Normalizing Post Translational Modification (PTM) Expression by Protein–Level Quantitation in Scaffold PTM

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Abstract

In many post translational modification (PTM) experiments quantitative variations of PTM peptides not only reflect the variation of PTM abundances but also the modified expressions of their substrate proteins. One strategy to deconvolute these changes entails performing parallel tandem mass spectrometry experiments with one targeted towards the protein level quantitation and the other focused on the quantitation of enriched PTM peptides. To accommodate this type of parallel experiment, we developed a new quantitative analysis method that normalizes PTM peptide abundances by their protein substrates expression for SILAC and label–free precursor intensity data sets. After normalization, statistical tests were developed to establish the PTM expression changes. This algorithm was tested using published datasets demonstrating both the accuracy and reproducibility of the method. This overall approach provides core lab researchers with a unified strategy to analyze both PTM and protein level expression simultaneously.

Methods

We analyzed label–free quantitative phosphoproteomics data for three biological replicates from a study by M. Mann *et al.*[1] HeLa S3 cells were left untreated or mitotically arrested with nocodazole and released. After proteins were lysed, each sample was divided in two. Each sample half was separated into six fractions with SAX or, for phosphoproteome analysis, SCX and TiO_2 microbeads (fig. 1). Fractions were analyzed with HPLC and a Q Exactive for a total of 72 MS/MS raw runs that were searched with MaxQuant.

Search and quantitation results were loaded into two Scaffold Q+ experiments for the proteome and phosphoproteome, respectively. In each experiment the data was grouped into two categories, Control and Nocodazole. Each sample's six fractions were loaded into one biological sample as a MudPIT. Data from the phosphoproteome experiment were imported into Scaffold PTM along with protein quantitation reports from the proteome experiment.

Scaffold PTM currently supports statistical validation for labeled quantitative data. To better support the label–free data in this experiment we implemented the Mann–Whitney *U*–Test in PTM. This test will be available in the next version of Scaffold PTM. We did not apply a multiple testing correction to computed p–values.

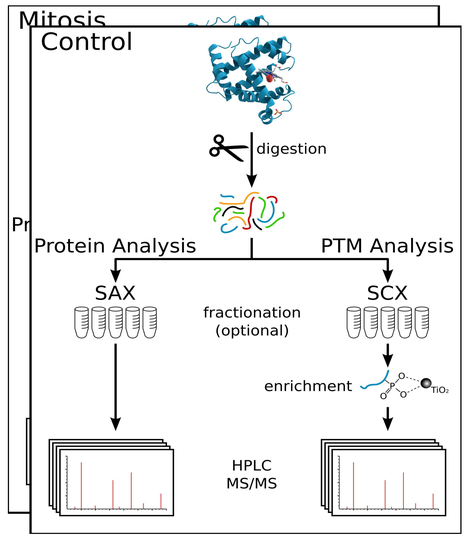

Figure 1: Data collection workflow for a parallel–quantitation experiment. Samples are analyzed twice; once to assess protein expression and once after a PTM enrichment step to ensure high coverage of modification sites.[1,2]

Results

Scaffold PTM was able to identify 23,709 phosphopeptides with localization confidence over 95%. Of these, 6,936 were observed in just a single MudPIT sample, and 2,867 were identified in every sample. Before protein normalization, we assessed statistical significance of expression changes for 2,139 sites, with 219 found significant at $p < 0.05$.

After normalizing by protein expression changes, we computed statistical significance for 1,013 phosphorylation sites, of which 99 were deemed significant.The remaining 1,126 sites were not considered due to missing protein expression values.

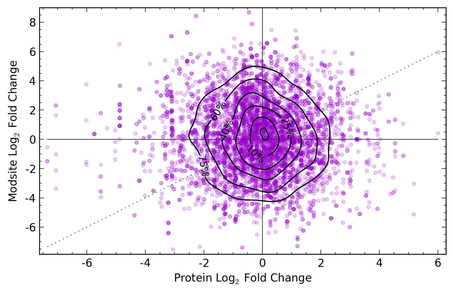

Figure 2: Scatter plot of whole–protein vs. site fold changes for 5,769 phospho sites from a single replicate.

Results (Contd.)

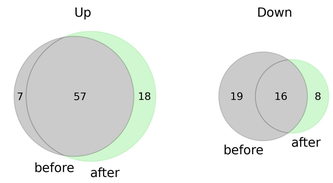

Figure 3: Summary of regulated phosphorylation sites ($p < 0.05$) before and after normalization by protein expression.

Normalization by protein expression has an important effect on the results we observe and greatly increases the accuracy of quantitative PTM analysis by measuring and correcting for confounding expression changes (fig. 3). Many more significantly regulated sites had positive fold changes, indicating that most regulation of phosphorylation was upwards, as expected during mitosis.[1] Additionally, protein normalization had a much greater impact on the statistical significance of downregulated sites. This is explained by a general reduction in protein levels we expect during mitosis which caused these sites to show significantly lower expression before normalization.

Protein normalization also improves reproducibility of measurements (fig. 5). Variation in site expression was reduced by deconvolving PTM and protein expression changes. Talin1 Ser425 phosphorylation is known to promote cell adhesion through $\beta 1$ integrin activation, as well as cancer metastasis.[3] This site is phosphorylated by the CDK5 kinase, one of several related kinases that regulate the cell cycle.

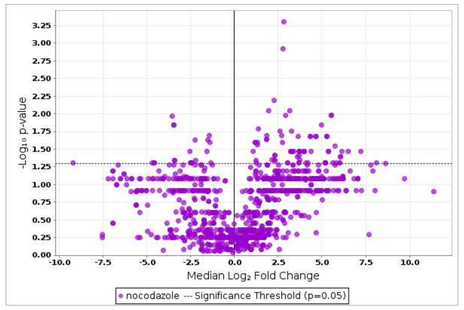

Figure 4: Volcano plot of Mann–Whitney *U*–Test results for protein-normalized phospho site fold changes showing significance threshold $p = 0.05$.

Conclusion

Our analysis demonstrates an easy–to–use approach for quantitative investigation of PTMs. Parallel analysis of proteome and PTM expression allows researchers to easily collect high–coverage PTM datasets *in vivo* and correct for changes in protein expression between conditions. Future work to reduce the number of missing values between samples will improve quantitative coverage and statistical confidence. The implementation of this workflow in off–the–shelf software and integration with statistical analysis simplifies analysis and allows for discovery and validation of significant results.

Phospho-only Site	Modification	Best Ascore	Localization Probability	Reference control_phospho_rep1	Reference control_phospho_rep2	Reference control_phospho_rep3	nocodazole nocodazole_phospho_rep1	nocodazole pvalue for nocodazole_phospho_rep1	nocodazole nocodazole_phospho_rep2	nocodazole pvalue for nocodazole_phospho_rep2	nocodazole nocodazole_phospho_rep3	nocodazole pvalue for nocodazole_phospho_rep3
T144	Phospho	17.20	98%	-0.16	--	--	--	--	--	--	--	--
T167	Phospho	1,000.00	100%	--	--	--	--	--	--	--	-1.20	--
S425	Phospho	102.11	100%	1.05	0.17	-0.03	2.81	0.0082	3.29	0.0404	3.35	0.0404
Protein Normalized												
T144	Phospho	17.20	98%	-0.15	--	--	--	--	--	--	--	--
T167	Phospho	1,000.00	100%	--	--	--	--	--	--	--	-1.21	--
S425	Phospho	102.11	100%	1.06	0.18	-0.03	3.55	0.0082	3.12	0.0404	3.34	0.0404

Figure 5: Quantitative values for sites in protein Talin1 before and after protein normalization.

References

[1] Sharma K., D'Souza R. C., Tyanova S., et al. Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr–based signaling *Cell Rep.* 2014;8:1583–1594.

[2] Wu R., Dephoure N., Haas W., et al. Correct interpretation of comprehensive phosphorylation dynamics requires normalization by protein expression changes *Mol. Cell Proteomics.* 2011;10:M111.009654.

[3] Jin J. K., Tien P. C., Cheng C. J., et al. Talin1 phosphorylation activates $\beta 1$ integrins: a novel mechanism to promote prostate cancer bone metastasis *Oncogene.* 2015;34:1811–1821.

Acknowledgements

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