

**“Selected Reaction Monitoring:  
A tool for quantifying proteins in biology and biomedicine”**



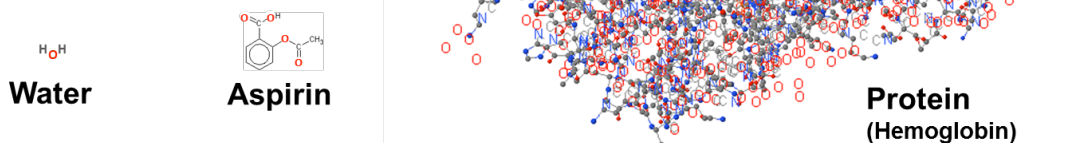
**Paola Picotti**

**Swiss Federal Institute of Technology, ETH Zurich**

## Introduction

The ability to quantify any protein or set of proteins of interest is an essential task in life science research. Proteins are the second most abundant molecule in humans, after water, and are crucial effectors and regulators of basically all biological processes in our organism. The human proteome contains more than ~20,000 different proteins, covering a broad range of abundances (up to 12-13 orders of magnitude)<sup>1</sup> and structures (Fig. 1). Measuring proteins is a crucial requirement in biomedicine, as they can change their abundance upon response to external or internal stimuli to an organism, for example in case of disease. This is exemplified by the protein PSA, which increases abundance in blood in case of prostate cancer and is routinely measured in the clinics as a marker for the disease. Quantifying proteins is also extremely important in biological sciences, for the understanding of basic cellular processes, since proteins supervise directly or indirectly all reactions occurring in the cells. Therefore, the development of methods and strategies to reproducibly, accurately and comprehensively measure proteins in biological and biomedical specimens is an urgent requirement.

**Figure 1. Complexity of protein structures.** The structures of a molecule of water, aspirin and a protein are shown on the same dimensional scale, to give an idea of the increasing structural complexity.



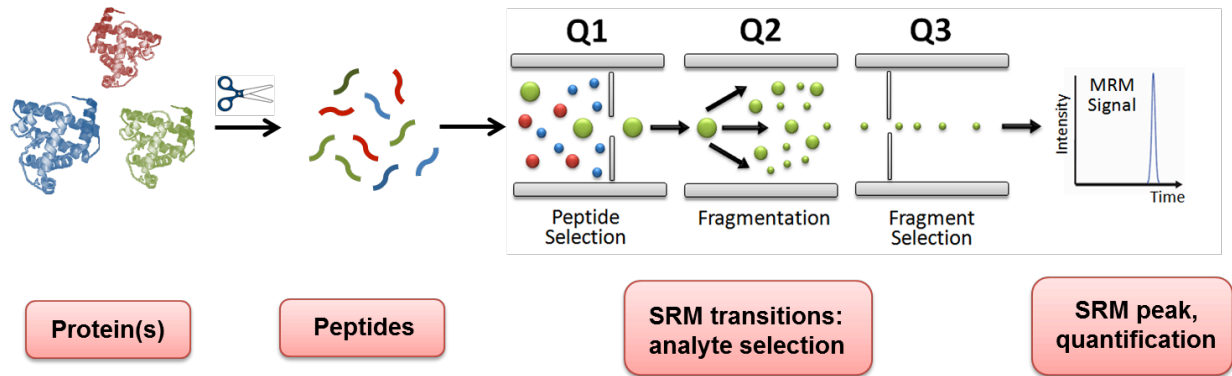
This has been attempted, but not accomplished, by experimental approaches based on affinity reagents, like antibodies<sup>2</sup>, which are able to specifically bind and eventually extract proteins of interest from complex mixtures. Multiple versions of antibody-based methods have been implemented. However, irrespective of the format used, the development of suitable antibodies remains challenging, expensive and arduous. The methods based on antibodies are therefore limited by slow assay development and, in

many cases, also by the inability to significantly multiplex detection of proteins in the same sample.

More recently, mass spectrometry based proteomic methods (shotgun proteomics) have been proposed as a surrogate to antibodies to quantify proteins in complex matrices<sup>3</sup>. However, these methods stochastically sample a fraction of the proteome that is usually biased towards the highest abundant proteins<sup>4</sup> and are characterized by a low reproducibility, which precludes the generation of complete datasets for the proteins of interest. In addition, such studies carry a significant experimental and computational overhead, and are therefore time/labor consuming and can be performed only in highly specialized laboratories. This makes such approaches impractical in cases that require the consistent quantification of sets of proteins of all abundances across different samples.

### **Selected reaction monitoring for targeted protein analysis**

To alleviate the limitations described above, during my post-doctoral work I developed an alternative, targeted, proteomic workflow based on a mass spectrometric technique called selected reaction monitoring (SRM, plural MRM)<sup>5, 6</sup> and performed on triple quadrupole mass spectrometers (Fig. 2). The essence of the approach, (Fig. 2), is the generation of specific, quantitative mass spectrometric assays for each analyte of interest and their subsequent application to the quantification of the analyte across multiple samples. First we developed a workflow to adapt the technique to the analysis of proteins, as follows. For each protein of interest proteotypic peptides (PTPs<sup>7</sup>) are selected and, for each PTP, a set of mass spectrometric coordinates are established, that characterize the PTP. These relationships are termed SRM or MRM transitions, and effectively constitute MS assays that identify and quantify a specific peptide and, by inference, the corresponding protein, in a complex protein digests. SRM data are acquired by setting the two mass analyzers of a triple-quadrupole mass spectrometer to predefined *mass-to-charge* ( $m/z$ ) values, corresponding to the multiply protonated ion and a specific fragment ion of the target peptide. The two-level mass filtering drastically increases selectivity, while the non-scanning nature of the technique accounts for a much higher sensitivity compared to conventional proteomic techniques. SRM assays are akin to sets of antibodies for ELISA or Western blot assays<sup>2</sup>, but with the advantage that they are multiplexed (many proteins can be analyzed within the same analysis) and quantitatively accurate. In addition, they can distinguish different protein isoforms.

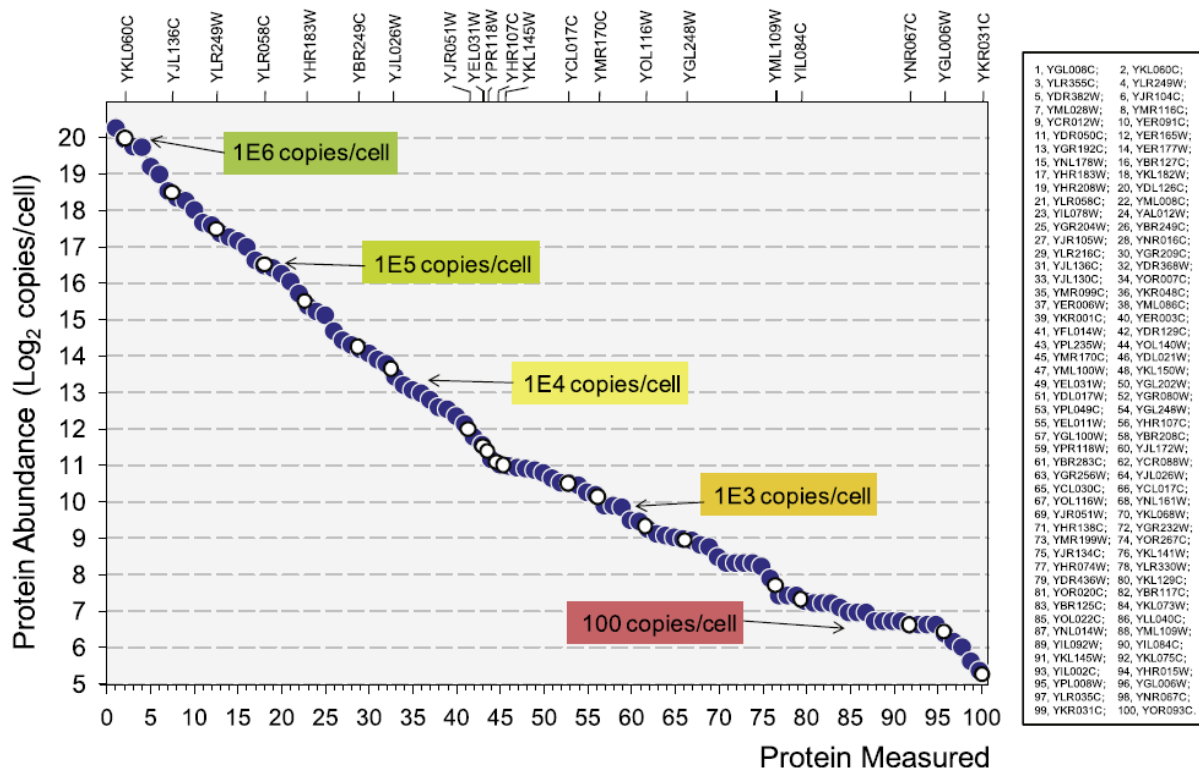


**Figure 2. Stages of an SRM experiment.** A protein sample is digested into peptides using a specific enzyme. Peptides are measured in a triple-quadrupole mass spectrometer. Molecular ions of a peptide of interest are selected in the Q1 analyzer and fragmented in a collision cell (q2). A fragment ion from the target peptide is selected in Q3, guided to the detector and counted over time, resulting in an SRM peak. The area of the SRM peak is proportional to the amount of target protein present in the sample.

### Sensitivity of SRM-based targeted proteomics

We probed the sensitivity of our SRM-based approach by applying it to the detection and quantification of proteins distributed across the whole range of cellular abundances, with the purpose of determining to what concentration (in copies per cell) proteins can be detected in a total cell extract. We used protein extracts from *S. cerevisiae* cells and applied a set of quantitative SRM assays for 100 proteins covering the whole range of cellular abundances (Fig. 3)<sup>6</sup>. We could demonstrate that:

1. SRM allows for a higher sensitivity compared to other mass spectrometry based proteomic techniques and is quantitatively accurate (average coefficient of variation < 20%)
2. The approach has the power to detect and quantify proteins expressed across the whole range of cellular abundances, down to a cellular concentration below 50 copies per cell;
3. Proteins can be measured in total cell lysates, without the need for sample fractionation or enrichment, making the technique fast and practical;
4. The technique is highly multiplexed, supporting the quantification of more than 100 different proteins, deliberately chosen, in a single (1 hour) analysis;
5. The technique allows to detect proteins which have been undetectable by classical mass spectrometry or antibody-based methods.



**Figure 3. Sensitivity of the SRM approach.** A set of proteins covering a broad range of abundances was detected (close circles) and quantified (open circles) using SRM assays. The experiment demonstrated the capability of SRM to measure proteins with abundances from over a million to below 50 copies per cell in total cell extracts, without the need for sample fractionation or enrichment.

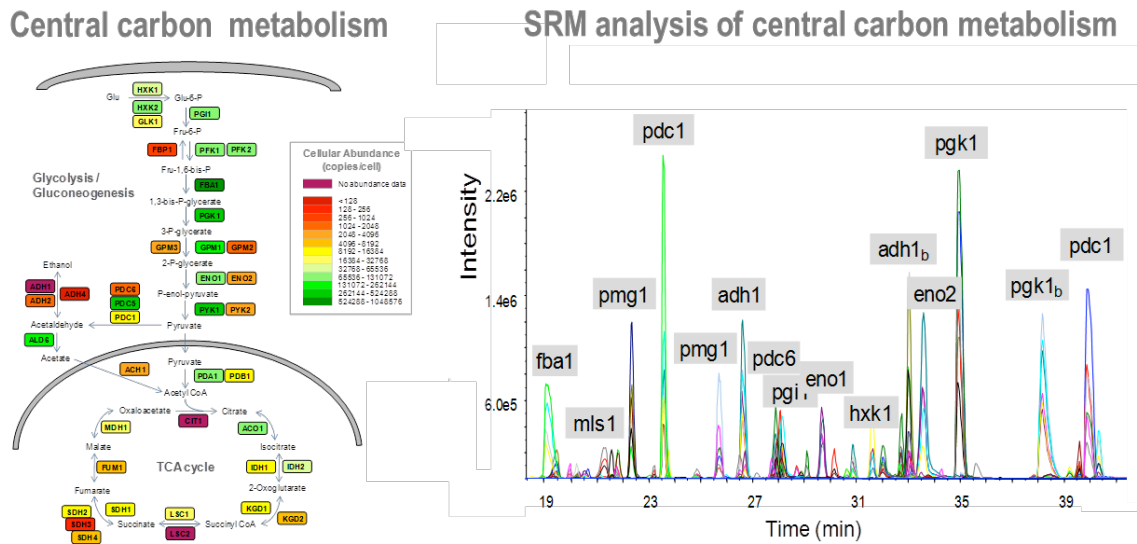
Overall these achievements demonstrated that SRM allows to comprehensively monitor complete protein networks in a 1-hour mass spectrometric run and thus to analyze in a reasonable time the effects of multiple perturbations on the protein system under study. In addition, we demonstrated the potential of SRM-based proteomics to provide assays for the consistent and reproducible detection and quantification of any set of proteins of interest in yeast cells.

### Application of SRM to the study of cellular metabolism

The previously described technical achievements prompted us to deploy the power of the technique for the analysis of crucial networks of proteins in cells, involved in nutrient metabolism. In a first application we measured proteins involved in the central carbon metabolism of *S. cerevisiae* (Fig. 4). We studied sugar metabolism, over a complete dynamic time-course of *S. cerevisiae* growth in glucose-rich medium to stationary phase, and throughout a series of different metabolic phases<sup>6</sup>. This resulted in the most complete quantitative dataset to date describing the responses of each metabolic protein to the series of events occurring during glucose-based growth, at high temporal resolution (Fig. 5). The

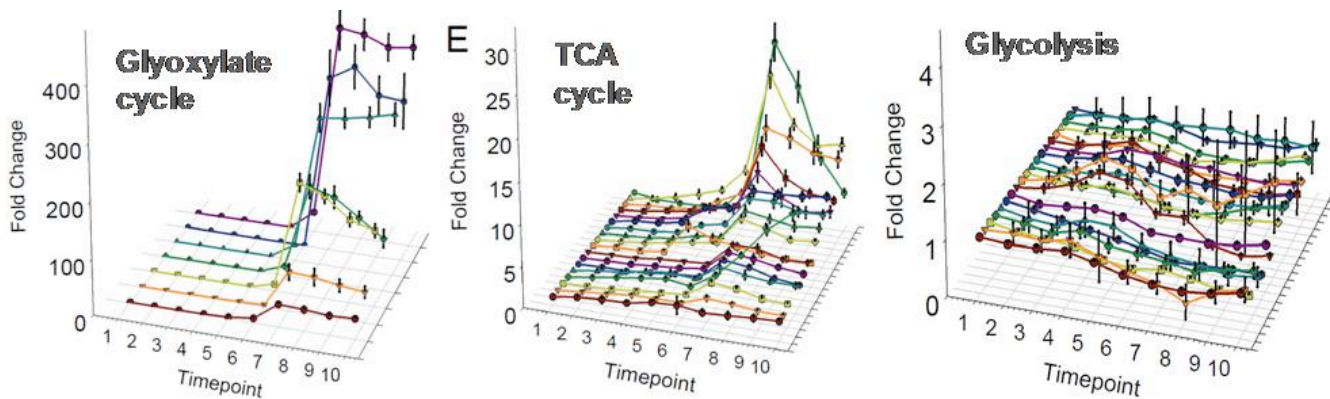


dataset provided a detailed picture of how carbon metabolism adapts to changing conditions of supply and demand of nutrients and an ideal framework for the advancement of mathematical models of metabolism during the diauxic shift in *S. cerevisiae*.



**Figure 4. SRM as a tool to study cellular pathways in biology.** *Left* - The ensemble of reactions constituting central carbon metabolism in (yeast) cells. Each reaction is controlled by at least one protein (enzyme). *Right* - A global SRM assay, quantifying all enzymes involved in carbon metabolism in 1-hour of instrument time, from a whole cell extract.

Next, in a collaboration with the group of Prof. Uwe Sauer, (ETH Zurich), we extended these measurements from central carbon metabolism to the whole metabolic proteome in yeast. We quantified metabolic proteins from different pathways through conditions inducing radically different metabolic setups. These dataset, interpreted through flux balance modeling, indicates that cells express superfluous proteins, not necessarily used in a particular metabolic condition. Further, the data allowed suggesting differential functionality for several metabolic isoenzymes<sup>8</sup>.



**Figure 5. The results of a typical SRM measurement.** The panels show the quantification of a set of metabolic enzymes throughout a time course of yeast growth in a glucose-based medium. Yeast cells experience exhaustion of glucose at time point 7 and switch to an ethanol-based metabolism (diauxic shift). The results are displayed as clusters of co-regulated proteins.

### A publicly accessible database of SRM assays

The development of these sets of SRM assays inspired me to develop a publicly accessible database that supports the collection, organization and dissemination of the developed assays. Once generated the SRM/MRM assays are universally useful and exportable. This resulted in the construction of the SRMatlas<sup>9</sup> (Fig. 6), a web-based resource to store the final coordinates of SRM assays and share them within the community (currently accessed > 25 times/day). The mass spectrometric coordinates required to reproduce the assays can be downloaded in a spreadsheet format and directly used to quantify proteins, in any sample of interest and in any laboratory equipped with a triple-quadrupole mass spectrometer.



**Figure 6. The SRMatlas.** Home page of the SRMatlas database. The database allows to download mass spectrometric assays to quantify target proteins of interest in biological or clinical samples.

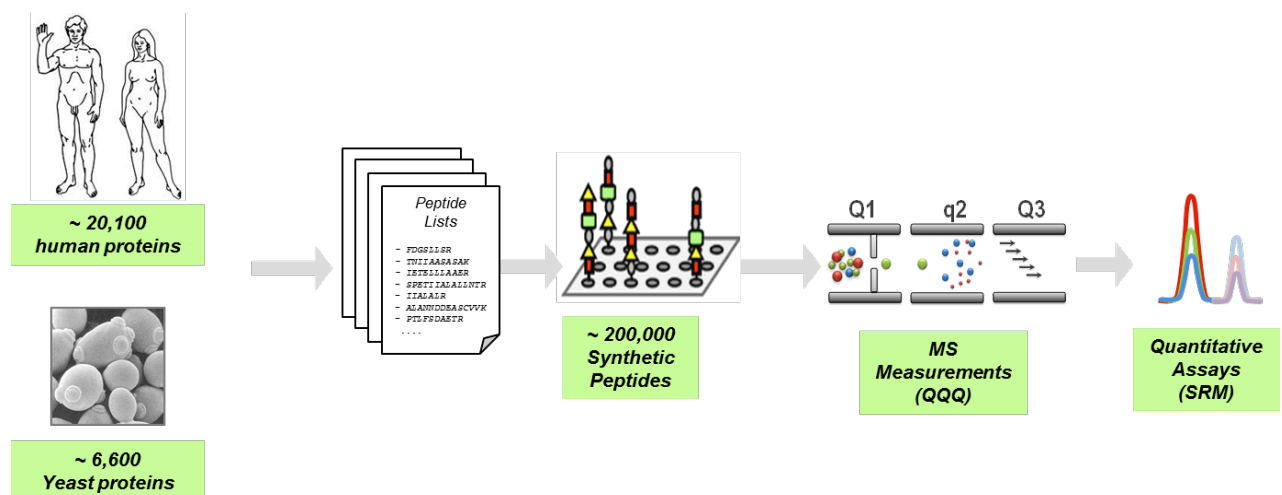
### Extension of SRM to complete proteomes

When the SRMatlas was generated, the effort required to design an MRM assay for a protein prevented us from completing the coverage of a whole proteome. The assay development process is based on a lengthy series of operations (e.g selection of appropriate fragment ions, optimization of MS parameters, etc) and it is based on a trial

and error approach. This can result in several days required to establish an assay for a single protein. To overcome this limitation and accelerate SRM assay generation, we developed a strategy based on crude, synthetic peptide libraries. It allows establishing assays at dramatically higher throughput compared to developing antibody-based assays (>100 assays can be generated in 1 hour). This technical breakthrough<sup>10</sup> enabled, for the first time, developing quantitative assays for a complete proteome. Therefore, using a set of ~30,000 synthetic PTPs, we could develop SRM assays for all putative proteins (>6,000, Fig. 7) in the *S. cerevisiae* proteome. This includes the ~2,000 yeast theoretical protein sequences for which there is still no experimental evidence. Akin to a complete library of antibodies for all yeast proteins, such SRM assays are an extremely powerful resource and will enable researchers to target the detection and quantification of any *S. cerevisiae* protein in cells grown under conditions of interest.

Similarly, we expanded the approach to the generation of SRM assays for 90% of the human proteome, using a set of 170,000 synthetic peptides (Fig. 7). These assays will soon be made publicly available through the SRMAtlas database (paper under evaluation). Such a resource will be a valuable tool in clinical research to facilitate the process of discovery and validation of novel disease biomarkers. The power of human protein SRM assays is demonstrated by a recent study, where SRM assays were used to measure ~50 candidate biomarker proteins for prostate cancer, in the blood of ~150 individuals. The study identified a 4-protein signature which, combined with the existing marker for prostate cancer PSA, increases both sensitivity and specificity of prostate cancer detection<sup>11</sup>.

The approach and the publicly accessible resources described above open new exciting avenues for the quantification of proteins in the context of biological and clinical research.



**Figure 7. Development of SRM assays for complete proteomes.** Using a library of ~200,000 crude synthetic peptides we generated assays for 90% of human proteins and 98% of the proteome of *S. cerevisiae*, a prototypical, commonly used microorganism in biological research.



## Acknowledgements

I am grateful to the International Latsis Foundation for encouraging and supporting young promising scientists. I would like to thank the Research Commission of ETH Zuerich for the interest in my work. Last, but actually first, I wish to thank my post-doctoral supervisor Prof. Ruedi Aebersold for being an outstanding mentor and a model for my growth as a scientist and as a person.

## References

1. Anderson, N.L. & Anderson, N.G. The human plasma proteome: history, character, and diagnostic prospects. *Molecular & cellular proteomics : MCP* **1**, 845-867 (2002).
2. Uhlen, M. Affinity as a tool in life science. *Biotechniques* **44**, 649-654 (2008).
3. Aebersold, R. & Mann, M. Mass spectrometry-based proteomics. *Nature* **422**, 198-207 (2003).
4. Picotti, P., Aebersold, R. & Domon, B. The implications of proteolytic background for shotgun proteomics. *Molecular & cellular proteomics : MCP* **6**, 1589-1598 (2007).
5. Anderson, L. & Hunter, C.L. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Molecular & cellular proteomics : MCP* **5**, 573-588 (2006).
6. Picotti, P., Bodenmiller, B., Mueller, L.N., Domon, B. & Aebersold, R. Full dynamic range proteome analysis of *S. cerevisiae* by targeted proteomics. *Cell* **138**, 795-806 (2009).
7. Kuster, B., Schirle, M., Mallick, P. & Aebersold, R. Scoring proteomes with proteotypic peptide probes. *Nat Rev Mol Cell Biol* **6**, 577-583 (2005).
8. Costenoble, R. et al. Comprehensive quantitative analysis of central carbon and amino-acid metabolism in *Saccharomyces cerevisiae* under multiple conditions by targeted proteomics. *Molecular systems biology* **7**, 464 (2011).
9. Picotti, P. et al. A database of mass spectrometric assays for the yeast proteome. *Nature methods* **5**, 913-914 (2008).
10. Picotti, P. et al. High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. *Nature methods* **7**, 43-46 (2010).
11. Cima, I. et al. Cancer genetics-guided discovery of serum biomarker signatures for diagnosis and prognosis of prostate cancer. *Proc Natl Acad Sci U S A* **108**, 3342-3347 (2011).