Peptide separations by on-line MudPIT compared to isoelectric focusing in an off-gel format: Application to a membrane-enriched fraction from C2C12 mouse skeletal muscle cells

Sarah Elschenbroich^{1,2}, Vladimir Ignatchenko¹, Parveen Sharma^{3,4}, Gerold Schmitt-Ulms⁵, Anthony O. Gramolini⁴, and Thomas Kislinger^{1,6}

¹Ontario Cancer Institute, University Health Network, Canada

²Department of Chemistry and Pharmacy, Friedrich-Alexander University Erlangen, Germany

³Banting and Best Department of Medical Research, University of Toronto, Canada

⁴Department of Physiology, University of Toronto, Canada

⁵Department of Laboratory Medicine and Pathology, University of Toronto, Canada

⁶Department of Medical Biophysics, University of Toronto, Canada

Abstract

High resolution peptide separation is pivotal for successful shot-gun proteomics. The need for capable techniques propels invention and improvement of ever more sophisticated approaches. Recently, Agilent Technologies has introduced the OFFGEL fractionator, which conducts peptide separation by isoelectric focusing in an off-gel setup. This platform has been shown to accomplish high resolution of peptides for diverse sample types, yielding valuable advantages over comparable separation techniques. In this study, we deliver the first comparison of the newly emerging OFFGEL approach to the well-established on-line MudPIT platform. Samples from a membrane-enriched fraction isolated from murine C2C12 cells were subjected to replicate analysis by OFFGEL (12 fractions, pH 3 – 10) followed by RP-LC-MS/MS or 12-step on-line MudPIT. OFFGEL analyses yielded 1398 proteins (identified by 10,269 peptides) while 1428 proteins (11,078 peptides) were detected with the MudPIT approach. Thus, our data shows that both platforms produce highly comparable results in terms of protein/peptide identifications and reproducibility for the sample type analyzed. We achieve more accurate peptide focusing after OFFGEL fractionation with 88 % of all peptides binned to a single fraction, as compared to 61 % of peptides detected in only one step in MudPIT analyses. Our study suggests that both platforms are equally capable of high quality peptide separation of a sample with medium complexity, rendering them comparably valuable for comprehensive proteomic analyses.

Introduction

Despite considerable technical and methodological advances during the past years, proteomic coverage of biological samples remains far from being exhaustive. The fundamental limiting factor in global protein profiling – sample complexity – spurs development of powerful methods to negotiate this challenge. The widely applied shotgun proteomics approach attempts to tackle the problem by dislocating analysis to the peptide level, separating peptides in multiple dimensions followed by mass spectrometry. Acquired data is subsequently matched against a protein sequence database by sophisticated search algorithms^{1, 2}, resulting in reported protein identifications. Shot-gun proteomics has proven to be a valuable tool in expression profiling, protein localization mapping, protein complex characterization and post-translational modification studies^{3–8}.

A critical determinant for shot-gun based studies is the value of the MS data as accurate protein identifications rely on high quality spectra. With hundreds of thousands of peptides per sample though, – e. g. a typical serum sample is expected to contain an estimated 400,000 – 600,000 peptides – sufficient fractionation before MS is one of the bottlenecks of this platform. Poor peptide separation leads to problems in ionization and spectra acquisition and low abundance species are prone to undersampling^{9, 10}. Diverse techniques have been employed to enhance peptide fractionation prior to LC-MS/MS, encompassing strong-cation exchange, size-exclusion chromatography, isoelectric-focusing (IEF), and reversed-phase (RP) LC, the latter often directly connected to the subsequent mass spectrometric analysis^{11–18}.

Methods are continuously being refined and improved in an effort to achieve maximum protein coverage. For example, classical gel-enhanced IPG-IEF of peptides is a technique that calls for recovery of analytes from gels after separation, thus being prone to sample loss¹⁹. In contrast, emerging gel-free and off-gel approaches allow for sample recovery from solution. One of different commercial devices conducting off-gel electrophoresis is the 3100 OFFGEL fractionator recently introduced by Agilent Technologies²⁰, which was used in the following experiments.

The OFFGEL device is comprised of multiple (12 or 24) chambers, which are opened at top and bottom extremities. Chamber frames are placed on top of IPG gel strips, in which the IEF takes places. Samples (in buffered solution) are placed into the chambers and all analytes will be charged according to their *pI* and the pH imposed by the underlying IPG gel. An electric field is then applied between the two electrodes located on both extremities of the gel and analytes will migrate according to their charge. As all chambers are connected only by the underlying gel, molecules have to permeate into the IPG gel strip in order to migrate within the electric field. Once species reach the chamber in which the pH equals their *pI*, they will lose their charge and thus can be recovered in the solution of the appropriate chamber^{20, 21}.

OFFGEL has proven to be a competitive fractionation tool in shot-gun proteomics for different sample types like human plasma²², tissue culture cell-derived secretions²², *E. coli* lysates²⁰, and rat muscular tissue²³. Its compatibility with iTRAQ labelling for quantitative

proteomics has recently been shown²². So far, only few studies addressed comparisons of OFFGEL to other fractionation techniques^{19, 23}.

Currently, one of the most widely employed platforms in shot-gun proteomics is Multidimensional Protein Identification Technology (MudPIT), comprised of online-SCX followed by RP-LC-MS/MS^{5, 18, 24, 25}. Nevertheless, to the best of our knowledge, no systematic comparison to the recently introduced OFFGEL fractionation has been performed to date. The aim of this study is therefore an assessment of performance of these two approaches.

We chose a membrane-enriched fraction from murine C2C12 myoblasts for our study, representing a sub-proteome of particular analytical and biological interest. This class of proteins constitutes an estimated 20 – 30 % of the human genome and membrane proteins play important roles in diverse cellular processes. Moreover, approximately 70 % of all protein based drug targets are active against membrane proteins^{26, 27}. Despite their importance, membrane proteomes are relatively understudied as compared to other sub-proteomes. Low abundance, hydrophobicity and insolubility are the main analytical obstacles, leading to considerable discrepancies between predicted and detected numbers of membrane proteins, and must, for a method to be successful, both be overcome^{8, 28–31}. Especially, the traditional 2D-PAGE-based proteomics studies are in general biased against membrane proteins³². Shotgun proteomics is a common platform for membrane protein analyses, with MudPIT currently being one of the most valuable options^{31, 33–35}. This approach has been successfully applied to the targeted analysis of membrane proteins of diverse origin, e.g. rat nuclear envelope⁸, *C. glutamicum*³⁶, and lung endothelial cells³⁷ each resulting in high numbers of membrane protein identifications. We therefore posed the question if the newly emerging OFFGEL approach might represent a valuable alternative for the analysis of membrane-enriched samples.

Using silica-bead coating³⁷, we isolated membrane and membrane-associated proteins from murine C2C12 myoblasts, and subjected replicates of this membrane-enriched fraction to either OFFGEL fractionation into 12 fractions (pH 3 – 10) followed by RP-LC-MS/MS, or a fully automated 12-step MudPIT^{5, 18} analysis as previously reported³⁸. Resulting data was thoroughly evaluated to deliver a comprehensive method comparison.

Materials and Methods

Materials

Ultrapure grade iodoacetamide, dithiothreitol (DTT), formic acid, LUDOX-CL colloidal silica, nycodenz, sucrose, polyacrylic acid (PAA), 2-(N-morpholino)ethanesulfonic acid (MES) buffer were obtained from SIGMA, Canada. Ultrapure grade urea, ammonium acetate, calcium chloride, sodium chloride, potassium chloride, potassium phosphate monobasic and potassium phosphate dibasic, TRIS, HEPES and Triton-X-100 were from BioShop Canada Inc., Canada. Protease inhibitor cocktail was obtained from Roche Diagnostics, Canada. HPLC grade solvents (methanol, acetonitrile and water) were obtained from Fisher Scientific, Canada. Recombinant, proteomics grade trypsin was from Promega, Madison, WI. Trifluoroacetic acid (TFA) was purchased from J. T. Baker, Phillipsburg, NJ.

Silica-bead Coating of C2C12 Cells

Plasma membrane-enriched fractions from C2C12 myoblasts (ATCC, Cedarlane Laboratories Ltd., Hornby, ON), containing membrane and membrane-associated proteins, were isolated using a modified cationic silica-bead plasma membrane isolation procedure³⁷. Specifically, murine C2C12 myoblasts were grown in cell culture dishes until differentiation was microscopically visible by formation of contractile microtubules (>90 % confluency). All of the following steps were performed on ice. Cells were rinsed with MES-buffered saline (MBS; 25 mM MES, pH 6.5, and 150 mM NaCl) and incubated with a 1 % ice-cold silica-bead solution for 10 min to allow attachment of the beads to the plasma membranes. After removal of the bead solution and three wash steps with MBS, 0.1 % polyacrylic acid was added to each plate to cross-link attached silica beads. Polyacrylic acid was removed after 10 min and 1 ml of a sucrose/HEPES solution (250 mM sucrose, 25 mM HEPES pH 7.4, 20 mM KCl containing 20 µl/ml Protease Inhibitor) was added to a first dish. Cells were harvested using a cell scraper and the cell suspension transferred to a second dish, in which cells were then scraped off in the same suspension. Thus, all dishes were harvested consecutively using the smallest volume possible. The cell suspension was centrifuged at 1,000g for 5 min and the supernatant (labelled subsequently homogenate H) was removed. The pellet was resuspended in 1 ml sucrose/HEPES solution and cells disrupted in a glass homogenizer using 20 pestle strokes. Nycodenz was added to the sample to a final dilution of 25 % and the solution placed on top of a discontinuous Nycodenz gradient (40 %, 35 %, 30 %, 27 % Nycodenz). Silica-bead-bound plasma membrane proteins were then purified by ultracentrifugation (32,000rpm for 1 h). The pellet was suspended in 500 µl NET buffer (1 % Triton-X-100, 400 mM NaCl, 25 mM HEPES) and proteins eluted from beads while rotating at 37 °C for 1 h. Centrifugation at 14,000g for 20 min yielded an enriched membrane protein fraction in the supernatant. Fractions from two biological replicates were isolated and each analyzed in duplicate (two technical replicates) resulting in four samples analyzed by both methodologies.

Preparation of Whole Cell Lysate from Human Embryonic Kidney (HEK) Cells

HEK cells (ATCC, Cedarlane Laboratories Ltd., Hornby, ON) were grown in cell culture dishes until confluency. Cell layers were rinsed with phosphate buffered saline (PBS, 136.8 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄), harvested using a cell scraper, and transferred to a test tube. To ensure complete cell harvest, dishes were rinsed with a small volume of PBS. Cell suspensions were centrifuged for 5 min at 1,000g and the resulting pellet was washed in 5 mL PBS and centrifuged a second time. Cell pellets were resuspended in 500 µL 10 mM HEPES buffer (pH 7.4) and placed on ice for 30 min. After incubation in the lysis buffer, cells were burst by sonication and Triton X-100 (final concentration 1 %) was added to solubilise proteins. Samples were rotated for 30 min at 4 °C. After centrifugation at 10,000g for 30 min at 4 °C, the protein containing supernatant was employed for the following experiments.

Protein Precipitation and Digestion

200 µg of protein per sample as determined by Bio-Rad protein assay (Bio-Laboratories, Mississauga, ON) were precipitated in 5 volumes ice-cold acetone at -20 °C overnight.

After centrifugation at 14,000 rpm for 15 min, the pellet was washed and centrifuged a second time. The supernatant was decanted and remaining acetone evaporated at 37 °C. The dried protein pellet was resuspended in 50 µl freshly prepared re-suspension buffer (8 M urea in 100 mM Tris pH 8.5 containing 2 mM DTT) and placed in an incubator at 37 °C for 30 min. Afterwards, free thiol moieties were alkylated by adding iodoacetamide (final concentration 8 mM) for 2 h at 37 °C. The sample was then diluted to a urea concentration of ~1.5 M with 100 mM ammonium bicarbonate buffer (pH 8.5) and calcium chloride was added to a final concentration of 2 mM. Proteins were digested with a 1:40 ratio of proteomics grade trypsin at 37 °C overnight.

Sample Clean-up

Protein digestion was stopped by addition of 50 µL 2.5 % TFA. For peptide concentration and removal of salts and detergents, samples were solid phase extracted with Varian OMIX cartridges (Mississauga, ON, Canada) according to the manufacturer's instructions. Eluted peptide mixtures were vacuum dried and reconstituted with 40 µl 5 % acetonitrile 0.1 % formic acid (for MudPIT) and 18 ml OFFGEL Peptide sample solution (for OFFGEL).

OFFGEL Fractionation of Peptides

For peptide IEF fractionation, the 3100 OFFGEL fractionator with a "Low Resolution Kit" pH 3 – 10 (Agilent Technologies) was used according to the manufacturer's instructions, employing the default peptide-focusing program. Solid phase extraction and salt removal for fractions recovered from OFFGEL were performed with C18 MiniSpin Columns (The Nest Group, Inc., Southboro, MA). After conditioning according to the manufacturer's instructions, fractions were loaded onto the columns. Five wash steps with 5 % acetonitrile 0.1 % TFA were necessary to remove residual reagents from the OFFGEL separation that interfered with subsequent LC-MS/MS analysis. Peptide mixtures were eluted, vacuum dried and reconstituted with 40 µl 5 % acetonitrile 0.1 % formic acid.

Mass Spectrometry

For MudPIT samples, a fully automated 12-cycle MudPIT was performed as recently described³⁸. Briefly, a nano-HPLC (Proxeon Biosystems, Odense, Denmark) was interfaced with a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA), which is equipped with a nano-electrospray source (Proxeon Biosystems, Odense, Denmark). An analytical column was made by pulling a 75 µm i.d. fused silica microcapillary (Innova-Quartz, Phoenix, AZ) column to a fine tip (allowing for electrospray formation) with a P2000 laser puller (Sutter Instruments, Novato, CA). The column was then packed with ~5 cm of 5 µm Magic C18 100Å reversed phase material (Michrom Bioresources Inc., Auburn, CA) with an in-house pressure vessel. A Kasil fritted pre-column (150 µm i.d.) was packed with ~4 cm of 5 µm Magic C18 100Å reversed phase material (Michrom Bioresources Inc, Auburn, CA) followed by ~4 cm of Luna® 5µ SCX 100Å strong cation exchange resin (Phenomenex, Torrance, CA) by means of an in-house pressure vessel. This vented-column set-up³⁹ was placed in-line with the EasyLC system and connected via a microsplitter tee (Proxeon Biosystems, Odense, Denmark) to which a distal voltage of 2.2 kV was applied. 15 µl of acidified sample was then automatically loaded from a 96-well microplate autosampler using the EASY-nLC system (Proxeon Biosystems, Odense,

Denmark). The sample loading was followed by a 120 minutes HPLC gradient consisting of buffer A (water/0.1 % formic acid) and buffer B (acetonitrile/0.1 % formic acid) at a flow of 400 nl/min, similar as recently reported³⁸. After the sample loading step, consecutive loading (“salt bumps”) of 8 µl of 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 200 mM, 250 mM, 300 mM, 400 mM, and 500 mM NH₄Ac was followed by the same HPLC gradient as above.

All OFFGEL fractions were subjected to a RP-LC-MS/MS on the same equipment as described for the MudPIT analyses. 15 µl of sample was automatically loaded, separated on an analytical column running the same HPLC-gradient as for MudPIT and introduced into the MS under the same conditions. The same vented column set-up³⁹ was used, although the pre-column contained only ~4cm of 5 µ Magic C18 100Å reversed phase material (Michrom Bioresources Inc, Auburn, CA).

The MS functions were controlled by the XCalibur data system (Thermo Fisher Scientific, San Jose, CA) and the chromatographic conditions by the Easy-LC software. All samples were analyzed on a LTQ-Orbitrap XL. The instrument method consisted of one MS full scan (400–1800 m/z) in the Orbitrap mass analyzer, an automatic gain control target (AGC target) of 500,000 with a maximum ion injection of 500 ms, 1 microscan and a resolution of 60,000 and using the preview scan option. Five data-dependent MS/MS scans were performed in the linear ion trap using the five most intense ions at 35 % normalized collision energy. The MS and MS/MS scans were obtained in parallel, with the ions selected for fragmentation (in the LTQ) from a preview scan and a more precise mass determination of a full MS scan (in the Orbitrap). AGC targets for the LTQ were 10,000 with a maximum ion injection time of 100 ms. A minimum ion intensity of 1,000 was required to trigger a MS/MS spectrum. The dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count with a repeat duration of 30 seconds and exclusion duration of 45 seconds.

Data Analysis

Raw data was converted to m/z XML using ReAdW and searched by X!Tandem against a locally installed version of a mouse (C2C12) or human (HEK) IPI (<http://www.ebi.ac.uk/IPI>) protein sequence database (version 3.41, released March 18, 2009). The search was performed with a fragment ion mass tolerance of 0.4 Da, a parent ion mass tolerance of ±10 ppm. Complete tryptic digest was assumed. Carbamidomethylation of cysteine was specified as fixed, and oxidation of methionine as variable modification. To estimate and minimize our false positive rate the protein sequence database also contained every IPI protein sequence in its reversed amino acid orientation (target-decoy strategy) as recently described^{40, 41}. For the presented study, we have set the value of total reverse spectra to total forward spectra to 0.5%, resulting in an infinitesimal number of decoy sequences in the final output (2 reverse proteins in 1,727 forward proteins for C2C12 samples, 0 reverse proteins in 3318 forward proteins for HEK samples). Only fully tryptic peptides ≥7 amino acids, matching these criteria were accepted to generate the final list of identified proteins. We only accepted proteins identified with two unique peptides per analyzed sample. To minimize protein inference we developed a database grouping scheme, and only report proteins with substantial peptide information, as recently reported^{40, 41}.

We used the ProteinCenter software suite (Proxeon, Denmark) for data analysis and annotation. Briefly, IPI protein accessions of identified proteins were imported into ProteinCenter for further data analysis. For prediction of transmembrane spanning peptides, we used the TMHMM algorithm (Center for Biological Sequence Analysis, Technical University of Denmark, <http://www.cbs.dtu.dk/services/TMHMM>).

Western Blot Analysis

Immunoblot analyses were performed using a standard SDS-PAGE chemiluminescent procedure. Blots were processed using commercially available antibodies: mouse monoclonal Na⁺/K⁺ ATPase (Atp1a2, a6F, Developmental Studies Hybridoma Bank, IA), chicken polyclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Ab14247, Abcam, Cambridge, MA), rabbit polyclonal glypican-1 (ab55971, Abcam), mouse anti-Xirp1 (xin actin-binding repeat containing 1, BD Biosciences, Mississauga, ON), and a rabbit polyclonal transketolase (a kind gift from Dr. Schimmer, University of Toronto).

Availability of the Mass Spectrometry Data

m/z XML files for all C2C12 samples (silica-bead surface enriched fractions) and HEK samples (whole cell lysates) were uploaded to the Tranche server (www.proteomecommons.org) and are available for download by others. To access the data, interested users need to access the 'search data' icon and search for 'Kislinger_Lab'.

Results

In the present study, we investigated a membrane-enriched proteome of murine C2C12 myoblasts with two state-of-the-art proteomic methods. Briefly, enriched membrane fractions were analyzed by both OFFGEL RP-LC-MS/MS and MudPIT. Equal amounts of protein (200 µg) were digested and peptides subsequently subjected to either OFFGEL analysis into 12 fractions over a pH range of 3 – 10 and recovered fractions analyzed by 1D-LC-MS/MS, or directly introduced into an on-line MudPIT comprised of 12 steps to allow for accurate method comparison (Fig. 1A). All analyses were performed in quadruplicate (two biological replicates) (**SI Table 1**).

Enrichment of the isolated membrane fractions was assayed by Western Blotting. Known membrane (Na⁺/K⁺ ATPase and glypican-1) and membrane-associated proteins (Xirp1) were shown to be enriched in the membrane fraction (M) and depleted in the homogenate fraction (H), whereas cytosolic proteins (GAPDH and transketolase) were found to be abundant in the homogenate but were significantly reduced or absent in the membrane fraction (Fig. 1B). We are aware, that silica-bead coating does not result in the isolation of absolutely pure plasma membrane fractions^{37, 42, 43}. Membrane-associated proteins and cytoskeletal proteins, tightly attached to the plasma membrane are also likely to be isolated, a problem especially prevalent in skeletal muscle cells with a tight myofibrillar network. Alternative technologies such as surface biotinylation⁴⁴ and cell-surface glycoprotein capture⁴⁵ could be applied in the future.

Protein and Peptide Identifications

As a result of four replicate analyses, 1,428 proteins were detected by on-line MudPIT, as compared to 1,398 proteins identified by OFFGEL fractionation (a deviation of only ~ 2 %). A similar number of average identifications was achieved by both methodologies, 937 proteins by MudPIT and 893 proteins by OFFGEL fractionation, respectively (Fig. 2A). Overall, comparison of the two platforms (Fig. 2B) demonstrated that the majority of detected proteins (1,099, 64 %) were independently identified by both methods. Importantly, as demonstrated in Figure 2C, a significant overlap was observed in-between the four replicate analyses for both techniques: Of the 1,428 proteins detected by MudPIT analysis, 543 (38 %) are observed in every run whereas 397 (28 %) only occur in a single sample. Comparably, with the OFFGEL approach, 478 proteins (34 %) were detected in all four and 423 (30 %) in one of four sample runs. The inter-sample variability is thus well within the range of random sampling commonly observed with shot-gun proteomics^{5, 9}.

Having probed a membrane-enriched fraction, we posed the question whether the two approaches perform equally in detecting this class of proteins. Transmembrane proteins (TMP) represent a subgroup of proteins expected to be present in our fractions and were chosen for a representative comparison. Roughly the same percentages of protein identifications (MudPIT 42 %, OFFGEL 41 %) were annotated as TMP, as analyzed by the ProteinCenter software suite, which is based on the TMAP algorithm⁴⁶. Further scrutinizing of this subgroup revealed an overlap of identified TMP of ~ 65 % between the two techniques - almost the same overlap as observed for total protein identifications (see Fig. 3A).

Just alluding to one example of this protein subgroup, we recorded 17 and 15 (non-redundant) peptides assigned to the Na⁺/K⁺ ATPase (Atp1a2), also immunochemically detected by Western Blotting (see Fig. 1B) with OFFGEL/RP-LC-MS/MS or MudPIT analysis, respectively. Interestingly, none of the identified peptides were part of the transmembrane region as predicted by the TMHMM algorithm (<http://www.cbs.dtu.dk/services/TMHMM>). In an additional approach to assess membrane protein specific performance, all 465 proteins with predicted TMDs by ProteinCenter and detected by both methods (Fig. 3A), were fed into the TMHMM algorithm, to specifically extract the amino acid sequences predicted as transmembrane regions. These sequences were then correlated to our in-house database containing all peptides identified with high confidence (152 protein sequences were extracted). Of these, less than ten percent (8 for MudPIT, 10 for OFFGEL) were detected by peptides predicted to span the membrane region (data not shown). Therefore, both technologies identified predicted TMD proteins mostly with peptides outside the membrane spanning region. From these simple examples, we conclude that both approaches perform similarly for the analysis of membrane-enriched proteins. Corroborating this conclusion, GO-analysis of protein identifications showed a highly comparable distribution for cellular compartment annotations for the four MudPIT compared to the four OFFGEL samples (data not shown).

As can be expected from the slightly higher number of protein identifications, MudPIT analysis also yielded a higher number of total unique peptides (11,078 identifying 1,428 proteins compared to 10,269 peptides identifying 1,398 proteins for OFFGEL, see Fig 3 B)

as well as average number of unique peptides per sample (5,959 for MudPIT, 5,128 for OFFGEL). The higher standard deviation observed for OFFGEL samples is possibly due to an additional clean-up step after the fractionation (see Materials and Methods), which could have led to marginal but non-reproducible sample loss.

Distribution of Peptides

In Figure 3C, the percentage of total peptides detected in a certain step/fraction is depicted. After OFFGEL separation, peptides are distributed over the whole pH range (pH 3 – 10) yielding a trimodal repartition with fractions 1/2, 5, and 12 being the most populated. In MudPIT analyses, peptides were found to elute mostly in the early steps (step 1–4), which might seem unexpected at first. From theory, peptides are assumed to bind to the strong cation exchange material during the first step and to elute upon increasing the salt concentration in subsequent steps, yielding an accumulation of eluting analytes in later steps. When running a similarly prepared sample of human embryonic kidney (HEK) whole cell lysate we observed a similar trend (see Supplementary Fig. 1) whereas samples of proximal fluid showed a shift of eluting peptides toward later steps (unpublished data). The distribution of peptides in MudPIT might thus be sample type specific.

Focusing Quality

Next, the focusing quality of both methods was assessed by calculating the number of steps/fractions an individual peptide was detected in. As to be expected for isoelectric focusing, OFFGEL achieves binning of analytes into only one fraction for 88 % of all identified peptides on average and constrains the remainder to 2 – 4 fractions as shown in Figure 4A. On the contrary for MudPIT analyses, “*peptide smearing*” is more commonly observed: On average 61 % of the identified peptides are confined to one step and other peptides can be found in up to seven steps with a higher percentage. The better focusing quality of OFFGEL is also clarified by the heat maps in Figure 4B. Note that peptides that are more abundant are more prone to occur in several fractions, as estimated by total spectra recorded for a given peptide. The heat maps also mirror the distribution of peptides across steps/fractions (as discussed above).

Discussion

Sufficient separation of peptides prior to mass spectrometric analysis is a prerequisite for high quality shot-gun proteomic analyses, thus urging development and improvement of capable methodologies^{5, 18, 24, 47–50}. The goal of this study was to compare the performance of two different state-of-the-art peptide fractionation methods, the well-established MudPIT and the recently introduced OFFGEL.

Our experiments show that the overall performance of both analytical platforms is highly comparable in terms of protein identifications for samples of medium complexity. The same holds true for the average number of identified proteins per sample and the reproducibility of protein detection. Furthermore, both methods yield about the same percentages of detected transmembrane proteins, thus rendering both approaches equally capable of sufficient membrane proteome coverage. Notably, OFFGEL outperformed MudPIT for a more

complex sample (HEK whole cell lysate, see below). Both techniques lead to reproducible protein identifications although the average number of detected proteins per sample differed more for the OFFGEL approach. This could very well, as explained earlier, be attributed to an additional sample handling step in the OFFGEL workflow, which could result in sample loss.

As expected from theory, as well as, from studies comparing OFFGEL to off-line SCX or GeLC-MS, the former leads to a better focusing of peptides^{19, 51}. However, the improved peptide separation observed did not result in an increased number of detected peptides/proteins in this study. This rather contradictory observation might be due to the medium complexity of the analyzed sample type. To further investigate this discrepancy, we also analyzed HEK whole cell lysate (see Supplementary Fig. 1 and **SI Table 1**). This experiment, performed in duplicates, indeed drew a different picture: OFFGEL analyses yielded a total of 3143 proteins, whereas only 2242 proteins were detected from MudPIT analyses of two samples. The fact that the OFFGEL technique outperforms MudPIT can likely be attributed to the higher focusing quality of the former (84 % of all peptides are only detected in a single fraction, as compared to 53 % for MudPIT). Comparing our results from two different sample types, we conclude that for samples of lower to medium complexity, both techniques are capable of peptide separation sufficient for comprehensive peptide identification. For higher complex samples, the superiority of peptide separation by OFFGEL fractionation becomes apparent, as more proteins are identified by the latter. Although we used the default parameters for the OFFGEL fractionation, further optimizations of the focusing parameters will also likely lead to even more enhanced peptide detections.

Several groups have evaluated the performance of OFFGEL in respect to other fractionation techniques. Waller et al. compared OFFGEL analysis of cerebrospinal fluid (CSF) to off-line SCX fractionation followed by 1D-LC-MS/MS⁵². The analyses of 227 µg CSF protein digest (each in triplicate) led to the identification of a total 156 proteins with OFFGEL, but only 115 with off-line MudPIT, 101 of which were overlapping between both methods. The OFFGEL approach might thus be advantageous for the particular sample type analyzed. Taking into account the inter-sample variability of protein detections (OFFGEL: 97, 135, and 99 proteins; SCX: 86, 94, and 83 proteins) though, the alleged superiority of OFFGEL could be within the range of statistical deviation.

A more comprehensive assessment of performance for off-line SCX and IPG-IEF was undertaken by Slebos et al.⁵¹. Paralleled analysis of peptide mixtures from 10 and 100 µg human colon adenocarcinoma cell lysate (RKO cells) by either SCX (10 fractions) or IPG-IEF (pH range 3.5 – 4.5, yielding 10 or 15 gel slices) both followed by RP-LC-MS/MS showed a substantially higher resolution quality of IPG-IEF (87 – 89 % of peptides only in one single fraction vs. 50 – 60 % for SCX). On the other hand, SCX yielded more detection on the protein and peptide level for both sample amounts: 1256 and 2188 proteins were identified whereas IEF only resulted in 440 and 1757 identifications; thus SCX proved especially advantageous for the smaller sample size. Although, this difference in the number of detected proteins might simply be due to the very narrow pH range chosen for the IEF analyses. These findings (higher focusing quality but fewer proteins with IEF) are in line

with our results when comparing technically similar methods using a membrane-enriched sample of medium complexity. Nine replicate analyses of 50 µg tissue digest from an adenocarcinoma with both techniques revealed that SCX, lead to more protein detections, but IEF reached a plateau in detected peptides and proteins with fewer replicates than SCX, mirroring a higher reproducibility of the former.

A recent study by Hubner et al. showed a substantial superiority of OFFGEL over GeLC-MS in terms of protein identifications¹⁹. Different amounts of yeast or HeLa cell lysate were either digested and subsequently separated by OFFGEL into 12 or 24 fractions (pH 3 – 10), or separated by SDS-PAGE, which in turn was cut into 12 or 24 slices and subjected to in-gel digest. All obtained fractions were analyzed by RP-LC-MS/MS. OFFGEL clearly outnumbered the conventional approach in protein identifications by almost 900 (~ 43 % more) for the yeast experiments, and as many as 1600 proteins (~ 68 % 12 fractions) and 900 (~28 % 24 fractions) for HeLa cell lysates. Mann and co-workers also concluded from these experiments that the OFFGEL 24-well format does not provide a gain proportional to the increase in sample size and separation: Despite doubling sample amount and machine time, separation into 24 fractions only led to the detection of 334/540 additional proteins. This finding is in line with our own experience (data not shown).

Our observation of unequal peptide distribution across fractions after OFFGEL fractionation is shared by other groups. Fraterman et al. obtained a very similar distribution after separation of peptides from rat extraocular and hind-limb muscle into 12 fractions²³. An accumulation of peptides in fractions 1/2, 5, and 12 was also obtained after fractionation of 50 µg of yeast whole cell lysate¹⁹. OFFGEL fractionation into 24 fractions (pH range 3 – 10) yields the same trimodal repartition of peptides for different sample types as might be expected from the similar pH distribution of fractions^{19, 20, 22}. By comparing results from an OFFGEL fractionation of human plasma and H358 human non-small lung adenocarcinoma cell line secretions (200 µg each) to the predicted distribution of peptides from the *in-silico* digest of all 9504 proteins from the Human Plasma Project after calculating a theoretical *pI* for each peptide, Seve and co-workers could show a strong correlation between predicted and experimental repartition²². The authors explain the gap of peptides in certain fractions with the lack of possible amino acid combinations that could build peptides with adequate *pI* values²².

The superior focusing quality of OFFGEL shown in our study could be expected, taking into account the different principles of the two methods: MudPIT is a continuous analysis, in which the steps follow one another after a fixed time, regardless of peptide elution from the columns. This means a group of identical peptides might reach the end of the SCX column within a step but not all peptides are completely eluted from it when the next step begins, thus smearing into the next fraction occurs. OFFGEL in contrast is a method that is not time-limited but finishes when all peptides are separated and it is only then when the second dimension of separation (i.e. RP-LC) begins. Thus, a better separation of peptides by OFFGEL comes to no surprise.

Lam et al. could by means of mathematical modeling show that the crucial determinant in peptide focusing is a peptide's charge gradient around the *pI*. Calculations with *in-silico*

digests of the proteomes of three different specimens (*Deinococcus radiodurans*, *Saccharomyces cerevisiae*, *Homo sapiens*) proved these proteins to have almost equal amounts of peptides with a charge slope steep enough to afford focusing into two fractions at the most for 90 % of all peptides²¹. In practice, focusing quality seems to some extent be dependent on the nature of the analyzed sample. A dependence of focusing quality on a peptide's *pI* – acidic peptide focus more precisely than neutral and basic ones – is also widely appreciated^{19, 20, 23}.

Better focusing quality of OFFGEL compared to other peptide separation techniques was observed in other studies as well. Mann and colleagues showed that focusing quality is inversely correlated to sample load and found for 100 µg and 250 µg yeast lysate only 62 % and 48 % of peptides binned to only one well when assayed in a 24-well format with fractions 1 – 5 subsequently analyzed¹⁹. In their study of human plasma and secretome samples, Chenau et al.²² achieved focusing into a single fraction (of 24) for 62 % and 77 % respectively of the peptides. Comparing our results of membrane-enriched samples from murine skeletal muscle cells (88 % of all peptides confined to just one fraction) to these studies and also to our own experience with other sample types (i.e. human proximal fluid, whole cell lysate), we conclude that focusing quality is dependent on the nature of the sample analyzed.

Aside from the reproducible protein identification and high focusing quality, we also experienced a drawback in working with the OFFGEL apparatus: Fractionated peptide samples contain interfering substances which negatively affected electrospray formation during subsequent LC-MS/MS analysis. This obstacle necessitated thorough fraction clean-up (as described in the Materials and Methods section), which in turn may lead to sample loss and increased bench time.

Taken together, our experiments demonstrate a highly comparable performance of both the well-established MudPIT and the emerging OFFGEL approach for the analysis of a membrane-enriched murine skeletal muscle sample. As shown in an experiment with HEK whole cell lysate, for samples of higher complexity (e.g. whole cell lysates) though, OFFGEL leads to more protein identifications, probably due to better peptide separation.

The demonstrated superiority of OFFGEL fractionation in terms of peptide focusing might not only be advantageous for shot-gun proteomics but could also drastically enhance downstream techniques. In quantitative biomarker studies for example, where oftentimes low abundance proteins are targeted, OFFGEL might pose an attractive alternative to purify and concentrate peptides from complex samples prior to targeted proteomics quantifications with SRM-MS, a strategy usually done by SISCAPA-SRM-MS^{53, 54}. Furthermore, the separation of peptides by *pI* offers the beneficial possibility of applying *pI* filtering to obtained MS data, which in turn can lead to improved protein identification and lower false positive rates in shot-gun proteomics^{12, 20, 21}.

Shortcomings of OFFGEL are an additional requirement for sample clean-up, possibly resulting in sample loss and additional work time, as well as the quite substantial price for IPG-strips, significantly increasing the cost per analyzed sample. In conclusion, the choice

of method depends on the requirements of the study in question: For set-ups with a need for good peptide separation, the more labor- and cost-intensive OFFGEL approach might be justified, whereas for high-throughput protein identification, possibly in limited sample volumes, MudPIT is the advisable method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Synopsis

In this study, we compared two high resolution peptide separation platforms, OFFGEL fractionation followed by 1D-LC-MS/MS, and MudPIT. We are able to demonstrate equal performance of both approaches in terms of peptide and protein identification for a membrane-enriched sample of medium complexity. We could furthermore show that OFFGEL fractionation has a higher peptide focusing quality which results in superior performance for a higher complex sample.

Figure 1.

A) Flow-chart of experimental design: After isolation via silica-bead coating from murine C2C12 cells, membrane-enriched samples were digested and subjected to either OFFGEL followed by RP-LC-MS/MS or MudPIT analysis **B)** Western Blotting shows high enrichment of typical proteins for the respective fractions homogenate (H) and membrane (M)

Figure 2.

A) Protein identifications are comparable between methods and across samples B) The majority of proteins identified with the two approaches are identical C/D) 4-way Venn-diagrams for OFFGEL and MudPIT analyses comparing protein identifications in all sample runs show comparable reproducibility

Figure 3.

A) Comparison of transmembrane proteins detected with both techniques shows the same percentage of overlap as the comparison of all protein identifications depicted in Fig. 2B B) The average number of peptide detections per sample for both platforms is comparable C) Visualization of peptide distribution into steps/fractions shows a trimodal repartition after OFFGEL separation whereas the majority of peptides are detected in the first steps of MudPIT analyses

Figure 4.

Pie charts and heat maps depict the superior focusing quality of OFFGEL: 88 % of all peptides are confined to one single fraction, whereas in MudPIT analyses only 61 % of all peptides are detected in a single step. More common detection of peptides in multiple steps is also mirrored in the heat maps, depicting summed spectral counts for all samples across steps/fractions