Chapter 2

Quantitative Peptide and Protein Profiling by Mass Spectrometry

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Summary

Proteomics may be defined as the systematic analysis of proteins expressed in a given organism (Electrophoresis 16:1090–1094, 1995). Important technical innovations in mass spectrometry (MS), protein identification methods, and database annotation, over the past decade, now make it possible to routinely identify thousands of proteins in complex biological samples (Nature 422:198–207, 2003). However, to gain new insights regarding fundamental biological questions, accurate protein quantification is also required. In this chapter, we present methods for the biochemical separation of different cellular compartments, two-dimensional chromatographic separation of the constituent peptide populations, and the recently published *Spectral Counting Strategy*, a label-free MS-based protein quantification technology (Cell 125:173–186, 2006; Anal Chem 76:4193–4201, 2004; Mol Cell Proteomics 4:1487–1502, 2005; Cell 125:1003–1013, 2006; Methods 40:135–142, 2006; Anal Chem 77:6218–6224, 2005; J Proteome Res 5:2339–2347, 2006). Additionally, highly accurate protein quantification based on isotope dilution, describing the isotope coded protein label (ICPL) – method shall be explained in detail (Mol Cell Proteomics 5:1543–1558, 2006; Proteomics 5:4–15, 2005).

Key words: Proteomics, Profiling, Mass spectrometry, Quantification, Spectral counting, Isotope labeling, MudPIT, ICPL.

1. Introduction

Proteomics is the detection of proteins expressed in a given biological system (e.g., an organism, tissue, cell, organelle, or protein complex) *(1)*. With the availability of the genomic sequences of human and many eukaryotic and prokaryotic organisms, the goal of proteome research is the qualitative, quantitative and functional analysis of protein expression. The tool of choice for the detection of proteins in systems biology is the mass spectrometer (MS) *(2)*.

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The many technical innovations of mass spectrometers in recent years have allowed scientists to rapidly and systematically detect hundreds to thousands of proteins in complex biological samples. Especially, the *Mu*lti*d*imensional *P*rotein *I*dentification *T*echnology (MudPIT), an elegant technology pioneered by the laboratory of Dr. John Yates III, has significantly increased to number of proteins detected by shot-gun proteomics *(3, 4)*. However, to fully understand the biological processes accurate quantification of proteins is required *(5)*. In 1999, Gygi and colleagues have developed a new approach for accurate protein quantification within complex mixtures using stable isotope labeling of proteins *(6)*. The method has shown to overcome several drawbacks of 2-DE based studies usually carried out for differential proteome analysis. Since then, the strategy has become increasingly popular and several groups have adopted the principle of this powerful methodology to generate additional strategies with their own strength and weaknesses. To date, three different ways of stable isotope labeling of proteins/peptides are utilized, that is chemically *(6–10)*, metabolically *(11, 12)* or enzymatically *(13)*. Since all methods are based on the same principle, we will describe the basic workflow and general challenges of protein quantification by stable isotope labeling on the basis of the recently developed isotope coded protein label (ICPL) approach *(7)*. ICPL is based on stable isotope tagging at the frequent free amino groups of isolated intact proteins and is therefore applicable to any protein sample, including membrane proteins, tissues extracts or body fluids. The following chapter we describe some of the recent developments in quantitative MS-based proteomics. The main focus will be on the detailed description of two alternative quantitative proteomics technologies (1) *Spectral counting* (SpC) in combination with MudPIT-based proteomics and (2) *ICPL* for quantitative proteomics with stable isotopes.

2. Materials

All materials used are of the highest quality. HPLC-grade solvents (water, methanol, acetonitrile) were from Fisher scientific. Proteomics grade enzymes (Endoproteinase Lys-C and trypsin) were obtained from Roche Diagnostics (Laval, QC, Canada). All standard proteins and *N*-(2-hydroxyethyl) piperazine-*N′*-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma. All solid chemicals were from Fluka and of the highest purity available. $^{12}C_6$- (light ICPL reagent), $^{13}C_6$-nicotinoyl succinimide (heavy ICPL reagent) were purchased from Serva (Serva, Heidelberg, Germany)/Bruker (Bruker Daltonics Inc., MA, USA).

a-Cyano-4-hydroxycinnamic acid (HCCA) was obtained from Bruker (Bremen, Germany). Tris-(hydroxymethyl) aminomethane (Tris) was purchased from Bio-Rad (Bio-Rad, Munich, Germany) and trifluoroacetic acid (TFA) was obtained from Applied Biosystems (Framingham, MA, USA).

2.1. MudPIT Analysis

1. Fused silica, 100 mm inner diameter (Polymicron Technologies, Phoenix, AZ).
2. P-2000 Laser puller (Sutter Instruments, Novato, CA).
3. Reversed phase beads – ZORBAX Eclipse XDB-C18 5 mm (Agilent Technologies, Mississauga, ON, Canada).
4. Partisphere strong cation exchange resin (Whatman, Clifton, NJ).
5. Pressure vessel – made in-house or commercially available from Proxeon Biosystems (Odense, Denmark) or Brechbuehler (Houston, TX).
6. OMIX solid phase extraction cartridges (Varian, Mississauga, ON, Canada).

2.2. HPLC Buffers for MudPIT Analysis

1. Buffer A: 95% water/5% acetonitrile/0.1% formic acid.
2. Buffer B: 80% acetonitrile/20%water/0.1% formic acid.
3. Buffer C: 500 mM ammonium acetate in Buffer A.

2.3. Tissue Homogenization and Protein Extraction

1. *Tissue homogenization buffer*: 250 mM sucrose, 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). DTT and PMSF are freshly added before every use.
2. *Sucrose cushion solution 1*: 0.9 M sucrose, 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF.
3. *Sucrose cushion solution 2*: 2.2 M sucrose, 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF.
4. *Nuclear extraction buffer 1*: 20 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.2 mM EDTA, and 25% glycerol.
5. *Nuclear extraction buffer 2*: 20 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.2 mM EDTA, 25% glycerol, and 1% Triton-X-100.
6. *Mitochondrial extraction buffer 1*: 10 mM HEPES, pH 7.8.
7. *Mitochondrial extraction buffer 2*: 20 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.2 mM EDTA, 25% glycerol, and 1% Triton-X-100.

2.4. Protein Digestion and Preparation for MudPIT Analysis

1. Approximately 150 μg of total protein extract in extraction buffer.
2. Ice-cold biotechnology grade acetone.
3. 8M urea, 50 mM Tris–HCl, pH 8.5.

4. 50 mM ammonium carbonate, pH 8.5.
5. Stock solution of 100 mM $CaCl_2$.
6. Endoproteinase Lys-C (Roche Diagnostics).
7. Trypsin, recombinant, proteomics grade (Roche Diagnostics).
8. OMIX solid phase extraction cartridges (Varian, Mississauga, ON, Canada).

2.5. HPLC Buffers and MALDI-Matrix for ICPL Labeling

1. Loading buffer: 0.1% trifluoroacetic acid.
2. Buffer A: 0.05% trifluoroacetic acid.
3. Buffer B: 80% acetonitrile/20%water/0.04% trifluoroacetic acid.
4. Matrix buffer 1: 10 mg/mL HCCA in 50% acetonitril/0.1% trifluoroacetic acid.
5. Matrix buffer 2: 10 mM ammoniumdihydrogen phosphate in 50% acetonitril/0.1% trifluoroacetic acid.

2.6. Cell Culture and Membrane Protein Preparation

1. Complete medium (1 L): 250 g NaCl, 20 g $MgSO_4$, 3 g sodium citrate, 2 g KCI and 10 g Oxoid Bacteriological Peptone L 37 H (Colab Laboratories, Glenwood, IL).
2. Basal salt buffer: 4.3 M NaCl, 81 mM $MgSO_4$ and 27 mM KCl.
3. 10 and 60% sucrose solution.
4. 1 M and 0.5 M NaCl solution.
5. Chloroform.
6. Methanol.

2.7. Isotope Labeling

1. *Sample buffer*: 6 M guanidine HCl and 0.1 M HEPES, pH 8.5 ± 0.1*.
2. *Reduction buffer*: 0.2 M tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 0.1 M HEPES, pH 8.5 ± 0.1*.
3. *Alkylation buffer*: 0.4 M iodacetamide and 0.1 M HEPES, pH 8.5 ± 0.1*.
4. *Alkylation stop buffer*: 0.5 M *N*-acetyl-cysteine and 0.1 M HEPES, pH 8.5 ± 0.1*.
5. *Labeling solution* (ICPL light): 0.15 M *N*-^{12}C-6-nicotinoyl-NHS in DMSO.
6. *Labeling solution* (ICPL heavy): 0.15 M *N*-^{13}C-6-nicotinoyl-NHS in DMSO.
7. *Stop solution*: 1.5 M hydroxylamine hydrochloride.

* Use 0.1 M NaOH or 0.1 M HCl for pH adjustment.

2.8. Protein Digestion and Sample Preparation for Mass Spectrometry

1. Approximately 100 µg of total labeled protein extract in labeling buffer.
2. 50 mM Tris–HCl, pH 8.5.
3. Trypsin, recombinant, proteomics grade (Roche Diagnostics).
4. Reprosil-Pur 120 ODS, C18 particles (Dr. Maisch GmBH, Germany, Cat#:r13.93).

3. Methods

Traditionally, two-dimensional gel electrophoresis (2-DE) followed by silver staining and MS identification of a separated gel spot, was the method of choice for proteome profiling. However, 2-DE is biased against the detection of membrane proteins, and proteins with extremes in molecular weight and isoelectric point. In recent years, several groups have presented gel-free approaches to overcome some of these limitations. Multidimensional protein identification technology (a.k.a. MudPIT) pioneered by the laboratory of John Yates III – allows for the systematic identification of hundreds to thousands of proteins in complex mixtures *(3, 4)*. However, the extreme complexity of the proteome of higher mammals (several 100,000 proteins including post-translational modifications), pushes modern proteomics to its limits. Even high resolution technologies such as MudPIT are thus not capable of identifying every protein present. Sample fractionation and minimization of sample complexity is therefore extremely important *(14)*.

We describe, below, a basic protocol for the fractionation of mammalian tissue (House mouse; *Mus musculus*) into defined organelle fractions, and their preparation for MudPIT analysis. The quantitative estimation of protein abundance based on *Spectral Counting* (SpC) is discussed *(14, 15)*. Additionally, a basic protocol for the isolation, isotopic labeling and quantitative analysis of membrane proteins from *Halobacterium salinarium* and the use of MALDI-TOF-MS/MS analysis for data dependent MS-analysis is discussed *(16)*.

3.1. Tissue Homogenization and Organelle Fractionation

1. Mice are CO_2-asphyxiated and sacrificed. The tissue of interest is removed, carefully minced with a razorblade and washed three times with ice-cold PBS. Minced tissue samples are homogenized in ice-cold *tissue homogenization buffer* in a dounce homogenizer, with at least 15 strokes. All subsequent centrifugation steps are performed at 4°C. Tissue lysate is centrifuged for 15 min at 800 × *g*. The supernatant (crude cytoplasm) is subsequently used for the isolation of mitochondria,

membranes and cytosol. The pellet contains crude nuclei, which are further purified. The crude nuclear pellet is resuspended in tissue homogenization buffer, layered onto *sucrose cushion solution 1*, and centrifuged for 15 min at 1,000 × *g*. To further purify nuclei, the pellet is resuspended in 8 mL of *sucrose cushion solution 1* and carefully layered onto 4 mL of *sucrose cushion solution 2* in a 13-mL ultracentrifugation tube. The mixture is pelleted at 100,000 × *g* for 60 min using a Beckman SW40.1 rotor. The pellet containing purified nuclei is resuspended in *nuclear extraction buffer 1* and incubated on ice for 15 min. Nuclei are lysed by ten passages though an 18-gauge needle, followed by centrifugation at 13,000 rpm, 21,000×g for 30 min. The supernatant is *nuclear extract 1*. The pellet is resuspended in *nuclear extraction buffer 2*, incubated on ice for 30 min, and then centrifuged at 13,000 rpm for 30 min. The resulting supernatant is *nuclear extract 2*.

2. Mitochondria are isolated by centrifugation of the crude cytoplasmic fraction at 8,000 × *g* for 20 min. The supernatant is collected for the isolation of mixed membranes and cytosol. The mitochondrial pellet is washed twice in *tissue homogenization buffer*, resuspended in *mitochondrial extraction buffer 1*, and incubated on ice for 30 min, followed by brief sonication. The solution is centrifuged at 13,000 rpm, 21,000×g for 30, min and the supernatant collected as *mitochondrial extract 1*. The pellet is resuspended in *mitochondrial extraction buffer 2* and incubated on ice for 30 min, followed by centrifugation at 13,000 rpm for 30 min. The supernatant is collected as *mitochondrial extract 2*.
3. Finally, the mixed membrane fraction is isolated from the crude cytoplasmic supernatant by centrifugation at 100,000 × *g* for 60 min (Beckman SW40.1 rotor), and the resulting pellet extracted in *mitochondrial extraction buffer 2* for 30 min on ice. This preparation is spun at 13,000 rpm 21,000×g, for 30 min and the supernatant collected as *mixed membrane extract*. The supernatant from the final ultracentrifugation at 100,000 × *g* is considered the *cytosol* (*see* **Notes 1** and **2**).

3.2. Protein Digestion and Preparation for MudPIT Profiling

1. One hundred fifty micrograms of total protein are precipitated over night at –20°C with 5 volumes of ice-cold acetone.
2. The solution is centrifuged at 13,000 rpm, 21,000×*g* for 15 min at 4°C.
3. The protein pellet is washed once with 150 mL of ice-cold acetone.
4. The protein pellet is carefully solubilized in 8 M urea, 50 mM Tris–HCl, pH 8.5, 2 mM DTT (dithiothreitol) at 37°C for 30 min.
5. IAA (iodoaceteamide) is added to a final concentration of 8 mM and the solution is incubated in the dark at 37°C for 30 min.

6. The sample is diluted to 4 M urea with 100 mM ammonium bicarbonate, pH 8.5 and digested with endoproteinase Lys-C (ratio 1:150) at 37°C for 6 h.
7. The digestion mixture is further diluted to 2 M urea with 50 mM ammonium bicarbonate, pH 8.5, $CaCl_2$ is added to a final concentration of 1 mM and the solution digested with trypsin (ratio 1:150) at 37°C over night.
8. The resulting peptide mixture is solid phase extracted with Varian OMIX cartridges according to the manufacturer's instructions and stored at –80°C until further use.

3.3. MudPIT Analysis

1. A fully automated MudPIT is used to analyze each sample, as previously described *(14, 15)*. Each MudPIT consists of several independent chromatography separation steps that form a sequence. Fused silica microcapillary columns (i.d. 100 mm) are pulled to a fine tip (~5–10 mm) using a Sutter P-2000 laser puller. The columns are custom packed with ~10 cm ZORBAX Eclipse XDB-C18 5 mm, followed by ~6 cm Partisphere SCX resin using a pressure vessel. Samples are loaded onto the column using the same pressure vessel and placed in-line with a capillary HPLC system (Agilent 1100 series or Thermo Finnigan Surveyor). The HPLC pumps are operated at a constant flow rate of 100 mL/min with a pre-column flow splitter. The effective flow rate at the column is ~200–400 nL/min. Peptides are directly eluted into the mass spectrometer. We use the Thermo Finnigan LTQ linear ion-trap equipped with a Proxeon Biosystems Nano Electrospray Ion Source. Chromatographic elution profiles and individual salt concentration steps for a typical MudPIT analysis are shown below **(Table 1)** (*see* **Note 3**).
2. The mass spectrometer is operated with the following settings.
   (a) Distal spray voltage: 2.3 kV.
   (b) Full scan mass spectrum: 400–1,400 *m/z*.
   (c) Six data-dependent MS/MS scans at a 35% normalized collision energy.

3.4. Sequest Searches and Validation by the STATQUEST Algorithm

1. MS data is searched using the Sequest algorithm, against publicly available protein sequence databases. We use the SwissProtein/TrEMBL (http://ca.expasy.org/) and IPI (http://www.ebi.ac.uk/IPI/IPIhelp.html) databases. To objectively estimate our false positive rate, we use the "target/decoy" database strategy *(14, 17)*, in which every protein sequence in the native "target" protein sequence database is reversed to generate a "decoy" database. Briefly, the number "decoy" proteins appearing in a list of protein identifications will provide a rough estimate of the false positive rate.

Table 1
Elution profiles

Time (min)	Buffer A (%)	Buffer B (%)	Buffer C (%)	Flow rate (mL/min)
0	100	0	0	100
2	100	0	0	100
2.01	90	0	10	100
7	90	0	10	100
7.01	100	0	0	100
12	100	0	0	100
12.01	95	5	0	100
85	30	70	0	100
88	100	0	0	100
90	100	0	0	100

Salt steps

Step	Buffer A (%)	Buffer B (%)	Buffer C (%)
1	100	0	0
2	90	0	10
3	80	0	20
4	70	0	30
5	65	0	35
6	60	0	40
7	50	0	50
8	45	0	55
9	40	0	60
10	20	0	80
11	0	0	100

2. Sequest search results are validated using an in-house probability-based algorithm termed STATQUEST *(14)*. This computer tool automatically assigns a defined percentage likelihood of correct peptide identification to every Sequest search result. For LTQ data we only accept peptide identifications with a confidence interval of ³99%.
3. As MudPIT profiling generates very large data files, we recommend parsing all search results and their associated Sequest and STATQUEST scores into a relational database. This step significantly speeds up subsequent data analysis.

3.5. Quantitative Proteomics Based on SpC

In principal, semi-quantitative proteomics based on SpC is a simple and straightforward methodology. It is important, however, that only spectra matching to peptides identified with high confidence (e.g., based on STATQUEST *(14)* or PeptideProphet *(18)*) are considered for comparison.

1. The number of peptides (or spectra) confidently identified and matched to a protein in the sequence database are used for quantitative comparison.
2. The number of spectra matching the same protein in different samples is compared.
3. In **Fig. 1** we present the data from a recently published manuscript *(15)*. Proteins identified in several subcellular fractions isolated from several healthy mouse tissues are clustered based on their SpC values (**Fig. 1a**). Western blotting results against specific cellular markers and their associated SpC values are presented (**Fig. 1b**). The two types of data are highly correlated (*see* **Note 4**).

3.6. ICPL Workflow

The complete workflow is shown in **Fig. 2** as recently published *(16)*. Membrane proteins obtained from aerobically and phototrophically grown cells, respectively, were first individually reduced, alkylated and labeled with either the "*light*" or "*heavy*" version of the ICPL reagent. After combining both mixtures, proteins were cleaved into peptides using trypsin and separated by nano-reversed phase HPLC. The eluting peptides were mixed with MALDI-matrix and directly spotted onto MALDI plates for data dependent MS/MS analysis. Since peptides of identical sequence derived from the two differentially labeled protein samples differ in mass. They appear as doublets in the acquired MS-spectra. From the ratios of the ion intensities of these sister peptide pairs, the relative abundance of their parent proteins in the original samples can be determined. Subsequently, isotopic peptide pairs that differ in abundance were automatically selected for collision-induced dissociation (CID) and identified by correlation with sequence databases using the MASCOT search algorithms *(19)*.

3.7. Bacterial Strain and Growth Conditions

Halobacterium salinarum (strain R1, DSM 671) was grown in complete medium *(20)* as previously described *(21)*. Briefly, for preparation of a starter culture, Halobacterium was grown aerobically in the dark at 37°C in 1 L of complete medium to the stationary phase. For protein preparation, Halobacterium was grown through three successive transfers to ensure uniform cell state. For the first two transfers, 35 mL of fresh medium was inoculated with 1 mL of the previous culture, for the third transfer 1 L medium in a 2 L flask was inoculated with 35 mL of the previous culture. The cells were grown to late log-phase (30–40 Klett units), either aerobically in the dark or phototrophically

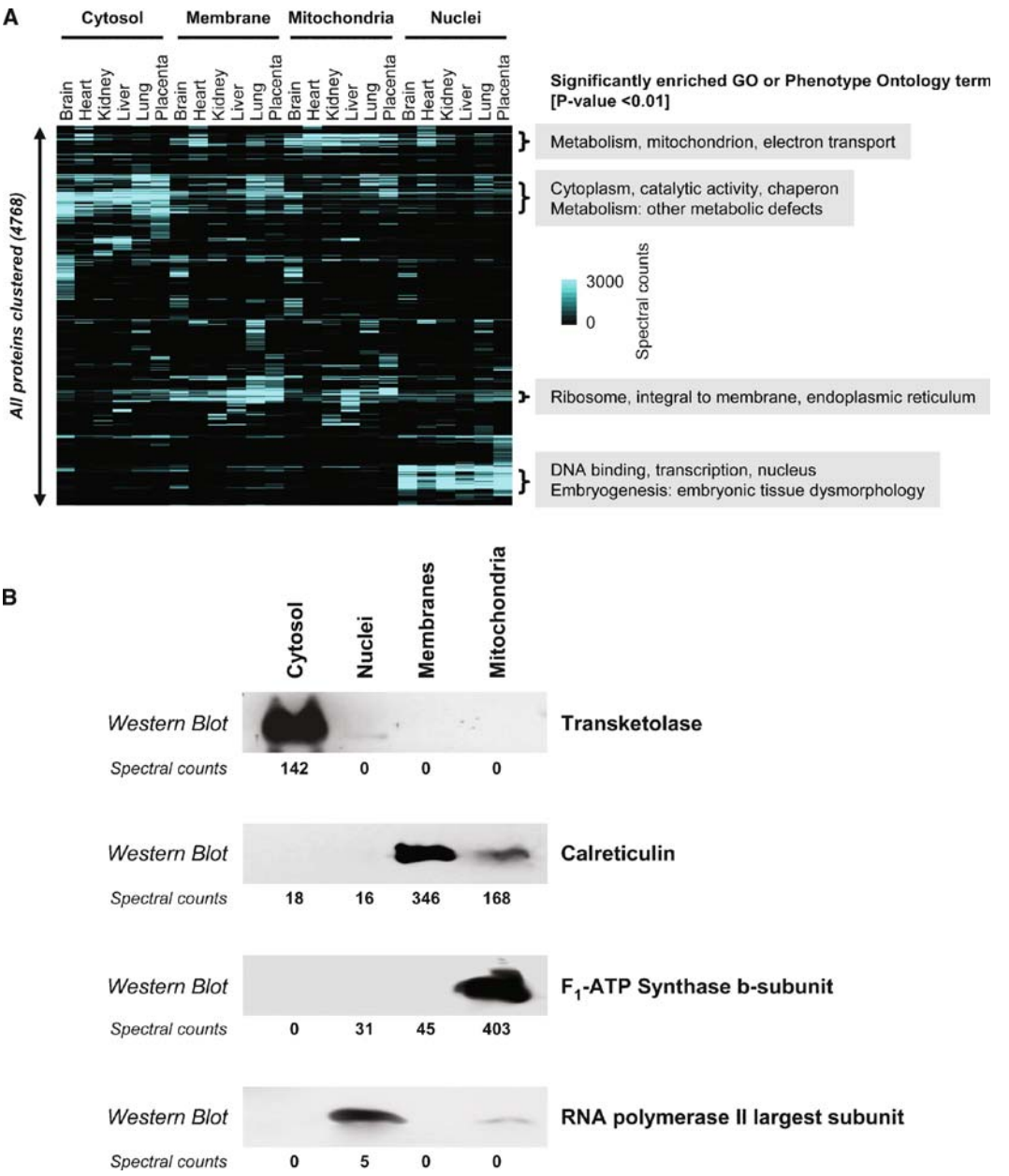

Fig. 1. Quantitative proteomics by SpC. (a) Confidently identified proteins are clustered based on SpC. (b) Selected marker proteins are shown by Western blotting together with their corresponding SpC.

with light as energy source. For the latter, flasks were closed after inoculation so that residual oxygen was consumed and growth continued under anaerobic conditions.

3.8. Membrane Protein Preparation

Two liters of cell culture were centrifuged for 50 min at 4,000 × *g* and cells were resuspended in 40 mL Basal salt buffer before cell

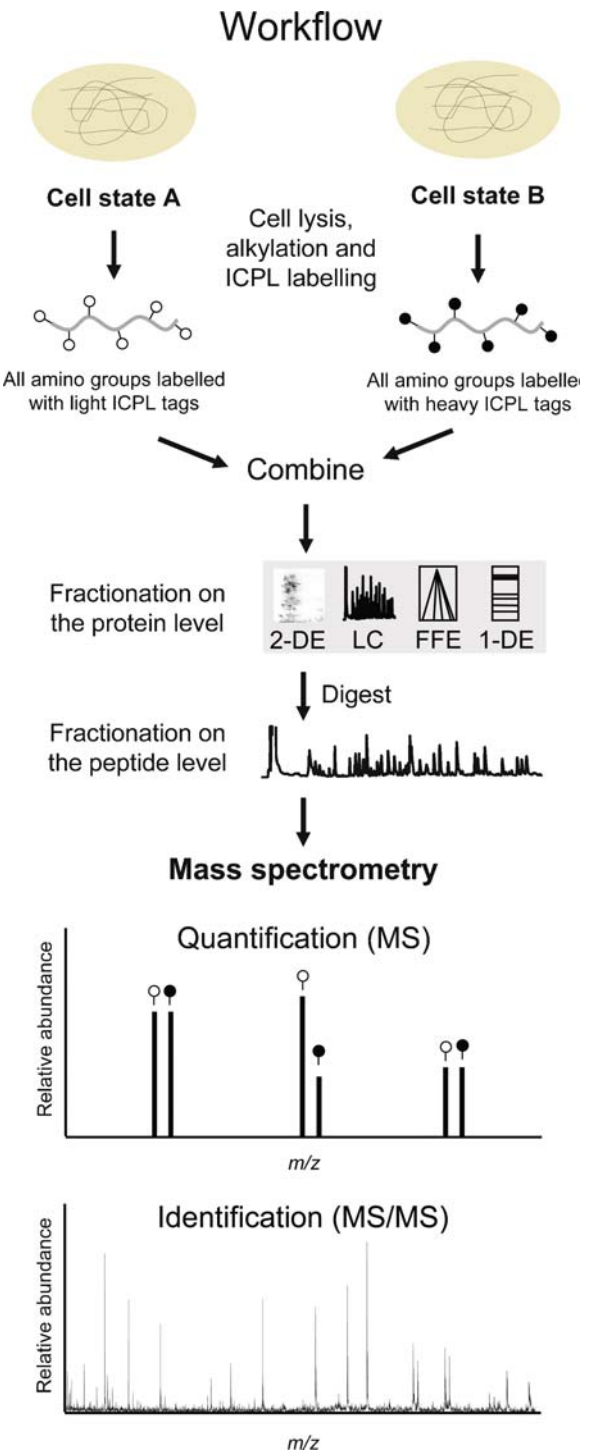

Fig. 2. Quantitative proteomics by ICPL. Protein extracts are labeled with either the "light" or the "heavy" version of the ICPL label. Protein extracts are combined, fractionated and analyzed by MS. Quantitation is achieved by comparing the relative intensity of each peptide pair in MS-mode.

rupture by sonication (3 × 1 min on ice, 50% Duty Cycle, Branson Sonicator). Solid debris was removed by a short centrifugation step (5,800 rpm, 10 min, 10°C, 4,000 × *g*). The vesicles were centrifuged (30,000 rpm, 1 h, 4°C, 65,000 × *g*), the pellet was resuspended in 2 mL Basal salt buffer, layered over a linear sucrose density gradient (10–60% sucrose in Basal salt buffer (w/w)) and centrifuged for 14 h (25,000 rpm, 4°C, 80,000 × *g*). The colored vesicle band was collected and sucrose was removed by dilution with 1 M NaCl and pelleting of vesicles by centrifugation. This step was repeated with 500 mM NaCl and the final pellet was resuspended in 2 mL H_2O. The membranes were delipidated with chloroform/methanol as described *(22)* and precipitated proteins were subsequently lyophilized (*see* **Notes 5** and **6**).

3.9. Isotope Labeling

One hundred micrograms of total protein obtained from aerobically and phototrophically growing cells are dissolved in 20 mL Sample buffer, respectively, and if necessary, the pH is adjusted to 8.5 ± 0.1 using 1 M NaOH or HCl.

3.9.1. Alkylation

Both samples are equally processed.

1. 0.5 mL of *Reduction buffer* is added and the solution kept at 60°C for 30 min.
2. After cooling and sinning down, 0.5 mL of *Alkylation buffer* is added and the solution is incubated in the dark at 25°C for 30 min.
3. The alkylation is stopped by adding 0.5 µL of *Alkylation stop buffer* and incubated for another 15 min at room temperature.

3.9.2. Isotope Labeling

1. Three microliters of *Labeling solution* light are added to aerobically grown sample and 3 mL of *Labeling solution* heavy are added to the phototrophically grown sample and the mixtures are incubated for 2 h at 25°C.
2. Then, 2 µL of Stop solution are added to each sample.
3. After 15 min at room temperature, the two samples are combined.
4. The pH is adjusted to 11.9–12.0 by adding 2 M NaOH and incubated for 20 min at 25°C.
5. Then, an equimolar amount of HCl is added to the sample to lower the pH to its original value (*see* **Notes 7** and **8**).

3.10. Sample Digestion and Preparation for LC-MS

1. The sample was diluted with 25 mM Tris (pH 8.5) to a final guanidine HCl concentration of 0.5 M and digested overnight at 37°C with trypsin (substrate-to-enzyme ratio = 50:1) (*see* **Note 9**).
2. The resulting peptide mixture was acidified with 10 mL 1% TFA and the volume reduced by evaporation to approximately 30 µL.

3. The resulting peptide mixture was solid phase extracted using self-made C18-columns packed with Reprosil-Pur 120 ODS, C18 particles (Dr. Maisch GmBH, Germany, Cat#:r13.93) as described *(23)*, dried under vacuum and stored at –80°C until further use.

3.11. LC-MALDI-TOF/TOF Analysis

1. All peptide separations were performed utilizing a capillary liquid chromatography system (Ultimate, LC Packings) containing a reversed-phase column (LC Packings Pepmap reversed-phase C18 column, 75 mm i.d., 15 cm) coupled directly online with a MALDI target spotter (Probot, LC Packings). A sample volume of 50 mL was injected and the peptides were trapped on a short reversed-phase column (300 mm i.d., 5 mm) using Loading buffer at a flow rate of 20 mL/min. For the separation of the peptides, a 65 min linear gradient from 10 to 45% B at a flow rate of 200 nL/min was used, followed by a 20 min wash step of the column with 100% B. The analytical column was directly connected to a MicroTee (Upchurch, Oak Harbor, WA) where the eluent was mixed with MALDI matrix solution (freshly prepared 1:1 mixture of Matrix buffer 1 and 2) at a flow rate of 1.3 µL/min and deposited onto a blank MALDI plate. The LC-eluent was automatically spotted in 10 s fractions over a time period of 66.66 min resulting in 400 spots per MALDI target plate. The sample spots were allowed to dry at room temperature.
2. The mass spectrometer is operated with the following settings.
   (a) 2,500 laser shots for each MS spectrum and 1,500 shots for each MS/MS spectrum.
   (b) Deflection cut off range was 700 *m/z*.
   (c) Focus mass was 2,100 *m/z*.
   (d) CID spectra were acquired using collision energy of 1 keV and nitrogen as collision gas.
3. MS/MS-data acquisition was done in a data dependent manner. First, MS-spectra of each LC-fraction were acquired. Then, isotopic labeled peptide pairs were automatically detected and quantified using the Peakpicker software (Applied Biosystems). To keep the analysis time as low as possible, only differentially regulated peptide pairs and from these only the more intense MS-peak were selected for MS-sequencing (*see* **Note 10**).

3.12. Data Analysis

1. All MS/MS-spectra obtained were searched against the Halobacterium protein sequence database that was exported from the HaloLex database (http://www.halolex.mpg.de) *(24)*, using an in house version of Mascot *(19)* in combination with the GPS-Explorer™ 2.0 software (Applied Biosystems). For the

database search, carbamidomethylation was set as a required cysteine modification, whereas oxidation of methionine was considered as a variable modification. Further potential modifications include $^{12}C_6$- and $^{13}C_6$-nicotinoylation of lysine and the protein N terminus. It is important to note that trypsin does not cleave the labeled lysines. Therefore, the enzyme Arg-C should be selected for database searching (*see* **Note 9**).

2. To objectively estimate our false positive rate, we use the "target/decoy" database strategy *(25)*, in which every protein sequence in the native "target" protein sequence database is reversed to generate a "decoy" database. Thereby, the false positive rate was estimated to be below 2%.

3.13. Calculation of Peptide Ratios

The ratio for each peptide pair was calculated using the program Peakpicker (Applied Biosystems). Ratios for each protein were determined by averaging all quantified peptides of one protein utilizing the DecisionSite 8.0 software (Spotfire AB, Goeteborg, Sweden) on the basis of the raw data. The median of the complete set of quantified peptides was determined and used for a computational normalization of the original ratios. Finally, regulation factors were computed for each protein such that the same extent of positive or negative regulation results in an identical absolute value of the regulation factor. To provide symmetric regulation factors these ratios were inversed and multiplied by –1. This scale excludes any values between 1 and –1.

The application of the ICPL methodology to the analysis of highly purified membranes of the halophilic archaeon *Halobacterium salinarum* resulted in the accurate quantification of over 150 membrane proteins *(16)*. Importantly, the comparison of the ICPL results to DIGE labeling in combination with an improved two-dimensional 16-BAC/SDS–PAGE procedure showed excellent correlation between both complementary technologies. In a proof-of-principle experiment two different growth conditions (aerobic vs. anaerobic/phototrophic) were compared by quantitative proteomics. Several differentially regulated proteins involved in photosynthesis and energy metabolism could be detected.

3.14. Conclusions

Advanced MS-based proteomics and allied bioinformatics tools enable biologists to confidently identify hundreds to thousands of proteins in complex biological samples. Direct quantitative comparison of different conditions (e.g., disease or development) on a global scale is very important to fully understand biological processes and mechanisms in a non-hypothesis driven manner. Several methodologies have been developed in recent years to accomplish this goal, possibly the simplest approach being SpC. Isotope labeling can provide significantly more reliable and accurate quantification over label-free quantitative approaches, but

the ongoing progress in both, instrumentation and software, will further increase the quality of such approaches. Therefore, it is to be expected that label-free quantification of proteins using methods based on spectral counting or MS-signal intensities in concert with their simple workflows and low costs will become widely used in the near future. The applications of these state-of-the-art technologies to diverse biological settings provide a unique opportunity for a more complete biological understanding.

4. Notes

1. The isolation of pure organelles is a challenging task, if not impossible. The protocol presented above was applied in our lab and works well with MS-based analysis. It provides reasonably clean or "enriched" organelle fractions, although some cross-contamination between the mixed membrane and mitochondrial fractions was observed. Nevertheless, one should always consider the possibility that some proteins are actually present in more than one cellular location, even if annotation is only available for one subcellular fraction.
2. Organelles should only be isolated from fresh tissues and cells.
3. The extreme complexity of mammalian proteome samples is daunting. Even high resolution procedures such as MudPIT are unable to detect every peptide present. The process is further complicated by the skewed range of overall protein abundance, with some proteins being present in very high abundance, and most proteins present in lower abundance. The detection of these "low abundance" proteins is like "finding a needle in a haystack." Several strategies for the enrichment of low abundance peptides have been presented in the scientific literature *(26–28)*.
   (a) Sample fractionation prior to MS analysis
      (i) Organelle fractionation
      (ii) Biochemical fractionations (e.g., ion exchange chromatography)
   (b) Repeat analysis of the same sample also increases the overall detection depth. We highly recommend analyzing several technical replicates: "random sampling effect" *(29)*.
4. Quantitative comparisons based on SpC are new and relatively unproven. Although several papers were recently published suggesting good correlation between relative protein abundance and SpC *(15, 29–34)*. We suggest caution in interpretation of quantitative data based on SpC. This is especially true if

low spectral counts and small differences between samples are observed. We suggest validating results using alternative methodologies (e.g., Western blotting, if antibodies are available). Normalization strategies of SpC values and statistical approaches for the comparison have also been described recently. (*(30, 31, 34, 38)*. Spectral counting is further complicated by the "protein inference" problem, where confidently identified peptides are shared among different database entries. Programs such as *ProteinProphet (35)* or *Isoform Resolver (30)* can help in grouping these proteins.

5. To effectively remove guanidine HCl, a charged molecule that impairs sample preparation steps, and to concentrate your sample for 1D-SDS–PAGE, acetone precipitation can be used. Therefore, the ICPL-labeled sample solution is diluted 1:1 (v/v) with water followed by 5 volumes (based on the volume of sample and water) of ice-cold acetone. After incubation at –20°C over night, the sample is centrifuged at 14,000 rpm, 21,000 × *g* for 30 min at 4°C. The supernatant is removed, the pellet is gently washed once with a ice-cold solution of 80% acetone and the sample is once more centrifuged at 14,000 rpm at 4°C for 15 min. Finally, the supernatant is discarded and the protein pellet dried under vacuum. The sample can either be stored at –80°C or further processed.
6. However, the comprehensive precipitation of hydrophobic membrane proteins is a difficult task. In our experiment, a significantly higher number of membrane proteins was identified by skipping the precipitation step and by direct digestion of the sample in solution like described above.
7. The pH of the solution is very important to obtain a specific and complete modification of all amino groups. It should therefore be tested and, if required, adjusted before adding the ICPL-reagent.
8. The labeling procedure has no impact on protein phosphorylation sites *(7)*. Therefore, the ICPL-method can be combined with phosphopeptide enrichment strategies, like IMAC *(36)* or TiO_2 *(11)*, to quantify changes of protein phosphorylation.
9. Since the all lysines are blocked, trypsin does not cleave after this amino acid. As a result, the enzyme Arg-C must be selected for database searches and endoproteinase Lys-C cannot be used at all to digest ICPL-labeled proteins. However, database searches with trypsin selected, offer an effective opportunity to determine the ICPL-labeling efficiency because no lysine terminating peptides should be identified, if all amino groups have been modified.

10. The number of modified lysines, can easily be calculated from the mass gap of each isotopic peptide pair, serves as a strong constraint for database searching and highly increases protein identification confidence of labeled peptides *(16)*.

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