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Filter-based Protein Digestion (FPD): A Detergent-free and Scaffold-based Strategy for TMT workflows

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Abstract

High-throughput proteome profiling requires thorough optimization to achieve comprehensive analysis. We developed a filter aided sample preparation (FASP)-like, detergent-free method - termed Filter-based Protein Digestion (FPD). We compared FPD to protein extraction methods commonly used in isobaric tag-based proteome profiling, namely trichloroacetic acid (TCA) and chloroform-methanol (C-M) precipitation. We divided a mammalian whole cell lysate from the SH-SY5Y neuroblastoma cell line for parallel protein processing with TCA (n=3), C-M (n=2), and FPD using either 10 kDa (n=3) or 30 kDa (n=3) molecular weight cut-off membranes. We labeled each sample with tandem mass tag (TMT) reagents to construct a TMT11-plex experiment. In total, 8,654 proteins were quantified across all samples. Pairwise comparisons showed very little deviation for individual protein abundance measurements between the two FPD methods, while TCA and FPD showed the most difference. Specifically, membrane proteins were more readily quantified when samples were processed using TCA precipitation than other methods tested. However, globally, only 4% of proteins differed greater than 4-fold in the most divergent pair of protein extraction methods (i.e., FPD10 and TCA). We conclude that the detergent-free FPD strategy, particularly using the faster-flowing 30kDa filter, is a seamless alteration to high-throughput TMT workflows.

Keywords

TMT; Lumos; FASP; sample preparation; precipitation; SPS-MS3

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Competing interest

The authors acknowledge no competing interests.

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Supplemental Methods 1: Detailed Methodology for liquid chromatography/tandem mass spectrometry and data analysis.

Supplemental Figure 1: Correlation across all samples for the TMT11-plex.

Supplemental Figure 2: Peptide dataset summary.

Supplemental Table 1: Proteins identified in this dataset

Supplemental Table 2: Peptides identified in this dataset

Introduction

Efficient protein isolation and digestion is vital for comprehensive proteome profiling. Irrespective of the experiment, obtaining a near complete peptide population is paramount to the success of any mass spectrometry-based global proteome analysis. Consistent and comprehensive protein isolation and digestion is important for downstream sample preparation and data analysis. For NHS-ester-based isobaric tagging experiments, e.g., TMT, small molecules with primary amines (e.g., glutathione and various neurotransmitters) must be removed to avoid quenching of the labeling reagent. Salts, nucleic acids, lipids, and detergents can interfere with protein digestion and mass spectrometric analysis. Isobaric labeling, such as TMT or iTRAQ, also present additional challenges as primary amines can quench these NHS-ester-based reagents, thereby necessitating some form of protein precipitation. As such, efforts must be directed toward generating protein samples devoid of interfering substances that may limit proteolytic digestion, adversely affect chromatography, or disrupt instrument performance, thereby facilitating high-throughput processing. Alternatively, protein isolation may be avoided by post-digestion protein desalting prior to TMT labeling. In this scenario, peptides could be lysed in 8M urea, the urea diluted, proteases added, and, peptides desalted prior to labeling. Here, however, we focused on pre-digestion protein isolation.

Recently, modifications to the general FASP concept have resulted in the development of several application-specific enhancements. For example, eFASP introduced passivation with Tween-20 and use of deoxycholic acid into the FASP workflow (1, 2). This passivation reduced peptide loss and increased digestion efficiency of enzymes. The slight differences in physicochemical properties of the peptides, showed complementarity of eFASP with the original method (3). iFASP incorporated isobaric tagging into the FASP protocol (4). Here the authors used the membrane filter for successful on-membrane TMT and iTRAQ isobaric labeling. However, iFASP, has not been widely adapted, possibly due to the greater than normal amount of label needed to achieve high labeling efficiency. In addition, FASP has also been modified to accommodate multi-well formats. For example, MStern extended the sample preparation into a 96-well plate (5). Unlike previous methods, MStern used a PVDF membrane to decrease centrifugation time, therefore increasing throughput. Moreover, further modifications have been made into the choice of membranes used for the 96-well plate format. Two groups recently introduced modified FASP procedures, pFASP (6) and q96FASP (7), which used polyethersulfone (PES) as the filter membrane. Both studies reached similar conclusions concerning the advantages in throughput of the PES membrane, with the former showing similarity in reproducibility and proteome coverage among all FASP-type methods. We have innovated this concept further to streamline TMT sample preparation, using the filter only as a scaffold for digestion in a detergent-free 96-well format.

Here we evaluate a modified FASP-based method, which we term “Filter-based Protein Digestion” (FPD) in a TMT11-plex experiment. We tested if FPD can substitute traditional TCA and C-M precipitation in TMT-based protein profiling experiments. Isobaric tag-based quantitative proteomics strategies leverage multiplexing capabilities to increase throughput and comprehensiveness of proteome coverage (8–10). However, many upstream sample

processing steps can be performed only sequentially and not in a parallel, high-throughput manner. Typical protein precipitation strategies, such as TCA and C-M precipitation, are simple and inexpensive to implement. However, such methodologies also require tedious, careful aspirations that are not amenable to automation, and can lead to user-based errors for large-scale processing. We aim to enhance the efficiency, save time, and limit sample loss by integrating detergent-free on-filter-based sample preparation into our TMT workflow.

In the FPD protocol, unlike traditional FASP, we perform the cell lysis in 8 M urea and reduction and alkylation occur prior to loading the sample onto the filter. These modifications enable us to forgo SDS-based cell lysis and associated buffer exchange centrifugations, thereby shortening the number of steps and the time for sample preparation. Moreover, filtration and digestion are performed in a 96-well plate format allowing as few as 1 and up to 96 digestion reactions at once per plate. While traditional FASP membranes are often composed of cellulose, we use a polyethersulfone (PES) membrane. This microporous material is hydrophilic, has very low protein binding affinity, and is amenable to fast flow rates. Here we show similar overall relative peptide measurements among the methods tested. The capability to simultaneously process dozens of protein samples in parallel is well-suited for isobaric tag-based protein profiling analysis.

Methods

Materials

Tandem mass tag (TMT) isobaric reagents were from ThermoFisher Scientific (Waltham, MA). AcroPrep™ 96-well Filter Plates (MWCO 10 kDa or 30 kDa were from PALL (Port Washington, NY). Water and organic solvents were from J.T. Baker (Center Valley, PA). Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were from LifeTechnologies (Waltham, MA). Trypsin was purchased from Promega (Madison, WI) and LysC from Wako Chemicals (Richmond, VA). Unless otherwise noted, all other chemicals were from Pierce Biotechnology (Rockford, IL).

Cell growth, harvesting, and lysis

Methods of cell growth and propagation followed previously utilized techniques (11, 12). In brief, cells were propagated in DMEM supplemented with 10% FBS. Upon achieving 80% confluency, the growth media was aspirated and the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS). Cells were treated with non-enzymatic cell stripper for 5 min and then pelleted by centrifugation at 3,000 × g for 5 min at 4°C. One milliliter of 200 mM EPPS, pH 8.5, 8M urea supplemented with 1X Roche Complete protease inhibitors) was added to each 15cm cell culture dish.

Cells were homogenized and DNA was sheared by 10 passes through a 21-gauge, 1.25 inch long needle. The homogenate was sedimented by centrifugation at 21,000 × g for 5 min and the supernatant was transferred to a new tube. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (ThermoFisher Scientific). Proteins were subjected to disulfide reduction with 5 mM tris (2-carboxyethyl)phosphine (TCEP), (room temperature, 30 min) and alkylation with 10 mM iodoacetamide (room temperature, 30 min in the dark).

Excess iodoacetamide was quenched with 10 mM dithiotreitol (room temperature, 15 min in the dark). The sample was divided into 100 μg aliquots and processed with four different precipitation methods: 1) trichloroacetic acid (TCA) precipitation (3 replicates), 2) chloroform-methanol (C-M) precipitation (2 replicates), 3) filter-based protein digestion with 10 kDa membrane (FPD10) (3 replicates), and 4) filter-based protein digestion with 30 kDa membrane (FPD30) (3 replicates).

Trichloroacetic acid (TCA) precipitation and protein digestion

In brief, 100% TCA was added to each designated sample to achieve a final concentration of 12.5%. The samples were vortexed, incubated on ice for 1 hr, and centrifuged at 20,000 RPM for 30 min at 4°C. The supernatants were aspirated and the sample was washed once with 1 mL of acetone. The samples were centrifuged at 20,000 RPM for 10 min at 4°C. The supernatant was aspirated and the samples were washed once with 1 mL of methanol and allowed to air-dry. Samples were digested at room temperature for 16 hr with LysC protease at a 100:1 protein-to-protease ratio. Trypsin was then added at a 100:1 protein-to-protease ratio and the reaction was incubated 6 h at 37°C.

Chloroform-methanol (C-M) precipitation and protein digestion

For chloroform-methanol precipitation, four parts neat methanol was added to each designated sample and vortexed. One-part chloroform was added to the samples and vortexed, and three parts water was added to the sample and vortexed. The samples were centrifuged at 20,000 RPM for 2 min at room temperature and after removing the aqueous and organic phases were subsequently washed twice with 100% methanol, prior to air-drying. As in the TCA precipitation protocol, samples were digested at room temperature for 16 h with LysC at a 100:1 protein-to-protease ratio, followed by trypsin at a 100:1 protein-to-protease ratio for 6 hr at 37°C.

Filter-based protein digestion (FPD)

After the cell lysate was reduced, alkylated, and quenched (as described above), 100 μg of total protein were loaded on AcroPrep™ 96-well Filter Plates (MWCO 10 kDa or 30 kDa, PALL). Lysates were spun down at 2,000 g at room temperature until lysis buffer was removed (~30 min for FPD30 and ~60 min for FPD10). Membranes were washed with 100 μL of 200 mM EPPS buffer (pH=8.5). Samples were digested by LysC (at 1:100 enzyme to protein ratio) in 50 μL of 200 mM EPPS buffer for 16 hr at room temperature, followed by trypsin digestion (at a 1:100 ratio) for 6 hr at 37°C. For elution, digests were spun down at 2,000 g at room temperature, followed by additional wash with 50 μL of 200 mM EPPS buffer.

Tandem mass tag (TMT) labeling

TMT reagents (0.8 mg) were dissolved in anhydrous acetonitrile (40 μL) of which 10 μL was added to the peptides (100 μg) along with 30 μL of acetonitrile to achieve a final acetonitrile concentration of approximately 30% (v/v). Following incubation at room temperature for 1.5 hr, the reaction was quenched with hydroxylamine to a final concentration of 0.3% (v/v). The TMT-labeled samples were pooled at a

1:1:1:1:1:1:1:1:1:1:1 ratio. The sample was vacuum centrifuged to near dryness and subjected to C18 solid-phase extraction (SPE) (Sep-Pak, Waters).

Off-line basic pH reversed-phase (BPRP) fractionation

We fractionated the pooled TMT-labeled peptide sample using BPRP HPLC. We used an Agilent 1260 pump equipped with a degasser and a single wavelength detector (set at 220 nm). Peptides were subjected to a 50 min linear gradient from 8% to 40% acetonitrile in 10mM ammonium bicarbonate pH 8 at a flow rate of 0.6 mL/min over an Agilent 300Extend C18 column (3.5 μm particles, 4.6 mm ID and 250 mm in length). The peptide mixture was fractionated into a total of 96 fractions which were consolidated into 24, from which 12 non-adjacent samples were analyzed (13). Samples were subsequently vacuum centrifuged to near dryness. Each fraction was acidified to 1% formic acid and desalted via StageTip, dried via vacuum centrifugation, and reconstituted in 5% acetonitrile, 5% formic acid for LC-MS/MS processing.

Liquid chromatography and tandem mass spectrometry

Data was analyzed as described previously (14). A detailed description may be found in the Supplementary Materials.

Results and Discussion

Over 8,600 proteins were quantified across all four-protein isolation/digestion methods tested

We designed a TMT11-plex experiment to determine if filter-based protein digestion (FPD) could be used an alternative to TCA or C-M precipitation in standard TMT quantitative protein profiling analyses (Figure 1). We appreciate that each technique has its advantages and caveats. Specifically, for large volumes, TCA may be optimal as the sample volume increases only 12–25%, whereas for C-M precipitation, the volume increases up to 8 times. Also, the consumable cost for chemical precipitation methods is lower than filter-based methods, due mainly to the relatively high price of the centrifugal filters. The time benefit for each analysis is dependent on the number of samples to be processed simultaneously. More specifically, for relatively few samples, such as 24–36 (which is commonly the capacity of standard table top centrifuges), chemical precipitations can generally be performed in less time than FPD centrifugations. However, when dealing with larger number of samples, a plate-based filter method can circumvent the multiple tedious pipetting and aspiration steps. As such, batch effects may be reduced and the procedure becomes amenable to robotic automation. We aim to integrate FPD into our high-throughput multiplexed TMT workflow, where TCA or C-M precipitation is commonly implemented.

For this experiment, we prepared eleven aliquots of 100 μg of protein from an SH-SY5Y mammalian cell lysate. This lysate had been reduced with TCEP, alkylated with iodoacetamide, and quenched with DTT prior to precipitation/filtration. Three aliquots were precipitated with TCA and two with C-M. These five samples were digested and processed further following typical TMT methodology (15–18). The remaining six samples were digested on-filter, three with 10kDa molecular weight cut-off (MWCO) and another three

with 30kDa MWCO filters. We refer to samples processed with the 10 kDa MWCO filters as FPD10, and those with the 30 kDa MWCO filters as FPD30. All samples were labeled with TMT, combined, and fractionated via basic pH reversed-phase chromatography.

A total of 8,654 proteins were quantified across all methods tested at a 1% protein false discovery rate (Supplemental Table 1). These proteins were assembled from a total of 85,155 peptides (Supplemental Table 2). Hierarchical clustering using the Euclidian distance matrix revealed the expected clustering pattern (Figure 2A). Specifically, replicates clustered most tightly together, as anticipated. The FPD methods were also closely associated as the first branchpoint was between FPD and the chemical precipitation methods (*i.e.*, TCA and C-M precipitation). These observations were supported by principal components analysis (PCA) (Figure 2B). When plotting the first two principal components against each other, the samples segregated by FPD versus non-FPD strategies in the first principal component and by method in the second. In all, 53.1% of the variance was explained by principal component 1 (PC1) and 21.6% by principal component 2 (PC2), which strongly supports the hierarchical clustering analysis (Figure 2A).

We extracted three clusters of proteins which exhibited the most relative differences in abundance from the heat map (Figure 2A). These clusters consisted of proteins that were 1) relatively lower in abundance in TCA and C-M samples (violet, n=986), 2) down in TCA samples (blue, n=560), and 3) down in FPD samples (green, n=612). We preceded to investigate some physicochemical properties - namely hydrophobicity, isoelectric point (pI) and length - of these clusters with respect to other clusters and to the whole dataset (grey, 8,654). Of the physicochemical properties investigated, we observed no overwhelming biases pertaining to any protein classifications in the clusters interrogated. We did, however, observe notable trends in the data. We examined the hydrophobicity of the proteins in each cluster by calculating the grand average of hydropathy (GRAVY) index, which is a measure of the overall hydropathy of amino acid residues in a protein (19) (Figure 2C). More specifically, higher GRAVY scores correspond to more hydrophobic proteins. The cluster with the highest mean and median GRAVY score consisted of proteins that were relatively lower in abundance in the FPD (green), namely, hydrophobic proteins, for example membrane proteins. Such a trend has been reported previously in the comparison between FASP and MStern (5). We suggest the potential use of a mass spectrometry-compatible detergent, such as RapiGest or ProteoMax to enhance digestion, as commonly added in non-filter-based methods (20). In addition, we examined the isoelectric point - the pH at which a protein carries no net electrical charge - of the proteins in these clusters. This analysis revealed that the cluster of proteins relatively lower in abundance in TCA (blue) have higher isoelectric points, implying a slight bias against proteins with high pI in TCA precipitations compared to the other methods investigated (Figure 2D). Finally, we examined length (and by extension, mass) of the proteins, those down in FPD were of similar length to the entire dataset (Figure 2E). However, the cluster of proteins with lower abundance in both TCA/C-M precipitated samples (violet) showed a trend toward smaller proteins. Such a result indicated that FPD was more likely to capture and digest small proteins that could not be precipitated by chemical means. Although trends with respect to the physicochemical properties investigated were present in the data, we observed no overwhelming bias in protein quantification among the methods tested.

Likewise, we conducted the distribution analyses above at the peptide level (Supplemental Figure 2). As with the protein analysis, we extracted three clusters of phosphorylation sites which exhibited the most relative differences in abundance from the heat map (Supplemental Figure 2A). These clusters consisted of phosphorylation sites that were 1) relatively lower in abundance in TCA and C-M samples (violet, n=4,295), 2) down in TCA samples (blue, n=7,200), and 3) down in FPD samples (green, n=3,996). Hierarchical clustering revealed that the peptides grouped similarly to the proteins (Supplemental Figure 2A). Moreover, PCA analysis showed that the first principal component explained over 48% of the variance and segregated the samples by precipitation method (Supplemental Figure 2B). The GRAVY and length distributions (Supplemental Figure 2B and 2D) were not altered substantially when comparing the peptides across different methods. Whereas, using FPD methods seemed to cover an expanded range of pI compared to TCA/CM (Supplemental Figure 2B). However, no major bias in either protein or peptide coverage were apparent in the protein isolation methods investigated herein.

Total peptides quantified and the coefficients of variation among methods investigated were similar

In a typical TMT protein abundance profiling experiment, the sum of the TMT intensities of all peptides in each channel are typically equated, as such the total amount of peptide in each channel is assured to be constant. As our goal was to examine how well each method can extract and digest protein, loading correction was not performed. We can, however, use these global protein correction factors (relative signal with respect to the minimum measurement across the 11 channels) to assess if any of the methods used precipitated significantly more or less total protein than any other. The analysis of the global protein normalization factors revealed that a similar amount of total protein was precipitated across all methods tested, with no pair deviating more than 20% (Figure 3A). This metric, however, provides only an indirect comparison of quantifiable proteins among the precipitation techniques investigated here. In multiplexed experiments, selection for sequencing is based on the sum of signal from all samples. When large fold-changes are observed among samples in TMT experiments, the sample of lower abundance may not have been selected for sequencing if it was to be analyzed alone. Alternatively, individual TMT experiments for each precipitation method may provide a better comparison of the proteome coverage among proteins and peptides. However, we are cautiously confident from previous label-free experiments, as well as data shown in Figure 3A, that proteomic depth will not be substantially different as a result of the precipitation method used.

In addition, we calculated the coefficient of variation (CV) of each method for all 8,654 proteins individually and examined the distributions of %CV across the dataset. The distributions of the %CV centered around 9% for the chemical precipitation methods and a bit less (at 6%) for the filter-based methods (Figure 3B). The slight decrease observed in the %CV for the FPD methods compared to TCA and C-M precipitation was consistent with the expected result, as the chemical precipitation methods required manual aspiration, which is more susceptible to human error. Even with great care, small particulates from the protein pellet might be aspirated inadvertently, and such losses will not be consistent from one sample to another. As such, the FPD protocol eliminated the human error associated with

aspiration. In general, all methods generated a similar number of peptides, however the %CV for FPD methods were lower, likely due to less manual sample processing.

Pair-wise comparison of different protein processing strategies revealed some method-specific bias in protein abundance measurements

We investigated further the effects of any precipitation bias by examining pairwise comparisons for each method. We plotted and overlaid the distribution of the average fold-change for each pair of sample preparation methods as probability density function-smoothed histograms (Figure 4A). As these distributions show, similar peak height and width, centered over zero, indicated only minor changes in protein abundance. Examining these data, we ranked the methods from most to least similar pairs as follows: FPD10 and FPD30, TCA and C-M, C-M and FPD30, C-M and FPD10, TCA and FPD30 and TCA and FPD10. These data agree with previous hierarchical clustering and PCA analysis (Figure 2).

We quantitatively assessed these data and determined the number of proteins that were altered 2-fold, 4-fold, or 8-fold (Figure 4B). Examining the extreme cases, we observed the largest deviation when comparing TCA and FPD10 and the most similarity when comparing FPD10 and FPD30. Of the 8,654 proteins identified, 1,124 differed more than 2-fold in abundance between TCA and FPD10. This number decreased more than 3 times to 364 for proteins that were more than 4-fold different. Only 82 proteins were more than 8-fold different in abundance. FPD10 versus FPD30, for example, showed few differences among quantified proteins. Only 45 proteins differed greater than 2-fold, 6 greater than 4-fold and only 1 greater than 8-fold. Many of these proteins were small and thus, were identified more readily in FPD10 than in FPD30, which likely did not retain these proteins. However, FPD30 has the advantage of allowing for faster processing, as centrifugation steps required half the time of FPD10, due to greater permeation of buffer through the larger membrane pores. As very few proteins differed between the two filter pore sizes, we suggest using the 30kDa filter over the 10kDa filter for high-throughput, quantitative proteomics profiling analysis to take advantage of faster sample processing.

In addition, we calculated the Pearson correlation matrix for relative protein abundance in this dataset (Supplemental Figure 1). We noted that the highest correlation was among the FPD samples ($r>0.99$), while replicates of the chemical precipitation samples were slightly less correlated ($r>0.95$). Although tight correlation among replicates was expected, we noted that across all comparisons, the lowest Pearson coefficient was $r=0.93$. While in individual cases, some proteins may be more readily precipitated with one technique than another, the overall dataset showed that comparable results may be obtained regardless of the protein isolation/digestion method used herein.

Some proteins are more readily isolated and digested with specific processing methods

TMT-based protein quantification has an advantage over other quantification methods as no missing values are observed across comparison groups. As such, a protein can be present at a generally undetectable amount in one channel if analyzed alone, but the presence of greater amount of the same protein in other channels will permit that protein to be identified and likely quantified. In the TMT11-plex experiment discussed herein, many proteins that

are observed to be >8-fold less abundant in one or more precipitation methods compared to the rest may not be selected for sequencing if analyzed individually by label-free quantification. As such, we emphasize that no method is comprehensive and *a priori* knowledge of certain proteins of interest may best guide the strategy for protein processing and digestion.

We examined protein-specific processing bias by highlighting several proteins having the largest method-specific abundance measurements differences. The TMT relative abundance (RA) profiles were examined for the following proteins: ENO1, EPDR1, HSBP1, PSMD2, SELK, and TMSB4X. ENO1 (enolase 1, alpha-enolase) was quantified readily in samples processed only with FPD methods, but not with chemical precipitation (Figure 5A). ENO1 is a glycolytic enzyme that is important in cancer research as it has been shown to bind to the c-myc promoter and has tumor suppressor function (21). In contrast, EPDR1 (ependymin related 1 transmembrane protein), a cell adhesion protein, was quantified mainly in samples precipitated with TCA (Figure 5B) (22). HSBP1 (heat shock factor binding protein 1) was quantified when FPD methods were used, but was observed to be less abundant when using TCA and marginally with C-M precipitation (Figure 5C). This protein binds to heat shock factor 1(HSF1), which is a transcription factor involved in heat shock response (23). HSBP1 is 76 amino acids long (mol. wt. 8,544 Da), so it is relatively small, and may remain in solution under chemical precipitation methods. PSMD2 (26S proteasome non-ATPase regulatory subunit 2) was quantified when C-M precipitation was used, and less with the other techniques investigated (Figure 5D). PSMD2 is responsible for substrate recognition and binding was implicated in proteasome function (23). SELK (selenoprotein K) was quantified when TCA or C-M techniques were used, but very little was measured from samples processed with FPD (Figure 5E). This result may be expected in part as SELK is a transmembrane protein localized to the endoplasmic reticulum (24). TMSB4X (Thymosin beta-4) was identified with FPD10, while the other three techniques struggled to quantify this protein (Figure 5F). TMSB4X is an abundant protein involved in the regulation of actin polymerization and has a role in proliferation, migration, and differentiation (25). However, TMSB4X is another small protein of 44 residues (mol. wt. 5,053 Da), and as such may be too soluble to precipitate with TCA or C-M, and small enough to permeate the FPD30 membrane. With these examples, we show that although, globally, we observed only minor differences in the proteins quantified by each method, individual proteins may preferentially be isolated by one method over the others. No universal and comprehensive method of protein extraction and digestion is suitable for all samples. As such, caution should be taken if the research is targeting a single protein or class of proteins to ensure that a compatible processing strategy is undertaken.

Conclusions

We showed that on-filter protein isolation and digestion can be integrated into a well-established TMT protocol. FPD provided a high-throughput, detergent-free platform that efficiently isolated and digested protein samples, while reducing the human error associated with multiple aspirations across samples. FPD is different from FASP as we perform the cell lysis in 8 M urea, reduction and alkylation occur prior to loading onto the filter, and PES membrane is used as the filter in either single or multi-well plate formats. These alterations

shorten the number of centrifugation steps and the time for sample preparation. Here, we compared the protein abundance profiles of over 8,600 proteins across four protein isolation/digestion methods in a TMT11-plex experiment. The data showed that the total peptides generated and the coefficient of variation among methods investigated were comparable. However, while similar amount of total protein was obtained with each method, slight differences were observed for specific protein processing strategies. Although, globally, differences due to protein precipitation were modest, we showed that individual proteins may be more compatible with certain processing methods than others. The integration of FPD into our TMT workflow was seamless, but minor caveats persisted. One caveat for using FPD in large high-throughput studies may be cost, as molecular weight cut-off filters are more expensive than the microliter amounts of TCA or chloroform needed for chemical precipitation. The costs, however may be offset by enhanced reproducibility and speed resulting from the multiplexing capability, particularly if using the 96-well microplate format. Downstream processing, such as TMT labeling and desalting, may also take advantage of the 96-well format, allowing for a more streamlined sample preparation strategy. We conclude that the FPD strategy, particularly using a 30 kDa filter, can be a simple-to-implement modification to any TMT workflow.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Erde J, Loo RR, Loo JA. Improving Proteome Coverage and Sample Recovery with Enhanced FASP (eFASP) for Quantitative Proteomic Experiments. Methods Mol Biol. 2017; 1550:11–18. [PubMed: 28188519]
2. Erde J, Loo RR, Loo JA. Enhanced FASP (eFASP) to increase proteome coverage and sample recovery for quantitative proteomic experiments. J Proteome Res. 2014; 13(4):1885–95. [PubMed: 24552128]
3. Nel AJ, Garnett S, Blackburn JM, Soares NC. Comparative reevaluation of FASP and enhanced FASP methods by LC-MS/MS. J Proteome Res. 2015; 14(3):1637–42. [PubMed: 25619111]
4. McDowell GS, Gaun A, Steen H. iFASP: combining isobaric mass tagging with filter-aided sample preparation. J Proteome Res. 2013; 12(8):3809–12. [PubMed: 23692318]
5. Berger ST, Ahmed S, Muntel J, Cuevas Polo N, Bachur R, Kentsis A, Steen J, Steen H. MStern Blotting-High Throughput Polyvinylidene Fluoride (PVDF) Membrane-Based Proteomic Sample Preparation for 96-Well Plates. Mol Cell Proteomics. 2015; 14(10):2814–23. [PubMed: 26223766]
6. Potriquet J, Laohaviroj M, Bethony JM, Mulvenna J. A modified FASP protocol for high-throughput preparation of protein samples for mass spectrometry. PLoS One. 2017; 12(7):e0175967. [PubMed: 28750034]
7. Yu Y, Bekele S, Pieper R. Quick 96FASP for high throughput quantitative proteome analysis. J Proteomics. 2017; 166:1–7. [PubMed: 28669814]

8. Dayon L, Hainard A, Licker V, Turck N, Kuhn K, Hochstrasser DF, Burkhard PR, Sanchez JC. Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. Anal Chem. 2008; 80(8):2921–31. [PubMed: 18312001]
9. Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem. 2003; 75(8):1895–904. [PubMed: 12713048]
10. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics. 2004; 3(12):1154–69. [PubMed: 15385600]
11. Paulo JA, Urrutia R, Banks PA, Conwell DL, Steen H. Proteomic analysis of a rat pancreatic stellate cell line using liquid chromatography tandem mass spectrometry (LC-MS/MS). J Proteomics. 2011; 75(2):708–17. [PubMed: 21968429]
12. Paulo JA, Urrutia R, Banks PA, Conwell DL, Steen H. Proteomic analysis of an immortalized mouse pancreatic stellate cell line identifies differentially-expressed proteins in activated vs nonproliferating cell states. J Proteome Res. 2011; 10(10):4835–44. [PubMed: 21838295]
13. Paulo JA, O'Connell JD, Everley RA, O'Brien J, Gygi MA, Gygi SP. Quantitative mass spectrometry-based multiplexing compares the abundance of 5000 S. cerevisiae proteins across 10 carbon sources. J Proteomics. 2016; 148:85–93. [PubMed: 27432472]
14. Paulo JA, O'Connell JD, Gygi SP. A Triple Knockout (TKO) Proteomics Standard for Diagnosing Ion Interference in Isobaric Labeling Experiments. J Am Soc Mass Spectrom. 2016; 27(10):1620–5. [PubMed: 27400695]
15. Paulo JA, Mancias JD, Gygi SP. Proteome-Wide Protein Expression Profiling Across Five Pancreatic Cell Lines. Pancreas. 2017; 46(5):690–698. [PubMed: 28375945]
16. Paulo JA, Gygi SP. Nicotine-induced protein expression profiling reveals mutually altered proteins across four human cell lines. Proteomics. 2017; 17:1–2.
17. Paulo JA, Gaun A, Gygi SP. Global Analysis of Protein Expression and Phosphorylation Levels in Nicotine-Treated Pancreatic Stellate Cells. J Proteome Res. 2015; 14(10):4246–56. [PubMed: 26265067]
18. Paulo JA, Gygi SP. A comprehensive proteomic and phosphoproteomic analysis of yeast deletion mutants of 14-3-3 orthologs and associated effects of rapamycin. Proteomics. 2015; 15(2–3):474–86. [PubMed: 25315811]
19. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol. 1982; 157(1):105–32. [PubMed: 7108955]
20. Pop C, Mogosan C, Loghin F. Evaluation of Rapigest Efficacy for the Digestion of Proteins from Cell Cultures and Heart Tissue. Clujul Med. 2014; 87(4):258–62. [PubMed: 26528033]
21. Zhu X, Miao X, Wu Y, Li C, Guo Y, Liu Y, Chen Y, Lu X, Wang Y, He S. ENO1 promotes tumor proliferation and cell adhesion mediated drug resistance (CAM-DR) in Non-Hodgkin's Lymphomas. Exp Cell Res. 2015; 335(2):216–23. [PubMed: 26024773]
22. Nimmrich I, Erdmann S, Melchers U, Chtarbova S, Finke U, Hentsch S, Hoffmann I, Oertel M, Hoffmann W, Muller O. The novel ependymin related gene UCC1 is highly expressed in colorectal tumor cells. Cancer Lett. 2001; 165(1):71–9. [PubMed: 11248421]
23. Satyal SH, Chen D, Fox SG, Kramer JM, Morimoto RI. Negative regulation of the heat shock transcriptional response by HSBP1. Genes Dev. 1998; 12(13):1962–74. [PubMed: 9649501]
24. Polo A, Colonna G, Guariniello S, Ciliberto G, Costantini S. Deducing the functional characteristics of the human selenoprotein SELK from the structural properties of its intrinsically disordered C-terminal domain. Mol Biosyst. 2016; 12(3):758–72. [PubMed: 26735936]
25. Gomez-Marquez J, Dosil M, Segade F, Bustelo XR, Pichel JG, Dominguez F, Freire M. Thymosin-beta 4 gene. Preliminary characterization and expression in tissues, thymic cells, and lymphocytes. J Immunol. 1989; 143(8):2740–4. [PubMed: 2677145]

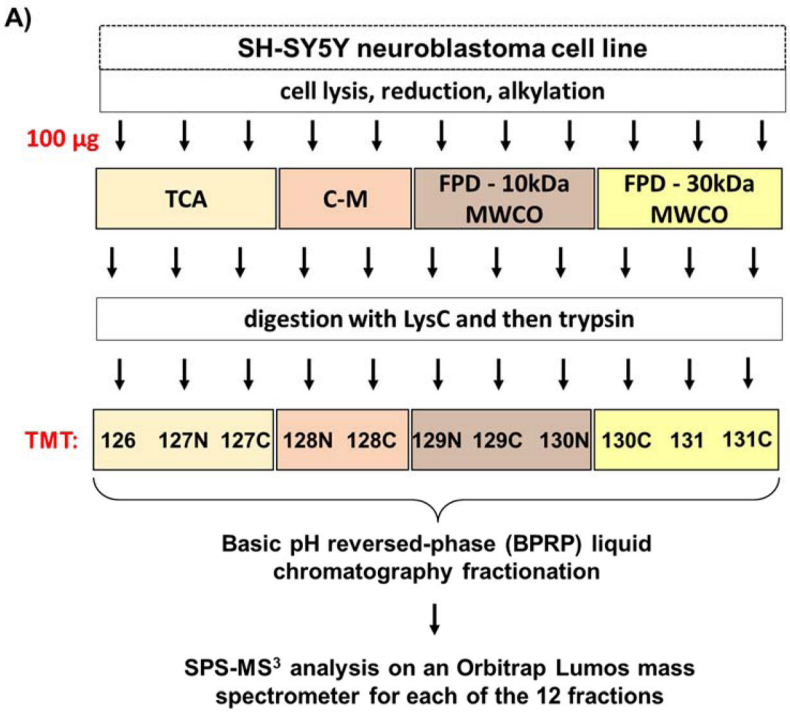

Figure 1. Experimental overview of the FPD/TMT strategy

SH-SY5Y cells were lysed in urea buffer, reduced with TCEP and alkylated with iodoacetamide. Proteins were extracted via precipitation or subjected to FPD and digested with LysC and trypsin. The resulting peptides were labeled with TMT, pooled, and fractionated via basic pH reversed-phase high performance liquid chromatography (BPRP-HPLC) prior to TMT analysis on an Orbitrap Fusion Lumos mass spectrometer. TCA, trichloroacetic acid; C-M, chloroform methanol; FPD, Filter-based protein digestion; TMT, tandem mass tag.

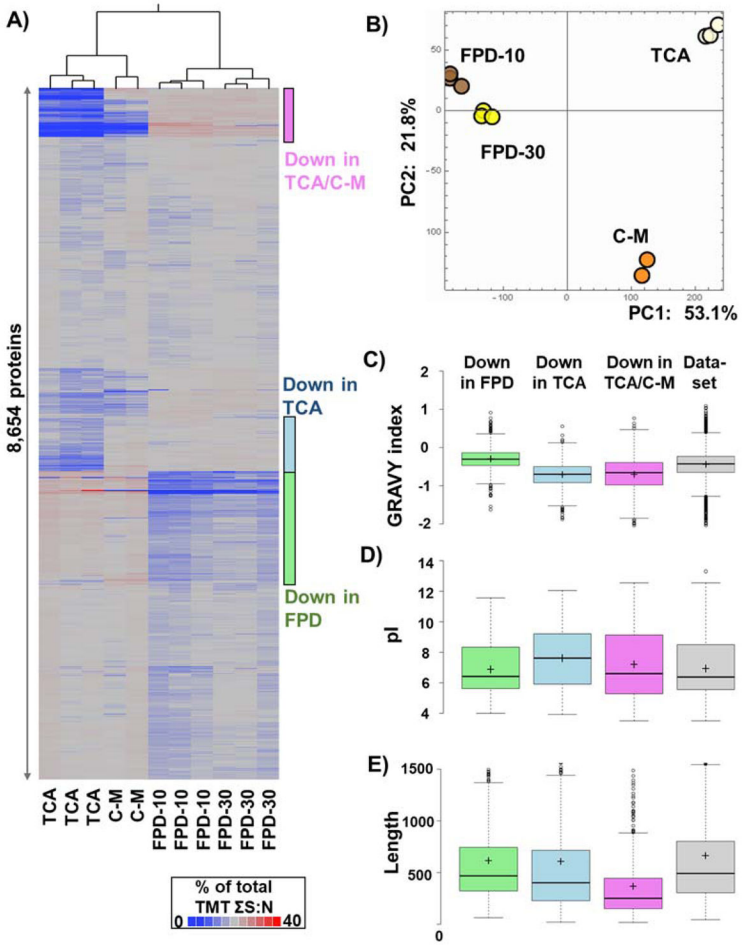

Figure 2. Protein dataset summary

A) Heat map and associated dendrogram of samples analyzed in the TMT11-plex experiment. Data shown were scaled such that the total TMT signal-to-noise across the TMT11-plex sums to 100. **B)** Principal components analysis showing segregation according to the protein extraction method utilized. Box and whisker plots display the distribution of the **C)** GRAVY index, **D)** isoelectric point (pI), and **E)** length of the proteins. The box colors correspond to the colored sections indicated on the right of the heat map. Grey boxes represent data from the entire dataset.

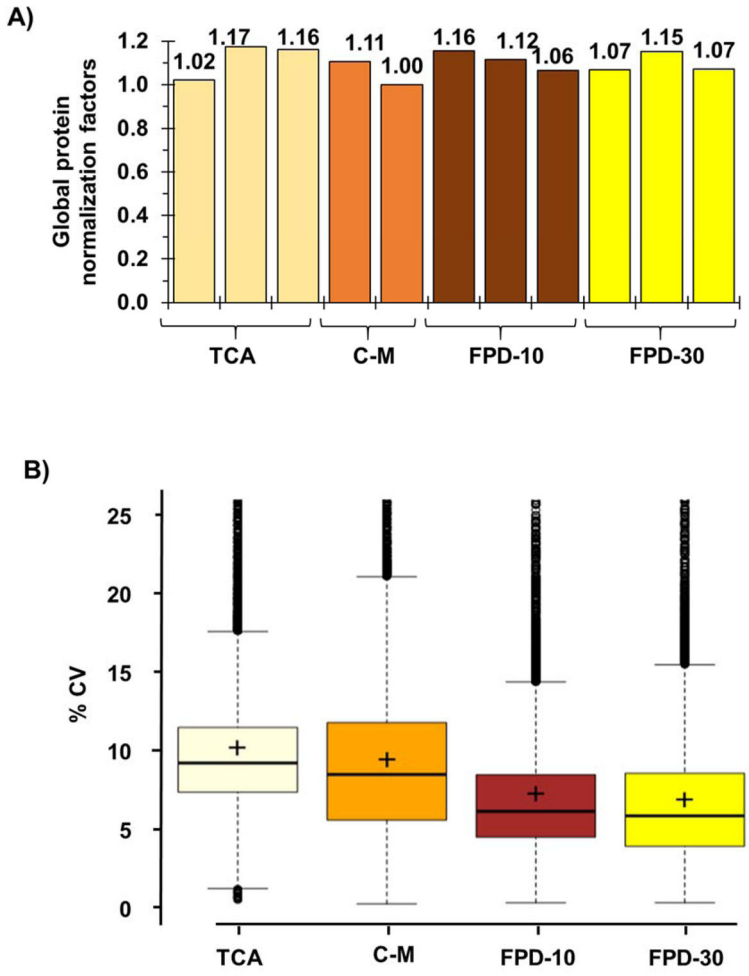

Figure 3. Coefficient of variation among the four protein extraction methods

A) Global protein normalization factors show little method-specific differences in the amount of protein analyzed by mass spectrometry**. B)** Box plots show the distribution of the percent coefficient of variation (%CV).

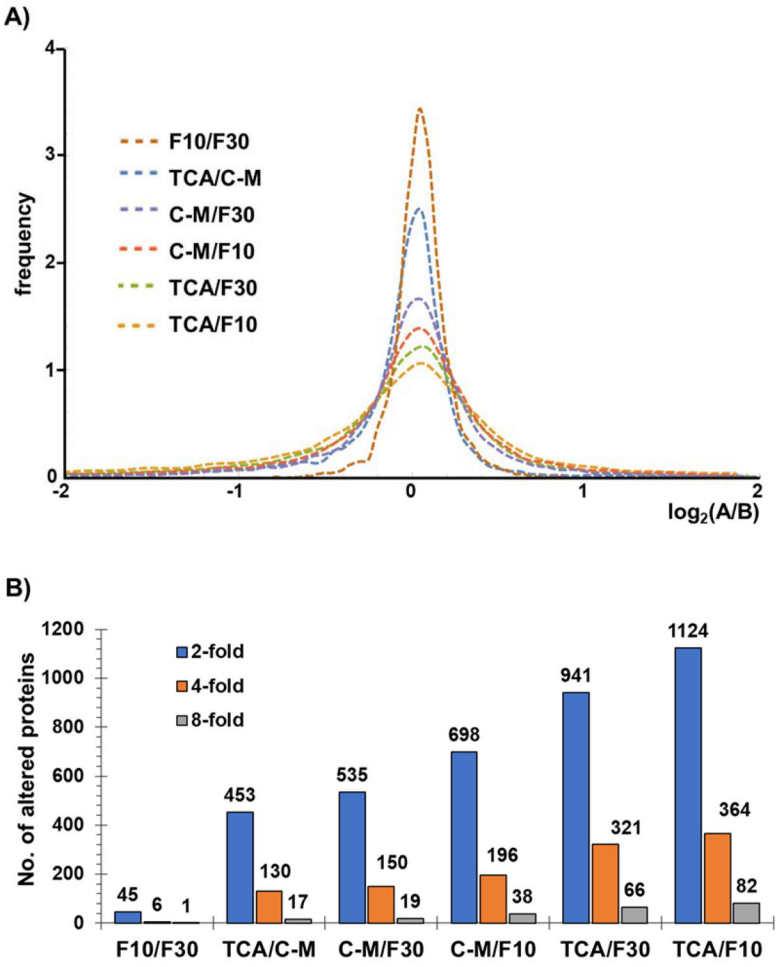

Figure 4. Protein fold changes across the four protein extraction methods

A) The histograms were smoothed using the probability density function and illustrate the $\log_2$ fold change for pair-wise comparisons of each protein extraction method. **B**) Bar chart shows the number of proteins with fold changes exceeding 2, 4, and 8 for each pairwise comparison of extraction methods. T, TCA; CM, chloroform-methanol; F10, FPD-10kDa; F30, FPD-30kDa.

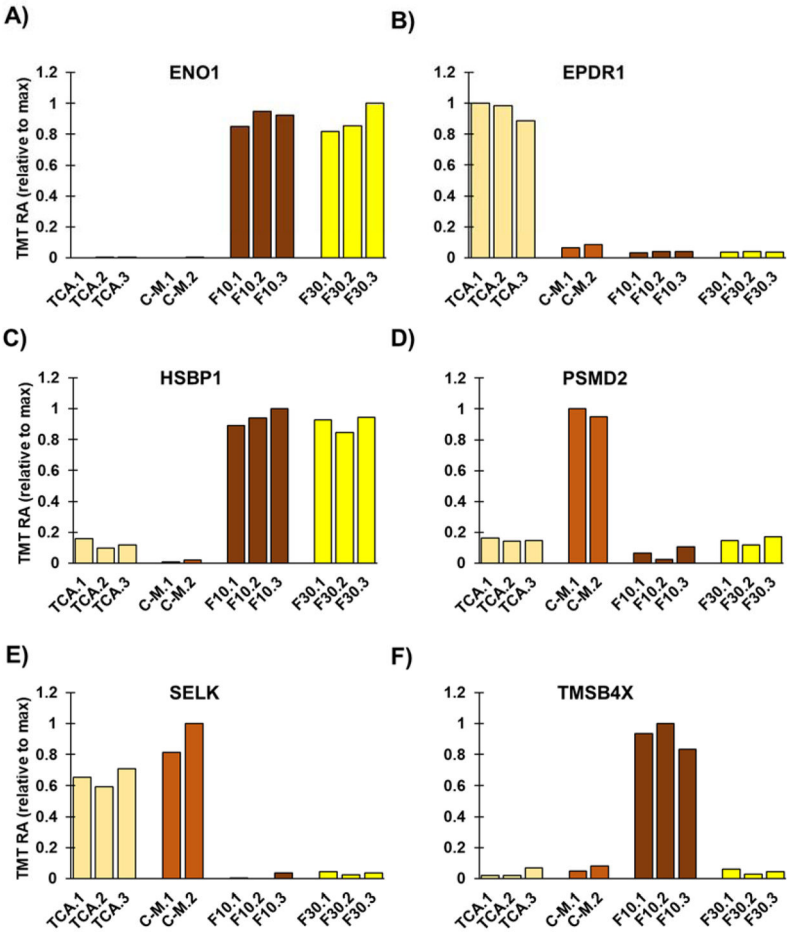

Figure 5. Example proteins showing changes in protein abundance across the different protein processing strategies

These bar graphs illustrate the TMT relative abundance (RA; i.e., the measurement relative to the maximum signal across all protein processing strategies for that protein) for: **A)** ENO1, **B**) EPDR1, **C)** HSBP1, **D**) PSMD2, **E)** SELK, and **F)** TMSB4X. TMT RA, tandem mass tag relative abundance; T, TCA; C-M, chloroform-methanol; F10, FPD-10kDa; F30, FPD-30kDa.