralink immobilized monomeric avidin from Pierce (Thermo Fisher Scientific).
3. Flint-glass Pasteur pipets (5-3/4 in).
4. Glass wool.
5. Sodium phosphate (dibasic), sodium chloride, (+)-biotin, and glycine.
6. 50 mM Ammonium bicarbonate, pH 8.
7. Acetonitrile (HPLC grade) and formic acid (ACS regent grade).
8. Siliconized polypropylene Eppendorf tubes.
9. Speed vacuum concentrator from Savant (Thermo Fisher Scientific).

2.8. Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS) Analysis

1. Acetonitrile (HPLC grade) and formic acid (ACS regent grade).
2. Water (18 MΩ) was purified using a Barnstead Nanopure Infinity water purification system (Dubuque, IA) or distilled and purified using a High-Q 103S water purification system (Wilmette, IL).

3. Methods

3.1. Enriching Integral Membrane Proteins from Bacteria

1. Harvest *Deinococcus radiodurans* cells by centrifugation at 3,200 × *g* for 10 min at 4°C.
2. Wash the cells with lysis buffer (use half the tube volume) and collect by centrifugation at 3,200 × *g* for 10 min at 4°C (*see* **Note 2**).
3. Resuspend the cells in lysis buffer using a 1:3 ratio of wet cell weight-to-buffer (mg/ml).
4. Rupture the cells using a French press with three presses at 16,000 psi (*see* **Note 3**).
5. Remove intact cells and debris by centrifugation at 3,200 × *g* for 10 min at 4°C.
6. Collect the supernatant (i.e., the lysate) and determine the protein concentration using the BCA assay *(12)* (*see* **Note 4**).
7. Dilute the lysate (which contained approximately 20 mg of protein) with ice-cold 100 mM sodium carbonate, pH 11, to produce a final protein concentration between 0.25 and 0.30 mg/ml (*see* **Note 5**).
8. Slowly agitate the solution using a shaker/rotisserie for 1.5 h at 4°C.
9. Centrifuge at 115,000 × *g* for 1 h at 4°C to pellet the membrane component.
10. Discard the supernatant and rinse the membrane pellet with deionized water, wash the pellet with 8 ml of lysis buffer and then centrifuge at 115,000 × *g* for 20 min.
11. Discard the supernatant. At this point the sample contains the enriched bacterial membranes and can be stored in the centrifuge tube at −80°C until further workup can be performed.

3.2. Solubilizing Membrane Proteins from Bacteria for Labeling and Proteolysis

1. Resuspend the membranes in 0.4 ml of 50 mM ammonium bicarbonate, pH 8, via intermittent vortexing and sonication using a sonicating bath (*see* **Note 6**) and transfer the sample into a siliconized polypropylene Eppendorf tube (1.5 ml or 2.0 ml).
2. Thermally denature the proteins by heating at 90°C for 20 min. After allowing the sample to cool to ambient temperature, transfer the tube to an ice-bath (*see* **Note 7**).
3. Dilute the sample with 0.6 ml of methanol to produce a composition of 60% organic solvent (*see* **Note 8**).
4. Determine the protein concentration using the BCA assay (*see* **Note 9**).
5. Solubilize the membrane proteins by intermittent vortexing and sonication using a sonicating bath.

6. At this point, the integral membrane proteins are suitable for the cysteinyl-labeling procedure (*see* **Note 10**).

3.3. Bacterial Membrane Protein Labeling and Proteolysis

1. Incubate the membrane protein sample already in 60% methanol/40% 50 mM ammonium bicarbonate in a boiling water bath for 5 min.
2. After cooling the sample to room temperature, add a 30-fold molar excess of tributylphosphine (TBP) to protein to reduce disulfide bonds and incubate the sample at 37°C for 30 min (*see* **Note 11**).
3. Biotinylate the thiolate groups of the cysteinyl residues by adding a 30-fold molar excess of iodoacetyl-PEO-biotin to protein and incubate the solution for 90 min at ambient temperature in the dark while using gentle agitation with a shaker/rotisserie (*see* **Note 12**).
4. After biotinylation, exchange the reaction buffer for 60% methanol/40% 50 mM ammonium bicarbonate using gravity-flow gel filtration chromatography with prepacked columns containing Excellulose from Pierce (*see* **Note 13**).
5. After buffer exchange, immediately perform proteolysis using a 1:20 or 1:50 (w/w) trypsin-to-protein ratio for 5 h at 37°C. Prepare a stock solution of trypsin by adding 20 µl of resuspension buffer (provided by the manufacturer) to the vial containing 20 µg of enzyme. An aliquot of this stock solution is added to the sample and the remaining stock can be stored at −20°C for future use (*see* **Note 14**).
6. Quench the digestion reaction by submerging the sample tube in liquid nitrogen. At this point, the sample can be stored at −80°C until biotin-avidin affinity chromatography is performed.

3.4. Enriching Integral Membrane Proteins from Plant Tissue

1. Homogenize about 20 g of plant tissue using a "kitchen" blender in 50 ml of lysis buffer for up to 10 min until it is a homogeneous mixture.
2. Remove cellular debris by centrifugation at 6,200 × *g* for 10 min at 4°C.
3. Transfer the supernatant to a centrifuge tube using a cheesecloth to filter any unpelleted debris.
4. Centrifuge at 100,000 × *g* for 2 h at 4°C to pellet the membrane component. The dark green microsomal pellet contains the endosomal membranes.
5. Resuspend and wash the pellet in 100 mM sodium carbonate (pH 11.0). Centrifuge at 100,000 × *g* for 1 h at 4°C to pellet the membrane component.
6. Discard the supernatant. At this point the pellet contains the enriched endosomal membranes and can be stored in the centrifuge tube at −80°C until further workup can be performed.

3.5. Solubilizing Membrane Proteins from Plant Tissue for Labeling and Proteolysis

1. Resuspend the microsomal pellet using 50 mM ammonium bicarbonate and transfer the sample into a polypropylene Eppendorf tube (1.5 ml or 2.0 ml). (*see* **Note 15**).
2. Use a sonicating probe to disrupt the membranes (*see* **Note 16**).
3. Determine the protein concentration using the BCA assay using BSA standards made with 50 mM ammonium bicarbonate to generate a standard curve. The protein concentration should be between 1 and 2 mg/ml so that the addition of methanol produces a protein concentration between 0.5 and 1 mg/ml. If the concentration is greater than 2 mg/ml, dilute the sample accordingly with 50 mM ammonium bicarbonate.
4. Dilute the sample with methanol to produce a composition of 60% organic solvent (*see* **Note 8**).
5. Determine the protein concentration using the BCA assay (*see* **Note 9**).
6. Solubilize the membrane proteins by intermittent vortexing and sonication using a sonicating bath. At this point, the integral membrane proteins are suitable for the cysteinyl-labeling procedure (*see* **Note 10**).

3.6. Plant Membrane Protein Labeling and Proteolysis

1. Incubate the membrane protein sample already in 60% methanol/40% 50 mM ammonium bicarbonate in a boiling water bath for 5 min.
2. After cooling the sample to room temperature, reduce disulfide bonds by adding a 30-fold molar excess of TCEP·HCl to protein and incubate the sample at 37°C for 30 min (*see* **Note 11**).
3. Biotinylate the thiolate groups of the cysteinyl residues by adding a 30 molar excess of iodoacetyl-PEO-biotin to protein and incubate the solution for 90 min at ambient temperature in the dark while using gentle agitation with a shaker/rotisserie (*see* **Note 12**).
4. After biotinylation, exchange the reaction buffer for 60% methanol/40% 50 mM ammonium bicarbonate using dialysis.
5. After dialysis, immediately perform proteolysis using a 1:20 or 1:50 (w/w) trypsin-to-protein ratio for 5 h at 37°C. Prepare a stock solution of trypsin by adding 20 μl of resuspension buffer (provided by the manufacturer) to the vial containing 20 μg of enzyme. An aliquot of this stock solution is added to the sample and the remaining stock can be stored at −20°C for future use (*see* **Note 14**).
6. The digestion reaction is quenched by submerging the sample tube in liquid nitrogen. At this point, the sample can be stored at −80°C until biotin-avidin affinity chromatography is performed.

3.7. Affinity Purification of Biotinylated Peptides

1. Bring the samples to ambient temperature and then heat them in a boiling water bath for 5 min to thermally denature the trypsin.
2. Once the sample has cooled to ambient temperature, add phenylmethanesulfonyl fluoride (PMSF) to the solution to make it 1 mM in PMSF and incubate the sample at room temperature for 20 min, a time sufficient to quench any remaining trypsin activity (*see* **Note 17**).
3. Prepare the immobilized avidin column (*see* **Note 18**). Add the slurry to a glass Pasteur pipette that contains a glass wool plug (*see* **Note 19**).
4. Equilibrate the avidin column. This procedure will vary according to each manufacturer's instructions. This step typically requires several buffers used in succession that will allow the biotin-labeled peptides to bind to avidin during loading and washing steps, but permit efficient elution of the labeled peptides without the need for detergents and boiling as is the case with streptavidin. A typical protocol consists of equilibrating the column with 15–20 column volumes (this is the bed volume of the column, which is typically 50% of the slurry volume used to transfer the suspended resin to the column) of 2X phosphate buffered saline (0.2 M sodium phosphate and 0.3 M sodium chloride, pH 7.2). During this equilibration, make sure to wash the walls of the pipette in order to direct all the avidin resin onto the column bed.
5. Block the irreversible biotin binding sites by treating the column with 4–5 column volumes of 0.2 mM (+)-biotin in 2X PBS. Add the solution carefully to the column so that the resin at the top remains undistributed during gravity flow.
6. Wash away the excess biotin with 4–5 column volumes of 0.1 M glycine at pH 2.8, and then wash the column with 15–20 ml of 2X PBS prior to sample addition (*see* **Note 20**).
7. Dilute the sample by adding 50 mM ammonium bicarbonate, pH 8, until a concentration of at least 10% methanol is achieved Alternatively, the concentration of methanol can be reduced by evaporation using a stream of nitrogen (*see* **Note 21**).
8. Add the sample onto the avidin column and once loaded, block the end of the column using aluminum foil that is held in place by parafilm. Make sure to not have the parafilm touch any of the solvent and incubate the column for 30 min (*see* **Note 22**). As an alternative to the 30 min incubation step, the flow-through can be reapplied to the column to allow the capture of biotin peptides that may not bind during the initial loading.
9. After the sample has been loaded onto the column, wash the column with 10 column volumes of 2X PBS, 10 column

volumes of 1X PBS, and then 5 column volumes of 20% acetonitrile in 50 mM ammonium bicarbonate, pH 8.

10. Elute the acetyl-PEO-biotin-labeled peptides using 2 ml of 30% acetonitrile/0.4% formic acid and collect into a siliconized polypropylene Eppendorf tube.
11. Lyophilize the sample or remove the solvent via vacuum centrifugation and store the labeled peptides at −80°C until LC/MS/MS analysis can be performed (*see* **Note 23**).

3.8. Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS) Analysis

1. The analysis of the biotin-labeled peptides can be performed using a micro or nanoflow high-performance liquid chromatograph coupled to an ion trap or tandem mass spectrometer.
2. To prepare the sample for analysis, solubilize the peptides in 5% organic mobile phase/95% aqueous phase used for the

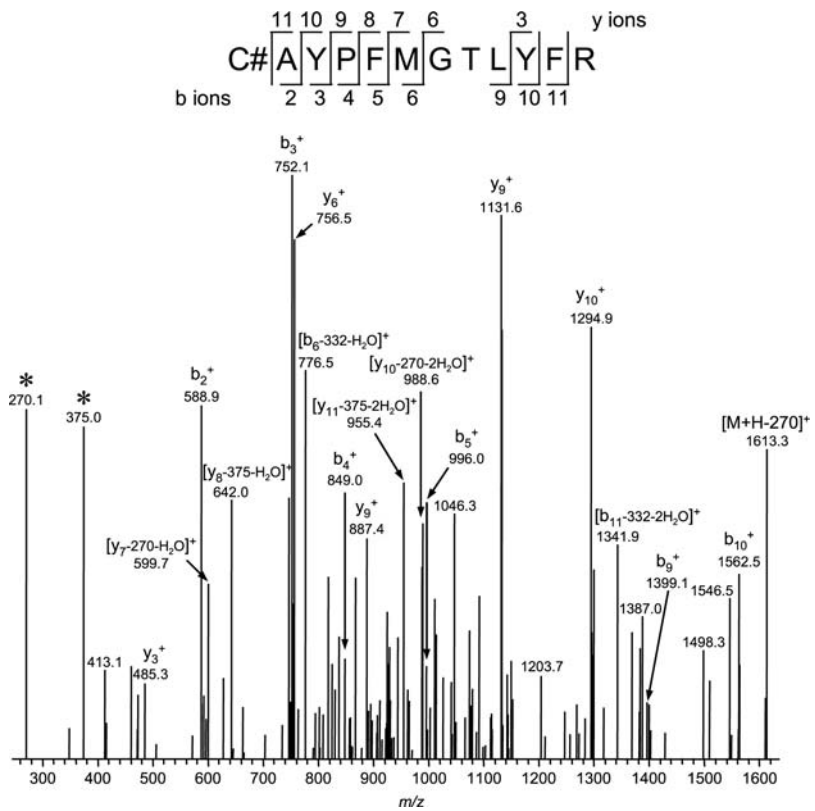

Fig. 22.2. Product ion spectrum of an acetyl-PEO-biotin-labeled cysteinyl-peptide. Using an LCQ Deca ion trap mass spectrometer (Thermo Electron, San Jose, CA; now Thermo Fisher Scientific) collision-induced dissociation of the $[M+2H]^{2+}$ precursor ion at *m/z* 942.8 produced b and y ions of the labeled peptide, including some label-specific fragments. The tryptic peptide identified C#AYPFMGTLYFR is from a leucine-rich repeat receptor-like kinase (At5g49760), an integral membrane protein from Arabidopsis, and is labeled with an acetyl-PEO-biotin tag at the cysteinyl residue (C#). Label-specific signature ions at *m/z* 270 and 375 are indicated with asterisks.

gradient separation to produce a concentration suitable for injection onto the reversed-phase column in use. To help solubilize the peptides, sonicate the sample for 1 min in a sonicating bath and use additional sonication if necessary or make the sample 10% (up to 30%) in the organic composition.

3. For the initial LC/MS/MS analysis use a 1% per min gradient of the organic mobile phase to determine the separation efficiency of the peptides contained in the sample. Based on this initial analysis, a more complex gradient can be utilized to achieve better separation of the peptides (*see* **Note 24**).
4. To aid in the interpretation of product ion spectra, detectable signature ions based on partial fragmentation of the acetyl-PEO-biotin label (singly charged ions at *m/z* 227.1, 270.1, 332.2, 375.2, 449.2) are present for most labeled peptides and can be used as a constraint to aid in identification *(10)* (*see* **Note 25**). An example of a product ion spectrum for a cysteinyl-tagged peptide of a leucine-rich repeat receptor-like kinase from Arabidopsis is presented in **Fig. 22.2**.

4. Notes

1. The described protocol for membrane protein enrichment and cysteinyl-labeling can be applied to any isolated cells or tissues once the sample has been effectively lysed or homogenized, respectively.
2. Additional washing steps can be performed if desired. However, subsequent steps that follow have additional washes of the membrane component, so just one washing step of the cells with the lysis buffer is usually sufficient.
3. Other steps of bacterial cell lysis, such as bead-beating, can be used. Based on our experience, better protein recovery was achieved using the French press method.
4. A standard curve for bovine serum albumin (BSA) protein standards prepared in the lysis buffer was generated between 0 and 0.5 μg/μl and used to determine the membrane protein concentration. Follow the manufacturer's protocol to make the necessary reagents for the BCA assay.
5. This method of membrane protein enrichment using carbonate extraction is a slightly modified version of those previously described *(13,14)*.
6. For sonication, the Eppendorf tube is placed in a float suspended in the sonicator containing the ice-bath. If sonication is performed without the ice, the water bath will heat during the 20 min of sonication. Every 3 or 4 min the sample is removed from the sonicator and vortexed for 30 s and then put back into the sonicator.

7. The thermal denaturation of the proteins is an optional step but was used according to a previous in-solution technique *(15)*.
8. The membrane proteins can also be subsequently thermally denatured by incubating in a boiling water bath for 5 min, but make sure the Eppendorf tube lid is securely closed (may have to seal with parafilm) before placing in the water bath. However, this thermal denaturation step is optional since 60% methanol is an effective denaturant as demonstrated on both hydrophobic and hydrophilic proteins *(8)*.
9. A standard curve for BSA protein standards prepared in 60% methanol/40% 50 mM ammonium bicarbonate was generated between 0 and 0.5 μg/μl and used to determine the membrane protein concentration.
10. Depending upon the nature of the proteomic study, the membrane proteins at this point of the protocol can be reduced and then (1) digested by trypsin with the resulting peptides analyzed by LC/MS/MS or (2) the cysteinyl residues labeled (or alkylated) prior to trypsin digestion and LC/MS/MS analysis.
11. The addition of TBP is made by adding the appropriate volume for a 0.1 M TBP stock solution made by dilating TBP with methanol. This stock solution can be stored at −20°C and can be repeatedly used for up to 1 or 2 months. As an alternative to TBP, tris-(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) can be used (make a 0.1 mM TCEP stock solution using water) and it can be repeatedly used for up to 1 or 2 months. It should be noted that TCEP is not volatile like TBP, and its use will add chloride ions to the solution and thus may affect subsequent work up and fractionation steps prior to LC/MS/MS analysis.
12. The addition of iodoacetyl-PEO-biotin can be performed by adding the appropriate amount of the solid reagent. The iodoacetyl-PEO-biotin is white and should be stored in a desiccator at −20°C and completely thawed out in a desiccator before it is weighed to prevent degradation. If the compound starts to exhibit a noticeable yellow color, it should be discarded. For most applications, the amount of iodoacetyl-PEO-biotin usually required is in the microgram range, an amount well below the accuracy of microbalances. Thus, an amount of 5 mg is weighed and dissolved in 50 μl of 60% methanol/40% 50 mM ammonium bicarbonate and then the appropriate aliquot removed and added to the sample. The remaining stock solution of iodoacetyl-PEO-biotin is discarded since it will degrade because of hydroxylation. Once the reagent has been added, perform the labeling reaction in the dark by covering the sample tube with aluminum foil.

This is necessary to prevent iodination of tyrosyl residues. Further details regarding the optimization of the cysteinyl-labeling reaction are described elsewhere *(10)*.

13. To perform gravity-flow gel filtration chromatography (size exclusion chromatography) to achieve buffer exchange, a medium compatible with 60% methanol must be used (such as Excellulose from Pierce). Two types of Excellulose columns can be purchased prepacked (2 ml and 5 ml), so an appropriate column can be selected based on the sample volume. Alternatively, dialysis could also be used in place of gel filtration (refer to Section 3.6).
14. Trypsin retains its specificity in the 60% methanol/40% 50 mM ammonium bicarbonate, although its activity is slightly reduced *(16)*.
15. Microsomal pellets obtained from Arabidopsis plants are green and reside at the bottom of the centrifuge tube as a thick viscous mass. Resuspend the microsomal pellet in a minimal volume of 50 mM ammonium bicarbonate (0.4 ml when starting with 20 g of Arabidopsis seedlings) to avoid producing a protein concentration below 0.5 mg/ml after methanol addition. Add just enough bicarbonate buffer to mediate transfer of the pellet to a siliconized polypropylene Eppendorf tube. An efficient way to perform the transfer is to use a small nylon brush (Royal 9250 No. 3 round brush) to facilitate the release of the pellet from the bottom of the centrifuge tube. The solution and pellet can then be easily transferred to the Eppendorf tube.
16. Use the sonicating probe in continuous mode at setting 5 and sonicate with four consecutive pulses of 1 min. The overall time of sonication depends upon the thickness of the pellet and its resuspension in solution. Sonication is complete when the sample becomes translucent.
17. The addition of PMSF is optional but is strongly suggested since any active trypsin will proteolytically cleave the immobilized avidin and interfere with effective biotinylated-peptide capture and produce avidin peptides that will contaminate the collected sample. Immobilized streptavidin can be used instead of monomeric avidin to capture the biotinylated-peptides; however, more aggressive methods are required to remove the bound peptides, such as using 2% SDS or guanidine hydrochloride with heating the sample in a boiling water bath, a process that would interfere with subsequent reversed-phase LC/MS/MS analysis.
18. The amount of resin needed is based on the binding capacity of the immobilized avidin and the amount of sample that was labeled. For a given proteome sample, assume that 30% of the peptides contain a cysteinyl residue and that each peptide has an average mass of 2000 Da, then calculate the μmoles of

biotinylated-peptides. According to this estimate, the avidin column to be made should be capable of binding an equivalent amount of biotin. Typically, the binding capacity of the immobilized avidin is reported as the amount of biotinylated BSA per volume of settled resin (mg/ml) or amount of biotin binding per volume of settled resin. Convert these masses to μmoles/ml of settled resin and add the amount of resin equivalent to the estimated μmoles of biotinylated-peptides. For most immobilized avidin preparations, the volume of the slurry (once the resin is resuspended) is 50%.

19. The glass pipette was found to work well, but it is possible for some peptides to be lost due to adsorption to the glass surface. A polypropylene pipette tip (1 ml) could be used as an alternative.
20. To save column preparation time for additional experiments, more avidin resin can be treated and stored in 2X PBS containing 0.02% sodium azide and stored at 4°C. In this manner, future columns can be made from this slurry and just equilibrated using 15 column volumes of 2X PBS prior to sample loading. Although some manufacturers indicated that the column can be regenerated and reused up to 10 times before binding capacity is diminished, it is strongly suggested that due to sensitivities of MS analysis, should the column be used once for each sample to prevent cross-contamination between samples.
21. Due to the hydrophobic nature of the peptides in the sample, removal of all the methanol via evaporation may cause some of the peptides to precipitate so be careful in reducing the volume of the sample. Depending on the nature of the sample, reducing the sample to $\leq$ 5% methanol may still be effective.
22. When loading the column collect the flow-through. This will contain the unbound peptide fraction that can be analyzed by LC/MS/MS to check for any biotin peptides that may not have bound. Since this fraction will contain residual PBS, an online trap column on the LC/MS/MS system or a longer wash after sample loading onto the reversed-phase column may be required to promote efficient electrospray ionization during gradient elution (depends on the LC/MS/MS system in use). Alternatively the use of 50 mM ammonium bicarbonate buffer instead of 2X PBS to equilibrate the column prior to sample loading on the avidin column can be used to eliminate the nonvolatile PBS buffer components, obviating the need for a desalting step or longer column wash.
23. Store the dried peptides in an air-tight container containing desiccant at −80°C. In this manner, the peptides can be

stored for months and should be shipped on dry ice if they are sent to a mass spectrometry facility for analysis.

24. For example, if most of the peptides elute between 35% and 70% of the organic mobile phase, then a steeper gradient up to 35% and after 75% can be used while making a shallower gradient between 35% and 70%. However, this depends on the nature of the sample. To achieve better peptide identification than just using reversed-phase chromatography, additional fractionation, such as strong-cation exchange, prior to the reversed-phase separation, can be used *(8)*.
25. The signature ions are due to fragmentation of the acetyl-PEO-biotin label that are typically present at low levels (relative to the b and y ions) and whose abundance will vary based on the sequence and size of the labeled peptide as well as the conditions used for collision-induced dissociation. As shown for the original ICAT reagent, these signature ions can sometimes be the only definitive marker using product ion spectra generated by an ion trap mass spectrometer to determine whether a peptide is tagged with a light or heavy ICAT label, particularly in regard to the $[M+3H]^{3+}$ precursor ions *(17)*.

Acknowledgments

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Chapter 23

Quantitative Proteomics Analysis of Pancreatic Zymogen Granule Membrane Proteins

Xuequn Chen and Philip C. Andrews

Abstract

Pancreatic zymogen granules (ZG) are specialized for digestive enzyme storage and regulated secretion in the exocrine pancreas and are a classical model for studying secretory granule function. To understand the function of this organelle, we have conducted a proteomic study to identify the ZG membrane proteins from ZGs purified by Percoll gradient centrifugation. By combining multiple separation strategies including two-dimensional gel electrophoresis and two-dimensional liquid chromatography with tandem mass spectrometry (TMS), we identified 101 proteins from purified ZG membranes including a large number of proteins previously unknown on ZG membranes. To distinguish intrinsic membrane proteins from soluble and peripheral membrane proteins, a quantitative proteomics strategy was developed to measure the enrichment of intrinsic membrane proteins through the purification steps by labeling crude, KBr-, and Na_2CO_3-washed ZG membranes with multiplexed isobaric tags (iTRAQ^{TM}), 114, 116 and 117, respectively. The proteins with 117:114 ratios greater than one correlated well with known or predicted intrinsic membrane proteins.

Key words: Pancreatic zymogen granule, Percoll gradient, 2D LC, tandem mass spectrometry, iTRAQ, membrane proteins, quantitative proteomics.

1. Introduction

The primary function of pancreatic acinar cells is to synthesize, package, and secrete digestive enzymes. This process is regulated by gastrointestinal hormones and neurotransmitters *(1, 2)*. In acinar cells, digestive enzymes are stored in zymogen granules (ZG). Stimulation of acinar cells by secretagogues triggers fusion of the ZG membrane (ZGM) with the apical plasma membrane and release of its content. In addition to fulfilling this

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important physiological role, zymogen granules have been used as a model system for studying secretory granule function in general. Identification of the component proteins of the ZG membrane is an essential first step in understanding the molecular architecture of the zymogen granule and its function. It is believed that ZGs share common mechanisms with other secretory organelles such as synaptic vesicles and chromaffin granules, in that two families of proteins, SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) and Rabs, govern vesicular trafficking from the Golgi network to the plasma membrane *(3, 4)*. However, many key components have not yet been identified on the ZGM. Early SDS-PAGE studies indicated a relatively simple protein composition for the ZGM because of limited resolution and sensitivity. Our recent proteomics analysis indicates a more complex scenario for ZG function *(5)*. In this study, we combined a well established ZG purification approach and comprehensive protein identification including 2D gel electrophoresis, 2D LC, and tandem mass spectrometry. Over 100 proteins have been identified on ZGM among which 73 have been localized on ZGs for the first time including multiple small GTP-binding proteins, SNARE proteins, and molecular motor proteins *(5)*. These observations bring new insights into the molecular mechanisms of ZG functions.

Another major effort in our study was to develop a quantitative proteomics strategy capable of monitoring intrinsic ZG membrane protein enrichment during purification. Since complete separation of soluble, membrane-associated proteins from intrinsic membrane proteins has been difficult to achieve for most cell fractions, we expected that the proteins identified on ZG membranes contained a mixture of all three types of proteins even after thorough washes. To enrich intrinsic membrane proteins, we extensively washed ZG membrane preparations using high salt, known to release extrinsic proteins as well as absorbed proteins from membrane preparations, and followed by high-pH buffers, the most satisfactory approach to separate ZG content from membrane *(6)*. Traditionally, to determine the enrichment of intrinsic membrane proteins or the removal of content or peripheral membrane proteins, Western blot or equivalent was needed for each individual protein to compare semiquantitatively the amount of protein remained in the membrane before or after each wash step. In the current study, using iTRAQ reagents *(7)*, we developed a quantitative proteomics strategy to obtain the same type of information systematically for all the proteins at the time of their identifications by TMS. The intrinsic membrane proteins determined by iTRAQ 117:114 ratio measurement correlated very well with known or TMHMM predicted membrane proteins *(5)*. This validated the potential of our iTRAQ in mass-spectrometry-based quantitative approach to obtain protein identification and

relative protein abundance simultaneously. Our method also provides a new quantitative proteomic approach to verify systematically intrinsic membrane proteins.

2. Materials

2.1. Isolation of ZGs and Purification of ZG Membranes

2.1.1. Isolation of ZGs

1. Typically, pancreases are obtained from 10 to 12 Sprague Dawley rats with body weight between 250 and 300 g.
2. Homogenization buffer: 0.25 M sucrose, 25 mM MES (2-Morpholinoethanesulfonic acid, monohydrate) pH 6.0 (adjusted with 1N HCl), 2 mM EGTA and 0.1 mM phenylmethylsulfonyl fluoride (*see* **Note 1**). It is made from stock solutions (2 M sucrose and 1 M MES, pH 6.0) stored at 4°C.
3. Teflon glass homogenizer with tight pestle (Thomas Scientific, Swedesboro, NJ).
4. Percoll (Amersham Biosciences).
5. Beckman ultracentrifuge with a Ti 70.1 rotor or similar.

2.1.2. Purification of ZG Membranes

1. Nigericin (Sigma) is dissolved in ethanol at a concentration of 10 mg/mL and stored at 4°C.
2. Protease inhibitor cocktail (Roche) is used according to company instruction.
3. ZG lysis buffer: 150 mM sodium acetate, 10 mM MOPS, pH 7.0, 27 μg/ml Nigericin, 0.1 mM MgSO4, 0.1 mM phenylmethylsulfonyl fluoride supplemented with protease inhibitor cocktail. The stock solutions of 1 M sodium acetate and 1 M MOPS, pH 7.0 (adjusted with 1N HCl), are stored at 4°C.
4. Beckman ultracentrifuge with a Ti 70.1 rotor or similar.
5. 250 mM KBr.
6. 0.1 M Na_2CO_3, pH 11.0. (adjusted with 1N HCl).

2.2. iTRAQ™ Labeling and 2D LC Separation of Tryptic Peptides

1. iTRAQ™ reagents from Applied Biosystems and stored at −80°C.
2. Strong cation exchange (SCX) buffer: 10 mM KH_2PO_4, 25% (v/v) acetonitrile with pH adjusted to 3.0 (adjusted with 1N HCl). It is stored at 4°C.
3. MicroSpin™ columns (PolyLC) are stored at room temperature.
4. Zorbax 300 SB C18 column, 75 μm × 150 mm, 3.5 μm particles (Agilent Technologies) is used for reversed-phase HPLC.
5. For reversed-phase HPLC separation, solvent A is 0.1% (v/v) TFA in water and solvent B is acetonitrile/water/TFA, 90/10/0.1% (v/v).
6. Agilent 1100 HPLC system for reversed-phase separation.

2.3. Tandem Mass Spectrometry

1. MALDI matrix solution: 10 mg/mL of α-cyano-4-hydroxycinnamic acid (Sigma Chemical) in acetonitrile/water/trifluoroacetic acid, 50/50/0.1 (v/v/v) is stored at −20°C.
2. The diluted MALDI matrix solution used for automated LC-spotting on a target plate is at 2 mg/mL α-cyano-4-hydroxycinnamic acid in the same solution as above.
3. MALDI-TOF/TOF instrument: Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems/MDX Sciex).
4. GPS explorer software (Applied Biosystems) for MS/MS data interpretation.

3. Methods

3.1. Preparation of Purified ZG Membranes

3.1.1. Isolation of ZGs

1. In a typical experiment, 10–12 rats are sacrificed by decapitation following CO_2 anesthesia. The blood is drained (*see* **Note 2**) and the pancreases are removed, minced and homogenized (two pancreases at a time) in 10 mL of ice-cold homogenization buffer. The homogenization is conducted for nine strokes on an electronic motor using a Teflon glass homogenizer.
2. Homogenates are combined and distributed in six 15-mL tubes and then centrifuged first at 300 g for 10 min at 4°C to remove unbroken cells and nuclei and the supernatant is transferred to six new tubes and spun down at 2,000 g to generate a white particulate pellet enriched in ZGs covered by a tan layer containing mainly mitochondria (*see* **Note 3**).
3. The particulate pellet is gently resuspended in 40 mL of homogenization buffer and mixed with 60 mL of Percoll solution containing 50 mL of Percoll, 9.4 mL of 2 M sucrose, and 600 μL of 1 M MES, pH 6.0.
4. The mixture is distributed in ten 10-mL ultracentrifugation tubes and centrifuged in a Beckman ultracentrifuge at 30,000 rpm (60,000 g) for 20 min using a Ti 70.1 rotor.
5. The dense white ZG bands near the bottom of the centrifuge tube are collected and diluted in 60 mL of homogenization buffer. In order to remove excess Percoll, the suspension is centrifuged to pellet ZGs at 1,000 g for 10 min.

3.1.2. Purification of ZG Membranes

1. To purify ZG membrane, the above ZG pellets are resuspended in 20 mL of ZG lysis buffer and incubated at 37°C for 15 min. The lysate, which becomes clear at the end of the incubation, is centrifuged at 38,000 rpm (100,000 g) for 1 hour in a Beckman ultracentrifuge using a Ti 70.1 rotor to pellet the ZG membrane.

2. To remove absorbed content proteins, the ZG membrane pellet is washed with 10 mL of 250 mM KBr and recentrifuged to pellet membrane. To further purify the membrane and remove some of the peripheral membrane proteins, the ZG membrane pellet is resuspended in 0.1 M Na_2CO_3 (pH 11.0) and incubated on ice for 30 min, then centrifuged for 1 hour at 38,000 rpm (*see* **Notes 4**, **5**).

3.2. iTRAQ™ Labeling and 2D LC Separation of Tryptic Peptides

3.2.1. iTRAQ™ Labeling

1. Multiplexed isobaric tags (iTRAQ™ reagents) 114, 116, and 117 are used to label tryptic peptides from crude, KBr- and Na_2CO_3-washed ZG membranes, respectively. The labeling procedure is according to the manufacturer as described previously *(7)* and more details are illustrated in **Fig. 23.1** and given below.
2. Proteins are extracted from crude, KBr- and Na_2CO_3-washed ZG membrane, respectively, by incubating with 0.5 M triethylammonium bicarbonate, 0.1% (w/v) SDS on ice for an hour followed by three 10-s sonications. The protein concentrations are determined using a Bio-Rad protein assay kit based on the Bradford method.
3. Twenty micrograms of protein in 20 μL from each fraction are reduced by adding 5 mM Tris-(2-carboxyethyl)phosphine and incubated at 60°C for 1 h, and cysteines are blocked by

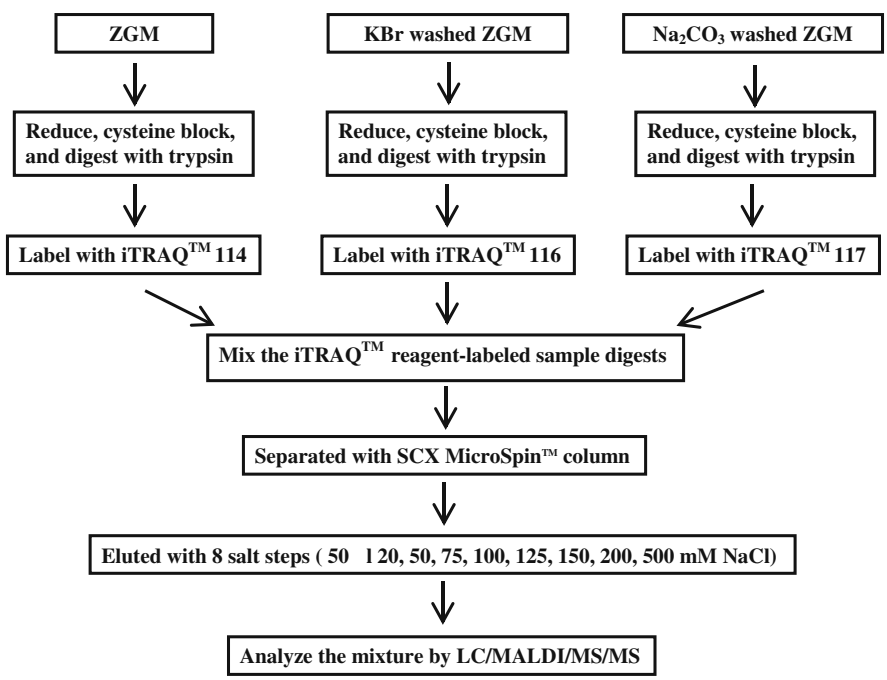

Fig. 23.1. Schematic representation of protocol for analysis of granule membrane proteins by iTRAQ.

adding 10 mM methyl methanethiosulfonate (MMTS) and incubated in the dark at room temperature for 10 min.

4. The proteins are digested with trypsin overnight (1:10, w/w, 37°C).
5. Dissolve the iTRAQ™ reagents by adding 70 μL of ethanol. The tryptic peptides are then mixed with 114, 116 and 117 iTRAQ™ reagents, respectively, at room temperature for an hour.
6. The three iTRAQ™-labeled peptide samples are mixed and diluted over 10-fold in strong cation exchange (SCX) buffer containing 10 mM KH_2PO_4, pH 3.0 and 25% (v/v) acetonitrile (*see* **Note 7**).

3.2.2. 2D LC Separation of Tryptic Peptides

1. The iTRAQ™-labeled peptide mixture is fractionated on a SCX MicroSpin™ column with sequential elution of bound peptides in eight salt steps: 50 μL of a 10 mM potassium phosphate buffer, pH 3.0 containing 25% (v/v) acetonitrile, and 20, 50, 75, 100, 125, 150, 200, and 500 mM NaCl are used successively.
2. The volume of the eluted material from each salt step is reduced in a SpeedVac and reconstituted with 40 μL of 0.1% (v/v) TFA in water.
3. Each reconstituted sample is separated by reversed-phase chromatography using a Zorbax 300 SB C18 column on an Agilent 1100 HPLC system. The flow rate is set at 300 nL/min and the following binary gradient is run: 0 min, 6.5% B; 9 min, 6.5% B; 12 min, 15% B; 92 min, 45% B; 97 min, 60% B; 102 min, 100% B; 104 min, 100% B; 105 min, 6.5% B; 115 min, 6.5% B.
4. The column effluent is mixed with diluted MALDI matrix solution (at 2 mg/mL α-cyano-4-hydroxycinnamic acid (*see* **Note 8**)) through a 25 nL mixing tee and spotted on 192-well MALDI target plates using Agilent 1100 series micro collection/spotting system.
5. The matrix is delivered to the mixing tee by an external infusion pump at a rate of 800 nL/min.

3.3. Tandem Mass Spectrometry

1. All MS and MS/MS spectra for both 2D gel and 2D LC samples are acquired on a MALDI-TOF/TOF instrument in positive ion reflection mode with a 200 Hz Nd:YAG laser operating at 355 nm. Accelerating voltage is 20 kV with 400 ns extraction delay. For MS/MS spectra, the collision energy is 1 kV and the collision gas is air.
2. Typical spectra are obtained by averaging 3,000 laser shots with the minimum possible laser energy in order to maintain the best resolution. Single-stage MS peptide mass fingerprints for the entire samples are collected first, and in each sample well, MS/MS spectra are acquired from the five most

intense peaks above the signal to noise ratio threshold of 30. Both MS and MS/MS data are acquired using the instrument default calibration, without applying internal or external calibration.

3. In the 2D LC-MALDI experiments, a MS survey scan is first performed for each salt fraction across the entire plate. After applying the exclusion list (*see* **Note 9**), the ten most intense peaks above S/N of 60 are selected from each well for MS/MS analysis.

3.4. Database Search and Data Analysis

3.4.1. Database search

1. Both MS and MS/MS spectra are processed in 4700 Explorer™ software (v2.0, Applied Biosystems) with Gaussian smoothing at filter width of 7 points.
2. For MS spectra, a S/N threshold of 30 whereas for MS/MS spectra, a threshold of 20 is used to detect peaks.
3. Monoisotopic peak lists are generated in Applied Biosystem's GPS Explorer™ v2.0 and submitted to the GPS Explorer™ v2.0 search tool (based on MASCOT) for protein identification.
4. The Nonredundant Protein Database, NCBInr, with 2,419,798 mammalian or 109,660 rodent sequences (National Center for Biotechnology Information, USA) is searched using the following parameters for 2D gels: 0 or 1 missed cleavage by trypsin, carboxyamidomethylation of cysteines as fixed modification, and methionine oxidations, N-terminal protein acetylation, Pyro-glu (N-term E), Pyro-glu (N-term Q) as variable modifications.
5. For iTRAQ™-labeled samples, iTRAQ modification of N-terminus and lysine is selected as fixed, together with MMTS for cysteines; methionine oxidation is selected as a variable modification and 0 or 1 missed tryptic cleavage is allowed.
6. For all searches, precursor ion mass tolerance is 100 ppm and fragment ion mass tolerance is 0.6 Da. For both 2D gel and 2D LC experiments, only MS/MS data are used for identification. Protein identifications are considered significant when they are based on at least two unique peptides, each of which has a Mascot score corresponding to $p<0.05$.

3.4.2. Data Analysis

1. In 2D LC experiments, a total of 2,498 peptides were selected for MS/MS analysis, and 630 peptides were assigned to 84 non-redundant proteins.
2. The peak areas of low mass reporter ions from the isobaric tags, 114, 116, and 117, are extracted from the spectra using 4700 Explorer™ and matched in an Excel datasheet to the identified peptides retrieved from the MS/MS summary table in GPS Explorer™ (*see* **Note 10**)
3. The complete list of identified peptides with reporter ion peak areas is then grouped into proteins for calculation of average

Table 23.1
Summary of iTRAQ ratios from identified ZG proteins*

Protein Name	Accession Number	# of spectra	116:114 ratios Mean ± S.D.		117:114 ratios Mean ± S.D.		TM[a] domains
ATP synthase alpha chain	114523	6	0.75	0.19	0.24	0.09	0
GP3 (PLRP-2)	17105374	21	0.88	0.29	0.43	0.15	0
Colipase	203503	11	1.11	0.08	0.44	0.16	0
Pancreatic lipase	1865644	21	0.96	0.05	0.44	0.05	0
Sterol esterase	1083805	17	0.86	0.14	0.47	0.21	0
Anionic trypsin precursor	67548	4	0.6	0.28	0.49	0.16	0
Syncollin	20806121	9	0.77	0.10	0.52	0.10	1
Alpha-amylase	62644218	26	0.72	0.12	0.60	0.17	0
ATP synthase alpha chain	114523	8	1.03	0.10	0.69	0.20	0
Carboxypeptidase A1 precursor	8393183	8	1.12	0.12	0.70	0.30	0
Carboxypeptidase A2 precursor	61556903	3	1.15	0.21	0.70	0.25	0
ZG16	19705541	6	1.99	0.20	0.70	0.15	0
Elastase 3B precursor	62649890	6	1.52	0.11	0.73	0.22	0
Caldecrin precursor (chymotrypsin c)	1705913	2	1.20	0.10	0.79	0.31	0
Protein disulfide isomerase precursor	129731	2	1.10	0.19	0.85	0.16	0
Pancreatic lipase related protein 1 (PLRP-1)	14091772	29	0.98	0.12	0.87	0.22	0
Clusterin	46048420	3	1.10	0.17	0.93	0.10	0
RAB27B	16758202	7	0.99	0.10	1.35	0.13	1(prenyl)
Rac1	54607147	3	1.05	0.11	1.40	0.15	1(prenyl)
21 kDa Transmembrane trafficking protein	3915137	2	1.23	0.30	1.40	0.19	1
Similar to osmotic stress protein	34856875	6	1.07	0.14	1.48	0.17	5
Ubiquitin	1050930	5	1.31	0.18	1.54	0.32	0

Table 23.1 (continued)

Protein Name	Accession Number	# of spectra	116:114 ratios Mean ± S.D.		117:114 ratios Mean ± S.D.		TM[a] domains
Vacuolar-type H^+-ATPase 115 kDa subunit, a1 isoform	13928826	7	1.13	0.23	1.74	0.14	6
Lysosomal-associated membrane protein 2	40254785	2	1.25	0.23	1.85	0.13	1(GPI)
Gamma-glutamyl transpeptidase	16758696	19	1.27	0.19	2.02	0.33	1
Rab8A	49522647	6	1.24	0.15	2.12	0.76	1(prenyl)
Rab6	62654200	6	1.19	0.18	2.35	0.73	1(prenyl)
Rab1	56605816	12	1.34	0.26	2.37	0.75	1(prenyl)
Signal sequence receptor, alpha	57114346	3	0.98	0.25	2.68	1.20	1
RAB3D	18034781	8	1.48	0.06	2.94	0.10	1(prenyl)
GP2	121538	28	1.64	0.67	3.06	1.11	1(GPI)
Protein transport protein SEC61 beta subunit	27714473	4	1.27	0.11	3.13	0.60	1
Integral membrane-associated protein 1(Itmap 1)	5916203	11	0.94	0.20	3.24	0.35	2
Dipeptidase 1	16758372	7	1.23	0.17	3.30	1.10	1
Voltage-dependent anion channel 2 (VDAC 2)	13786202	2	1.48	0.21	3.48	0.48	1(β-sheet)
Voltage-dependent anion channel 1 (VDAC 1)	48734887	5	1.50	0.21	3.58	0.62	1(β-sheet)
Myosin Vc	62653910	7	1.10	0.10	3.67	0.71	0

[a] Numbers of TMHMM predicted transmembrane (TM) domains are shown with the known posttranslational modifications in the parenthesis.

* This table is a modified version of Table 2 in our recent publication *(15)*.

ratios and standard deviations. Although the same peptides identified from multiple wells in the LC-MALDI experiments are counted as only one unique peptide, the iTRAQ^{TM} values obtained from the fragmentation of these peptides are considered independent measurements for the calculation of average ratios and standard deviations.

4. Abundance ratio calculation includes corrections for overlapping isotopic contributions (both natural and enriched ^{13}C components).
5. The iTRAQ ratios of identified ZG proteins are summarized in **Table 23.1**. They are grouped based on whether their 117:114 ratios are less than or greater than one and the two groups were highlighted with light and dark grey colors, respectively. Proteins with 117:114 ratios greater than one (the minimal ratio measured on a known membrane protein, Rab27B, is 1.35) correlate well with intrinsic membrane proteins that contain either known or predicted transmembrane domains, whereas proteins with ratios less than one do not have known or predicted membrane domains or posttranslational modifications for membrane insertion (*see* **Note 11**).
6. To test our iTRAQ^{TM} ratio-based classification independently, we annotated all the proteins in **Table 23.1** for the presence of known or predicted transmembrane structures. The annotations were based on either database and literature information or the predictions by a widely used algorithm, TMHMM, for transmembrane helixes *(8, 9)*. The result of this annotation is summarized in the column *TM domains*.

4. Notes

1. Because phenylmethylsulfonyl fluoride is not stable, it is added to the buffer from a 0.2 M stock solution stored at −20°C immediately before use and every hour thereafter.
2. The blood needs to be drained as completely as possible. Large amounts of residual blood in the pancreas tissue may contaminate ZG preparation and is apparent as a reddish pellet at the very bottom of the white pellet after the second low-speed centrifugation. This blood contaminant is hard to separate efficiently by Percoll gradient centrifugation.
3. The loose layer of tan pellet containing mainly mitochondria can be largely removed by rinsing the pellet with homogenization buffer several times. The residual mitochondria appear in the top band of the centrifuge tubes after Percoll gradient ultracentrifugation.
4. The high salt wash alone is not sufficient to remove absorbed ZG content proteins. In Coomassie stained 2D gels of 300 μg of KBr-washed ZGM proteins, only abundant membrane proteins such as GP2 and dipeptidase and contaminant content proteins could be detected. In contrast, small GTPases such as Rab27B and Rab11 became apparent in Coomassie stained 2D gels of 300 μg of Na_2CO_3-washed ZGM proteins.

5. ZG membrane pellets can be used immediately after preparation, or alternatively, stored in the centrifuge tubes at −80°C with the caps sealed with parafilm.
6. In our experience, ASB-C8ϕ is a key detergent in the buffer to improve the solubility of ZGM proteins. As an alternative to dissolving ZGM pellets directly in IEF buffer, we have tried to dissolve ZGM pellets in Tris-HCl buffer with 0.5% (v/v) Triton X-100 and precipitate ZGM proteins by acetone/TCA precipitation. The precipitant pellets were washed and then dissolved in IEF buffer. Direct solubilization in IEF buffer seemed to give better result.
7. If the SCX step is not planned immediately after tagging, the labeled samples can be kept separately or mixed and diluted immediately in SCX buffer. The iTRAQ tagging reaction proceeds rapidly and to completion under the conditions described here, so a specific quenching step is generally not required, however, effective quenching may be attained, if desired, by addition of 50 μL of 100 mM ammonium bicarbonate.
8. A reduced concentration of α-cyano-4-hydroxycinnamic acid is used to prevent matrix crystals from clogging the capillary in the nanoflow pump.
9. Prior to the 2D LC-MALDI MS/MS experiments, a 1D LC-MALDI MS/MS run is performed using 5 μg of the same sample mixture in order to identify the most abundant proteins in the sample. From this experiment, an exclusion list is generated containing 70 peptides from the abundant proteins in the sample including GP2, GP3, syncollin, colipase, pancreatic lipase, sterol esterase, and amylase.
10. GPS Explorer™ v3.0 allows the iTRAQ ratios to be calculated automatically without the need to export the peak areas of low mass reporter ions.
11. In contrast to the clear-cut results from 117:114 ratios, the 116:114 ratios were less informative in distinguishing membrane from soluble proteins, although there were clusters of content and membrane proteins with 116:114 ratios below and above 1.0, respectively. In fact, this lack of efficiency is consistent with our observation that KBr wash alone did not remove a significant amount of proteins from crude ZGM, whereas Na_2CO_3 wash removed almost two-thirds of the proteins. It was also observed that a large amount of content proteins whereas very few membrane proteins were identified on 2D gel of KBr washed ZGM.

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