MSstatsPTM: Statistical relative quantification of post-translational modifications in bottom-up mass spectrometry-based proteomics

Devon Kohler[1], Tsung-Heng Tsai[2], Erik Verschueren[4], Ting Huang[1], Trent Hinkle[3], Lilian Phu[3], Meena Choi*[3], and Olga Vitek*[1]

[1]Khoury College of Computer Science, Northeastern University, Boston, MA, USA
[2]Kent State University, Kent, OH, USA
[3]MPL, Genentech, South San Francisco, CA, USA
[4]ULUA BV, Arendstraat 29, 2018 Antwerp, Belgium
*Corresponding Authors

Abstract

Liquid chromatography coupled with bottom up mass spectrometry (LC-MS/MS)-based proteomics is increasingly used to detect changes in post-translational modifications (PTMs) in samples conditions. Analysis of data from such experiments faces numerous statistical challenges. These include the low abundance of modified proteoforms, the small number of observed peptides that span modification sites, and confounding between changes in the abundance of PTM and the overall changes in the protein abundance. Therefore, statistical approaches for detecting differential PTM abundance must integrate all the available information pertaining to a PTM site, and consider all the relevant sources of confounding and variation. In this manuscript we propose such a statistical framework, which is versatile, accurate, and leads to reproducible results. The framework requires an experimental design, which quantifies, for each sample, both peptides with post-translational modifications and peptides from the same proteins with no modification sites. The proposed framework supports both label-free and tandem mass tag (TMT)-based LC-MS/MS acquisitions. The statistical methodology separately summarizes the abundances of peptides with and without the modification sites, by fitting separate linear mixed effects models appropriate for the experimental design. Next, model-based inferences regarding the PTM and the protein-level abundances are combined to account for the confounding between these two sources. Evaluations on computer simulations, a spike-in experiment with known ground truth, and three biological experiments with different organisms, modification types and data acquisition types demonstrate the improved fold change estimation and detection of differential PTM abundance, as compared to currently used approaches. The proposed framework is implemented in the free and open-source R/Bioconductor package $MSstatsPTM$.

Introduction

Signaling mechanisms allow cells to mount a fast and dynamic response to a multitude of biomolecular events. Signaling is facilitated by the modification of proteins at specific residues, acting as molecular on/off switches [1, 2, 3]. Characterizing relative abundance of a modification site's occupancy repertoire across experimental conditions provides important insights [4]. For example, meaningful patterns of changes in post-translational modifications (PTMs) abundance can serve as biomarkers of a disease [5]. Alternatively, distinguishing the quantitative changes in a PTM from the overall changes of the protein abundance helps gain insight into biological and physiological processes operating on a very short timescale [6][7]. This helps to distinguish between relative site occupancy changes at steady-state protein levels, typical for short time-scale signaling events, and observed relative changes of PTMs as a result of underlying gene expression or protein abundance levels.

Bottom-up liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is a tool of choice for unbiased and large-scale identification and quantification of proteins and their PTMs [8, 9]. However, LC-MS-based interrogation of the modified proteome is challenging, for a number of reasons. First, the relatively lower abundance of modified proteoforms dictates that a global interrogation can only be achieved through large-scale enrichment protocols with modification-specific antibodies or beads [10]. Variability in the enrichment efficiency inevitably affects the reproducibility of the number of spectral features (e.g., peptide precursor ions or their fragments) and their intensities. Second, contrary to the often large number of identified peptides that can be used to quantify protein abundance, there are relatively few representative peptides that span a modification site, and there may be multiple modified sites on a single peptide [4]. Third, unless early signaling events are interrogated, the interpretation of the relative changes in modification occupancy are inherently confounded with changes in the overall protein abundance, complicating the interpretation of the results [11][12]. Finally, technological aspects of bottom-up MS experiments, such as presence of labeling by tandem mass tag (TMT), introduce additional sources of uncertainty and variation.

The technological difficulties in PTM identification and quantification increase the uncertainty and the variation in the data, and challenge the downstream statistical analyses. Frequently data from these experiments are analyzed using statistical methods that were not originally designed for this task. Researchers use methods such as t-test[13], Analysis of Variance[14], or Limma[15], by taking as input the intensity ratios of modified and unmodified peptide features, and comparing the mean abundance of different PTM sites. Such approaches do not fully account for all the sources of uncertainty. As the result, these approaches are either not directly applicable to experiments with non-trivial designs (such as experiments with multiple conditions, paired and time course designs, and experiments with labeling), or require the analysts to exercise non-trivial statistical expertise.

This manuscript proposes a versatile statistical analysis framework that accurately detects relative changes in post-translational modifications. The framework requires an experimental design, which quantifies, for each sample, both the peptides with post-translational modifications, and peptides from the same proteins with no modification sites. The framework supports data-dependent acquisitions (DDA) that are label-free or tandem mass tag (TMT)-based. The statistical methodology separately summarizes the abundances of peptides with and without the modification sites, and fits separate linear mixed effects models that reflect the biological and technological aspects of the experimental design. Next, model-based inferences regarding the PTM and the protein-level abundances are combined to account for the confounding between these two sources.

We evaluated the proposed framework on two datasets from computer simulations, one benchmark controlled mixture, and three biological investigations. The datasets illustrate a diverse set of organisms, modification types, acquisition methods and experimental designs, showing the applicability of the framework to a variety of situations. By appropriately leveraging the information from the unmodified peptides, the proposed approach improved the accuracy of the estimates of PTM fold changes, and produced a better calibrated false positive rate of detecting differentially abundant PTMs as compared to existing methods. In particular, accounting for the confounding from protein abundance allowed us to characterize the true effect of the modification, avoiding the need for more manual and time intensive follow-up investigation.

The proposed approach is implemented as a freely available open source R package $MSstatsPTM$, as part of the $MSstats$ family of packages [16, 17], and is available on Bioconductor.

Experimental procedures

Data overview and availability

Table 5.1 summarizes the experiments. Two computer simulations had known ground truth, and varied in experimental realism. The first simulation produced a perfectly clean dataset, with many replicates and no missing values. The second simulation introduced real-world characteristics, such as limited modified features and missing values. Details of computer simulations are available in Supplementary Section 2.1 and on GitHub (`https://github.com/devonjkohler/MSstatsPTM_simulations`).

One spike-in experiment also had known changes in modified spike-in peptides, but had real-world experimental characteristics. Finally, three biological experiments demonstrated the applicability of the proposed approach across different biological organisms, modifications, experimental designs and acquisition strategies. The experimental data, R scripts with $MSstatsPTM$ analysis, and results of the statistical analysis are available in MassIVE.quant (`https://massive.ucsd.edu/ProteoSAFe/static/massive-quant.jsp`) [18].

Dataset 1: Computer simulation 1 - Label-free

Simulation design: The simulation represented an idealistic case. 24 synthetic label-free datasets were generated with different experimental designs and different biological variation. In each dataset, 1,000 proteins had 10 unmodified features per protein. Each of the 1,000 proteins had one PTM. Each PTM was represented by 10 modified features. The PTMs of 500 proteins had a differential fold change between conditions, while the other 500 proteins were generated with no changes in abundance between conditions. Furthermore, the fold changes of half of the 500 differential PTMs were fully masked by changes in the unmodified protein. Finally, the fold change of half the 500 non-differential PTMs was entirely due to changes in the unmodified protein. All the differential PTMs were generated with an expected log base 2 fold change of 0.75 between conditions.

Each simulation was generated with random biological variation. The observed peptide abundances were simulated by adding random noise $\mathcal{N}(0, \sigma^2)$ to the deterministic abundances described above. Two values $\sigma^2 = \{.2, .3\}$ were motivated by the experimental datasets in this manuscript.

Evaluation: We evaluated the ability of the statistical methods to correctly detect differentially abundant PTMs. We gauged the methods ability to avoid false positives (i.e. specificity), accurately estimate the fold change between conditions, and analyzed the sensitivity of detecting differentially abundant PTMs. The evaluation was performed both in the presence of confounding with changes in the unmodified protein and after applying adjustment to correct for the confounding.

Dataset 2: Computer simulation 2 - Label-free missing values and low features

Simulation design: The data were simulated as above, while providing a more realistic representation of the experiments. The feature counts and the proportion of missing values were as observed on average over all the the experimental datasets in this manuscript. Specifically, PTMs were simulated with 2 modified peptide features, and unmodified proteins were simulated with 10 features. Additionally, 20% of observations for both modified and unmodified peptides were missing completely at random.

Evaluation: The methods were evaluated as above. We evaluated their ability to correctly detect PTM's specificity, fold change estimation, and sensitivity. These statistics were analyzed both in the presence of, and without, confounding with the overall changes in protein abundance.

Dataset 3: SpikeIn benchmark - Ubiquitination - Label-free

Experimental Design: Figure 1(a) overviews the experimental design. Four mixtures (i.e., conditions) were created with varying amounts of human lysate, background *E. coli* lysate, and human spike-in ub-peptide mixture. Unmodified peptides from human lysate were viewed as the global proteome. Background *E. coli* lysate were used to equalize total protein levels prior to enrichment or global protein profiling between Mix 1 and 3, and between Mix 2 and 4. 50 heavy-labeled KGG motif peptides from 20 human proteins were used as

spike-in peptides in a mixed background of lysates from *E. coli* and a human cell line. Quantitative changes in protein and site abundance of these 20 human proteins were the target of the benchmark. In particular, we distinguished the changes in the abundances of the modified peptides (i.e., unadjusted changes) and the changes of their abundance relative to the changes in the abundances of the human lysate (i.e., protein-level adjusted changed). The true log-fold changes between the relevant components of the relevant mixtures are summarized in **Figure 1**(b). Two replicate mixtures were created per condition.

Data acquisition: Each mixture was analyzed with KGG enrichment, and without KGG enrichment (i.e., in a global profiling run), with label-free LC-MS/MS. There was a 90.2% overlap of protein identifications between the identified background modified peptides and proteins that were quantified in the global profiling run.

Evaluation: We expect the spike-in peptides to change corresponding to the values in Figure 1(b). These peptides are treated as positive controls in all comparisons except Mix 1 vs Mix 4, which was treated as a negative control. Additionally, the background *E. coli* lysate peptides were treated as negative controls and were not expected to change in any comparison. We evaluated the statistical methods ability to avoid false positives, as well as their sensitivity in detecting the differentially abundant spike-in peptides and accurately estimate their expected fold change.

Dataset 4: Human - Ubiquitination - 1mix-TMT

Experimental Design: Luchetti et al. [19] profiled human epithelial cells engineered to express IpaH7.8 under a dox inducible promoter. Uninfected cells were measured at 0 and 6 hours, while cells infected with *Shigella flexneri* (*S. flexneri*) bacteria were measured at 1, 2, 4, and 6 hour increments, resulting in six total conditions. 11 samples were allocated to 1 TMT mixture in an unbalanced repeated measure design. All conditions had two biological replicates except for the Dox1hr condition, which was allocated one replicate.

Data acquisition: The ubiquitinated peptides, and the total proteome (i.e., global profiling) were each conducted in a single LC-MS/MS run. There was a 95% overlap between the identified modified peptides and proteins that were quantified in the global profiling run.

Evaluation: We evaluated the ability of the statistical methods to detect changes in the abundance of modified peptides both before and after adjusting for changes in global protein abundance. The six condition were labeled Dox1hr, Dox2hr, Dox4hr, Dox6hr, NoDox0hr, and NoDox6hr. All conditions were compared with each other, resulting in 15 pairwise comparisons. Since the dataset was a biological investigation, the true positive modifications were unknown. Shigella ubiquitin ligase IpaH7.8 was shown to function as an inhibitor of the protein gasdermin D (GSDMD). GSDMD was actively degraded when IpaH7.8 expression was induced by dox treatment in human cells. We expect IpaH7.8 to function as an inhibitor of GSDMD in the global profiling run.

Dataset 5: Mouse - Phosphorylation - 2mix-TMT

Experimental Design: Maculins et al. [20] studied primary murine macrophages infected with *S. flexneri*. The experiment quantified the abundance of total protein and of phosphorylation in wild type (WT), and in ATG16L1-deficient (cKO) samples, uninfected and infected with *S. flexneri*. The abundance of total protein and post-translation modifications were quantified at three time points, uninfected, early infection (45-60 minutes), and late infection (3-3.5 hours). 22 biological samples were allocated to 2 TMT mixtures in an unbalanced repeated measure design, with 11 samples allocated to each mixture. 16 replicates were spread equally between the early and late WT and cKO conditions, resulting in four replicates per condition. Both the uninfected WT and cKO contained 3 replicates, with mixture one allocating one replicate to uninfected WT and two replicates to uninfected cKO. Conversely, mixture two contained one replicate of uninfected cKO and two uninfected WT.

Data acquisition: This experiment included a total proteome (i.e., a global profiling run) and a phosphopeptide enrichment run. There was a 90% overlap between the identified modified peptides and proteins that were quantified in the global profiling run.

Evaluation: We evaluated the ability of the statistical methods to detect changes in the abundance of modified peptides both before and after adjusting for changes in global protein abundance. The six condition were labeled KO_Uninfect, KO_Early, KO_Late, WT_Uninfect, WT_Early, and WT_Late. 9 total comparisons were made, namely KO_Early-WT_Early, KO_Late-WT_Late, KO_Uninfected-WT_Uninfected, KO_Early-KO_Uninfected, KO_Late-KO_Uninfected, WT_Early-WT_Uninfected, WT_Late-WT_Uninfected, Infected-Uninfected, and KO-WT. Since the dataset was a biological investigation, the true positive modifications were unknown.

Dataset 6: Human - Ubiquitination - Label-free no global profiling run

Experimental Design: Cunningham et al. [21] investigated the relationship between USP30 and protein kinase PINK1, and their association with Parkinson's Disease. The experiment profiled ubiquitination sites, and analyzed changes in the modified site abundance. The experiment had four conditions, CCCP, USP30 over expression (USP30 OE), Combo, and Control. Cell lines were used to create two biological replicates per condition. The abundance of modified peptides was quantified with label-free LC-MS/MS.

Data acquisition: This experiment did not include a separate global profiling run to measure unmodified peptides. In addition to low feature counts for unmodified peptides, this lead to substantially fewer matches between modified and unmodified peptides. There was a 41.9% overlap between the identified background modified peptides and proteins that were quantified in the global profiling run.

Evaluation: We evaluated the ability of the statistical methods to detect changes in the abundance of modified peptides both before and after adjusting for changes in global protein abundance. All the conditions were compared with each other in a full pairwise comparison, resulting in 6 comparisons. Since the dataset is a biological investigation, the true positive modifications were unknown.

Background

Goals of PTM characterization, input to statistical analyses, and notation

Consider a label-free LC-MS/MS experiment in the special case of a balanced design with I conditions and J biological replicates per condition. For simplicity, we assume that the experiment has no technical replicates, such that each biological replicate corresponds to an LC-MS/MS run. Figure 2 schematically illustrates this data structure for one PTM site, in a special case of $I = 2$ conditions and $J = 2$ biological replicates per condition. For one protein, the PTM site is represented by K spectral features (i.e., peptide ions, distinguished by their cleavage residues and charge states). The number of modified and unmodified features typically varies across proteins. Some log-intensities may be outliers, and some spectral features can be missing. The $\log_2$-intensity of Feature k, in Replicate j of Condition i is denoted by y^*_{ijk}. Conversely, the unmodified protein is represented by L spectral features, and the log-intensity of Feature l from the unmodified peptides in the same run is denoted by y_{ijl}. The features can be quantified as part of a same mass spectrometry run, or in separate enrichment and global proteome profiling runs.

The population parameter of interest is the difference between the abundances of a PTM site in Condition i and Condition i', denoted by μ^*_i and $\mu^*_{i'}$ respectively. We are interested in testing the null hypothesis

$$H_0 : \Delta_{PTM} = \mu^*_i - \mu^*_{i'} = 0 \text{ vs } H_a : \Delta_{PTM} = \mu^*_i - \mu^*_{i'} \neq 0 \quad (1)$$

Unfortunately, the population parameter is inherently confounded with the overall changes in protein abundance. To account for this, it is advantageous to consider a different null hypothesis:

$$H_0 : \Delta_{adj} = (\mu^*_i - \mu_i) - (\mu^*_{i'} - \mu_{i'}) = 0 \text{ vs } H_a : \Delta_{adj} = (\mu^*_i - \mu_i) - (\mu^*_{i'} - \mu_{i'}) \neq 0 \quad (2)$$

where μ_i and $\mu_{i'}$ reflect the overall protein abundances in Condition i and Condition i'. These quantities are estimated using protein features without with and without the modification site.

Existing statistical methods for detecting differentially abundant PTMs

[OV: even though we say 't-test', the model that we describe is in fact an ANOVA (i.e., nothing limits us to 2 conditions. Should we change it to ANOVA directly?)]

Two-sample t-test based on modified $\log_2$-intensities

[OV: this doesn't look right, as it seems that all the features and replicates equally contribute to the error. Do people do some kind of summarization of the features first? Let's first describe the summarization] [DK: I think we should just delete this part entirely. It isn't how we compared the method and is not really used in the literature from what I can tell.] [OV: below in the results we use the notation y_{i++} etc. Alternatively, since we put a hat on $\hat{y}_{ij}$, maybe we do not need '++', as it is clear that this is a summary. I removed '++' in the results section for now. Let's use the same notation in this section consistently]

The basic two-sample t-test [ref - ideally from this literature if you can find] is based on the model

$$y^*_{ijk} = \hat{\mu}^*_i + \epsilon^*_{ij},\ \epsilon^*_{ij} \overset{iid}{\sim} \mathcal{N}(0, \sigma^{*2}),\ i = 1, \ldots, I,\ j = 1, \ldots, J,\ k = 1, \ldots, K \tag{3}$$

The model allows us to estimate $\hat{\Delta}$ and its standard error. Based on the model, the approach tests the null hypothesis in Eq. (1), by comparing the test statistic against the Student distribution with $df = 2J - 2$ degrees of freedom in balanced designs. Unfortunately, this approach is fundamentally flawed as it does not account for the confounding between changes in the PTM abundance and the overall changes in the abundance of the protein.

Two-sample t-test based on ratios of modified and unmodified $\log_2$-intensities

The basic t-test can be extended to account for the confounding of changes in PTM abundance and overall changes in protein abundance [22, 23, 24]. Typically this is done by first summarizing the feature intensities in each run, separately for modified and unmodified features, e.g. with the sum on the original scale, dividing the summary of the PTM abundance by the summary of the protein abundance and then taking a log. Specifically, for condition i and biological replicate j, the approach calculates

$$u_{ij} = \log\left(\sum_{k=1}^{K} 2^{y^*_{ijk}}\right) - \log\left(\sum_{l=1}^{L} 2^{y_{ijl}}\right) \tag{4}$$

The approach then models these values as

$$u_{ij} = (\mu^*_i - \mu_i) + \epsilon'_{ij}, \text{where } \epsilon'_{ij} \overset{iid}{\sim} \mathcal{N}(0, \sigma'^2), i \in \{1, 2\} \tag{5}$$

The model allows us to estimate $\hat{\Delta}_{adj}$ and its standard error. Based on this model, we can test the more relevant null hypothesis in Eq. (2), by comparing the test statistic against the Student distribution with [how many] degrees of freedom in balanced designs.

Although effective, the approach is somewhat simplistic. It is not applicable to experimental designs with more complex sources of biological and technological variation, such as experiments with repeated measurements, experiments with multiple batches or experiments with multi-run multiplexing. Since Eq. (4) performs the adjustment on the replicate level, the experiment must contain a matching number of replicates in both the modified and unmodified runs. Technological artifacts such as missing values further undermine the calculation of u_{ij} in Eq. (4). Finally, the lack of a self contained, straightforward implementation of the method challenges the applications [OV: is the last statement true? Seems simple enough]. [DK: I meant more that there was no simple package implementation. Researchers need to program the methods themselves. I cant find an implementation at least]

Limma

The two versions of t-test [OV: ANOVA?] above can be expanded with Empirical Bayes moderation in Limma [15, 22, 25, 26, 27, 28]. In particular, the ratio-based version models the ratios $\hat{u}_{ij}$ in Eq. (4). Limma then fits a linear model using the transformed inputs.

$$\hat{u}_{ij} = \beta_0 + c_i\beta_1 + +r_j\beta_2 + \epsilon''_{ij}, \text{where } \epsilon''_{ij} \overset{iid}{\sim} \mathcal{N}(0, \sigma''^2/w_j), i \in \{1, 2\}, j \in \{1, 2\} \tag{6}$$

[Essentially, Limma can fit all kinds of models - with or without the bio replicate, including the ones in the equations above, it depends on the user. The only difference from the approaches above is the EB moderation. I am confused by r_j do you mean $I_{biorep=j}$? Since replicate id takes continuous values, the model above is a regression with replicate id as a single predictor / 1 df - not sure that's right; I am confused by w_j - this is not how Limma model is specified. Also what are the β and why we only have one of them? Let's check which versions of the models the references use and how Ting describes Limma in her paper.]

The goal of Limma is to increase the sensitivity of detection of differential abundance when the experiment has few biological replicates. This is done by means of Empirical Bayes moderation, i.e. by borrowing the information regarding the variation across all the modification sites. Specifically, [OV: this should be another model equation, rather than the text]

For each modification site, the model yields the same estimate $\hat{\Delta}_{adj}$ as Eq. (5), but a different standard error. To estimate $\widehat{SE(\hat{\Delta})}$, Limma leverages empirical bayes moderation [29] to adjust the model variance of the individual PTM using the shared variance of all PTMs modeled in the experiment. [DK: I introduced a new subscript here for the PTM p. It feel awkward to add it across the other methods so I only included it in the moderation describtion.] For a single PTM p with an estimated fold change $\hat{\Delta}_p$, the estimated variance is calculated as $var(\hat{u}^*_{pij}) = \sigma^2_p / w_{pj}$ where σ^2_p is the variance resulting from the linear model of PTM p and w_{pj} is the global variance across all PTMs in replicate j. The statistical significance is then determined using $\hat{\Delta}_p$ and $\widehat{SE(\hat{\Delta}_p)}$ calculated using the adjusted variance $var(\hat{u}^*_{pij})$.

Since Limma only improves upon the methods above in estimation of variation, it has the same limitations in terms of the experimental designs. The method is only directly applicable to experiments with at most two variance components, meaning that in more complex experiments it cannot account for all sources of variation. Additionally, it still requires the same experimental design for the modified and unmodified features so that the ratio calculation can be performed. Finally, there is no self contained implementation of the methods to PTMs, requiring manual transformation and application by the user.

Isobar-PTM Isobar-PTM was also proposed for experiments with LC-MS/MS quantitative strategies that employ isobaric labels such as TMT or isobaric tag for relative and absolute quantification (iTRAQ)[30]. Isobar-PTM expresses MS measurements with a linear model and performs adjustment with respect to protein abundance using the difference between log-ratio of modified peptides in two channels and log-ratio of protein level. Unfortunately, this statistical modeling framework is not applicable to either label-free workflows or experiments with complex designs.

Statistical modeling and parameter estimation in MSstats

MSstats [16] and *MSstatsTMT* [17] are a series of R/Bioconductor packages designed for protein significance analysis and statistical relative quantification of proteins and peptides in global, targeted and data-independent proteomics. Models in these packages takes as input a list of $\log_2$-intensities y_{ijk}. For each protein, the feature $\log_2$-intensities are first summarized into a single value per protein per run using Tukey's median polish [31]. These summarized values are then used as input to fit a flexible family of linear mixed-effects models [32, 33, 34]. The specific model instances depend on the experimental design and data acquisition. For example, for an experiment with [group comparison or time course design?] *MSstatsTMT* fits the following model [OV: could we keep the notation as consistent as possible across this section? Above conditions had subscripts i and bio reps had subscripts j. Could we keep a consistent notation below (or change the notation elsewhere in the manuscript?)] [Should we maybe give a model for label-free, to stay consistent with the example in figure 2? Just use I and J etc for the indices...]

$$y_{mtcb} = \mu + Mixture_m + TechRep(Mixture)_{t(m)} + Condition_c + Subject_{mcb} + \epsilon_{mtcb}, \quad (7)$$
$$\text{where } Mixture_m \sim^{iid} \mathcal{N}(0, \sigma^2_M), TechRep(Mixture) \sim^{iid} \mathcal{N}(0, \sigma^2_T),$$
$$\sum_{c=1}^{C} Condition_c = 0, \ [Subject \overset{iid}{\sim} \mathcal{N}(0, ?)] \ \epsilon_{mtcb} \sim^{iid} \mathcal{N}(0, \sigma^2)$$

Parameters of the model are estimated using restricted maximum likelihood (REML) [35]. In turn, the parameters allow us to estimate the relevant quantity $\hat{\Delta}_{Protein}$ [this has not been defined, Define here, using

the notation above?] and its standard error. Similarly to *Limma*, *MSstatsTMT* includes an optional Empirical Bayes moderation of the standard error [17]. [OV: maybe elaborate on that?] Based on the model, the approach tests the null hypothesis in Eq. (1), by comparing the test statistic against the Student distribution.

The *MSstats* framework has a number advantages over the methods above. First, unlike [t-test (or ANOVA?) and *Limma*], the family of linear mixed effects models is applicable to many complex experimental designs, including designs with multiple sources of variation, and unbalanced designs. Second, the approach is applicable to various data acquisition types, including label-free DDA and DIA, and experiments with TMT labeling, and is compatible with various data processing tools such as [XXX] Third, the run-level summarization has been shown to be more robust to outliers and missing values as compared to feature averages [16]. Finally, the custom *MSstats* and *MSstatsTMT* implementation accounts for potential data artifacts, and is numerically scalable and stable, and [is available through both command line and a dedicated graphical user interface]. Unfortunately, MSstats estimates the difference in overall protein abundance and tests the null hypothesis in Eq. (1). It does not account for the confounding between the changes in PTM abundance and the overall changes in protein abundance. In other words, it cannot directly test the null hypothesis in Eq. (2).

Results

Statistical methods in MSstatsPTM

Detection of changes in PTMs in balanced design with one source of variation

We propose a statistical approach for detecting changes in PTMs. The method takes as input the modified spectral features y^*_{ijk}, and the corresponding unmodified features y_{ijk}. Ideally, the modified features are acquired separately, after an enrichment to maximize the information content in the resulting dataset, and the unmodified features are acquired separately as part of a global proteome profiling. However the method can also take as input a combination of modified and unmodified features acquired within a same run.

We leverage the existing methods in *MSstats* and *MSstatsTMT* and extend them to remove confounding between changes in the PTM and the overall changes in protein abundance. Each feature type is summarized separately using *MSstats* or *MSstatsTMT*, resulting in run level values for the PTM, $[\hat{y}^*_{ij}]$ [OV: I am confused: since we summarize per run, we need at least an index for a condition and an index for bio rep. Could we use this consistently across all the sections?], and unmodified peptide. The estimated summaries of the modified features are then used as the input to the model such as in eq XXX in *MSstats* or *MSstatsTMT*. Similarly, the estimated summaries of the unmodified features are used as the input to the corresponding model in *MSstats* or *MSstatsTMT*.

For the modified features, the model-based estimates in *MSstats* and *MSstatsTMT* include $\hat{\Delta}_{PTM} = \hat{\mu}^*_i - \hat{\mu}^*_{i'}$, and its standard error $\widehat{SE}(\hat{\Delta}_{PTM})$. Similarly, for the unmodified features, the model-based estimates in *MSstats* and *MSstatsTMT* incluse $\hat{\Delta}_{protein} = \hat{\mu}_i - \hat{\mu}_{i'}$, and its standard error $\widehat{SE}(\hat{\Delta}_{protein})$. Therefore, the adjusted difference $\hat{\Delta}_{adj}$ in Eq. (2) can be estimated as

$$\hat{\Delta}_{adj} = (\hat{\mu}^*_i - \hat{\mu}_i) - (\hat{\mu}^*_{i'} - \hat{\mu}_{i'}) = (\hat{\mu}^*_i - \hat{\mu}^*_{i'}) - (\hat{\mu}_i - \hat{\mu}_{i'}) = \hat{\Delta}_{PTM} - \hat{\Delta}_{protein} \tag{8}$$

Assuming that the unexplained by the model sources of variation in the modified features are independent from the corresponding sources of variation in the modified features, the standard error $\widehat{SE}(\hat{\Delta}_{adj})$ is obtained by combining the standard errors from the unmodified and modified model fits.

$$\widehat{SE}(\hat{\Delta})_{adj} = \sqrt{\widehat{SE}(\hat{\Delta}_{PTM})^2 + \widehat{SE}(\hat{\Delta}_{protein})^2} \tag{9}$$

The estimated standard error is larger than the standard errors associated with each distinct feature type, reflecting the increased uncertainty inherent to combining the uncertainty in two estimators. Finally, the degrees of freedom associated with Eq. (9) are obtained via the Satterthwaite approximation [36][OV: maybe

site a more recent exp design book?]

$$\left(\widehat{SE}(\hat{\Delta}_{PTM})^2 + \widehat{SE}(\hat{\Delta}_{protein})^2\right)^2 \Big/ \left(\frac{\widehat{SE}(\hat{\Delta}_{PTM})^4}{\mathrm{df}(\widehat{SE}(\hat{\Delta}_{PTM}))} + \frac{\widehat{SE}(\hat{\Delta}_{protein})^4}{\mathrm{df}(\widehat{SE}(\hat{\Delta}_{protein}))}\right) \tag{10}$$

To test the null hypothesis in Eq. (2), the test statistic $\hat{\Delta}_{adj}/\widehat{SE}(\Delta)_{adj}$ is compared with the Student distribution with the degrees of freedom in Eq. (10). The p-values of the comparison are adjusted for multiple testing using the approach by Benjamin and Hochberg [37].

By leveraging the implementations in *MSstats* and *MSstatsTMT*, the proposed approach is very versatile. It is applicable to a wide variety of experimental designs, including group comparison, paired designs, time course designs and unbalanced designs. It is applicable to label-free data acquisition strategies such as DDA and DIA and experiments with TMT labeling. It is compatible with data processing tools [XXX]. The proposed approach is also applicable to experiments where the experimental designs for PTM profiling and global proteome profiling vary in properties such as number of biological replicates, data acquisition strategies and runs. [OV: We do have a requirement that the scale of measurements is comparable between TMT and PTM, correct? Otherwise the SE may not be comparable]

Design of balanced PTM experiments with one source of variation

The proposed statistical framework allows for design of PTM experiments in terms of sample size calculation and power analysis. Sample size calculation takes as input a) q, the desired false discovery rate, b) β, the average Type II error rate, c) Δ, the minimal log-fold change in adjusted PTM abundance that we would like to detect, d) $m_0/(m_0+m_1)$, the fraction of truly differentially modified PTM sites in the comparison, and e) $\sigma^2_{\gamma^*}$ and σ^2_{γ}, the anticipated variances associated to modified and unmodified peptide features, respectively. The variances can be derived based on the dataset being analyzed, assuming similar quantitative properties and variations. With these values and a user-specified number of conditions, the corresponding number of technical replicates per condition can then be derived, as described in [38]. Given the above quantities, the minimal number of replicates J is determined by the variance of the estimated log-fold change $\mathrm{SE}^2(\hat{\Delta})$ as [OV: hmm... For what design and what model? It is unclear where eq 11 came from. This will be quite specific to the model and the design. Maybe refer to the model in Eq 7? (Which we should probably simplify to reflect the experiment in Figure2, while keeping the general indeces)]

$$\widehat{SE}(\hat{\Delta})^2_{adj} = \left[\frac{2}{J}\left(\hat{\sigma}^2_{\gamma^*} + \hat{\sigma}^2_{\gamma}\right)\right] \leq \left(\frac{\Delta}{t_{1-\beta,df} + t_{1-\alpha/2,df}}\right)^2 \tag{11}$$

where

$$\alpha = (1-\beta)\cdot\frac{q}{1+(1-q)\cdot m_0/m_1} \tag{12}$$

and $t_{1-\beta,df}$ and $t_{1-\alpha/2,df}$ are the $100(1-\beta)^{\text{th}}$ and the $100(1-\alpha/2)^{\text{th}}$ percentiles of the t distribution, with $df = I(J-1)$ degrees of freedom in balanced designs.

Solving for J, the number of biological replicates per group is calculated as

$$J \geq \frac{(2\hat{\sigma}^2_{\gamma^*} + 2\hat{\sigma}^2_{\gamma})(t_{1-\beta,df} + t_{1-\alpha/2,df})^2}{\Delta^2} \tag{13}$$

Details on how the proposed method can be used to run sample size calculations and power analysis on experiments with complex design can be seen in **Supplementary Sec. 1.1**. More details on sample size calculation can be found in [39].

Implementation of MSstatsPTM

[OV: I feel that some parts of this section are either specific to MSstats/MSstatsTMT, or a bit redundant with the methods. For the parts that we do not implement anew but reuse, could you move them to the 'Existing methods'/MSstats section? Here let us focus only on things that we had to develop from scratch for this project. If we implement the methods, we do not need to re-describe the methods]

[OV: could you make this section more specific to PTM converters? E.g., I remember that the input should be different, there are some parts relevant to the localization of PTMs. This was not part of the base MSstats. Could you emphasize that?] The proposed methods are implemented in the open source R package *MSstatsPTM*. An overview of the steps of the package are illustrated in Figure 3. *MSstatsPTM* includes converters for multiple spectral processing tools, including MaxQuant, Progenesis, and Spectronaut. The converters take as input the raw data from the tool, identify the modification site for modified peptide, and put the data into the correct format for analysis in *MSstatsPTM*. Conversion is done separately for the modified peptide and global profiling runs. If the global profiling information is not available, the package can still analyze the modified run, but will do so without adjusting for changes in unmodified protein abundance. This amounts to testing the null hypothesis in Eq. (1); which amounts to running the methods seen in *MSstats* on modified peptide level data [16] [17].

[OV: this is the part that looks redundant with the MSstats section. In the MSstats section, do not repeat the methods, just focus on the specifics of the implementation. Relavant parts would be that we fit simplified models if the base model is not applicable. In this section, could you just say that we do the same as MSstats (including fitting simplified models). But there are some caveats, e.g. assumptions of missing values due to low abundance are not necessarily appropriate here so we recommend to not use that option? Also specialized sample size calculations] After using the converters, the next step is peptide/protein summarization and missing value imputation. For the modified run, the package summarizes features which include the same modification together. Features with multiple modification sites are not included with single site features and are summarized separately. For the global profiling run all unmodified features from the same protein are summarized together, up to the protein level. Additionally, the summarization includes global median normalization and normalizes between MS runs. The package can optionally impute missing values using an AFT model [40]. Missing value imputation should be performed with care when applied to PTM data. PTMs generally exhibit low feature counts and may be missing due to reasons other than low abundance. These issues can cause the missing value imputation to be unstable.

The final step of the package is modeling the summarized dataset. A linear mixed effects model is fit for both the summarized modified and global profiling runs. This model is automatically adjusted depending on the experimental design and acquisition method. The comparisons of interest can either be predefined or a full pairwise comparison will be tested. After fitting a model to both the modified and unmodified data, the modified model is adjusted for changes in unmodified protein abundance, using the methods described above.

[OV: similarly, some of this belongs to MSstats. Could you focus on project-specific implementation. E.g., we separately visualize modified and unmodified peptides. (Btw, sequence-specific visualization such as the one you did for MSstatsLiP would be awesome if there is a chance)] Beyond the core functionality, the package also includes functions for plotting the results. These include summarization plots to help with quality assurance and identifying sources of variation (such as quality control plots and profile plots), as well as modeling plots (such as volcano plots and heatmaps), which evaluate the fold change between conditions and peptides.

The package relies on functionalities from the R packages *MSstats* [16] and *MSstatsTMT* [17]. The statistical modeling relies on the functionality from the R packages *lme4* [41] and *lmerTest* [42]. The code is available on Bioconductor, `http://www.bioconductor.org/packages/release/bioc/html/MSstatsPTM.html`, and Github, `https://github.com/Vitek-Lab/MSstatsPTM`.

Evaluation

We evaluated *MSstatsPTM* on simulated and spike-in datasets with known ground truth in terms of true positives (TP), false positives (FP), true negatives (TN), and false negatives (FN) differentially abundant PTM. The true positives were defined as PTMs with changes distinct from the overall changes in protein abundance. The true negatives were defined as PTM which, after accounting for the changes in the overall protein abundance, were not differentially abundant. Additional summaries were the false discovery rate $FDR = FP/(TP+FP)$, $Recall = TP/(TP+FN)$, and $Accuracy = (TP+TN)/(TP+TN+FP+FN)$. For biological experiments with unknown ground truth, we compared the differentially abundant PTM with and without adjusting for changes in unmodified protein abundance.

The evaluations were based on the *MSstatsPTM* [any details of the options? E.g., was Empirical Bayes

moderation used in MSstatsPTM?] *MSstatsPTM* was compared [with methods XXX - why chosen? We did not compare to IsobarPTM because XXX. Do we say t-test or ANOVA?] All the evaluations were done at the adjusted p-value cutoff of $\alpha = .05$. More details are in **Supplementary Sec. 2**.

[OV:Most of the following sections simply re-state the results that can already be read off the figures. The sections would have more value if in addition/instead of describing the figures, we could provide the reasons/insight into why we see this performance. Could you try? In general, no need to repeat what we see in the figure - this is what the figures are for.]

[OV: Also, maybe combine two sections that make a similar point?]

[OV: also avoid the terms 'significant'. Replace it with 'differentially abundant'. Instead of 'bias' and 'convolution', use 'confounding']

In clean simulations, MSstatsPTM corrected for high FDR of differentially abundant PTM

[OV: maybe say something about the fact that this is a group comparisons design that doesn't require many variance components, and as such is favorable to Limma and t-test? Later when you have repeated measurements, clarify that these situations are more challenging for the existing methods and therefore the difference in the results is more pronounced.]

Figure 4a illustrated the high FDR observed on the simulated datasets when not accounting for the overall changes in protein abundance. After the adjustment, the FDR of all methods improved. In a clean simulation, recall and accuracy of *MSstatsPTM* and *Limma* performed similarly. [As expected when not using EB moderation in MSstatsPTM?,] Limma performed slightly better in simulations with fewer biological replicates. In contrast, the performance of the two-sample *t*-test method lagged behind. This is due to the fact that the *t*-test only uses data within the groups of interest while ignoring the remainder of the data. In contrast, *MSstatsPTM* and Limma leverage information across comparisons, resulting in improved power in simulations with more comparisons.

In noisy simulations, MSstatsPTM [had a higher metric XXX than] the existing methods

[This section just describes the figure. Could you emphasize why we see these results? What are the reasons for differences in performance?] In simulations with missing values and few features, *MSstatsPTM* outperformed *t*-test and *Limma*. Changes in unmodified protein level still needed to be accounted for to control the FDR. Once controlling for changes in the unmodified protein, the proposed method outperformed the other methods, as seen in **Figure 4b**. The proposed method calibrated model accuracy and recall rate well, even when the number of replicates were low. Additionally, when comparing the fold change estimation across all modified peptides, the proposed method showed a tighter distribution of estimated fold changes around the true fold change, as compared to *t*-test and *Limma*. Specifically, the inner quartile range (IQR) of the estimated fold change for the proposed method was on average 21.8% smaller than *Limma* IQR and 10.4% smaller than *t*-test's IQR. This fold change comparison can be seen in **Supplementary Figure S6**.

In the label-free benchmark experiment, MSstatsPTM [had a higher metric XXX than] the existing methods

[This section just describes the figure. Could you emphasize why we see these results? What are the reasons for differences in performance?] In this experiment all models incorrectly estimated the fold change of the modified spike-in peptides before adjusting for changes in global protein abundance. After adjustment, the spike-in peptides' fold change was generally in line with expectation in all methods, however the distribution of estimated fold changes was visibly wider (**Figure 5a**). Of the three approaches, the proposed method showed the tightest distribution around the true log fold change. Comparing the IQR of the spike-in peptide's log fold change, the proposed method's IQR was 36.78% smaller than *Limma* and 32.98% smaller than *t*-test's. While Limma and *t*-test generally estimated the correct fold change, the proposed method's estimation was more consistent across all modifications.

In **Figure 5b** we can clearly see the fold change of the red labeled spike-in peptides was only correctly estimated when accounting for changes in the unmodified protein abundance. Additionally, the background peptides, serving as the null model, show many false positives before adjustment. After adjustment the number of false positives substantially decreased. Specifically, for the proposed method, the number of

false positives went from 20.88% to 1.84% after adjustment was applied. While the proposed method and *Limma* both correctly estimated the fold change of the spike-in peptides, using *Limma* resulted in many large adjusted p-values. Using *Limma* would have resulted in missing the majority of differential PTMs.

In the group comparison TMT experiment, MSstatsPTM corrected for bias [OV: 'corrected for bias' is unclear. We talk about confounding. Maybe use a more specific term?]

[This section just describes the figure. Could you emphasize why we see these results? What are the reasons for differences in performance?] The results of this experiment are summarized in **Figure 6**. **Figure 6a** shows the number of significant modified peptides before and after adjustment. More peptides lost than gained differential abundance after the adjustment. 3,236 modified peptides became insignificant, 1,352 became significant, while 4,282 were significant in both models. For the peptides that became insignificant in the adjusted model, their change in abundance was mainly due to changes in global protein abundance. In contrast, for peptides that became significant after adjustment, their true abundance change was masked by underlying changes in the unmodified protein. Both of these issues were corrected in adjustment, and the unconfounded abundance change is shown. An additional question that must be addressed is if the decrease in significant peptides is due to the increased variance that comes from adjustment. This was tested by looking for modified peptides whose adjusted log fold change was within 10% of the unadjusted log fold change but became insignificant after adjustment. In other words, the fold change was the same between models but variance increased. When this test was applied on this experiment, only one peptide became insignificant due to an increase in variance. Thus we can conclude that the drop off in significant peptides was due to changes in global protein abundance.

In **Figure 6b**, the modification of protein $GSDMD$ at site $K62$ showed the advantage of the proposed method. The modified peptide originally showed no abundance change between the infected 1 hour, 4 hour, and 6 hour conditions. This was contrasted with a strong negative change in the global profiling run between the same conditions. Looking at the Dox4hr vs Dox1hr conditions and modeling the modified peptide without adjusting for changes in the global profiling, the fold change was $-.501$ and the adjusted p-value was insignificant at .0644. After adjusting for changes in the global protein abundance, the fold change was much higher, 2.79, and the adjusted p-value became significant, $5.25e-8$. In this case the effect of the modified peptide was strongly confounded with changes in the global protein. The proposed method allowed us to remove this confounding and estimate the true effect.

In the two-mixture TMT experiment, MSstatsPTM removed confounding [OV: between what and what? Is it similar to the previous section?]

[This section just describes the figure. Could you emphasize why we see these results? What are the reasons for differences in performance?] The results of this experiment are summarized in Figure 7. Figure 7a shows the number of significant modified peptides before and after adjustment. Again more PTMs became insignificant after adjustment than became significant. 19,286 peptides became insignificant, 4,947 became significant, and 41,552 were significant in both models. Again we tested if the decrease in significant peptides was due to removing confounding with changes in the umodified protein or increased variance after adjustment was applied. This was tested by looking for modified peptides whose adjusted log fold change was within 10% of the unadjusted log fold change but became insignificant after adjustment. When this test was applied, 548 peptides became insignificant due to an increase in variance. This is only 3.4% of the total peptides that became insignificant after adjustment. Thus we can conclude that the drop off in significant peptides was mainly due to changes in global protein abundance.

In Figure 7b the profile plot of protein TTP modified at site $S178$ showed the power of the proposed method. Without adjustment, there was a large positive log fold change of 2.9 between the WT_Late and WT_Uninfected conditions. However, the global profiling run showed a similar log fold change of 2.014 between the same conditions. This indicated that the abundance change in the modified peptide is nearly entirely due to changes in the global protein. When adjusting for the global protein the modified peptide's adjusted p-value became insignificant, going from .0009 to .248. Correcting for changes in global protein abundance allowed us to see the true impact of the modification at $S178$ which would have otherwise been challenging to perceive.

In label-free experiment without a separate global profiling run, MSstatsPTM eliminated the confounding due to changes in the unmodified protein, albeit less effectively than in the presence of a global profiling run

[This section just describes the figure. Could you emphasize why we see these results? What are the reasons for differences in performance?] As discussed in **Section 2**, there was no unmodified global profiling run performed in this experiment. Once identification and quantification of the Ubiquitination profiling was performed, peptides which were unmodified were extracted and used in place of a global profiling run. This resulted in a lack of overlap between modified and unmodified peptides. Any modified peptide without a corresponding unmodified protein could not be adjusted. Of the 10,799 ubiquitin sites identified, only 4,526 had a corresponding unmodified protein and could be adjusted. Additionally, the lack of a separate global profiling run resulted in low feature counts for the unmodified protein model compared to other experiments, seen in Table 5.1.

The results of this experiment are summarized in **Figure 8**. After adjusting for changes in the unmodified protein, there were fewer significant modified peptides. In total, 2709 modified peptides became insignificant, while only 547 became significant. However, this was mainly due to not having a global profiling run, resulting in a lack of overlap between modified and unmodified peptides. In the bottom plot, only modified peptides that could be adjusted are shown. Here there were much fewer peptides that became insignificant after adjustment. 726 modified peptides became insignificant, 547 became significant, and 1,078 were significant in both models. Only 25 PTMs became insignificant due to increased variance from adjustment.

Discussion

We proposed a general statistical modeling framework and implementation for PTM characterization. The framework is designed for bottom-up MS workflows, which are characterized with variations from multiple confounded sources, frequent missing data, and associated uncertainty in the conclusions. The framework is general and is applicable to a variety of experimental designs. It outperforms the ad-hoc methods underlying *t*-test and Limma, and yields accurate results in the broad type of experimental circumstances, including the presence of missing values, changes in protein abundance, few representative peptides, and different acquisition methods. The framework allows us to plan for subsequent experiments, and choose the appropriate number of replicates in consideration of adjustment with respect to protein abundance. The implementation allows for straightforward application of the methods discussed and allows for reproducible experimental analysis.

Our results show that the proposed approach for modeling and summarization leads to more sensitive PTM significance analysis and more accurate and precise quantification. The gain is due to a more efficient use of the data, and to a more accurate understanding of the systematic and random variations. The proposed framework can be extended beyond the experimental designs with variation from multiple sources discussed above. Although demonstrated here on DDA, is also applicable to DIA, SRM and PRM acquisitions. Additionally, the approach can handle experiments with modified peptides processed using label-free methods and unmodified peptides processed using TMT labeling, or vice versa. In this case summarization and modeling is still done separately for both the modified and unmodified data, and then combined after modeling.

A potential limitation of the proposed framework is the assumption that all the peptides are correctly mapped to the underlying proteins and PTM sites, and the features are informative of the abundances of underlying protein and PTM. Also, characterizing PTMs with current data-dependent acquisition workflows is prone to being under sampled, leading to a sparse dataset with a large number of missing values for the analysis. Statistical methods accounting for effects due to experimental units and missing values introduced in this manuscript help interpret the data in a more objective manner. The latest development of targeted acquisition and data-independent acquisition methods are expected to further alleviate these issues.

Additionally, abundance levels of PTM sites can be confounded with each other if there are multiple modification sites per peptide, or confounded with changes in the unmodified peptide (as opposed to the unmodified protein). In the current implementation the effect of a specific modification in a peptide with multiple modifications cannot be quantified. One potential solution to this is to measure the abundance of peptides with one modification and use this to adjust the peptide with multiple sites to remove the

confounding. However, this method would likely run into challenges due to sparsity of features for modified peptides with both a single and multiple modification sites. A more complex approach to addressing this problem is likely necessary.

Overall, the proposed approach balances accuracy and practicality, and enables the analysis of complex experiments in high throughput. Future work is to carry out the inference and testing for not only the relative change of PTM abundance, but also the fraction of the protein that is modified at the particular site (site occupancy, or stoichiometry), and attempt to remove the confounding of individual PTMs in peptides with multiple modifications.

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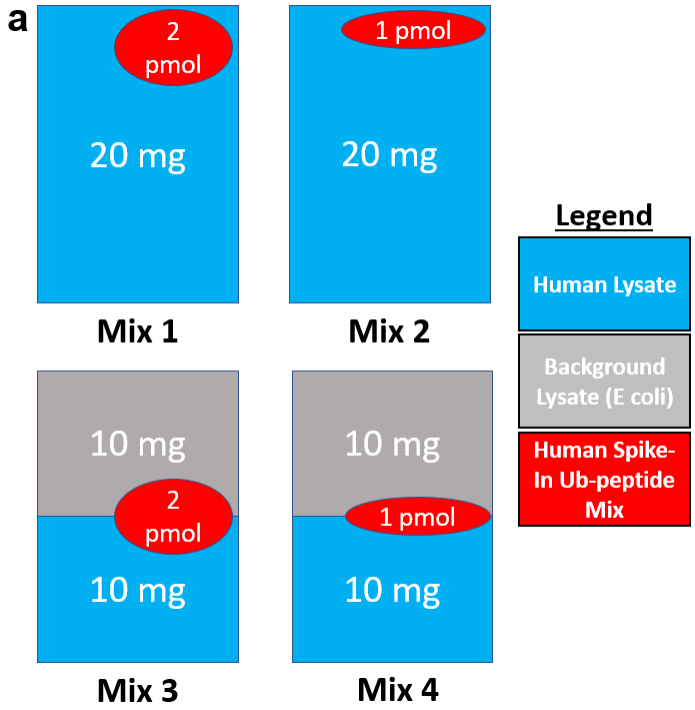
Tables

Experimental Datasets Overview

	Dataset	No. of Conditions	No. of bio. replicates	No. of mod. peptides	No. of mod. features/site	No. of unmod. features/protein	Data availability	Analysis
Known Ground Truth	Computer simulation 1 - Label-free	2/3/4	2/3/5/10	1,000	10	10	Github	
	Computer simulation 2 - Missing and low features	2/3/4	2/3/5/10	1,000	2	10	Github	
	SpikeIn benchmark - Ubiquitination - Label-free	4	2	12,137	1.37	10.17	MSV000088971	TBD
Biological Experiment	Human - Ubiquitination - 1mix-TMT	6	2 or 1	8,848	1.21	11.01	MSV000088966	RMSV000000356
	Mouse - Phosphorylation - 2mix-TMT	6	4 or 3	26,433	1.67	11.61	MSV000085565	RMSV000000357
	Human - Ubiquitination - Label-free	4	2	10,799	1.40	1.65	MSV000078977	RMSV000000358

Table 5.1: **Simulated and experimental datasets.** "Dataset" is the dataset code name. "No. of bio. replicates" shows the number of biological replicates per condition. Simulations were generated with different numbers of replicates. The designs of two biological experiments were unbalanced with unequal replicates per condition. "No. of mod. features/site" is the number of features (i.e., peptide ions) used to estimate the abundance of a single modification. "No. of unmod. peptides/protein" is the number of peptide ions without modifications that were used to estimate the global protein abundance. "Data availability" is the ID of the MassIVE.quant repository or the GitHub repository. "Analysis" is the ID of the MassIVE.quant reanalysis container, containing analysis code and modeling results. All the experiments were conducted in data-dependent acquisition (DDA) mode.

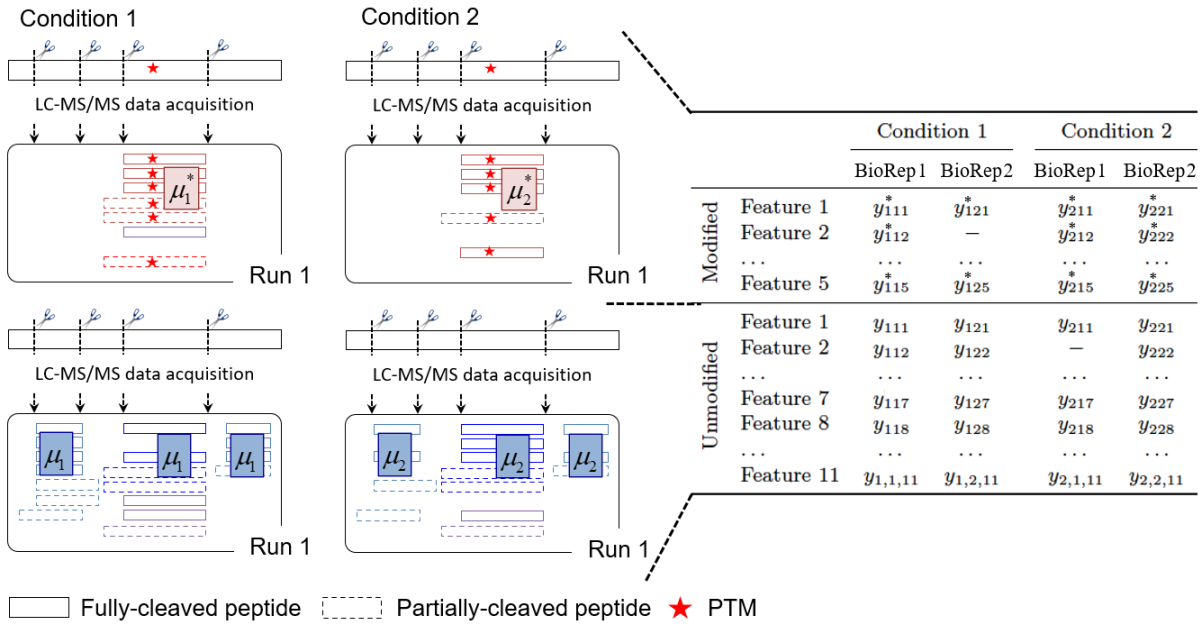
Figures



b

Comparison	Unadj. true FC	Adj. true FC
mix1-mix2	-1	1
mix1-mix3	0	-1
mix1-mix4	-1	0
mix2-mix3	1	-2
mix2-mix4	0	-1
mix3-mix4	-1	1

Figure 1: **Dataset 3: SpikeIn benchmark - Ubiquitination - Label-free**. (a) Relative amounts of human lysate, background *E. Coli* lysate, and spike-in peptides. Volumes of each mixture totaled 20mg, with 10mg of background *E. Coli* lysate used to normalize mix 3 and mix 4 to make all mixtures directly comparable. 2 pmol of spike-in peptides was added to mix 1 and mix 3, while 1 pmol was added to mix 2 and mix 4. (b) The expected fold change of the spike-in peptides in each comparison. "Unadj. true FC" is the fold change of the spike-in peptides (red) without adjusting for changes in human lysate (blue). This was calculated by directly comparing the amounts of spike-in in each condition. "Adj. true FC" is the fold change of the spike-in peptides when adjusting for changes in human lysate. This was calculated by determining the ratio of spike-in peptides to human lysate in each mixture and then using the ratio to calculate the true fold change across comparisons.



		Condition 1		Condition 2	
		BioRep1	BioRep2	BioRep1	BioRep2
Modified	Feature 1	y^*_{111}	y^*_{121}	y^*_{211}	y^*_{221}
	Feature 2	y^*_{112}	–	y^*_{212}	y^*_{222}
	...	...	...	...	...
	Feature 5	y^*_{115}	y^*_{125}	y^*_{215}	y^*_{225}
Unmodified	Feature 1	y_{111}	y_{121}	y_{211}	y_{221}
	Feature 2	y_{112}	y_{122}	–	y_{222}
	...	...	...	...	...
	Feature 7	y_{117}	y_{127}	y_{217}	y_{227}
	Feature 8	y_{118}	y_{128}	y_{218}	y_{228}
	...	...	...	...	...
	Feature 11	$y_{1,1,11}$	$y_{1,2,11}$	$y_{2,1,11}$	$y_{2,2,11}$

Figure 2: Schematic representation of one PTM site, in a special case of $I = 2$ conditions and $J = 2$ biological replicates per condition. We are interested in estimating the difference between the population-level PTM abundance between Condition 1 and Condition 2 (i.e, $\mu_1^* - \mu_2^*$), relative to the population-level difference of the overall protein abundance (i.e., $\mu_1 - \mu_2$). These quantities are characterized by the observed spectral Features (boxes), i.e. peptides of different charge states. The peptides can be fully cleaved (solid lines), or partially cleaved (dashed lines). The $\log_2$-intensities of the modified peptides in Condition i, Run j, and Feature k are denoted by y^*_{ijk}. The $\log_2$-intensities of Feature k corresponding to the unmodified peptide in Condition i and Run j are denoted by y_{ijk}.

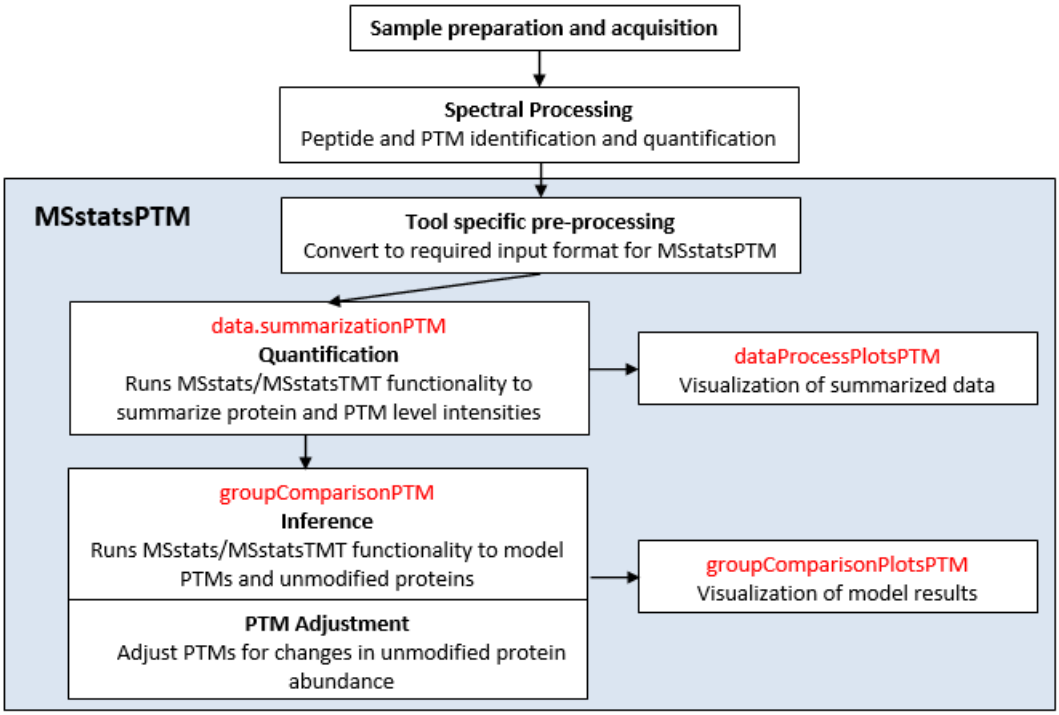
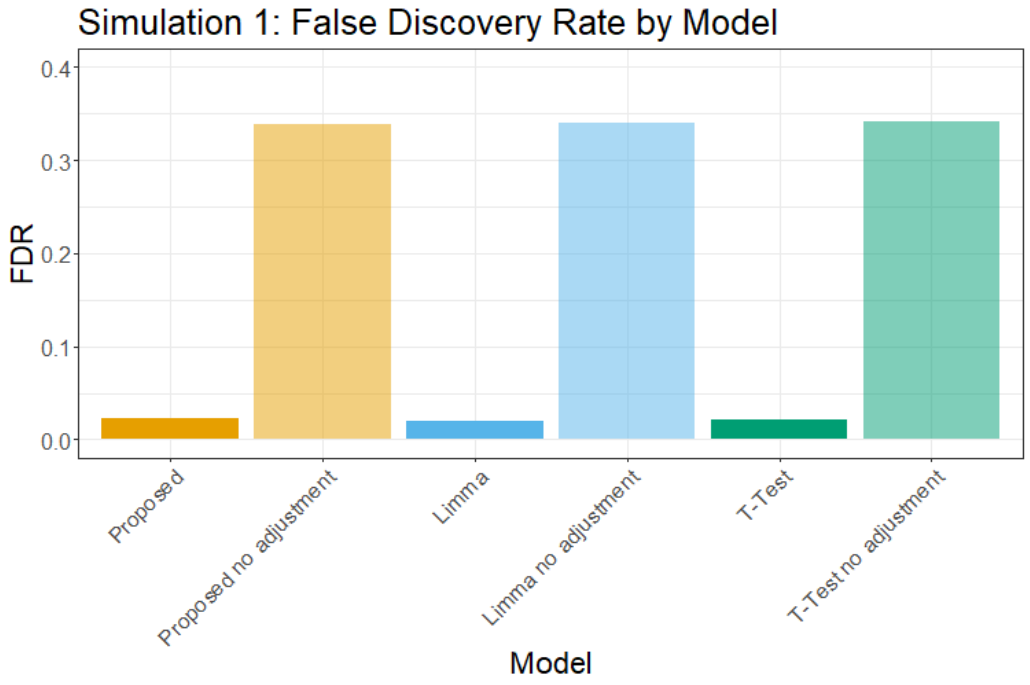
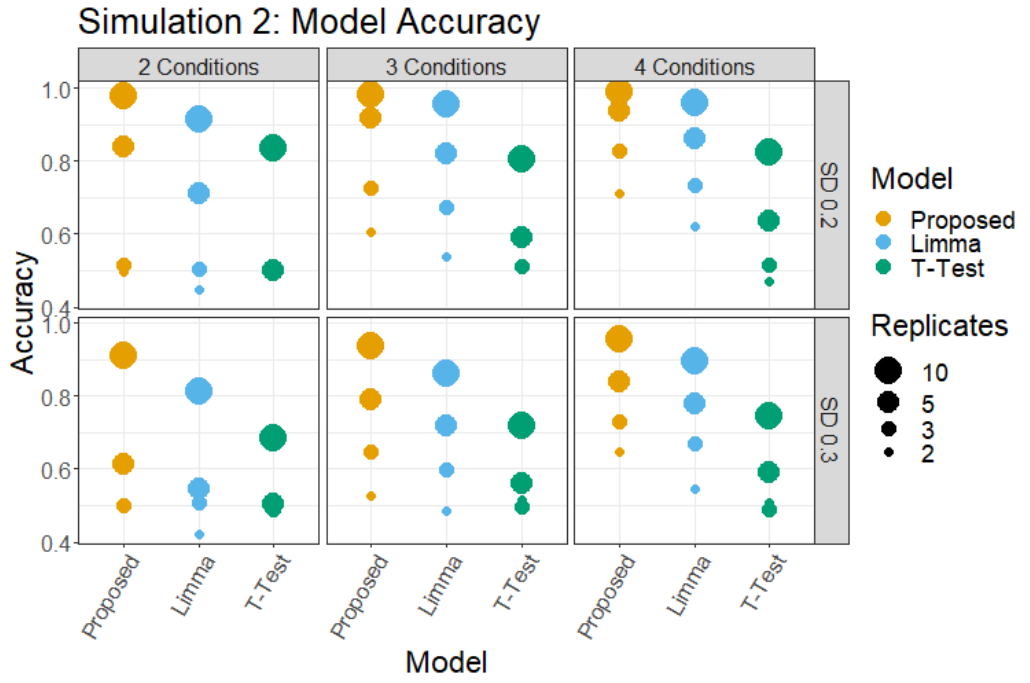


Figure 3: The workflow of $MSstatsPTM$ and how it fits into the experimental pipeline. The name of the R functions used for each step are highlighted in red. The package's workflow starts after modified and unmodified peptide quantification. First tool specific pre-processing is performed, this includes modification site identification, general data cleaning, and formatting the data into the format needed for the package. The next step is feature level summarization, which summarizes features up to the modification level for the PTM data, and the protein level for the protein data. In the final step a model is fit to identify differential PTMs and unmodified proteins across conditions and the PTM model is adjusted for changes in the unmodified protein. After both the summarization and group comparison steps, plots can be created to summarize the results.

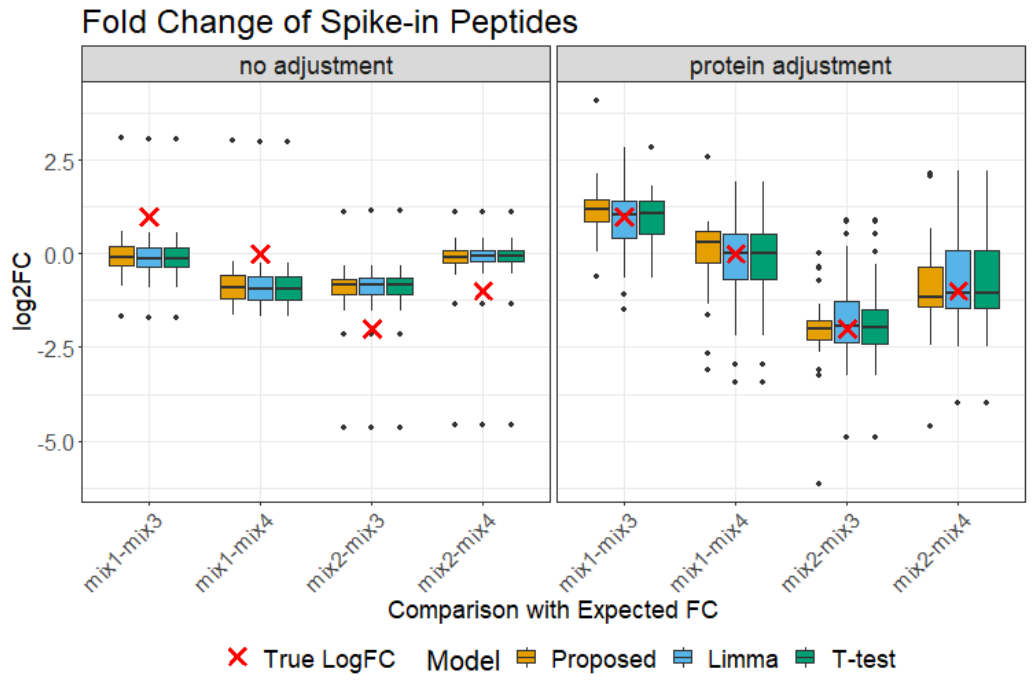


(a)

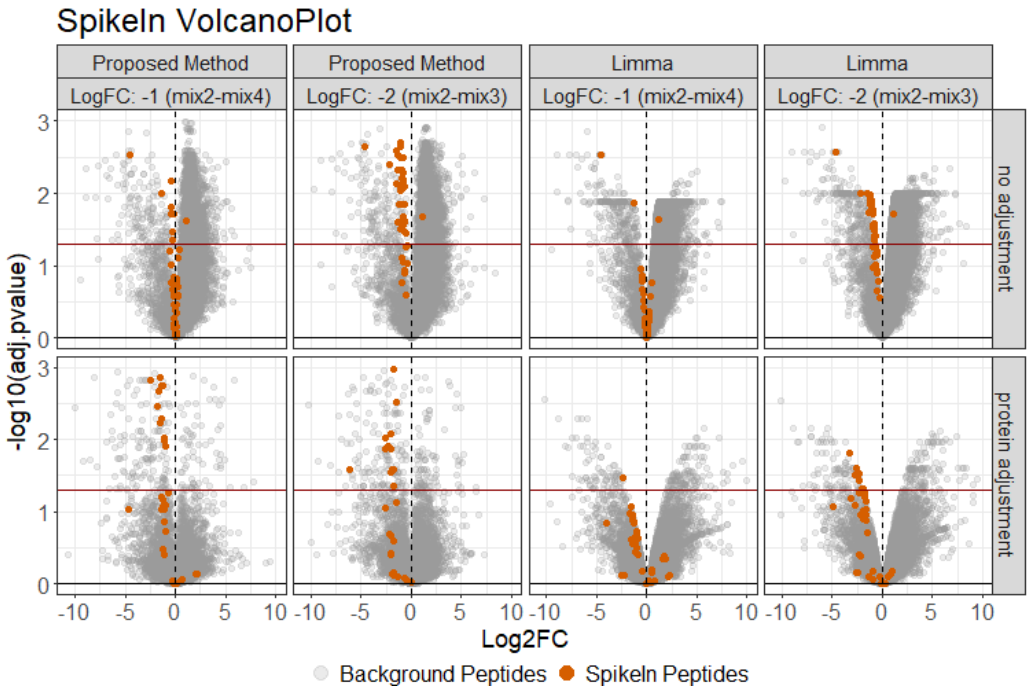


(b)

Figure 4: Dataset 1 & 2: Computer simulation. a) All the considered methods in the first computer simulation correctly calibrated FDR when adjusting for changes in protein abundance. In comparison, the methods without accounting for the protein-level changes resulted in off-target, high false positive rates. b) The advantage of using the proposed approach was apparent when including limited observations and missing values. Looking at accuracy, the proposed method outperformed Limma and t-test in nearly every model.

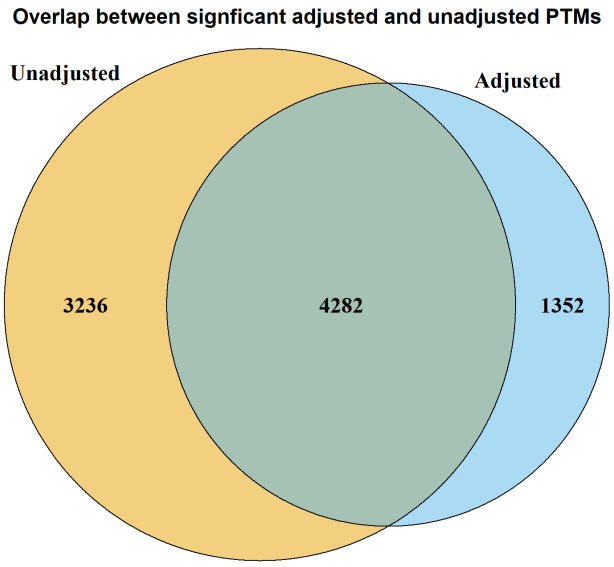


(a)

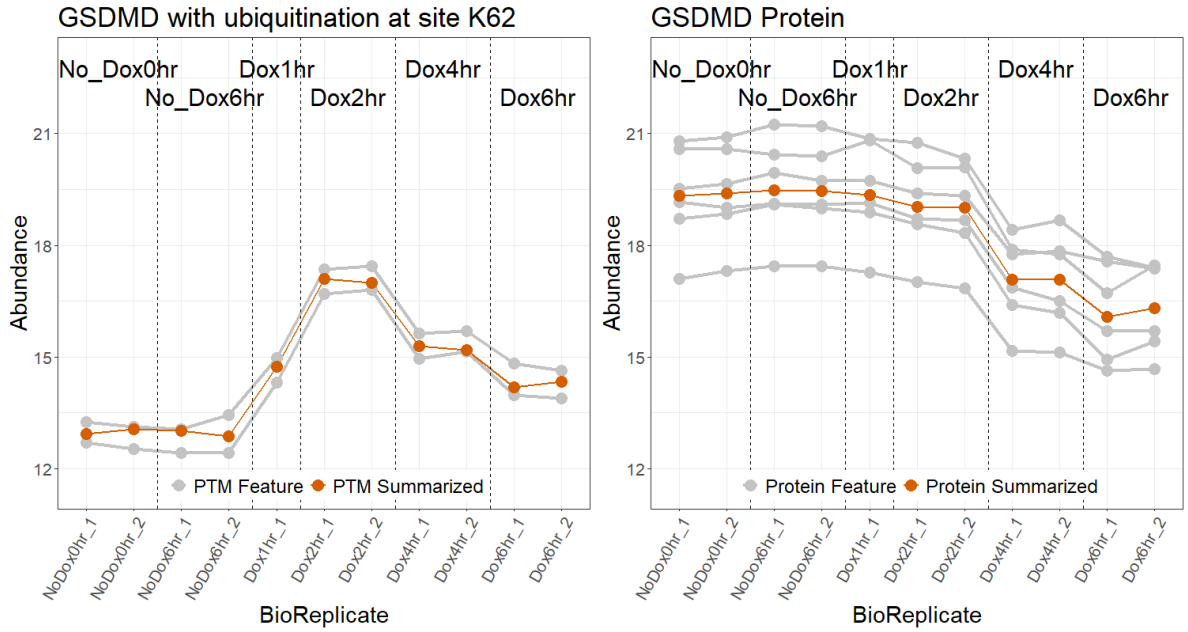


(b)

Figure 5: Dataset 3: SpikeIn benchmark - Ubiquitination - Label-free. a) Before adjustment the fold change of the spike-in peptides' were systematically different from the expected fold change in all models. After adjustment, this systemic difference was removed, however the inner quartile range of the Limma and *t*-test models was wider than the proposed method. b) Before adjustment the spike-in peptides (colored red) did not follow the expected log fold change; after adjustment, the spike-in peptides were more in line with expectation. Using Limma, the spike-in peptides followed the expected log fold change after adjustment, however the majority of spike-in peptides did not have a significant adjusted p-value.

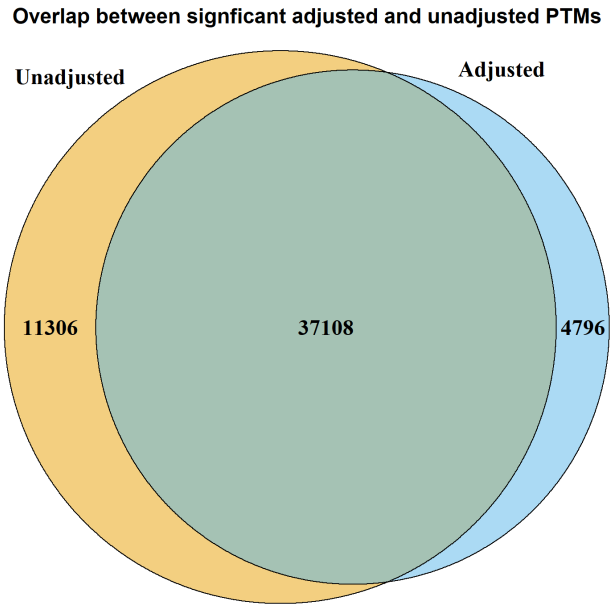


(a)

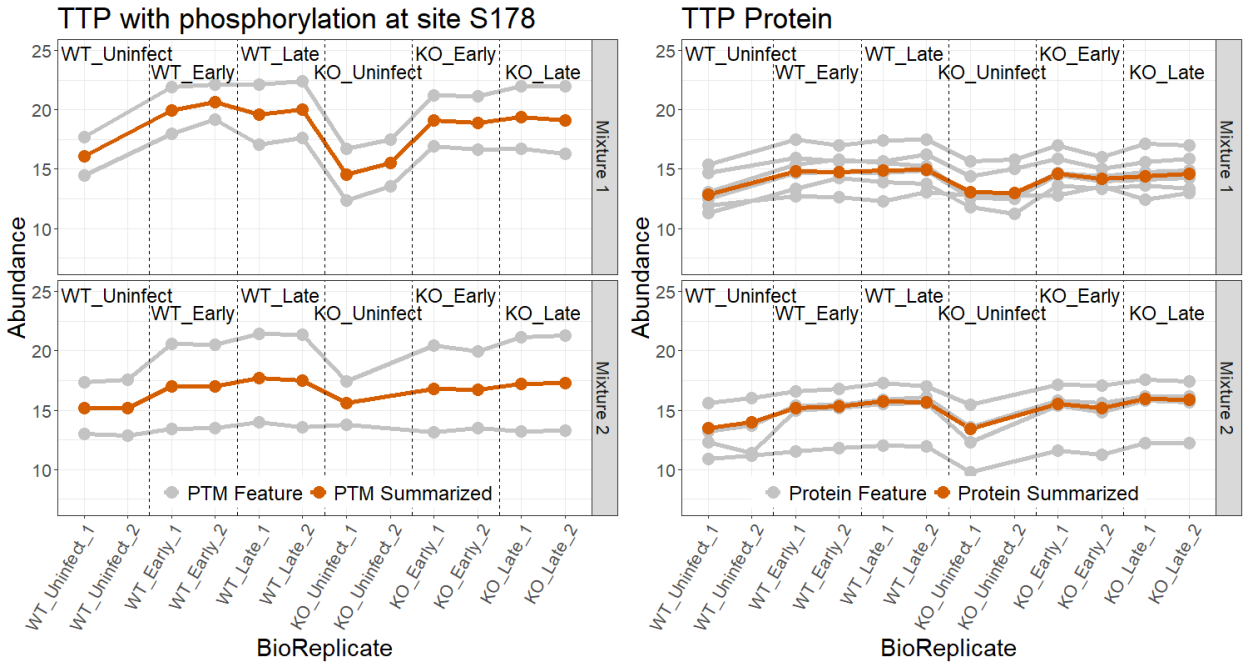


(b)

Figure 6: Dataset 4: Human - Ubiquitination - 1mix-TMT. a) The overlap of differential modified peptides for the PTM model with and without global protein level adjustment across all comparisons. More PTMs became insignificant after adjustment than became significant. For the peptides that became insignificant in the adjusted model, their change in abundance was driven by changes in the global protein. In contrast, peptides that became significant after adjustment had their true abundance change masked by underlying changes in the unmodified protein. b) Comparing the global profiling of protein $GSDMD$ with the ubiquitination of the protein at site $K62$. When looking at the summary of the modification and global protein it was clear the conditions follow different trends. Specifically, there appeared to be no change in abundance between Dox1hr and Dox4hr in the modified plot, however there was a large negative change when looking at the unmodified plot. This indicated the modification was confounded with changes in the unmodified protein.

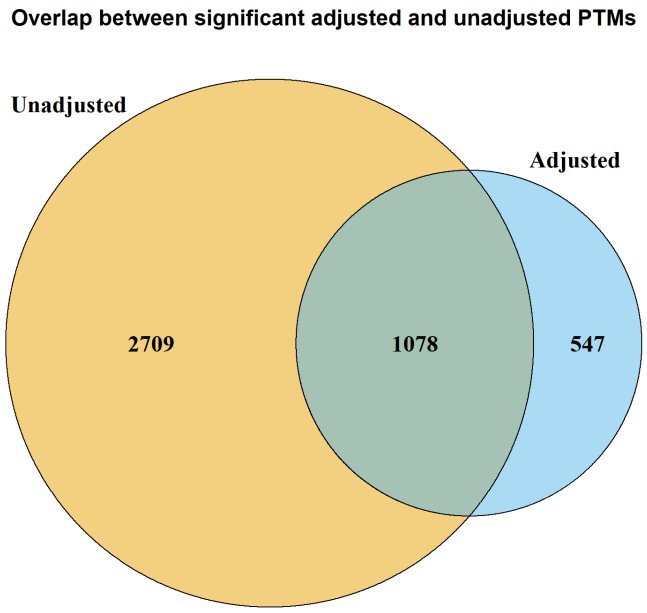


(a)

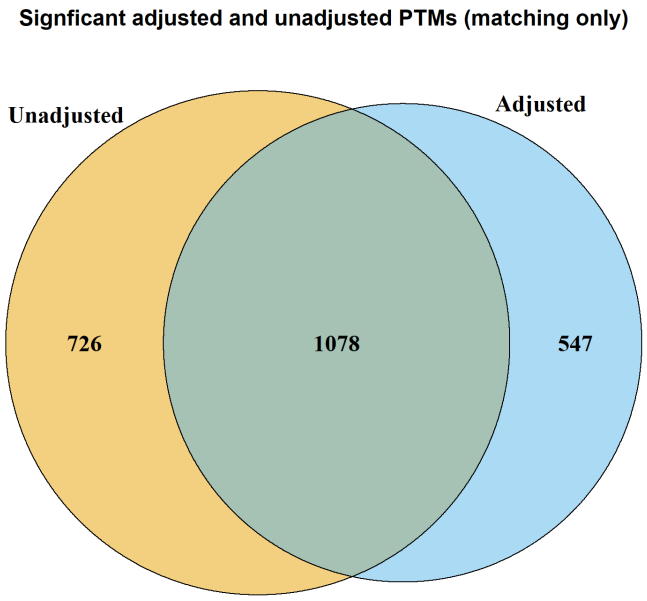


(b)

Figure 7: Dataset 5: Mouse - Phosphorylation - 2mix-TMT. a) The overlap of differentially modified peptides between the PTM model with and without global protein level adjustment across all comparisons. Again more PTMs became insignificant after adjustment then became significant. b) Comparing the global profiling of protein TTP with the modification of the protein at site $S178$. When looking at the summary of the modification and global protein it was clear the difference between conditions followed the same trend. Specifically, there was a positive adjustment in abundance when comparing WT_Uninfect to WT_Late in both the modification and global profiling run. This indicated the movement was driven by changes in global protein that was only accounted for in the model after adjusting for global protein abundance change.



(a)



(b)

Figure 8: Dataset 6: Human - Ubiquitination - Label-free no global profiling run. a) The overlap of differential modified peptides for the PTM model with and without global protein level adjustment across all comparisons. More PTMs became insignificant than became significant after adjustment. This was due to not having a global profiling run, resulting in a lack of overlap between modified peptides and unmodified proteins. b) Here we made the same comparison but only looked at modified peptides where adjustment could be performed, ie they had a matching unmodified protein. In this case there were significantly less peptides that became insignificant after adjustment. This highlighted the need for a global profiling run.