Analyzing the neuronal phosphoproteome: A systematic comparison of Fusion Lumos and timsTOF Pro data.

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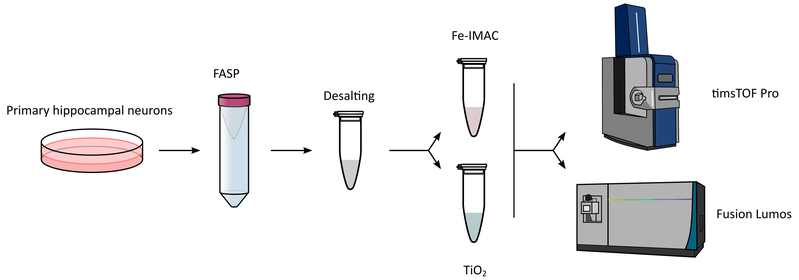
Overview

Of all cell types, neurons display the highest diversity and depth of proteome-wide phosphorylation. While inherently challenging to analyze, the comprehensive detection and quantification of phosphorylation states can provide important new insights into the molecular mechanisms underlying synaptic function, plasticity, and learning and memory. Due to recent advances in instrumentation and preparation techniques, complex phosphoproteome mixtures can now be analyzed. However, no systematic comparison of Orbitrap and timsTOF Pro data is available. Here, we performed both single-shot analyses of primary hippocampal neurons. Our results show that both instruments can identify and quantify distinct sets of phosphopeptides, and that by using a combination of both approaches an unprecedented depth of phosphosite coverage can be achieved.

Methods

Primary hippocampal cells were cultured in 20 dishes of 6 cm diameter (10 million cells in total) for 21 days. A whole cell lysate of the hippocampal neurons containing 1.25 mg protein was enzymatically digested in a modified FASP protocol using Amicon filter units (30 kDA MW cut-off, Ultra-15). Phosphopeptides were enriched in a concatenated enrichment strategy using Fe-NTA and TiO2 (High Select™ Kits, Thermo Scientific). Phosphopeptides were nano-HPLC separated on nanoElute (Bruker Daltonics) on a 250 mm column (IonOpticks, Australia) or Ultimate 3000 (Dionex) on a 500 mm easySpray column (Thermo Scientific). MS-analyses were performed on a timsTOF Pro mass spectrometer (Bruker Daltonics) using the PASEF acquisition method or an orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). At the timsTOF Pro, a PASEF cycle of 1.1 s was used equating to a 100 ms TIMS MS scan followed by ten 100 ms PASEF MS/MS cycles each fragmenting up to 16 precursors.

Data were analyzed using MaxQuant (Jürgen Cox, MPI of Biochemistry), PEAKS Studio X (Bioinformatic Solutions Inc.) and Sequest (Thermo Proteome Discoverer 2.3).



In brief, spectra were matched to a Rattus norvegicus database downloaded from uniprot.org (Proteome ID: UP000002494) and a contaminant database. Tryptic peptides with a minimum length of 7 amino acids and a maximum of 2 missed cleavage sites were included. In MaxQuant for the Fusion Lumos precursor mass tolerance was set to 20 ppm in the first search and 4.5 ppm in the main search. For the timsTOF Pro, the mass tolerances were set to 20 ppm in the first search and 10 ppm in the main search. Analogous to this, in Proteome Discoverer precursor tolerance was set to 20 ppm/ 10 ppm and 0.07 Da/ 10 ppm for Fusion Lumos and timsTOF, respectively. All searches were carried out with a static modification of Cys residues (carboxyamidomethylation) and variable modifications of Ser, Thr and Tyr residues (phosphorylation) and N-termini (acetylation). The number of modifications was limited to 5 events per peptide. For all search results of the different search engines a FDR at PSM level of 0.01 was applied. In MaxQuant protein and peptide FDRs were additionally set to 0.01. In Proteome Discoverer 2.3, peptides were filtered for high confidence for the analysis.

References

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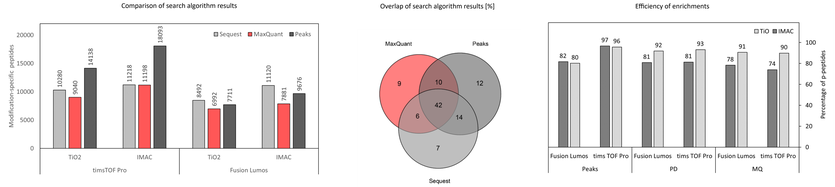
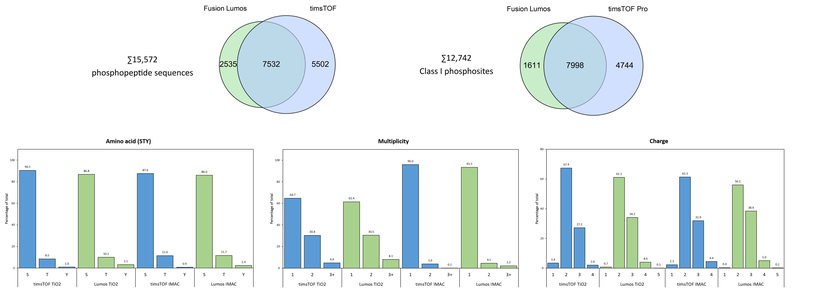
Results: The hippocampal phosphoproteome

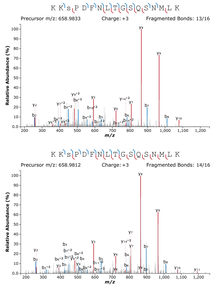
Here we analyzed primary cultured hippocampal neurons to compare the two mass spectrometry systems. In our first experiments, we tested both setups in single-shot experiments. To obtain identical samples, the digested and purified phosphopeptides were enriched by both IMAC- and TiO_2-affinity purification and then split for Orbitrap and TIMS-MS measurements (with 625 µg of starting material each). Both mass spectrometers were coupled to different liquid chromatography systems (Ultimate 3000 and nanoElute), but with identical injected peptide amounts and gradients that had previously been tested to ensure optimal performance of the systems.
Both dataset were then searched with MaxQuant 1.6.2.3, PEAKS Studio X and Proteome Discoverer 2.3 with different sets of parameters (see Methods). Notably, PEAKS Studio X identified the highest number of phospho-peptides and class I sites using the standard parameter set.



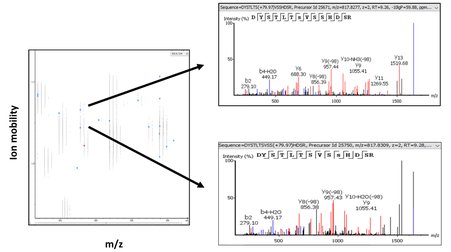
For subsequent analysis, we focused on stringently-filtered MaxQuant data. We identified 10,067 phosphopeptides on the Fusion Lumos mapping to 9,609 Class I phosphosites and 13,034 phosphopeptides on the timsTOF Pro mapping to 12,743 Class I phosphosites. Combining the results from both mass spectrometry systems, we identified 15,572 phosphopeptide sequences. In these data, we found an overlap of 48 % of Fusion Lumos and timsTOF Pro data. The Fusion Lumos data showed 2,535 unique phosphopeptides and the timsTOF Pro 5,502 unique phosphopeptides.



Comparison of MS/MS spectra for identical peptides:
MS/MS spectra for selected peptides displayed similar fragmentation patterns in both instruments. Example peptide derived from protein Grin2a, Andromeda Score: 185.92/ 218.03 on Fusion Lumos/timsTOF.



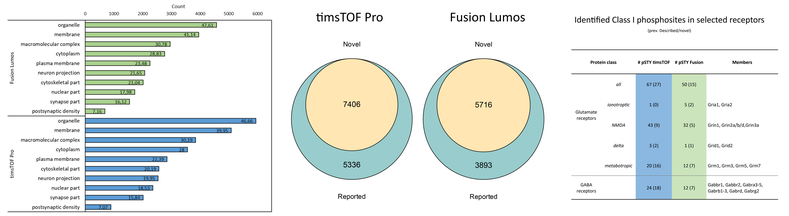
Separation of isobaric phosphopeptides using ion mobility
Isobaric phosphopeptides with different modified phosphorylation positions can be separated using ion mobility. Example phosphopeptide from Integrin-4:



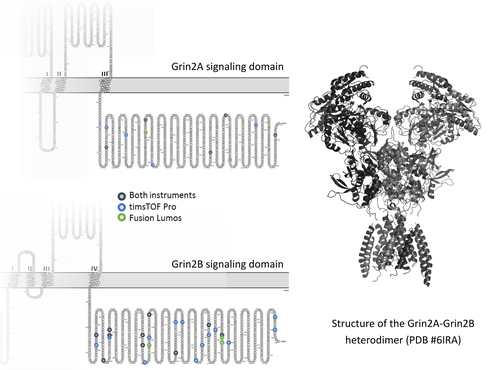
Peptide: DYSTLTSVSSHDSR, z=+2, derived from Integrin-4

Results: Distribution and depth of data

We then performed a GO enrichment analysis on both datasets to investigate if there were any systematic, physiological differences in the data from both instruments. Both datasets showed almost identical distributions of GO term enrichments.
We then investigated how many of the phosphosites had been described previously. Only a minority of the identified phosphosites had been previously described in literature (e.g. on Phosphosite Plus). These novel sites were detected on multiple proteins essential for plasticity, synaptic transmission and cellular homeostasis.



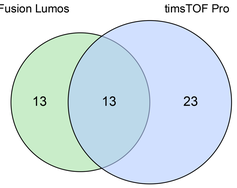
We then investigated specific proteins known to play essential roles in neurobiological processes. One of these proteins comprises the NMDA receptor, a membrane protein complex of which individual subunits are regulated by phosphorylation in their intracellular C-terminal domains. Figure X displays a schematic view of the regulatory C-terminal domain of the NMDA receptors 2A and 2B (Grin2A, Grin2B). Phosphosites exclusively identified with the Fusion Lumos or with the timsTOF Pro are marked in green and blue, respectively. Phosphosites identified on both instruments are displayed in dark grey.



Structure of the Grin2A-Grin2B heterodimer (PDB #6IRA)

Grin2A/2B receptor

Fusion Lumos	26 class I sites (2 unreported)
timsTOF Pro	36 class I sites (5 unreported)
Both:	49 class I sites (7 unreported)



Conclusions

Here we present a systematic comparison of Fusion Lumos and timsTOF Pro phosphoproteomics data. We analyzed identical samples, using both IMAC– and TiO_2-based purification approaches, on both instruments in single shot experiments without peptide fractionation. Depending on database search stringency, we identified up to 32,231 phosphopeptides. Using stringent filters, we identified 15,572 phosphopeptide sequences. In our experiments, the timsTOF Pro achieved higher identification rates than the Fusion Lumos (12,742 class I phosphosites identified in total, with 7,988 (62.7%) sites identified using both instruments and 1,611 (12.6%) exclusively on the Fusion Lumos and 4,744 (37.2%) exclusively on the timsTOF Pro. While overall fragmentation patterns appeared similar, complementary sets of phosphopeptides were identified on both instruments (typical technical overlap in these setups: 80-85% for class I sites). On the timsTOF Pro, ion mobility-based separation allowed us to differentiate sets of isobaric phosphopeptides with different phosphorylation positions. In addition, we observed complementary data in IMAC- and TiO2-based phosphopeptide enriched samples and observed a high number of phosphosites that were previously not reported.
We are currently investigating large sample cohorts (both cell and tissue culture as well as brain tissue-derived preparations) to elucidate if both instruments also deliver complementary data using long gradients and pre-fractionated phosphopeptides. We then plan to make use of these approaches to investigate functional changes in phosphorylation states in different treatment paradigms and model systems.

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