

# Signal enhanced proteomics

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# Signal Enhanced Proteomics: a Biological Perspective on Dissecting the Functional Organisation of Cell Proteomes

Running title: Signal Enhanced Proteomics


## Abstract

Proteomes are highly dynamic and can respond rapidly to environmental and cellular signals. Within cells, some proteins can form distinct ‘pools’, i.e. where a subset of the protein shows different functions and/or properties, such as subcellular location. This means that for a given protein ‘A’, a subset (pool) of that protein can differ in a value that is measured for the total population of molecules of protein ‘A’. However, in quantitative proteomics studies it is common to measure averaged values for proteins that do not reflect variations that may occur between different protein isoforms, different subcellular compartments, or in cells at different cell cycle stages. Here we review experimental approaches that can be used to enhance the signal from specific pools of protein that may otherwise be obscured through averaging across protein populations. This signal enhancement can help to reveal functions associated with specific protein pools, providing insight into the regulation of cellular processes. We review different strategies for proteomic signal enhancement, with a focus on the analysis of protein pools in different subcellular locations. We describe how MS-based proteome analyses can be combined with a general physico-chemical cell fractionation procedure that can be applied to many cultured cell lines.


## Introduction

The field of proteomics has seen tremendous advances that have improved the efficiency of protein detection at multiple levels, including experimental design, sample preparation workflows, LC-MS instrumentation and computational analysis. As a result, it is now possible not only to identify a large proportion of a steady state cell proteome in a single experiment, either with, or without, fractionation [1,2] but also to describe additional proteome dimensions, such as protein turnover rates, cell-to-cell variation, post-translational modifications, and subcellular localization.

From a biological perspective, a limitation of most shotgun proteomics experiments is that protein extracts are typically prepared from heterogeneous populations of cells, e.g. either from tissues, whole organisms, or from unsynchronised cells at different cell cycle stages. The resulting quantitative data represent an averaged value across all of the pools of each protein. However, to characterise biological regulatory mechanisms, it is important not only to quantify protein expression levels, but also to resolve protein groups into separate isoforms. This “isoform inference” problem is inevitably associated with bottom-up proteomics; i.e. where proteins extracted from a cell, tissue or an organelle are identified following their digestion into peptides, which are then analysed using LC-MS/MS. Furthermore, information from additional proteome ‘dimensions’ e.g. describing the subcellular distribution of proteins and *cross-correlating* this with data on post-translational modifications (PTMs), protein complexes, rates of protein synthesis and turnover, can provide valuable insights into regulatory mechanisms and generate hypotheses that can be evaluated in follow-on experiments. This combined analysis approach has been referred to as either “Next Generation Proteomics”, or, “Multidimensional Proteomics” [3].
In this manuscript, we will review examples of methods that make it possible to enhance proteomic signals and thereby detect protein-level changes that would not have been detected in standard one-dimensional analyses.

**Signal enhanced proteomics vs Classical proteomics**

Most proteomics approaches have tended to provide measurements that describe an averaged view, or steady state proteome. Protein expression levels are measured from the combined analysis of different sub-populations of protein molecules extracted from homogenized cell or tissue extracts. In turn, the extracts are generated from pools of cells, which typically include cells at different stages of the cell cycle and may include cells that have shown different response levels to external stimuli. While this is useful in providing general information on the proteome and its remodelling, as described below, the averaging can obscure the detection of changes in proteins that occur specifically in subsets of the global proteome (Figure 1).

To address the population averaging problem, several enrichment strategies at either cellular, or subcellular levels, can be applied to link the proteomics information more

effectively with cell biology. For example, immunologists have long used surface markers to label and sort different subpopulations of immune cells, using Fluorescence Activated Cell Sorting (FACS). With recent improvements in sensitivity and throughput for MS analysis, it has become technically feasible and cost effective to combine FACS sorting with proteomics analysis, which helps to target protein detection in specific cell subsets [4,5]. Recently, Ly et al. extended this approach to using FACS also to isolate cell subpopulations defined by immunolabelling intracellular, rather than cell surface antigens [6]. Using this strategy, termed PRIMMUS (PRoteomic analysis of Intracellular iMMUnolabelled cell Subsets), Ly et al. were able to separate interphase and mitotic cells, and also able to isolate populations of FACS-sorted cells enriched for specific mitotic subphases in sufficient quantities for detailed MS-based proteomic analysis, as shown schematically in Figure 1. For example, using PRIMMUS allowed the identification of 115 protein phosphorylation sites that increased during G2, including the phosphorylation of serine S738 on TPX2, which was shown to be important for TPX2 function and mitotic progression. This demonstrates that even minor subsets of cells in a population, which exist only transiently, can be isolated and protein responses detected that would otherwise be obscured in the bulk analysis of the proteome in extracts prepared from mixtures of cells at different cell cycle stages. New technology platforms are now being developed, based on FACS, to allow proteomics analysis from low numbers of cells, even aiming at single cell analysis. For example, Zhu et al. described the use of FACS to deposit cells into a newly developed nanodroplet sample processing chip for nanoLC-MS analysis [7]. This allowed the identification of ~ 670 protein groups from a single HeLa cell. They further demonstrated that this single cell platform can differentiate cell types from enzyme-dissociated human lung primary cells and identify specific protein markers for epithelial and mesenchymal cells.

**Isoform specific proteomics**

In higher eukaryotes, many genes encode two or more protein isoforms, which behave as distinct pools of related proteins and that may differ in terms of subcellular localization, interaction partners and function. For example, alternative splicing of pre-mRNA transcripts is commonplace, generating multiple mRNAs from the same gene, which in turn gives rise to proteins that can differ in structure and function. Isoforms can also arise via differential protein processing, e.g. cleaving the original translation

product into shorter forms. In other cases, protein isoforms can arise from expression of closely related, duplicated genes.

Whatever the mechanism, a common feature of closely related protein isoforms is that they usually share extensive regions of protein sequence identity and consequently have many shared peptides. The corollary is that many peptides that are identified in the typical bottom-up MS-based proteomics workflows cannot reliably be assigned to only one specific protein species for quantitation (Figure 2). If the structure of different protein isoforms is known experimentally, or predicted from genomic and transcriptomic studies, peptides that are either unique to a specific isoform, or shared between different isoforms, can be identified and used for MS-based quantification.

However, the identities of all protein isoforms in different cell types and organisms are not always known in advance. To address the isoform problem experimentally, without relying on accurate genome annotations, a protein size fractionation step can be included in the workflow, e.g. using SDS-PAGE, or size exclusion chromatography (SEC), prior to digestion [8-10]. This can enrich for protein isoforms that differ in size, which in turn reduces the isoform inference problem in subsequent MS analysis.

Computational approaches can also be used to distinguish protein isoforms. For example, Ahmad et al. [11] used a candidate approach, combined with sub-cellular fractionation, to detect protein isoforms that showed differential behaviour in separate subcellular compartments. Here, average values for protein intensity are first calculated using all of the peptides detected from a given gene, irrespective of isoforms. Next, the potential protein coding region is subdivided along its length and protein intensity is re-calculated, either using the peptides from the respective amino terminal, middle and carboxy-terminal ‘thirds’ of the protein sequence (‘rule of thirds’ approach), or else protein intensity calculated using groups of adjacent peptides, moving sequentially along the protein length from the amino to carboxy terminus. The principle is that if a protein isoform has a region of peptide sequence that is not present in other isoforms (e.g. resulting from inclusion of a differentially spliced exon), that may result in a protein intensity value for the isoform that differs from the average value obtained using all of the peptides matched to the protein group. Using the unbiased approach described above, Ahmad et al. [11] detected candidate protein isoforms via the analysis of peptide subsets and showed that the expression of some of these isoforms differed between the respective cytoplasmic and nuclear compartments of cultured human cell lines.

Ahmad et al. [11] also analysed correlations between protein post-translational modifications (PTMs) and protein localisation and turnover rates. For example, this showed that while the presence of one or more phosphorylated residues had little or no effect on the mean protein turnover rate, a subset of proteins were identified for which phosphorylation correlated with altered turnover rates. Interestingly, in HeLa cells this also correlated with protein localisation. Thus, a larger fraction of nucleolar proteins showed effects of phosphorylation on turnover rates, as opposed to either cytoplasmic, or nuclear proteins. Gene ontology analysis showed further that the proteins whose turnover rates are most affected by phosphorylation were enriched for ATP and nucleotide binding proteins, multiple cell cycle regulated proteins and proteins involved in apoptosis and cell death response mechanisms.

An alternative approach to identifying protein isoforms is to use a ‘top down’ MS strategy, rather than the more common ‘bottom up’ shotgun MS strategy discussed above. The top down strategy relies upon the ability to identify intact proteins by MS, rather than digested peptides [12]. The analysis of intact proteins can provide more accurate information, e.g. on the size of the proteins, on the presence of splice forms and PTMs [13-17]. Moreover, this approach can potentially provide more accurate quantification by overcoming many of the problems inherent in quantification using bottom up strategies. Currently, application of the top down strategy is still limited, at least in part, by the resolution and throughput capacity of modern MS instruments. However, the ability to resolve intact proteins by MS has improved significantly in recent years and we anticipate that further improvements will result in a wider adoption of the top down strategy in future.

**The subcellular proteome**

While most proteomic analyses have studied whole cell extracts, avoiding the issue of subcellular protein localization, some studies have focussed on analysing the proteomes of purified organelles and more recently global approaches have been developed to tackle the spatial dimension of the proteome. These global proteome localization approaches can be divided into two groups: first, targeted studies, which attempt to isolate biologically defined compartments (organelles), using fractionation methods that yield relatively pure fractions; second, global studies where multiple fractions are generated, using characteristics of subcellular compartments, e.g.

density and solubility to detergents, as the basis for fractionation. Subsequently, cross-gradient profiles are used to group proteins and assign them to compartments, based on co-fractionating markers.

Examples of methods from the first group include the classic nucleolar extraction protocol, which has been used in the “spatial proteomics” workflow (Figure 3A) [18] as well as methods using detergents of increasing strength to target compartment proteomes, based on their accessibility and solubility [10]. Methods which belong in the second group include Protein Correlation Profiling (PCP) [19] and Localization of Organelle Proteins by Isotope Tagging (LOPIT) [20], both of which rely on statistical methods to unravel the pattern of distribution of compartment proteomes in mixed populations separated using a gradient fractionation approach. The LOPIT workflow has been used successfully to investigate how the proteome, at steady state, is partitioned between multiple organelles and compartments. LOPIT has taken advantage of isobaric labelling, such as iTRAQ [21] and TMT[22], which allows simultaneous analysis of up to ten samples in a single MS run. Isobaric labelling is particularly well suited for analysis of fractionation experiments, because physically combining fractions early in the workflow makes the analysis internally controlled and improves data quality by reducing the problem of missing values.

Hyperplexed LOPIT [23] leverages new technological development, both in the TMT-technology and MS instrumentation, allowing more accurate quantification of an increased range of reporter tags. In addition to higher multiplexing capabilities, HyperLOPIT features improved subcellular fractionation protocols, which aim to preserve as many sub-organelles as possible [24]. Another important part of any spatial proteomics approach is the computational workflow used to combine the proteomics data with the subcellular fractionation/organelle compartment information. Accordingly, considerable effort has been dedicated to the development of computational packages for spatial proteomics in recent years. For example, Breckels et al., have recently described an example of a spatial analysis workflow combined with a step by step analysis guide [25]. An overview of a data analysis workflow for spatial proteomics is illustrated in Figure 3B.

In addition to characterising organelle/compartment proteomes at steady state, it is also important to understand the dynamic remodelling of the subcellular proteome when cells respond to stimuli, e.g. stress, or viral infection. This can result not only in changes in protein abundance, but also in protein translocation between

compartments. Recently, Cristea and co-workers extended the methods described above to study virus-induced spatial cell remodelling. By combining label-free and isobaric labelling, they measured the abundance levels for host and viral proteins and their localization throughout the time course of human cytomegalovirus (HCMV) infection, providing a comprehensive resource for understanding host and virus biology during HCMV pathogenesis [26,27]. They reported global reorganisation of proteins across different cellular compartments, including reorganization and processing of lysosomal proteins into distinct pools and translocations of individual proteins between organelles at specific timepoints. They also demonstrated that translocation of an unconventional myosin, MYO18A, from the plasma membrane to the viral assembly complex, is necessary for efficient HCMV replication.

The extent of proteome relocalization is also affected by the cell genotype, as first shown by comparing the response to stress induced by DNA damage in human HCT116 cells that were either wild type, or null, for the tumour suppressor p53 [28]. Using a MS-based proteomics approach, combined with subcellular fractionation, the distribution of the proteome between the nucleus and cytoplasm was compared before and after DNA damage induced by etoposide treatment, in both p53 +/+ and p53 -/- HCT116 cells. Few p53-dependent differences in proteome localization were detected under normal cell growth conditions, but clear differences after induction of DNA damage, particularly affecting the ability of ribosomal proteins to accumulate in nucleoli. This study illustrates how the unbiased proteomic analysis of part of the role of p53 in the DNA damage response was only revealed after linking MS-based proteome measurements with subcellular fractionation.

### Protein turnover analysis

Early biochemical studies of protein turnover relied on detecting the incorporation of radiolabeled amino acids into newly translated proteins [29]. Typically, proteins were labelled with [$^{35}$S] methionine and pulse-chase experiments used to determine their rate of degradation, after adding drugs to block protein synthesis. Nowadays, MS-based proteomics allows the measurement of turnover rates for large numbers of proteins simultaneously, for example by using pulse labelling experiments combined with stable isotope labelling by amino acids in cell culture (SILAC). The principle of pulse SILAC is to metabolically label proteins with heavy isotope substituted amino

acids and then to quantify how the isotope-labelled protein population changes over time (Figure 4). We and others have used pulse SILAC to study protein synthesis and turnover [10,30-36].

An alternative method is metabolic labelling using bioorthogonal amino acids [37], such as azidohomoalanine (AHA), which is incorporated into newly synthesised proteins instead of methionine [38]. AHA contains an azide group, enabling capture of newly synthesised proteins via click chemistry [37]. This, combined with SILAC, enables short pulse times [39,40].

Recently, protein turnover has been studied in high throughput by an MS-based approach combining SILAC and TMT labelling [41], which helped to address the problem of missing data between the time points, while allowing different proteoforms to be resolved by providing peptide-level measurements of turnover rates. Another example of peptide level turnover data was recently reported by Ly et al. in immortalised human breast epithelial cells [42], who used pulse-SILAC and cellular assays to study the activation of v-Src tyrosine kinase activity in untransformed MCF10A cells. v-Src induced rapid oncogenic transformation, with the cells showing major phenotypic changes within 48-72 hours, affecting their morphology, motility and invasiveness. Over this time course, the expression and/ or turnover levels of only ~3% of proteins changed by 2-fold or more. Furthermore, since many of the transformation-responsive proteins were low abundance, oncogenic transformation affected only ~ 1.5% of the total protein molecules in the MCF10A proteome.

Protein turnover rates can vary for separate pools of the same protein located in different subcellular compartments (Figure 4). For example, this was identified for ribosomal proteins in HeLa cells using a combination of pulsed stable isotope labelling with SILAC and fluorescence microscopy [33,43]. There was a higher rate of ribosomal protein turnover in HeLa cell nuclei than in the cytoplasm. Newly translated ribosomal proteins are immediately imported into the nucleus, ready for assembly into nascent ribosomal subunits in the nucleolus. If not bound to rRNA, however, free ribosomal proteins in this nuclear pool are rapidly degraded. Ribosomal protein stability was dramatically increased upon assembly into ribosome subunits and export to the cytoplasm [43].

Another example showing how different pools of the same proteins can exhibit differential turnover rates was provided in a study on the assembly of RNA polymerase II complexes by Boulon *et al.* [44] By using pulsed SILAC to analyse protein turnover

rates, combined with subcellular fractionation, they studied the assembly of RNA polymerase II, which occurs predominantly within the cytoplasm. After its assembly is completed, RNA polymerase II is transported into the nucleus, thereby preventing partially assembled and potentially non-functional sub-polymerase complexes competing for binding to gene promoters. The pulse-SILAC data showed that, similar to the situation with ribosomal proteins, protein turnover rates for subunits of the large polymerase complexes are higher in the cytoplasmic compartment, where assembly takes place, as opposed to the nucleus, where the complex functions (note the roles of these compartments is reversed for ribosome subunits).

Systematic proteome level analyses of the relation between protein turnover levels and subcellular localization were carried out, using unbiased MS approaches in U2OS cells, by Larance et al., 2013 [10]. This study systematically compared protein turnover levels in the respective nuclear, cytosolic, membrane and cytoskeletal compartments, revealing an important feedback mechanism, whereby inhibition of protein degradation by the proteasome resulted in a rapid inhibition of new protein translation, mediated by induced phosphorylation of eIF2alpha. Importantly, all of these studies together show that protein half-life values based only on analyses of whole cell extracts provide average values that can mask the existence of pools of protein with different properties.

## Concluding Remarks

In this manuscript we have highlighted some of the practical issues involved in integrating data from MS-based proteomic studies with functional studies in cell biology. In particular, we have focussed on how proteomics can be used to study subcellular localization and to identify pools of protein and distinct protein isoforms that can exhibit differences in structure and properties (e.g. turnover rate, interaction partners and PTMs) in different subcellular compartments. This information can be critical for understanding biological regulatory and response mechanisms, but is often lost or obscured in proteomic studies because of the effect of cell and protein population averaging when whole cell or tissue extracts are analysed.

Subcellular fractionation approaches ([20,23,24,26,27]) can be conveniently applied in a multi-dimensional proteomics strategy to improve the functional analysis of cell proteomes [24] and in the characterization of spatial remodelling following a

perturbation such as viral infection [26,27]. As shown for the role of p53 in the cellular response to DNA damage [28] characterising how subcellular proteome dynamics is affected by genotype will also be important and can now be analysed more systematically in human cells thanks to the availability of genome engineering technology with CRISPR/Cas9. We anticipate that in future more detailed studies examining the composition and dynamic remodelling of organelle proteomes at a multidimensional level will help to reveal new insights into the specific protein complexes and functional pools of proteins and isoforms that participate in cell regulatory mechanisms and metabolism.

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**Legends**

**Figure 1**

Schematic comparing classic ‘averaged’ proteomics analysis and signal enhanced proteomics. When a population of cells in culture is subjected to external stimuli, some cells may respond differently, resulting in a heterogeneous population, shown here as red and blue cells. In a classical proteomics experiment, cell extracts are prepared from the mixed population, resulting in averaged protein signals. With ‘Signal Enhanced Proteomics’, the two populations of cells are separated prior to proteomics analysis, thus reducing the dilution of the signal for proteins in a specific subpopulation of cells. For example, in a classic proteomics experiment, without an enrichment step, the peptide denoted with (*) was not selected for fragmentation because it was below the intensity threshold (red dashed line). However, after cell enrichment, the intensity of this peptide was now above the threshold intensity required for sequencing and hence could be detected.

**Figure 2**

Isoform-specific Proteomics. A schematic representation of how a single gene can encode two protein isoforms as a result of alternative splicing. These isoforms extensively share their protein sequence, but differ in specific segments, exemplified here by the blue and red peptide sequences. The two protein isoforms may have different properties, such as different subcellular localizations, which will only be resolved if sub-cellular fractionation is used prior to the proteomics analysis.

**Figure 3**

An example of a spatial proteomics workflow. A) A detailed subcellular fractionation protocol, based on the nucleolar isolation protocol, but with the number of subcellular fractions collected extended, thus increasing the resolution of proteomics measurements. Total cell lysate (TCL) is included as a control in subsequent analyses. B) A data analysis workflow, including a protein size separation step, using SDS-PAGE, of extracts from subcellular fractions, followed by LC-MS/MS. Raw files are analysed in MaxQuant [45,46], then peptide files re-assembled from individual evidence entries, analysed by Re-Fraction [47] to resolve protein groups into single protein IDs wherever possible. Protein cross-fraction profiles are generated from assigned peptide profiles. Protein profiles are clustered, using either a hierarchical, or k-means algorithm and predictive localization(s) assigned, based on their distance to co-clustering markers in the abundance space.

**Figure 4**

Compartment-specific protein turnover measurements. Pulse SILAC can be combined with sub-cellular fractionation to measure protein turnover in different subcellular compartments. A) Cells are cultured in different SILAC media, containing either “light” (L, K0R0), or “medium” (M, K4R6) isotope substituted forms of the amino acids arginine and lysine, until all proteins are fully labelled. The culture medium of the cells growing with the “medium” amino acids is then replaced with a culture medium containing “heavy” (H, K8R10) substituted versions of arginine and lysine. Finally, cells are harvested at different time points, along with the control cells growing in the culture medium containing normal, “light” (i.e. unsubstituted) arginine and lysine. Equal numbers of control and pulsed cells are then combined, prior to protein isolation and analysis. Either whole cell extracts can be prepared cells from each time point, or

extracts prepared from cells that are fractionated, e.g. into cytoplasmic, nuclear and nucleolar fractions, as illustrated. The subsequent proteomics analyses allow the measurements of rates of protein synthesis, degradation and turnover. B) For a given protein, the change in isotope ratios over time measure, respectively, (i) the rate of protein degradation (M/L isotope ratio), (ii) the rate of protein synthesis (H/L isotope ratio) and (iii) the rate of net protein turnover (H/M isotope ratio). C) Turnover data can be collected for different subcellular compartments, revealing proteins and protein isoforms that differ in their turnover rates according to their localization.

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