High Sensitivity Phosphoproteomics using PASEF on a TIMS-QTOF mass spectrometer

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Introduction

High resolution mass spectrometry-based proteomics has become a powerful tool to study signal transduction pathways. Sensitivity, sequencing speed and peak capacity are prerequisites for deep identification and quantification into the phosphoproteome. Here, we present trapped ion mobility spectrometry (TIMS) in combination with QTOF technology using the parallel accumulation – serial fragmentation (PASEF) [1] acquisition mode for deep phosphoproteomics analysis at record acquisition speeds. Using this approach in the analysis of primary hippocampal neurons has enabled us to obtain comprehensive data from his dynamic and complex phosphoproteome.

Methods

Whole cell lysate of primary cultured hippocampal neurons (DIV 21, 5 million cells in total) was proteolytically digested in a modified FASP protocol. Phosphopeptides were enriched from the desalted peptides using TiO2- and Fe-NTA-based affinity purification strategies (High Select™ Enrichment Kits, Thermo Scientific™).

Phosphopeptides enriched from proteolytic digests of HeLa cells or primary hippocampal neurons were nano-HPLC separated (nanoElute, Bruker Daltonics) on a 250 mm pulled column (IonOpticks, Australia) and analyzed on a high-resolution timsTOF Pro mass spectrometer (Bruker Daltonics) using the PASEF acquisition method. 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 12 precursors. Data were analyzed using PEAKS 8.5 (Bioinformatics solution Inc.) and MaxQuant (Jürgen Cox, Max Planck Institute of Biochemistry).

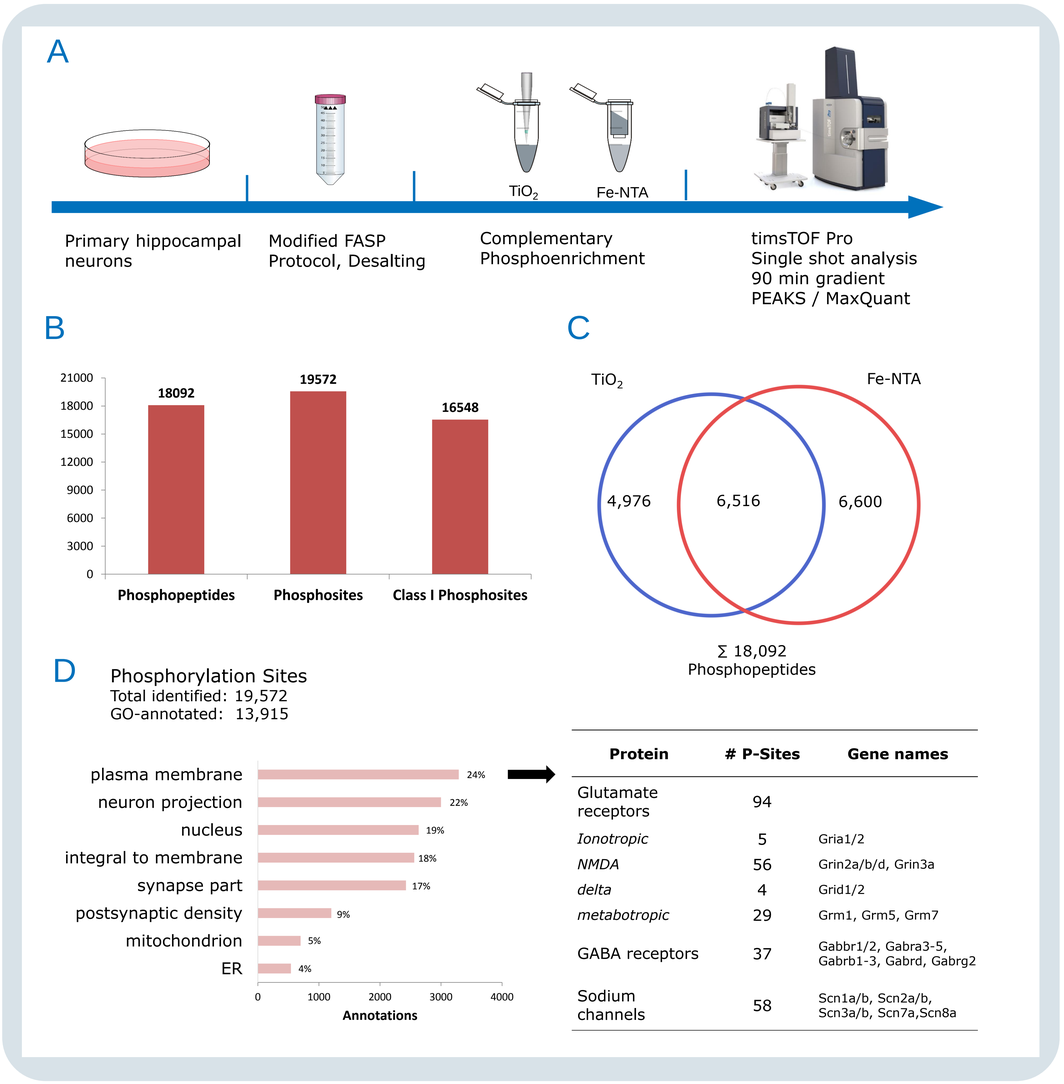

Figure 3: Deep phosphoproteomics of primary neurons
A) Workflow for phosphoproteome analysis of primary neurons. Downstream analysis was performed using MaxQuant and Perseus. B) Unique phosphopeptides and phosphosite identification combining IDs of TiO_2 and Fe-NTA enrichments. C) Overlap of identified phosphopeptides for both enrichments. D) GO-annotation of the identified phosphosites. Listing of phosphosites on major groups of channels and transporters at synapses.

Results

The increased peak capacity from the extra dimension of separation provided by TIMS and increased sequencing speed of the PASEF method enables very large number of phosphopeptide identifications from short gradients and low sample amounts. More than 17,400 unique phosphopeptides were identified using a 90 min gradient (Figure 1).

Co-eluting peptides that only differ slightly in m/z can be resolved using TIMS resulting in clean MS/MS spectra. In the example below the peptides differ only in their site of phosphorylation. They could be separated by their collisional cross sections. This would not be possible on a conventional non-TIMS mass spectrometer.

Figure 1: Single shot phosphoproteomics at high depth and sensitivity
Unique phosphopeptides (1% FDR) identified from enriched HeLa cell digests (200 µg) using 15, 30, 60 and 90 min gradients. Data analysis was performed using PEAKS studio.

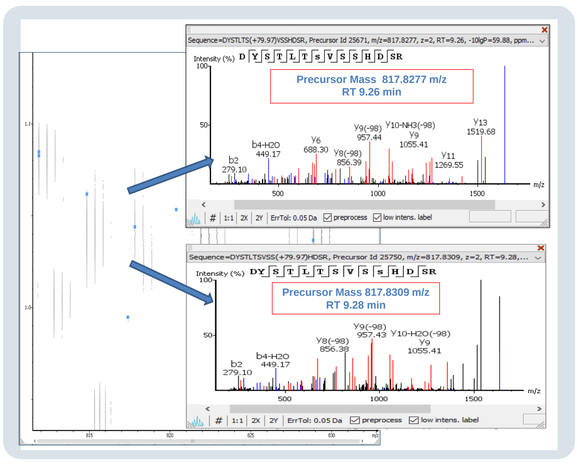

Figure 2: Separation of isobaric peptides
Co-eluting phosphopeptides that differ only by the phosphorylation localization site are separated by TIMS, resulting in non-chimeric MS/MS spectra that enable localization of the different sites of phosphorylation

In the phosphoproteome analysis of primary cultured hippocampal neurons 19,572 phosphorylation sites on 18,092 unique phosphopeptides were identified in a single shot analysis (90 min gradient, Figure 3), with accurate site localization to a single amino acid for 16,584 phosphosites (Class I sites, $p > 0.75$). A concatenated and complementary enrichment strategy (TiO_2 and Fe-NTA) was used to increase the total number of identified phosphopeptides. The majority of the phosphopeptides (64%) were exclusive for either one of the enrichments. The depth of the timsTOF Pro data enables comprehensive analysis of signaling pathways in hippocampal neurons even with limited sample amounts. We found > 3000 phosphosites (24% of the data) in the plasma membrane (based on GO annotation), including phosphorylation of all major groups of channels and transport proteins important for neuronal function and synaptic plasticity.

Conclusions

- PASEF on a timsTOF Pro instruments offers the possibility to investigate samples to an unprecedented depth
- Sensitive PTM analysis to investigate signal transduction pathways
- High-throughput; PASEF enables deep proteomics analysis on short gradients

Reference

(1) Meier et al.; Jounral of Proteomics Reasearch

timsTOF Pro