Farm animal proteomics 2013

Proceedings of the 4th Management Committee Meeting and
3rd Meeting of Working Groups 1, 2 & 3 of COST Action FA1002
Košice, Slovakia – 25-26 April 2013

edited by:
André de Almeida
David Eckersall
Elena Bencurova
Saskia Dolinska
Patrik Mlynarcik
Miroslava Vincova
Mangesh Bhide

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Farm animal proteomics: going to the European capital of culture 2013

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It was almost a year ago, that the management committee of COST Action FA1002 decided to take Farm Animal Proteomics from the westernmost point of Europe, the beautiful coastal harbor of *Vilamoura*, to the vibrant central European city - Kosice, also the European Capital of Culture for 2013.

On behalf of the organizers and FA1002, we have great pleasure in welcoming you to the 3rd annual meeting on Farm Animal Proteomics. We are sure that this meeting will once more immerse you all in the excellent research activities. This year, joined by eminent speakers from most Europe countries, as well as Australia and New Zealand. We hope that this meeting will serve as a platform to present and discuss the newest results and build scientific networks and collaborations in the form of scientific literature and research projects, and very importantly, friendship bonds. Following the lectures of invited speakers, we hope that the meeting will allow time for listening and meeting scientists in the earlier stage of their careers, particularly short-term-scientific mission (STSM) grantees. With fourteen key speeches, seventy five extended-abstracts, thirty eight posters and twenty two scientific lecture presentations from young scientists, the event has numerous scientific themes to share, learn and collaborate.

We are sure that the academic and cultural heritage of Kosice will infuse a sense of the pursuit of excellence. Additionally, the architecture and rich history of the city, a legacy of the numerous civilizations and cultures that passed through Kosice will provide a magical charm and truly fairy-tale memories to take back home. Košice, the metropolis of Eastern Slovakia, laying in the valley of the Hornad River in the basin that shares its name, is the regional administrative centre and Eastern Slovakia's hub of industry, commerce, science and culture. Košice is a city with an eventful and illustrious past, its earliest recorded mention dating from 1230, when it is referred to as 'Villa Cassa'. The coat of arms is the oldest in Europe, a fact attested to by a letter dated 1369. The city's historic sights - from various eras - are concentrated in the historic centre, which is an Urban Heritage Area.

We also hope you will enjoy the social event and engage in productive interactions with bouquet, color and taste of *Tokaj* Wine. So, enjoy the gorgeous wine cellars in *Tokaj*, descend into its mysterious silences, and coolness, covered with mold and have a look into dark galleries dug in bare volcanic rock. Listen carefully to the whisper of a thick wine, slowly poured into the glass as it was tired of waiting for so long until some Scientist dares to taste it ... 'A rush of wine to the head' ... You will never forget.

Part I
Invited plenary communications

Mining deeper into the proteome: pros and cons of pre-fractionation and depletion

Ingrid Miller
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Previous studies on the complexity of proteomes have estimated protein concentrations spanning over at least 10 orders of magnitude for serum/plasma, and of 6-8 orders of magnitude for cells. At the same time, marker proteins characteristic for particular diseases are supposed to be found in the low to trace abundance range. With conventional proteomic methods 2-4 orders of magnitude may be covered, thus making it necessary to look for strategies for digging deeper into the proteome.

For two-dimensional electrophoresis (2-DE) and without changing the method of sample preparation, a first attempt to gain sensitivity could be the use of larger gels, increased amount of loaded protein, application of more sensitive detection methods, as well as focusing on a narrower pH-range in the first dimension.

Pre-fractionation and depletion both change the composition of the original sample, and are additional steps prior to separation. They aim at reducing the complexity of the sample, either by removing high abundance proteins or by enriching proteins with particular properties. This starts with the buffer selection for sample extraction, which can also be performed in multiple steps with solutions of increasing dissolving power. Subcellular fractionation in a density gradient separates the organelles present in a cell, allowing the study of their subproteomes. Separation based on physico-chemical properties of the proteins may be achieved by chromatography. The most commonly applied principles are ion-exchange and reversed-phase chromatography, as well as affinity-based reactions (for instance enrichment of phosphoproteins). Preparative electrophoresis can also be a versatile tool, especially if it applies conditions different to a subsequent one- or two-dimensional separation (for instance, under native conditions, compared to denaturing and reducing 2-DE).

Similar principles are applied in depletion approaches, usually with the aim of removing 'unwanted' highly abundant proteins. For serum, this is often performed for albumin and IgG, but there are an increasing number of commercial products designed for removal of several plasma proteins. Most of these products are developed for human proteins, and need to be carefully tested for their usefulness in animal samples.

It is important to note that those sample pretreatment steps do not always give clearcut separations: a protein may be present in more than one fraction, or it may appear in another

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fraction when its properties change (for instance, in the course of a disease). Thus, care has to be taken to check and optimize the respective methods for the samples to be investigated.

Examples will be presented from different fields, mainly for 2-DE. A more detailed overview is given in Miller (2011).

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Detection and annotation of common post-translational modifications in mass spectrometry data

Julien Mariethoz[1], Oliver Horlacher[1], Frederic Nikitin[1], Matthew P. Campbell[2], Nicolle H. Packer[2], Markus Muller[1], Frederique Lisacek[1]
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Objectives

Glycosylation is probably the most important post-translational modification in terms of the number of proteins modified and the diversity generated. In spite of such a central role in biological processes, the study of glycans remains isolated, protein-carbohydrate interactions are rarely reported in bioinformatics databases and glycomics is lagging behind other -omics. Recent progress in method development for characterising the branched structures of complex carbohydrates has now enabled higher throughput technology. Automation then calls for software development. Adding meaning to large data collections requires corresponding bioinformatics methods and tools. Current glycobioinformatics resources do cover information on the structure and function of glycans, their interaction with proteins or their enzymatic synthesis. However, this information is partial, scattered and often difficult to get to for non-glycobiologists.

Phosphorylation is essential for the regulation of cellular processes. The CID-MS/MS fragmentation patterns of phosphopeptides differ from those of the non-phosphorylated peptides and MS/MS search engines often do not consider these differences. Further, even when the true peptide was found, the correct positioning of the phosphate on a residue is hampered by neutral loss of the phosphate, incomplete fragmentation, and coelution of peptides with different phosphate attachment sites.

Material and methods

In partnership with expert international research groups we are involved with the development of the UniCarb KnowledgeBase (UniCarbKB), an effort to develop and provide an informatic framework for the storage and the analysis of high-quality data collections on glycoconjugates. UniCarbKB (http://unicarbkb.org) is an initiative designed to support research in systems biology by complementing proteomics with glycomics (Campbell *et al.*, 2011). It aims to: (1) organise data to enable user-friendly interaction and querying by adopting standardisation and controlled vocabulary guidelines; (2) build a platform that will support the inclusion of new data mining tools and connect disparate existent glycobiology resources; (3) integrate

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functional data through cross-linking with sugar-binding information. The framework adopts agreed standards to store structural and metadata content including the translation of GlycoSuiteDB structure entries (Cooper *et al.*, 2003) into the GlycoCT format (Herget *et al.*, 2008) offering a comprehensive structure database.

In order to tackle phosphate positioning issues we developed a new algorithm, which takes the neutral loss peaks into account and reports a positioning score and false localization rate for all potential phosphorylation sites. The algorithm is self-learning and can adapt itself to new MS/MS data (Horlacher *et al.,* unpublished data).

Results and discussion

UniCarbKB offers a unique approach to access the most comprehensive biocurated overview of existing glycoinformation associated with proteins in a site-specific manner both from the attachment and the recognition perspective. The initiative is driven as a community-endeavour to promote data sharing in glycobiology and ensure its future development and growth. As mass spectrometry has become the most common method for solving glycan structures and identifying glycopeptides, there is now a substantial range of software tools that are available for analysing MS data produced in glycomics. Tools integrated in the UniCarbKB platform benefit from this work.

Our phosphate-positioning algorithm outperforms the AScore algorithm, which is widely used in the proteomic community. On test datasets, our algorithm yielded a false localisation rate (FLR) between 6.1% and 8.8% depending on preset conditions, whereas the AScore method had a localisation error of 16.6%. Overall, AScore proved to be less sensitive at a given FLR. At a FLR of 1% or higher it identified 30% less correct phosphosites. These results need be confirmed using further large-scale phosphopeptide datasets. This algorithm is to be integrated in the QuickMod (Ahrne *et al.*, 2011) platform that processes spectra for the accurate detection of PTMs in MS/MS data and based on annotated spectral libraries.

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Applied bioinformatics in the structural, post-genomic era

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Objectives

The sheer size of genomic data is overwhelming and has recently emerged as the major bottleneck in the field of modern bioinformatics. Nowadays, there are huge genomic databases available that remain unexploited due to the lacking of efficient mining algorithms and processing power (Dobson *et al.*, 2004; Illergård K, 2009). Even so, the quest of joining the dots between sequence and biological function is still primarily based on outdated primary sequence comparisons, which do not cover similarity searches adequately. Provided that secondary, tertiary and quaternary structure is far more conserved in proteins than their corresponding primary amino acid sequence, there is a dear need for new structure-based similarity searching approaches (Kolodny *et al.*, 2005). All evolutionary relationships of proteins, protein structure-function predictions, molecular docking and comparative modeling should all be based on analyses, searches and databases containing structural information (Berbalk *et al.*, 2009).

Material and methods

Herein, we report a new tool that we developed towards this direction; to perform protein similarity searches based on protein secondary structural elements rather than just primary sequence information. The query input sequence can either be of known or unknown structure. In both cases the primary amino acid sequence is converted to amino acid Structural Features Sequence (PSSP) format. The PSSP format is 'per residue' annotation method based on the structural conformation of each amino acid in the query sequence (α-helix, β-sheet, coil, etc). If the query input sequence is of unknown structure, it is subjected to secondary structure prediction algorithms and the SFS format is consequently deduced. Our algorithm is broken down in three parts; in part 1, the query sequence is converted to PSSP format, in part 2 the Query-PSSP is structurally aligned against the reference PSSP databases and finally, in part 3, the structural similarity results are combined with classic primary sequence based results and output to user.

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Results and discussion

Our methodology aims to provide the tools essential for performing similarity searches amongst proteins using structural rather than primary sequence information. The query input sequence may either be of known or unknown tertiary or quaternary structure (Figure 1). In either case the primary protein sequence must be transformed to amino acid PSSP format. The PSSP format is a residue-annotation method based on the structural conformation of each amino acid in the query sequence. This type of secondary structure annotation has previously been introduced by the DSSP suite.

All residues that form an a-helix will be substituted with an 'H', a beta-sheet with an 'S', and a coil formation with a 'C' until all query residues have been annotated with a PSSP value. In the case, where the query input sequence lacks 3D structural information, using STRAP it is subjected to secondary structure prediction, where the PSSP format will be subtracted. The exact same PSSP formatting methodology with or without secondary prediction algorithm will be applied to the full NCBI Genomic database. On the other hand, due to the fact that all entries in RCSB database encompass secondary structural information, the transformation to PSSP format may be achieved without performing secondary structure predictions. The proposed algorithm is summarized in Figure 1.

Due to the fact that sequence-based data is by default incomplete (in the case of genomic sequences), which means that they lack structural information, a fast and efficient secondary structure prediction algorithm will be generated and applied. Nonetheless, even upon

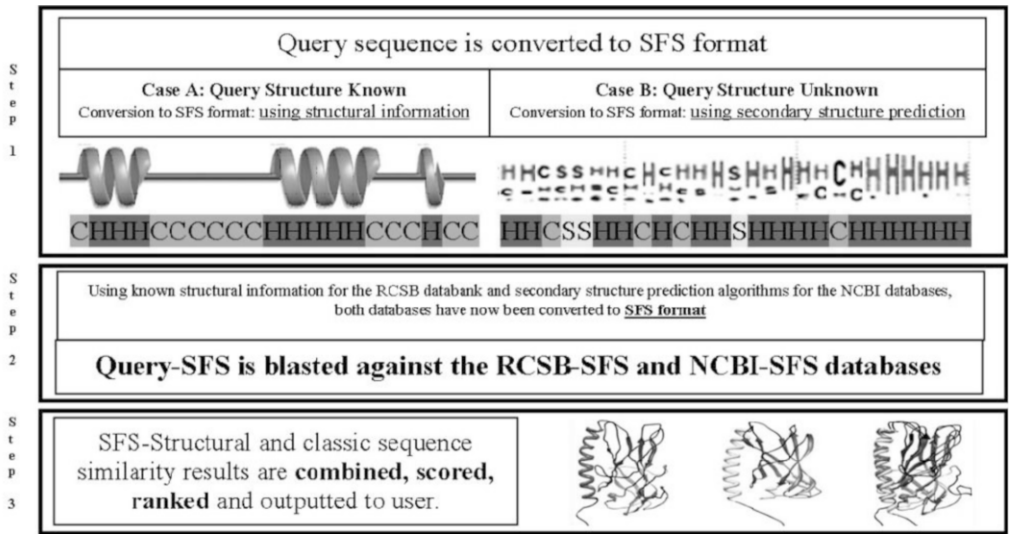

Figure 1. Diagram of the proposed approach.

application of the secondary structure predictions it is still possible to obtain 'unclear' data in the cases where the secondary prediction score do not indisputably indicate a certain secondary structural element. Therefore, the plan is to develop a strategy comprising of two different lines that tackle this issue: First line is the usage of multiple algorithms, which perform secondary structure predictions. The application of a repertoire of different algorithms and approaches on the same sequence string will ensure that eventually the user will end up with a 'consensus prediction', which is statistically more reliable. Second line, is the development of a fast routine, which aims to recognize and in the same time annotate the origin and functionality of each query DNA sequence string using artificial intelligence and machine learning techniques. Then using comparative three dimensional modelling a final prediction of the various structural elements will be made. This way a set of secondary elements will be attributed to the query sequence. Statistically, the weight ratios used by the secondary prediction and homology modelling algorithms will be user defined, optimized for the given needs of the experiment. For instance, obtaining unclear data from a query DNA sequence that has been found to contain conserved features of a designated transcription factor family, which is made up by alpha-helical repeats, will inform and train our algorithm to expect similar patterned alpha-helical conformations too.

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PTMomics – a potpourri of experimental approaches

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The modification of proteins post-translationally is of great importance in protein activity and cellular metabolism regulation. The protein amino acids may be modified after translation, altering the proteins and peptides molecular masses, which can complicate the identification of proteins, in particular from organisms that do not have their genomes sequenced.

There are over 400 post-translational modifications of proteins described. The most common and naturally occurring PTMs include phosphorylation, glycosylation, cleavage, formylation, methionine oxidation and ubiquitination. There are multiple experimental approaches that can be used for their detection and for the characterization and quantification of the generated protein isoforms. In order to achieve these aims it is important to individualize the isoforms either at the protein or at the peptide level. In that respect, 2D electrophoresis has been a helpful separation method, which can be coupled with specific stains or antibody interactions for PTM extensive detection and quantification and with mass spectrometry (MS) for isoform characterization and localization of modified amino acid residues.

Depending on the type of PTM under study different MS strategies tend to be selected mainly due to their lability and chemical specificities. Additionally, in some cases it is indispensable to perform previous enrichment protocols.

Several PTMomics case studies reported by our group and involving PTM identification, characterization and quantification will be discussed (Gomes *et al.,* 2006; Zhenjia *et al.,* 2007; Antunes *et al.,* 2010; Franco *et al.,* 2012).

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Gel-free quantitative proteomics approaches, current status

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Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has been used for the last forty years as a powerful technique to separate proteins and to compare their relative quantities in complex matrices. A major evolution of the technique was brought by the introduction of Differential in Gel Electrophoresis (DIGE) method. The latest evolution allowed up to 3 protein extract co-separations, by using labeling with different fluorescent dyes enabling their selective detection. The quantitation is performed by densitometry measured on gel images and the gel spots of interest are submitted to enzymatic digestion followed by mass spectrometry analysis and database searches for protein identifications.

Meanwhile, the technological evolution of liquid chromatography separations, mass spectrometry techniques, software and bioinformatics allowed gel free differential protein relative abundance analysis of complex protein mixtures to reach suitable power and availability to compete with gel based methods. Multidimensional (ion exchange, reverse-phase or affinity) *nano*-or capillary chromatography in combination with high resolution, high accuracy and fast scanning mass spectrometers, provide exceptional tools that generate unambiguous protein identification and their accurate relative quantification for discovery studies. Specific hardware and software solutions for selective quantification workflow have also been extensively developed.

This lecture will review these recent advances in MS based methodologies for proteomics studies using gel free techniques.

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New proteomics strategies applied to clinical studies

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Advances in proteomics have always been tightly linked to the development of mass spectrometry instrumentation. While for over one decade liquid chromatography/mass spectrometry based proteomics has relied on shotgun approaches, new hypothesis-driven methods have emerged. The latter allows more systematic analyses of peptides (used as surrogate for the proteins) with a high degree of sensitivity. Thus, they facilitate large-scale quantitative clinical studies, aiming at evaluating and validating biomarker panels in bodily fluids, such as blood or urine.

The new hybrid quadrupole-orbitrap mass spectrometer offers unique capabilities in quantitative proteomics, including high-resolution, accurate mass measurements, and fast acquisition. These features, combined with stable isotope dilution, have enabled the emergence of a novel quantification methods to detect and quantify large sets of peptides in complex mixture. Quantification in clinical samples has been performed either on the precursor ions or on the fragment ions.

In order to perform precise quantification, accounting for the variations occurring during the sample preparation and the LC-MS analysis, isotopically labeled internal standards are systematically used. The approach was applied to precisely quantify potential biomarkers of bladder cancer recurrence in urine, and lung cancer markers in blood samples. The analyses were carried out on a quadrupole-orbitrap mass spectrometer operating in full scan (SIM) and MS/MS modes, in conjunction with polypeptides internal standards. To evaluate the performance of the method, the isotopically labeled internal standards were spiked in the clinical samples before and after the proteolysis. Based on calibration curves of the reporter, the qualified markers were quantified in the low amol concentration range. These results demonstrate proof-of-principle and allow assessing the robustness of the method by benchmarking the results with SRM analyses performed on a triple quadrupole instrument. The data stress the benefit of using the high resolution and accurate mass to increase the selectivity of quantitative assays in very complex matrices, through reduction of signal interferences.

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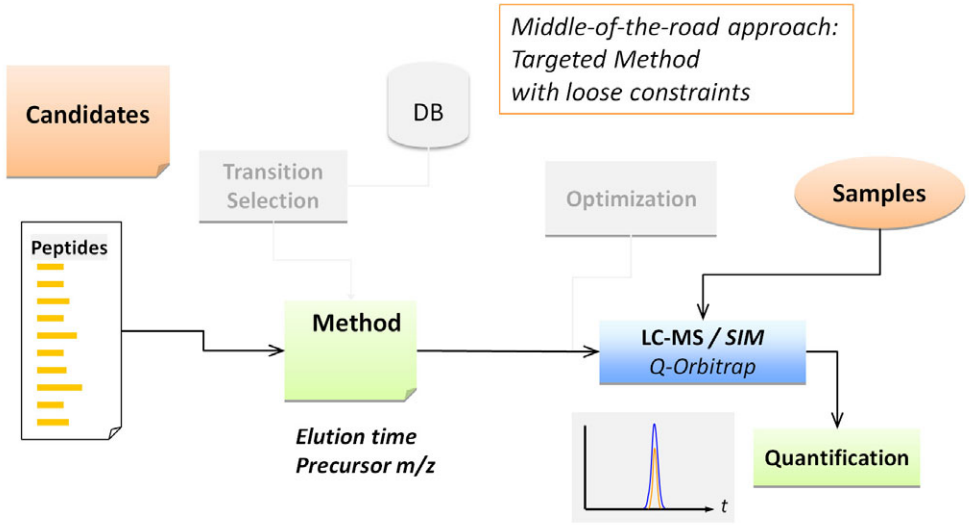

Figure 1. High-resolution / accurate mass workflow applied to quantitative proteomics.

Neuro-immune proteomic crosstalk in health and disease: partners in love, partners in divorce

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The permanent neuro-immune dialogue is required for normal brain function. Both systems use the same molecular language; they are covered with similar receptors for neurotransmitters, cytokines, chemokines and trophic factors. Neuronal cells actively participate in the brain immune response whereas glial cells are involved in neurotransmission. In disease condition, this dialogue is significantly impaired, the neuro-immune signaling is not properly regulated, neurons can either overactivate glial cells or on the other hand they can downregulate glial immune response. The abnormal signaling can be further modulated by genetic background, which can explain the different sensitivity of patients to human neurodegenerative disorders. In our study we focused on the 'ON' and 'OFF' signaling which is widely used by neurons to switch on or switch off the glial activity. The lecture will act as a guided tour through the highly variable neuro-immune kingdom, where sometimes partners can live in peace but sometimes they do not understand each other.

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Top-down proteomics: 2D gels are an integral part of the process

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Two-dimensional gel electrophoresis (2DE) remains widely regarded as a gold-standard for proteomic analyses. Nonetheless, issues with the method have been routinely noted in the 'review' literature, although there has been little to substantiate these claims and many do not seem plausible when the 2DE technique is considered from the perspective of its underlying chemistry and that of proteins. As (or perhaps *because*) gel-based proteomics is a 'mature' technology, factors contributing to possible reductions in performance are known and thus it is possible to better optimize ongoing analyses by targeted refinement of the technique; in contrast, issues with other approaches that have been popularised over the last decade are only now being more widely recognised. Here I briefly review efforts from my group to quantitatively improve every stage of 2DE analysis, from sample preparation and protein extraction, to in-gel spot 'fractionation' for further improving overall protein resolution, through to improved in-gel protein detection approaches for the enhancement of total proteome coverage. As the overall objective is to provide optimal analyses of (patho)physiological mechanisms, we have applied this refined 2DE protocol in proteomic investigations of human preterm labour, spinal cord injury, and a number of other conditions/samples relevant to both basic and clinical sciences.

Initially we sought to address issues of sample handling and extraction, and the resolution of hydrophobic proteins using 2DE. We suspected that membrane proteomes were regarded in the literature as difficult to resolve by 2DE due at least in part to poor extraction and the substantial amounts of soluble protein in total extracts. We initially addressed existing issues in six ways. First, we tested for ourselves and adopted specific protocol amendments that were quantitatively well supported by the available literature; in particular, this has included sample alkylation and reduction before both the first and second dimensions of 2DE (Herbert *et al.*, 1998, 2001). Second, stacking gels are routinely used in the second dimension, and this SDS-PAGE step is carried out in a cold room (i.e. 4 °C) to fully optimise protein resolution (Coorssen *et al.*, 2002). Third, detailed analyses established that automated frozen disruption (AFD), in which tissue samples are routinely and reproducibly powdered in the deep frozen state, was the best starting point for protein extraction (i.e. providing the highest possible yields) and that this was particularly advantageous for samples that were widely regarded as difficult to handle (e.g. muscle and plant tissues) (Butt and Coorssen, 2006). Fourth, as the least disruptive of prefractionation strategies, we introduced a simple physical separation, using hypotonic lysis and ultracentrifugation to fractionate samples into total soluble and total membrane proteins; each is then resolved separately in our proteomic analyses (Butt and

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