Letter

Instrument Logic Increases Identifications during Mutliplexed Translatome Measurements

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ABSTRACT: Pulsed Stable Isotope Labeling in Cell culture (SILAC) approaches allow measurement of protein dynamics, including protein translation and degradation. However, its use for quantifying acute changes has been limited due to low labeled peptide stoichiometry. Here, we describe the use of instrument logic to select peaks of interest via targeted mass differences (TMD) for overcoming this limitation. Comparing peptides artificially mixed at low heavy-to-light stoichiometry measured using standard data dependent acquisition with or without TMD revealed 2−3-fold increases in identification without significant loss in quantification precision for both MS^2 and MS^3 methods. Our benchmarked method approach increased throughput by reducing the necessary machine time. We anticipate that all pulsed SILAC measurements, combined with tandem mass tagging (TMT) or not, would greatly benefit from instrument logic based approaches.
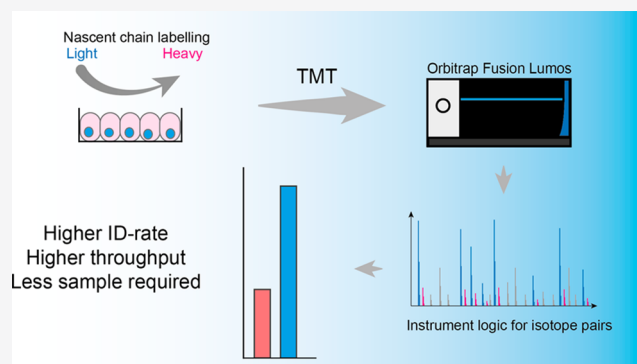
Most protein dynamics measurements by mass spectrometry rely on metabolic labeling, such as Stable Isotope Labeling in Cell culture (SILAC) or unnatural amino acid analogues.[1−3] Combining these labeling approaches with tandem mass tagging (TMT) enables highly reproducible multiplexed measurements.[4−9] However, a major challenge when using pulsed SILAC (pSILAC) approaches to study protein dynamics in a time-resolved manner is the low stoichiometry of labeled peptides, preventing their identification and quantification. We recently described multiplexed enhanced PROtein Dynamics (mePROD) proteomics that use a booster channel (peptides derived from fully SILAC labeled cells) to specifically increase the signal of labeled peptides only, to allow their quantification.[10] However, labeled peptides remain at low stoichiometry compared to their nonlabeled counterparts, resulting in only a small fraction of mass spectrometer measurement time to be used to measure labeled peptides.

The new generation of Orbitrap mass spectrometers, such as the Orbitrap Fusion, Orbitrap Fusion Lumos, Orbitrap Eclipse, and Exploris480, offer the possibility to use instrument logic workflows, which we reasoned could potentially overcome this problem to a large extent. These filters enable on-the-fly identification of label pairs in the survey scan, such as that produced by SILAC, and subsequent targeting of one or both ions for MS^2. We hypothesized that low stoichiometry SILAC samples would greatly benefit from targeted mass difference (TMD) based instrument methods. A similar rationale has previously been used for targeted experiments[11] but, to our

knowledge, not for data dependent acquisition (DDA) measurements.

Here, we assessed whether TMD can improve identification rates of TMT-multiplexed pSILAC samples. We found that label identification increased to approximately 2.5-fold in an unfractionated sample, without influencing quantification accuracy.

■ EXPERIMENTAL SECTION

Cell Culture. HeLa cells were cultured as described previously.[10]

Sample Preparation. Lysates for MS sample preparation were obtained and TMT-SILAC samples prepared as previously described.[10]

Liquid Chromatography Mass Spectrometry. Samples were prepared for mass spectrometry analysis as described previously.[10] After sample preparation, 1 μg of peptide was resuspended in 2% acetonitrile (ACN)/1% formic acid (FA) and separated on an Easy nLCII (Thermo Fisher Scientific) using a 25 cm long, 75 μM inner diameter fused-silica column packed in house with 3 μM C18 particles (ReproSil-Pur, Dr.
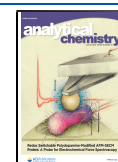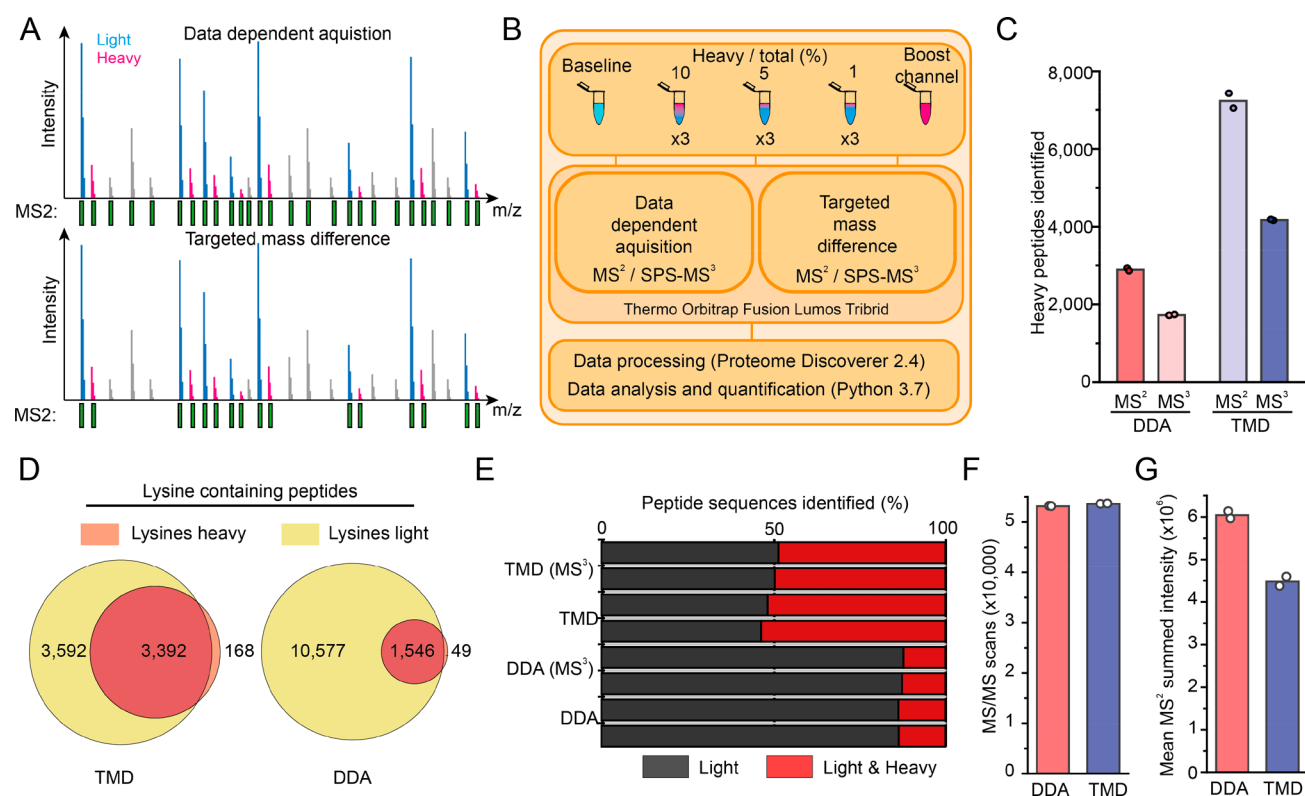
Figure 1. Instrument logic measurements increase identification and quantification rate in pulsed SILAC-TMT experiments. (A) Scheme of instrument logic based methods. Isotope pairs are identified online and subsequent scans only performed on identified pairs. (B) Experimental scheme, low stoichiometry SILAC ratios were mixed and combined with baseline and boost channel, labeled with TMT11 and measured either by data dependent acquisition (DDA) or targeted mass difference (TMD) with MS^2 or MS^3 acquisition settings. Data processing was performed using Proteome Discoverer 2.4 and Python 3.7. (C) Number of identified heavy peptides for all tested methods ($n = 2$). Bar represents mean. (D) Comparison between heavy and light lysines identified. (E) Percentage of heavy light distribution over all replicates and methods. (F) Number of MS/MS scans performed during same gradient time by DDA and TMD ($n = 2$). (G) Mean MS^2 summed intensity for DDA and TMD based methods ($n = 2$). Bars represent mean of replicates.

Maisch) and kept at 45 °C. Peptides were eluted by a nonlinear gradient from 4 to 40% ACN over 120 min and sprayed into an Orbitrap Fusion Lumos mass spectrometer. The exact settings for each of the examined methods can be found in the Supporting Information.

Data Analysis. Raw files were analyzed using Proteome Discoverer (PD) 2.4 (ThermoFisher Scientific). Spectra were selected using default settings and database searches performed using the SequestHT node in PD. Database searches were carried out with databases of trypsin digested proteomes (*Homo sapiens* SwissProt database [TaxID:9606, 2017-06-07/2018-11-21]) and FASTA files of common contaminants ("contaminants.fasta" provided together with MaxQuant) for quality control. Fixed modifications were set as TMT6 at the N-terminus and carbamidomethyl at cysteine residues. As dynamic modifications, TMT6 (K), TMT6+K8 (K, +237.177), Arg10 (R, +10.008), and methionine oxidation were set. After a search, posterior error probabilities were calculated and perfect spectrum matches (PSMs) were filtered using Percolator using default settings. Consensus Workflow for reporter ion quantification was performed with default settings, except the minimal signal-to-noise ratio was set to 5. Results were then exported to Excel files for further processing. Excel files were used as input for a custom-made in-house Python pipeline. Python 3.6 was used together with the following packages: pandas 0.23.4,[12] numpy 1.15.4,[13] and scipy 1.3.0., as described previously.[10] For injection time normalization,

summed TMT intensities per PSM were divided by the injection time for that PSM, scaled by a factor of 1000, and split on the individual channels by their original ratios.

Isolation interference was calculated by summing the intensities for all TMT channels per PSM and dividing the baseline value by the sum. Assuming isolation interference to be stochastically even across all channels, the resulting value was multiplied by the number of channels used.

RESULTS AND DISCUSSION

During mePROD or comparable experimental measurements, the signal of interest (e.g., the heavy peptide, representing newly synthesized proteins) usually ranges up to around 10% of the corresponding light peptide intensity. During DDA, the top N most intense ions are selected for the dependent scan(s), which are most likely nonlabeled peptides, due to their inherently higher intensity. Thus, we hypothesized that identifying the isotope pair in the survey scan and targeting both ions of the pair for MS^2 or MS^3, respectively, would increase the amount of measured signal of interest while enabling normalization on total protein level (Figure 1A).

To assess whether instrument logic based workflows using TMD targeting are beneficial for pSILAC-TMT methods, we constructed a mePROD experiment with artificially mixed, substoichiometric heavy peptide ratios (Figure 1B). The sample contained a mix of peptides derived from lysates of nonlabeled or heavy (SILAC) labeled HeLa extracts at heavy/
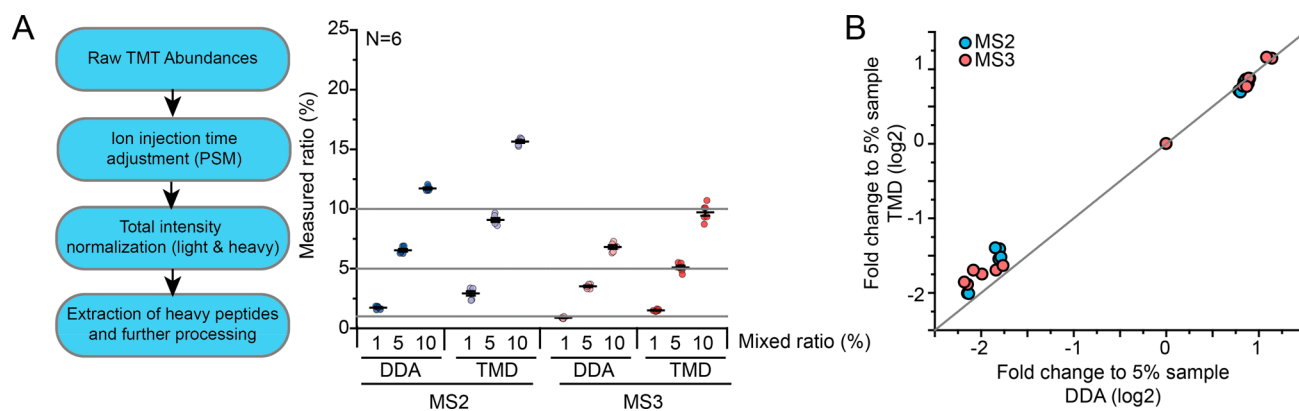
Figure 2. Absolute and relative quantification accuracy of TMD measurements. (A) Scheme of normalization approach used for determination of heavy/total ratio. Median measured ratio of all heavy PSMs compared to fully labeled booster ($n = 6$, over two multiplexes) for DDA and TMD measurements. Gray lines indicate mixed ratios, black lines indicate mean of replicates. (B) Comparison of dynamic range of MS^2 and MS^3 based relative quantification to reference samples (5% mixed ratio) for TMD and DDA approaches. Gray line indicates reference diagonal.
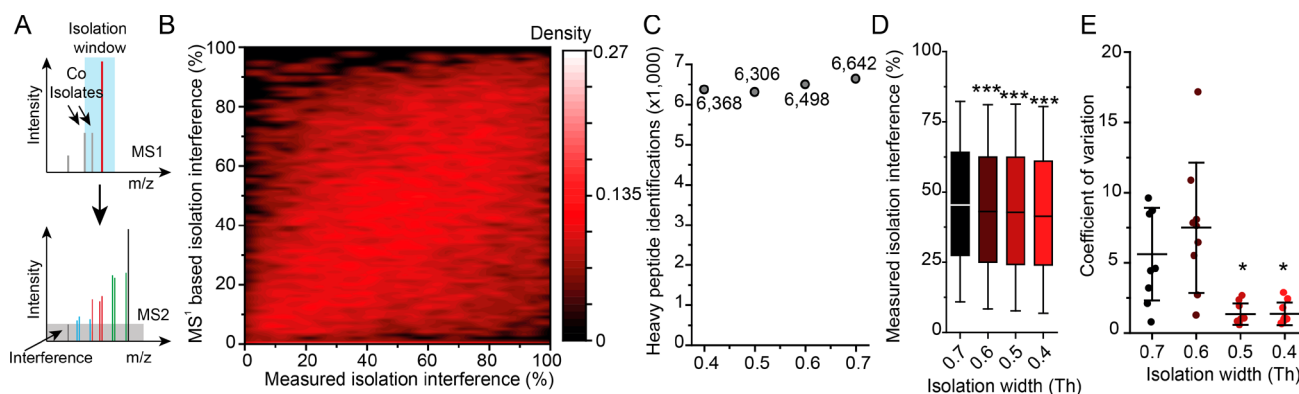

Figure 3. Narrower isolation window reduces isolation interference and variation while maintaining identification rate. (A) Scheme of experimental determination of coisolation for each PSM. Light-only baseline channel abundance represents coisolated light peptides in heavy PSM. Noise subtraction can overcome ratio compression. (B) Density plot for measured isolation interference and isolation interference predicted from survey scan (PD 2.4). (C) Heavy peptide identifications by TMD in dependency of isolation width. (D) Measured interference in dependency of different isolation windows. ***$P < 0.001$. Boxes represent 25−75% quantiles, and whiskers indicate SD. (E) Coefficient of variation of measured heavy/total ratios with different isolation windows. *$P < 0.05$ ($n = 9$, one multiplex). Middle bars represent median, and error bars indicate SD.

total ratios between 1 and 10% in triplicate. In addition, we included a TMT-labeled booster channel and a nonlabeled noise channel for a mePROD measurement setup that allows heavy peptide signal amplification and noise determination, respectively.[10] We observed a 2.5-fold increase in identified heavy peptides from the same sample when comparing TMD to standard DDA measurements, irrespective of acquisition by MS^2 or MS^3 methods (Figure 1C). The majority of measured heavy peptide sequences was also identified in their non-isotopic form (Figure 1D). TMD improved the percentage of isotope labeled peptides identified relative to total by about 3-fold to about 50% in MS^2 and MS^3 measurements (Figure 1E). The total number of scans performed by the spectrometer remained the same (Figure 1F). We observed a drop in mean MS^2 intensities consistent with a higher percentage of scans carried out for low abundant peptides (Figure 1G).

Next, we examined the accuracy of heavy peptide quantification by comparing the signal of all channels after data analysis to the booster channel. Total intensity normalization of the full data set (light and heavy) resulted in an overestimation of the measured heavy to total ratios in both MS^2 and MS^3 (Figure S1). We expected this to be the result of the normalization procedure, rather than the measured data. Due to the constrained nature of the population of ions

measured in a TMT experiment,[14] the extracted intensities of the reporter ions are not faithfully reflecting the peptide intensity. For MS/MS scans, a fixed amount of ions is collected, independent of the precursor abundance, thus creating technical constraint for reporter ion intensities that can be reached. As a result, normalization by summing TMT intensities during SILAC hyperplexing might distort the calculated results. We therefore included an additional calculation in the data analysis: measured abundances were corrected for the injection time, which was needed to reach the AGC-target value, as an experimentally determined value approximating ion abundance (Figure 2A). Subsequently, corrected values were normalized and processed as before. This additional correction step reduced ratio distortion observed in MS^2 and MS^3 TMD measurements, with only minor effects on DDA-based quantifications. While TMD-MS^2 measurements still showed a 1.5-fold overestimation of the heavy to total ratio, TMD-MS^3 exhibited the most accurate quantification over the whole range of ratios tested. When assessing relative fold changes instead of absolute values (i.e., heavy/total ratios), DDA and TMD methods performed equally well, irrespective of MS^2 or MS^3 measurements used (Figure 2B). In most experimental setups, only relative quantification is required. For these, we recommend using

MS[2] methods as these outperform MS[3] in the number of identifications.

A common problem during MS[2] and (to a lesser extent) MS[3] based quantification is the isolation of interfering peptides distorting quantification results. Precursor ion contamination in MS[2] results in ratio compression and was overcome by multinotch MS[3] approaches.[15] We recently showed that the inclusion of a baseline channel for background subtraction in mePROD measurements is sufficient to overcome ratio compression.[10] Strikingly, the baseline channel in mePROD experiments provides with an experimental system to measure isolation interference by determining isolation interference from highly abundant light peptides (Figure 3A). Notably, we observed that the measured isolation interference did not correlate with the isolation interference predicted from survey scans for filtering of perfect spectrum matches (PSMs) (Figures 3B and S2A/B). This was irrespective of MS[2] or MS[3] methods being used (Figure S2A/B). Using an MS[3] method reduced the measured isolation interference while still observing a broad range of interference values among heavy PSMs. TMD approaches in general exhibited a higher isolation interference as expected due more low abundant peaks being targeted for subsequent scans (Figure S3B). We next examined whether isolation window narrowing reduces measured isolation interference. Narrowing the isolation window to 0.4 Th significantly reduced isolation interference while only resulting in a slight reduction of heavy peptide and protein identification (Figures 3C/D and S2C). Combining post-acquisition baseline correction with isolation window narrowing significantly increased accuracy with smaller variation observed (Figure 3E).

Isolation window reduction resulted in higher median MS[2] fill time and decreased mean summed MS[2] intensities (Figure S2D). We next asked whether increasing maximum fill times would result in an additional benefit for quantification. Increasing maximum MS[2] injection time (while keeping the isolation window fixed at 0.4 Th) resulted in a smaller number of identified peptides as expected due to the increase in cycle times (Figure S2E). Overall, the loss in MS[2] intensity was only partially rescued with median summed intensities increased by 28%, while the injection time was tripled (Figure S2F). Therefore, we concluded that the reduction of the isolation window can enhance the data quality and does not need to be supplemented by higher injection times, resulting in most efficient use of the cycle time.

Taken together, we showed that instrument logic based measurements significantly increase the identification of low abundant pSILAC signals while maintaining relative quantification accuracy. This significantly reduces the machine time required and improves identifications and quantification. The benefit of TMD was particularly pronounced in conditions with large ratio differences between the used SILAC labels. In equally distributed samples, TMD increased identification and ensured measurement of both labels to calculate ratios.

We showed that experimental setups such as those in mePROD experiments allow one to experimentally assess co-isolation interference in isobaric-multiplexed experiments and could provide the bases for statistical approaches to reduce ratio compression. We anticipate that instrument logic based approaches are beneficial for any pSILAC experiment (including triple SILAC labeled samples), especially when requiring high throughput or low sample input amounts.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c01749.

Instrument settings for Orbitrap Fusion Lumos for DDA and TMD based methods; overestimation of heavy to total ratios using classical total intensity normalization; effect of isolation window narrowing on the measured isolation interference in pulsed SILAC experiments (PDF)

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https://pubs.acs.org/10.1021/acs.analchem.0c01749

Author Contributions

K.K performed the experiments and analyzed data. K.K. and C.M. designed experiments, analyzed the data, and wrote the manuscript. C.M. conceived and supervised the study. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[16] partner repository with the data set identifier PXD018445.

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