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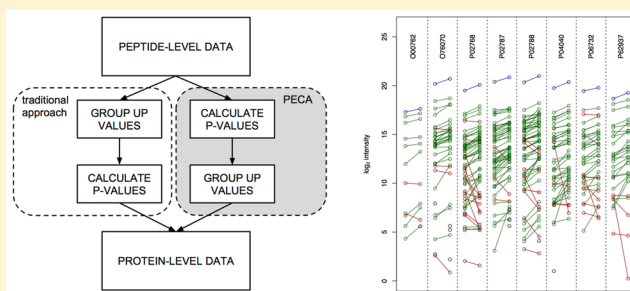
Using Peptide-Level Proteomics Data for Detecting Differentially Expressed Proteins

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S Supporting Information

ABSTRACT: The expression of proteins can be quantified in high-throughput means using different types of mass spectrometers. In recent years, there have emerged label-free methods for determining protein abundance. Although the expression is initially measured at the peptide level, a common approach is to combine the peptide-level measurements into protein-level values before differential expression analysis. However, this simple combination is prone to inconsistencies between peptides and may lose valuable information. To this end, we introduce here a method for detecting differentially expressed proteins by combining peptide-level expression-change statistics. Using controlled spike-in experiments, we show that the approach of averaging peptide-level expression changes yields more accurate lists of differentially expressed proteins than does the conventional protein-level approach. This is particularly true when there are only few replicate samples or the differences between the sample groups are small. The proposed technique is implemented in the Bioconductor package PECA, and it can be downloaded from <http://www.bioconductor.org>.

KEYWORDS: PECA, peptide-level, differential expression, label-free, protein-quantification.



1. INTRODUCTION

Mass spectrometers are high-throughput devices that can be used for quantifying complex protein mixtures. Proteins have traditionally been quantified using stable isotope labels, but in recent years, statistical label-free methods have been introduced.^{1–3} More commonly, only relative protein abundances are inferred, either from spectral counts^{4–8} or from peptide peak intensities.^{9–11} Before any statistical approach can be applied to determine differentially expressed proteins, there are several preparative steps, including the identification of peptides using database searches, data normalization, and quality assessments.¹² In this study, we focus on the algorithms for detecting differential expression, assuming that all the preceding steps have been done appropriately.

Quantitative proteomic studies have some characteristics similar to those of high-throughput gene expression studies, and the resulting measurements can sometimes be analyzed using the same computational tools. Currently, however, a more limited selection of statistical tools is used in proteomics studies than in gene expression studies. Proteomics is also considered a more difficult problem compared to gene expression because there are issues such as limited and degrading sample material, vast dynamic range, and post-translational modifications.¹³ As the tools and methods have been maturing, there has been a shift toward precise

quantitative proteomics. For recent developments in mass spectrometry (MS)-based proteomics, see Becker et al., Wasinger et al., and Richards et al.^{14–16} For an overview of applying machine learning techniques in various stages of proteomic workflow, see Kelchtermans et al.¹⁷

In most cases, protein is the desired unit for differential expression detection, although the measurements are made at the peptide level.¹⁸ Accordingly, the measured peptide-level intensities are typically combined into protein-level intensity estimates for further analysis. There are various methods to choose from, including simple approaches such as arithmetic mean¹⁹ or sum,²⁰ as well as more complex approaches such as linear models^{9,18,21} and identifying temporal patterns.²² These methods are discussed in more detail in Carillo et al.²³ Several issues make the estimation of protein abundance challenging, including experimental and biological artifacts that cause peptides from the same protein to behave differently. What is surprising is that it remains a common practice to analyze the differential expression between sample groups using these combined values with the possibility of losing valuable information.²⁴ Alternatively, it has been suggested to detect differences separately for each peptide,¹⁸ which does not

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provide a direct solution to the protein-level detection, or to use ANOVA-based methods with peptide-level measurements.^{9,25}

By combining multiple statistical detections (one for each peptide) from the same protein, we hypothesize that improvement of the robustness of detections can be achieved, especially when the number of replicate samples is low or the changes between the sample groups are small. A similar peptide-level approach has been used in MaxLFQ procedure, albeit using ratios instead of statistical tests.²⁶ Overall, the peptide-centric methods have shown great promise.²⁷ Here, the improved performance of the proposed method is demonstrated in controlled spike-in experiments. Additionally, we introduce a novel Bioconductor package, PECA (www.bioconductor.org/packages/release/bioc/html/PECA.html), to perform differential expression analysis using the available low-level measurements.

2. MATERIALS AND METHODS

2.1. Peptide-Level Expression Change Averaging Procedure

We present the peptide-level expression-change averaging procedure, named PECA, which determines differential protein expression using peptide-level measurements from MS-based proteomic data sets. The method differs from the common approach, where protein expression intensities are precomputed from the low-level peptide data. In the proposed method, an expression change between two groups of samples is first calculated for each measured peptide. The corresponding protein-level expression changes are then defined as medians over the peptide-level changes. Figure 1 shows the steps of

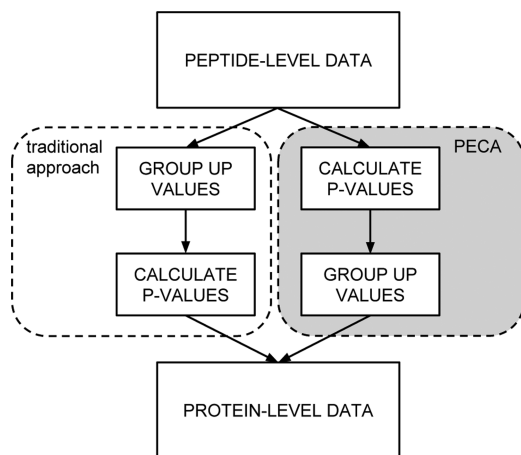


Figure 1. Schematic illustration of the peptide-level expression change averaging procedure in mass-spectrometry studies.

PECA in comparison to the conventional protein-level approach. We have previously demonstrated the utility of low-level measurements when detecting differential gene expression.^{28–30}

In its current implementation, PECA starts with an optional step of quantile or median normalization that can be selected by users working with unnormalized data. This is followed by \log_2 transformation. PECA then tests the significance of the peptide-level expression changes with the ordinary or modified t statistic. The ordinary t statistic is calculated by the Bioconductor *genefilter* package using the `rowttests` function,

and the modified t statistic is calculated by the linear modeling approach of the Bioconductor *limma* package using `lmFit` and `eBayes` functions. Both paired and unpaired tests are supported. Median t statistics are used to calculate p values, thus taking into account the direction of change. The p values are determined from the beta distribution.³¹ This is based on the fact that, under the null hypothesis, the p values of the n peptides corresponding to a protein follow the uniform distribution $\mathcal{U}(0, 1)$ and that order statistics from that distribution have beta distributions. The median t statistic, the corresponding median p value (score), and the p value from beta distribution are all reported for each protein.

Users can also choose to aggregate results using Tukey's biweight instead of the median. The statistical significance of the protein-level detection is then based on a simulated distribution, which is created by repeatedly storing Tukey's biweight values from a set of random p values based on the total number of peptides in the given protein. The quality control and filtering of the data (e.g., based on low intensity) are left to the user.

2.2. Spike-in Data

The spiked yeast data contained different concentrations of the Universal Proteomics Standard proteins (UPS1, equimolar amounts of 48 proteins, Sigma-Aldrich, St. Louis, MO, United States), which were dissolved in in-solution digestion buffer, reduced and alkylated, and digested with trypsin. After digestion, the peptide mixture was desalted using C18 pipet tips, evaporated to dryness, and resuspended in 0.1% formic acid. Digested UPS1 mixture was spiked into a yeast proteome digest to create the spiked concentrations of 2, 4, 10, 25, and 50 fmol/ μ L. The amount of yeast peptides per injection was 100 ng.

A total of three runs per spiked concentration were analyzed on LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, United States) coupled to an EASY-nLC nanoflow liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, United States). The LC gradient length was 110 min, and the flow rate was 300 nL/min. Peptides were separated on an in-house-built C18 analytical column and ionized by ESI. Data-dependent analysis was used, with the top 20 ions selected for fragmentation by CID. The mass scan range was 300–2000 m/z . Dynamic exclusion was enabled with a repeat count of 1, a repeat duration of 30, an exclusion list size of 500, and an exclusion list duration of 60. The Mascot algorithm in the Proteome Discoverer software (Thermo Fisher Scientific) was used to perform database search (UniProt KB/SwissProt release 2011_03, 525997 entries with UPS protein sequences appended). The Mascot score corresponding to 95% probability was used as a cutoff value for peptide identifications. Search was done for peptides formed by trypsin digestion, where one miscleavage was allowed. Cysteine carbamidomethylation was selected as a fixed modification and methionine oxidation was selected as a dynamic modification. The accepted precursor mass tolerance was set to 5 ppm and the fragment mass tolerance to 0.5 Da. Listing of peptide and protein-level intensity values was generated using Progenesis LC-MS software (Nonlinear Dynamics), which also performs normalization of the data. It selects the run with the greatest similarity to others as a reference and calculates the scaling factors for all of the other runs. Relative protein quantitation using nonconflicting peptides was used in Progenesis, which sums up the peptide-

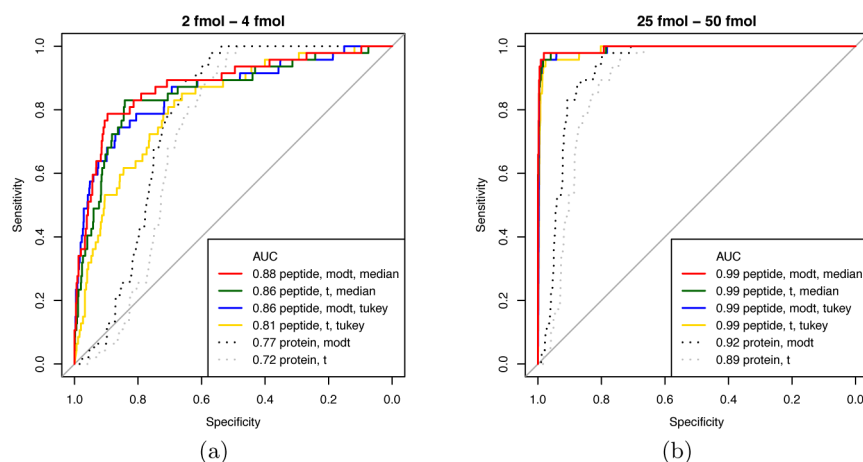


Figure 2. Sensitivity and specificity of the peptide-level analysis compared to the protein-level analysis using UPS spike-in data comparison of (a) 2 fmol vs 4 fmol and (b) 25 fmol vs 50 fmol. ROC curves and the estimated area under the curve are given for different variants of PECA and the protein-level approach.

level intensities. Peptides not used in the protein-level summary were also filtered out for peptide-level analysis. Peptides detected with different precursor ion charge states were treated as separate cases in subsequent tests during the calculation of peptide-level differential expression using PECA.

From the different spiked concentrations, we chose 2-fold comparisons of 2 fmol versus 4 fmol and 25 fmol versus 50 fmol to be tested. Similarly, we also tested 5-fold comparisons of 2 fmol versus 10 fmol and 10 fmol versus 50 fmol. Scatterplots of the mean intensity values across all replicates for the selected comparisons are shown in Figures S1 and S2. Results that are shown here are focused on the 2-fold comparisons because detecting differential expression in them is considered harder than in 5-fold comparisons. They are also the cases where the selection of method has the largest impact on the results, thus being the most interesting ones. The 5-fold comparisons are shown in the Supporting Information.

3. RESULTS

3.1. Sensitivity and Specificity

Performance analysis was done using receiver operating characteristic (ROC) curves, which are created by plotting the fraction of the true positives (detected UPS proteins) out of the total actual positives against the fraction of false positives (detected non-UPS proteins) out of the total actual negatives at various p value thresholds. Figure 2 shows the ROC curves of the different methods in both the 2 fmol versus 4 fmol and the 25 fmol versus 50 fmol comparisons. For other tested comparisons, see Figure S3. Table 1 summarizes the area under curve (AUC) values for the comparisons with different PECA parameters and the presumed protein-level values. The same statistical methods were used with the presumed protein values and with the peptide-level values: the t statistic by using rowtests of the *genefilter* package and the modified t statistic by using the linear modeling approach of the *limma* package. Progenesis itself provides ANOVA, but because the t test is a special case of ANOVA, the results were therefore not included. The ROC analysis revealed that the PECA method led to higher accuracy than the conventional protein-level analysis regardless of the statistic or aggregation method used. The best ROC curves were obtained using a PECA-modified t test with the median as aggregation method (first row of Table

Table 1. AUC Values from ROC Curves for Comparisons of Samples with Spiked Protein Amounts^a

level	statistic	aggregation	2 fmol vs 4 fmol	25 fmol vs 50 fmol	2 fmol vs 10 fmol	10 fmol vs 50 fmol
peptide	modt	median	0.88	0.99	1.00	1.00
peptide	t	median	0.86	0.99	1.00	1.00
peptide	modt	Tukey	0.86	0.99	1.00	0.99
peptide	t	Tukey	0.81	0.99	0.99	1.00
protein	modt	–	0.77	0.92	0.99	0.99
protein	t	–	0.72	0.89	0.98	0.98

^aNo aggregation method is available on protein-level comparisons because the values are precombined.

1). Table 2 shows the statistical significance of the difference between the best ROC curve and the others. In each comparison, the chosen method differs clearly from the protein-level results (DeLong's test, $p < 0.001$).

3.2. Differences between PECA and the Protein-Level Method

To investigate the differences between the results of PECA and the protein-level method, we compared the estimated p values of differential protein expression. A scatterplot for the 2 fmol versus 4 fmol comparison is shown in Figure 3a, where spike-in UPS proteins are highlighted. The scatter plots for the other comparisons are shown in Figure S4. In these plots, the majority of spike-in proteins have smaller p values when the analysis is done on the peptide level instead of the conventional protein-level analysis (points below the diagonal), whereas many of the background yeast proteins (true negatives) were correctly detected as clearly nondifferentially expressed with PECA (horizontal line at the top). This was not the case with protein-level analysis. The histogram of differences between the peptide-level and protein-level p values is shown in Figure 3b. Although the highest frequencies were found in the low p value difference regions as expected, there were still a great number of pairs for which the calculated p values differed remarkably. Figure 3c shows the number of true and false positives as a function of p value on the 2 fmol versus 4 fmol comparison. For example, when using a threshold of 0.05, the total number of detected proteins is 38 for the peptide-level approach and 55 for the protein-level approach. The number of UPS proteins

Table 2. DeLong's Test p Values for ROC Curves of Peptide-Level-Modified t Test Compared to Other Methods

level	statistic	aggregation	2 fmol vs 4 fmol	25 fmol vs 50 fmol	2 fmol vs 10 fmol	10 fmol vs 50 fmol
peptide	modt	median	1.00000	1.00000	1.00000	1.00000
peptide	t	median	0.00132	0.05594	0.15653	0.31310
peptide	modt	Tukey	0.09661	0.01619	0.00253	0.00021
peptide	t	Tukey	0.00010	0.10088	0.00068	0.03205
protein	modt	—	0.00023	0.00000	0.00033	0.00055
protein	t	—	0.00000	0.00000	0.00015	0.00019

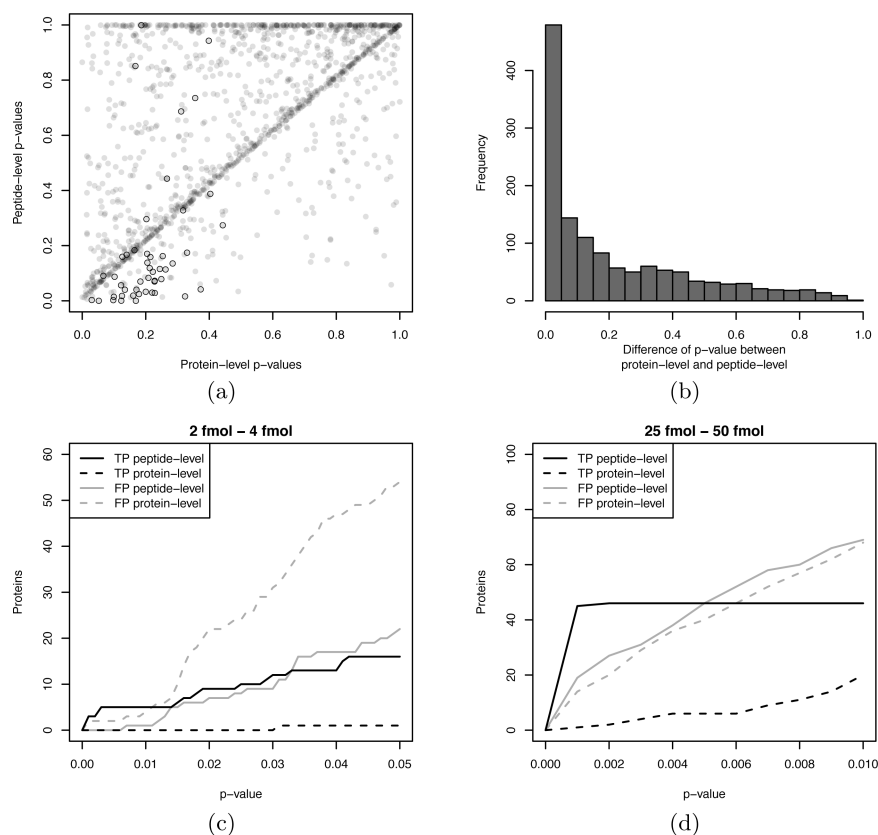


Figure 3. (a) Scatterplot of p values from peptide-level (PECA) and protein-level analysis from the 2 fmol vs 4 fmol comparison, where values are calculated using a modified t test and peptide-level statistics are aggregated using the median approach. The black circles correspond to the spike-in UPS proteins. Points below the diagonal correspond to proteins that are more significant using the peptide-level approach compared to the protein-level approach. (b) Distribution of p value differences between the peptide-level and protein-level approaches. (c) Number of true and false positives as a function of the significant p value threshold on the 2 fmol vs 4 fmol comparison. (d) Number of true and false positives as a function of the significant p value threshold on the 25 fmol vs 50 fmol comparison.

among the detections is 16 out of 38 on the peptide level and only 1 out of 55 on the protein level. This demonstrates the improved ability of PECA to detect true UPS spike-in proteins as differentially expressed. Figure 3d shows the same numbers on the 25 fmol versus 50 fmol comparison. Although the number of false positives between the methods remain similar, PECA clearly outperforms the protein-level approach in terms of true positives.

Figure 4 illustrates the detected differentially expressed UPS proteins from the 2 fmol versus 4 fmol comparison that have their p values below 0.05 using PECA. Even though all of the protein-level values have their total intensities increasing toward the 4 fmol sample, they do not stand out from the background noise because there are also similar changes in the nonspiked proteins. However, by looking at the peptide-level values, one can see that there is a clear difference. In the UPS proteins, the majority of peptides have systematically higher intensities in the 4 fmol sample than in the 2 fmol sample, and these repeating

patterns distinguish them from the non-UPS proteins. In the non-UPS proteins, on average, the number of peptides showing positive and negative changes is close to even.

3.3. Effect of Reducing the Number of Replicates

We also tested the effect of reducing the number of available replicates used for differential expression analysis (Figure 5). On the 25 fmol versus 50 fmol comparison, there is not much difference in performance when using only two replicates. On the 2 fmol versus 4 fmol comparison, the curves clearly show the poor performance when the number of replicates was reduced to only two. Notably, however, PECA with two replicates remained better than the protein-level method with three replicates at low false positive rates, which are of practical interest in proteomic studies when searching for good candidates for further validation experiments. However, having only two replicates available should be avoided and is becoming rare. In cases where some samples are deemed unsatisfactory

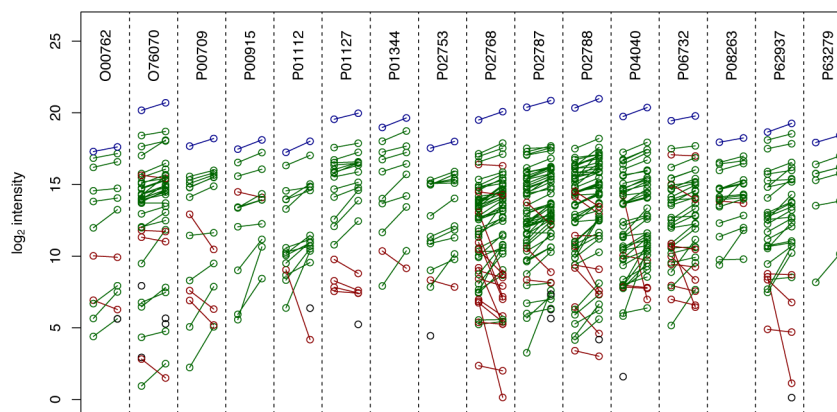


Figure 4. Visualization of normalized log-scaled peptide intensities and corresponding protein-level values of UPS proteins identified by PECA as differentially expressed in the 2 fmol vs 4 fmol comparison. For each protein, the mean intensity value from the 2 fmol replicates is on the left, and the mean value from the 4 fmol replicates is on the right. Plotted pairs are colored green in cases where the measured intensity is higher on the 4 fmol sample and red otherwise. The highest pair (blue) represents the summed protein intensity values.

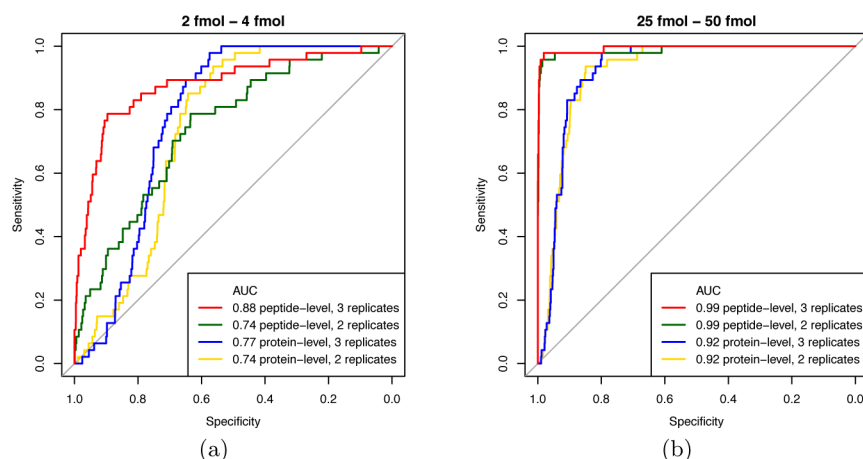


Figure 5. Receiver operating characteristic curves with the area under curve of PECA and protein-level analysis when the number of replicates is reduced on UPS spike-in data sets (a) 2 fmol vs 4 fmol and (b) 25 fmol vs 50 fmol. The rankings determined for plotting the ROC curves with a reduced number of replicates were calculated as median p values from all possible reduced replicate combinations that could be chosen.

from a quality control point of view, new experiments are usually preferred instead of relying on smaller number of replicates.

3.4. Comparison to Other Analysis Software

Finally, we compared the proposed PECA method to common proteomic analysis software platforms that are currently used, in addition to other tasks such as visualization, to calculate differential expression between sets of samples. These tools were selected on the basis of their ability to accept peptide-level values as an input similar to that of PECA. Figure 6 shows the performance of PECA in comparison to that of *MSstats*²⁵ and *InfernoRDN* (previously *DAnTE*³²) on the 2 fmol versus 4 fmol and on the 25 fmol versus 50 fmol data sets using default settings and all three replicate samples. The peptide-level method proposed by Karpievitch et al.⁹ is similar to *MSstats* and is not included in this comparison. Because these methods have internal filtering mechanisms, only those differential expression estimates that were common between the methods were used. From the original list of 1387 proteins, 947 were left for benchmarking due to filtering. In *InfernoRDN*, the peptides were combined to protein measurements using the three different rollup methods available with their default settings. RRollup uses the peptide with the most presence across all the

samples as a reference when calculating scaling factors for each peptide belonging to that particular protein. ZRollup scales peptides by using standard deviation of their median-centered values across the samples. In both cases, the protein abundance is defined as the median of the scaled peptide abundances. With the QRollup method, 33% of the top peptides were considered in median calculation without any scaling beforehand. In *MSstats*, the default settings were used except the normalization procedure, which was omitted because it was performed earlier to ensure compatibility. In these comparisons, PECA clearly outperformed the other tested methods. Comparisons for the other spike-in concentrations are shown in Figure S5.

4. CONCLUSIONS AND FURTHER RESEARCH

We tested the viability of the peptide-level expression-change averaging in proteomics data and showed that the method has good potential in determining the differentially expressed proteins. The spike-in experiments showed that the method works better in comparison to other tested methods, especially when the differences between the sample groups are small. Our results in the spike-in proteomic study showed that while the results improved overall, the largest improvement was found in the most difficult case, i.e., the case where the difference

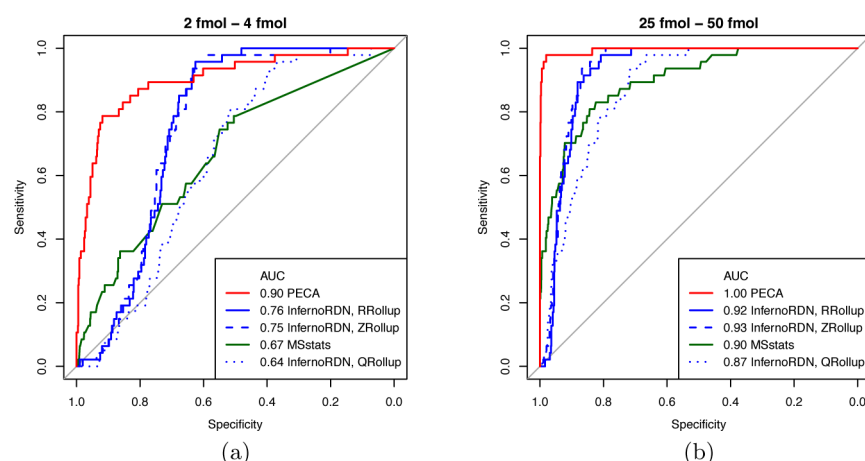


Figure 6. Receiver operating characteristic curves with area under curve of PECA, *MSstats*, and *InfernoRDN* using the UPS spike-in data comparison of (a) 2 fmol vs 4 fmol and (b) 25 fmol vs 50 fmol.

between the spiked concentrations was smallest of all possible combinations (2 fmol versus 4 fmol). As the spike-in quantities get substantially larger, they are more easily separated from the background noise. It is therefore likely that by increasing the concentrations further, a level will be reached where the peptide-level analysis no longer achieves better results over the standard protein-level results. It is also a typical goal in this type of proteomics studies to produce a reliable ranking of the proteins according to the significance of the change and not an accurate quantification. In practice, small changes between samples can contain biologically relevant information and, therefore, their reliable detection is important.

We also showed that only by looking at the peptide-level values can we distinguish patterns on the differentially expressed proteins that would otherwise be missed. This also suggests that selecting any subset of peptides to represent the intensity of the whole protein can lead to a loss of valuable information. For example, when selecting only a fraction of the peptides having the highest intensities, as sometimes is done, the fraction could also contain outliers. In our spike-in data, there are proteins for which some of the highest-intensity peptides have opposite-signal log ratios compared to others (e.g., Figure 4, P06732; fourth protein from the right and second peptide from the top). This might not be an issue when the differences between the sample groups are large, but our goal is to push the limits of finding the statistically significant changes in protein expression levels even when the differences between the sample groups are small or the number of replicates is low.

The effect of using a 1% FDR threshold instead of the default 5% for Mascot identifications in the 2 fmol versus 4 fmol comparison was also tested (Figure S6). The use of this stricter threshold for peptide identifications slightly improves the overall AUC values of ROC curves, but the relative order of the curves remains the same. The most notable improvements were found among the top-ranked proteins with small estimated *p* values, which is of highest practical interest. This reflects the limitations of the different methods to robustly mitigate the possible misidentifications made at earlier stage. The use of peptide median in PECA makes it robust against outliers.

We are currently using peak intensity values to calculate the differential expression of proteins, but one possibility is to use spectral counts instead. This is an example where modifications to PECA would be necessary because such data frequently

contains a large number of zero values. Further research could also include testing PECA on various other data sets, possibly using a large-scale test bed for benchmarking against multiple other methods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00363.

Figure S1: twofold scatter plots from UPS spike-in data. Figure S2: fivefold scatterplots from UPS spike-in data. Figure S3: ROC curves using different PECA parameters on UPS spike-in peptide-level data. Figure S4: Scatter plots of *p* values from UPS spike-in data. Figure S5: ROC curves comparing different methods on UPS spike-in data. Figure S6: ROC curves comparing different methods on 2 fmol versus 4 fmol comparison when using Mascot FDR thresholds of 0.05 and 0.01. (PDF)

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Notes

The authors declare no competing financial interest.

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