Chapter 11

Improving the Phosphoproteome Coverage for Limited Sample Amounts Using TiO_2-SIMAC-HILIC (TiSH) Phosphopeptide Enrichment and Fractionation

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Abstract

Obtaining high phosphoproteome coverage requires specific enrichment of phosphorylated peptides from the often extremely complex peptide mixtures generated by proteolytic digestion of biological samples, as well as extensive chromatographic fractionation prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Due to the sample loss resulting from fractionation, this procedure is mainly performed when large quantities of sample are available. To make large-scale phosphoproteomics applicable to smaller amounts of protein we have recently combined highly specific TiO_2-based phosphopeptide enrichment with sequential elution from immobilized metal affinity chromatography (SIMAC) for fractionation of mono- and multi-phosphorylated peptides prior to capillary scale hydrophilic interaction liquid chromatography (HILIC) based fractionation of monophosphorylated peptides. In the following protocol we describe the procedure step by step to allow for comprehensive coverage of the phosphoproteome utilizing only a few hundred micrograms of protein.

Key words Phosphopeptide, Phosphoproteomics, Protein phosphorylation, TiO_2, Capillary HPLC, HILIC, TiSH, Enrichment, Fractionation

1 Introduction

Mass spectrometry (MS)-based proteomics have improved tremendously within the last decade, leading to a rapid progression in our knowledge of cellular signaling processes. Particularly in the field of post-translational modifications (PTMs), MS has become an indispensable tool for large-scale characterization and quantification of protein phosphorylation, glycosylation, ubiquitination, acetylation, etc. As a result of the generally low stoichiometry of these PTMs, specific enrichment steps are absolutely necessary to achieve adequate coverage of the modified sites in the samples of interest. While immobilized metal affinity chromatography (IMAC) initially was the method of choice for phosphopeptide enrichment [1, 2], TiO_2-based enrichment strategies [3–7] have in many laboratories

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become the de facto standard in phosphoproteomics due to their high specificity and robustness towards detergents and salts [8]. Furthermore, the combination of the two enrichment techniques (in a procedure called "*S*equential elution from *IMAC*"—SIMAC) has been shown to facilitate separation of mono- and multi-phosphorylated peptides [9]. However, in spite of increasingly sensitive and fast MS instruments facilitating identification of thousands of phosphorylation sites in a few hours, sample complexity still poses a challenge in the quest for achieving a complete coverage of the cellular phosphoproteome. Five years ago hydrophilic interaction liquid chromatography (HILIC) was introduced as a prefractionation step in large-scale phosphoproteomics [10] and is now, along with strong cation exchange and high pH reversed phase chromatography, commonly used as the first dimension of fractionation prior to reversed phase (RP) nanoLC-MS/MS analysis of phosphopeptides. Amongst the advantages of HILIC are a salt-free buffer system and high orthogonality with the second dimension RP LC separation [11]. However, HILIC has been shown to deliver a lower resolution for multi-phosphorylated peptides than for singly phosphorylated species [12, 13]. Furthermore, as the first dimension of chromatography traditionally has been performed prior to phosphopeptide enrichment, the process of enriching all the collected fractions lowers the throughput of the setup. Moreover, fractionating the small amounts of purified phosphopeptides using standard HPLC columns (2–4.6 mm i.d.) can lead to higher phosphopeptide losses due to adsorption to column and plastic surfaces. Performing peptide fractionation prior to phosphopeptide enrichment can also complicate the experimental workflow if the highest possible specificity is aspired: The ratio of TiO_2 beads to peptide starting material is very important for obtaining a selective purification of phosphopeptides and the addition of too much TiO_2 resin to a peptide fraction of unknown quantity will lead to a significant co-purification of non-modified peptides [12, 14].

To circumvent these drawbacks, while still utilizing the advantages of TiO_2, SIMAC, and HILIC, we have combined all three enrichment/fractionation methods into an integrated method dubbed TiSH—*Ti*O_2-*S*IMAC-*H*ILIC [12] (*see* Fig. 1). The TISH-strategy relies on a TiO_2-based pre-enrichment step, removing most non-phosphorylated peptides prior to separation of mono- and multi-phosphorylated peptides. The sample containing multi-phosphorylated peptides is less complex and contains a smaller amount of peptide and is therefore analyzed directly by RP nanoLC-MS/MS. The sample containing mono-phosphorylated peptides is fractionated using capillary scale HILIC (320 μm i.d. in-house made column (*see* Fig. 2)). Fraction collection is performed directly into a 96-well microplate, in which the samples can be dried by vacuum centrifugation and dissolved in an RP compatible buffer and directly analyzed by nanoLC-MS/MS. Using

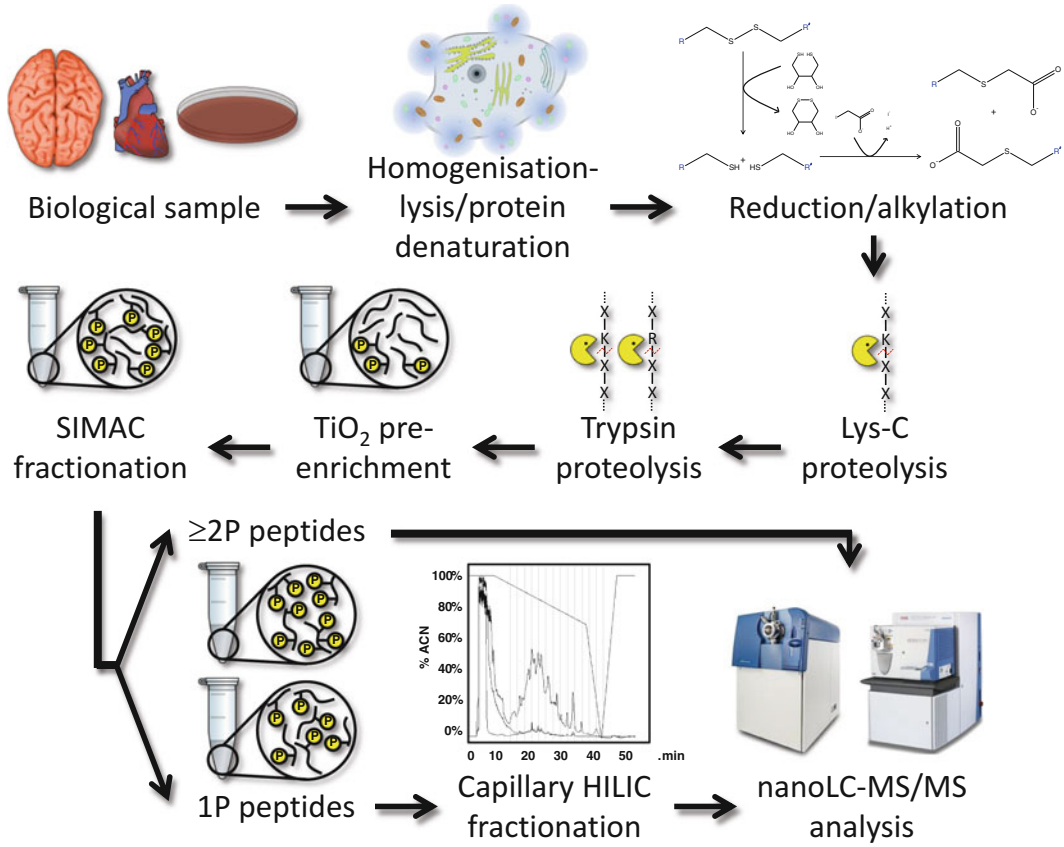

Fig. 1 Outline of the overall TiSH phosphopeptide enrichment and fractionation strategy. After an initial integrated homogenization/lysis-protein denaturation of the tissue or cells, the protein sample is reduced and alkylated prior to proteolytic digestion using endoproteinase Lys-C, followed by tryptic proteolysis. The resulting peptides are subjected to an initial TiO_2 enrichment to remove most non-phosphorylated peptides before the SIMAC fractionation of mono-phosphorylated and multi-phosphorylated peptides. While the latter are analyzed directly by LC-MS/MS, the mono-phosphorylated peptides are further fractionated using capillary HILIC before LC-MS/MS analysis

this setup, 6600 unique phosphopeptides could be identified in a quantitative duplex dimethylation experiment with starting amounts of 300 µg of insulinoma cell line protein per condition, which was analyzed on an LTQ-Orbitrap Velos using a standard (0.075 mm × 20 mm column) nanoLC setup [12].

The exact procedure for cell lysis and protein extraction is highly dependent on the nature and amount of biological sample. The lysis and digestion procedure presented here is suited for low to medium amounts (100 µg to 2 mg protein) of soft tissue or cultured cells and is focused on minimizing sample losses by avoiding protein precipitation steps etc., but can be substituted with other lysis/extraction protocols if necessary as TiO_2-based phosphopeptide enrichment is highly robust towards various salts, buffers and denaturing reagents [8].

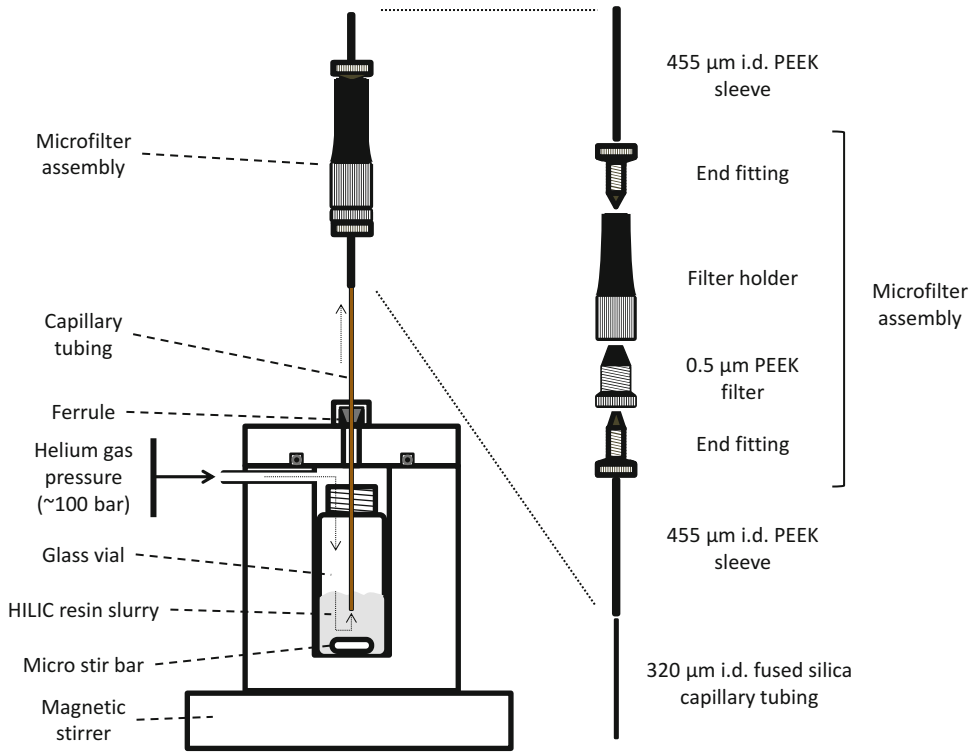

Fig. 2 Schematic illustration of the in-house made capillary HILIC column and the column packing process. The column consists of a 0.5 µm PEEK micro filter connected to a 320 µm i.d. fused silica capillary using a 455 µm i.d. sleeve. The column is packed with the TSKGel Amide-80 HILIC resin slurry from a vial in a high-pressure packing device into which the upstream end of the fused silica capillary is inserted. A magnetic stirrer underneath the device and a micro stir bar in the vial keeps the resin in suspension and by applying a helium gas pressure (~100 bar) to the device, forcing the slurry into the capillary, the resin is trapped by the micro filter and the column packed. When the column has packed to the desired length, the pressure can be released very slowly, and the column removed and attached to the capillary HPLC system

When processing protein amounts outside the 0.1–2 mg range, adjusting the quantities of chromatographic resin/columns, volumes, etc. is necessary for optimal efficiency and specificity of the protocol. For a simpler and faster procedure, the protocol can be performed without the SIMAC mono-/multi-phosphopeptide fractionation step, but will potentially result in a lower number of multi-phosphorylated peptide identifications.

2 Materials

All solutions are prepared using ultrapure water and analytical grade chemical reagents. To minimize protein and peptide adsorption to plastics surfaces, all microcentrifuge tubes and tips should be of the "low-binding" type.

2.1 Cell Lysis, Protein Extraction and Digestion

1. Ultrapure water (18 MΩ-cm).
2. 8 M urea.
3. Phosphatase Inhibitor Solution.
4. 1 M dithiothreitol (DTT).
5. 0.5 M iodoacetamide (IAA).
6. Ultrasound probe sonicator with micro probe (e.g., Branson 250A Sonifier Ultrasonic Processor Cell Disruptor with Micro Tip).
7. 1 M triethyl ammonium bicarbonate (TEAB) (*see* **Note 1**).
8. Protein concentration measurement method such as amino acid composition analysis or other technique capable of measuring low microgram amounts of protein/peptides such as the Pierce Micro BCA Protein Assay Kit (Thermo Fisher Scientific) (*see* **Note 2**).
9. 0.05 AU/μL endoproteinase Lys-C in H_2O.
10. 1 μg/μL Sequencing Grade Modified Trypsin in 50 mM TEAB (*see* **Note 3**).
11. 10 % trifluoroacetic acid (TFA).

2.2 Phosphopeptide Pre-enrichment

1. Ultrapure water (18 MΩ-cm).
2. Acetonitrile (ACN, HPLC grade).
3. Trifluoroacetic acid (TFA).
4. Methanol (MeOH).
5. Titansphere 5 μm TiO_2 resin (GL Sciences).
6. Glycolic acid.
7. Ammonium hydroxide 25 % (NH_4OH).
8. TiO_2 Loading Buffer: 1 M glycolic acid in 80 % ACN, 5 % TFA.
9. TiO_2 Washing Buffer 1: 80 % ACN, 1 % TFA.
10. TiO_2 Washing Buffer 2: 20 % ACN, 0.2 % TFA.
11. TiO_2 Elution Buffer: 1 % NH_4OH, pH 11.

2.3 Separation of Multi- and Mono-phosphorylated Peptides Using Sequential Elution from IMAC (SIMAC)

1. PhosphoSelect IMAC resin (Sigma-Aldrich).
2. 200 μL gel loader tips.
3. 1 mL plastic syringe with home-made adaptor facilitating attachment to gel loader tip.
4. Formic acid (FA).
5. Titansphere 5 μm TiO_2 resin (reuse beads from phosphopeptide pre-enrichment) (*see* Subheading 3.2, **step 9**).
6. Trifluoroacetic acid (TFA).
7. Acetonitrile (ACN, HPLC grade).

8. SIMAC Loading Buffer: 50 % ACN, 0.1 % TFA.
9. SIMAC Acid Elution Buffer: 20 % ACN, 1 % TFA (*see* **Note 4**).
10. SIMAC Basic Elution Buffer: 1 % NH_4OH, pH 11.
11. pH meter with micro pH electrode.
12. Gauge 25 blunt HPLC syringe needle.
13. POROS Oligo R3 (Life Technologies).
14. 3 M Empore C18 extraction disc (3 M).
15. Reversed Phase (RP) Conditioning/Elution Buffer: 70 % ACN, 0.1 % TFA.
16. Reversed Phase (RP) Washing Buffer: 0.1 % TFA.
17. Vacuum centrifuge.

2.4 Desalting of Non-phosphorylated Peptides

1. Sep-Pak C18 Plus Light cartridge (Waters).
2. Reversed Phase (RP) Conditioning/Elution Buffer: 70 % ACN, 0.1 % TFA.
3. Reversed Phase (RP) Washing Buffer: 0.1 % TFA.

2.5 Packing of Hydrophilic Interaction Liquid Chromatography (HILIC) Capillary Column

1. TSKGel Amide 80 3 μm HILIC resin (Tosoh Bioscience, from HPLC column).
2. Methanol (MeOH).
3. Polyether ether ketone (PEEK) inline microfilter (Upchurch Scientific).
4. MicroTight Sleeve Black (F-186), 455 μm i.d. (Upchurch Scientific).
5. 0.32 mm (inner diameter (i.d.))/0.435 mm (outer diameter (o.d.)) fused silica capillary tubing (Polymicro Technologies).
6. Vertical high-pressure capillary column packing device (e.g. Nanobaume (Western Fluidics) or PC77 Pressure Injection Cell (Next Advance, Inc.)).
7. Vespel ferrule (0.5 mm (i.d.) × 3.2 mm (o.d.) × 3.7 mm) (SGE Analytical Science).
8. 2 mL glass vial.
9. Micro stir bar.
10. Magnetic stirrer.
11. Compressed helium.

2.6 Peptide Fractionation via Hydrophilic Interaction Liquid Chromatography (HILIC)

1. Capillary flow (1–20 μL/min) high-performance liquid chromatography system with UV detector. In this protocol an Agilent 1200 (Agilent Technologies) equipped with an autosampler with a 40 μL loop, a UV detector with an 80 nL flow cell and a micro fraction collector was used.
2. In-house made 0.32 × 20 mm 3 μm resin TSKGel Amide 80 HILIC capillary column.

3. Dimethyl sulfoxide (DMSO).
4. Ultrapure water (18 MΩ-cm).
5. Acetonitrile (ACN, HPLC grade).
6. Trifluoroacetic acid (TFA).
7. HILIC Solvent A: 0.1 % TFA.
8. HILIC Solvent B: 90 % ACN, 0.1 % TFA.

2.7 Analysis by Mass Spectrometry

1. Mass spectrometer capable of performing MS/MS—preferentially a high-resolution/high mass accuracy instrument (recent Q-TOF, or Orbitrap based mass spectrometer) interfaced to a nanoHPLC with a 50–100 μm i.d. RP capillary column setup for highly sensitive online peptide separation.
2. Software for processing of raw mass spectrometry data files and generation of peak lists for searching against a protein database (e.g. Uniprot) such as Mascot/Mascot Distiller (Matrix Science, London, UK) (data from most vendors and instruments), Proteome Discoverer (Thermo Scientific, Bremen, Germany) (data from Thermo instruments), MaxQuant [15] (high resolution data from Thermo Orbitrap instruments and certain Bruker and ABSciex Q-TOFs) and the TransProteomicPipeline [16] (vendor independent).

3 Methods

3.1 Cell Lysis, Protein Extraction and Digestion

1. Prepare lysis buffer by making an 8 M urea solution in 50 mM TEAB and add phosphatase inhibitors to a 1× final concentration. Add the minimum amount of lysis buffer necessary for full lysis of the cells on ice. Keeping the volume down is important as the sample has to be diluted to ≤1 M urea (≥8× dilution) prior to tryptic digestion. Vortex extensively until a clear solution is obtained.
2. If sample volume is large enough to allow for sonication: Sonicate the sample for 3 × 10 s (with 10 s breaks for the sample to cool) on ice (*see* **Note 5**) at the highest intensity possible without causing the sample to foam.
3. Measure protein concentration of a small aliquot of the sample while keeping the rest of the sample on ice to reduce endogenous protease activity.
4. Perform combined reduction of protein disulfide bonds and Lys-C proteolysis by adding 1 M DTT to a final concentration of 10 mM and Lys-C (0.05 AU Lys-C per 100–300 μg) to the sample and incubate at room temperature (*see* **Note 5**) for 3 h.
5. Alkylate reduced cysteine residues by adding 0.5 M IAA to a final concentration of 20 mM, incubating the sample in the dark at room temperature for 20 min.

6. Dilute the sample 8× in 50 mM TEAB to a final concentration of 1 M urea.
7. Optional—if no sonication was performed in **step 2**: Sonicate the sample on ice (*see* **Note 5**).
8. Add trypsin in a 1:50 trypsin–protein ratio and incubate the sample at room temperature (*see* **Note 5**) overnight.
9. At this step the sample(s) can be subjected to chemical stable isotope labeling such as reductive dimethylation [17], iTRAQ [18] or TMT [19] for relative quantification (*see* **Note 1**).
10. Adjust the sample to a pH of below 3 using 10 % TFA or FA, centrifuge the sample at 14,000×*g* to precipitate lipids and transfer the supernatant to a new 1.5 mL microcentrifuge tube.
11. Optionally, at this step a fraction of the sample can be removed for later LC-MS/MS analysis if protein-level identification/quantification is required. Alternatively, non-phosphorylated peptides can be obtained from the TiO_2 pre-enrichment flow-through fraction (*see* Subheading 3.4 and **Note 6**).

3.2 TiO_2 Pre-enrichment

1. Adjust the peptide sample to 1 M glycolic acid in 80 % ACN, 5 % TFA. For example, if the sample is present in 150 µL add 50 µL 100 % TFA, 800 µL ACN and 76.05 mg of glycolic acid (*see* **Note 7**). Vortex to dissolve the glycolic acid.
2. Add TiO_2 resin—0.6 mg per 100 µg of peptide—and incubate under vigorous shaking for 10 min. Pellet the resin by brief centrifugation (2000×*g* for 1 min) and transfer the supernatant to a new tube (Optional: If enriching for other PTMs after phosphopeptide enrichment or analysis of the non-modified peptides, save the flow-through for later desalting in Subheading 3.4).
3. Optional second incubation for improved recovery of phosphopeptides: Add TiO_2 resin—0.3 mg per 100 µg of peptide—to the supernatant in the new tube and incubate under vigorous shaking for 10 min. Pellet the resin by brief centrifugation (2000×*g* for 1 min) and transfer the supernatant containing non-phosphorylated peptides to a new tube (can be stored at −80 °C or directly subjected to vacuum centrifugation prior to desalting)—(*see* **Note 6** and Subheading 3.4).
4. Add 500 µL of TiO_2 Loading Buffer to the first tube containing the TiO_2 resin and mix by vortexing. If a second incubation was performed (**step 3**), transfer the suspension to the tube containing the TiO_2 resin and transfer the resin pool to a new 1.5 mL micro centrifuge tube to leave behind non-phosphorylated peptides absorbed to the tube.
5. Vortex briefly and pellet the resin by brief centrifugation (2000×*g* for 1 min). Discard the supernatant.

6. Wash the resin in 500 µL TiO_2 Washing Buffer 1, vortex briefly, and pellet the resin by brief centrifugation (2000 × *g* for 1 min). Discard the supernatant.
7. Wash the resin in 500 µL TiO_2 Washing Buffer 2, vortex briefly, and pellet the resin by brief centrifugation (2000 × *g* for 1 min). Discard the supernatant, and dry the resin briefly in a vacuum centrifuge to evaporate residual acidic buffer.
8. Elute the phosphorylated peptides off the TiO_2 resin by adding 100 µL TiO_2 Elution Buffer to the resin, and incubate the slurry with mixing for 15 min followed by 1 min of centrifugation at 14,000 × *g* (*see* **Note 8**). Transfer as much supernatant as possible to a new tube without aspirating any beads.
9. Add another 40 µL of TiO_2 Elution Buffer and 10 µL ACN to the resin, mix briefly and pellet the resin by centrifugation for 1 min at 14,000 × *g*. Aspirate as much of the supernatant as possible without disturbing the resin and pool the liquid with the first eluate (*see* **Note 9**). Wash the TiO_2 resin in 500 µL TiO_2 Washing Buffer 1, vortex briefly, and pellet the resin by brief centrifugation (2000 × *g* for 1 min). Discard the supernatant and save the resin for the later TiO_2 enrichment step (*see* **Note 10**).
10. Dry the eluate to completeness in a vacuum centrifuge (*see* **Note 11**).

3.3 Separation of Multi- and Mono-phosphorylated Peptides Using Sequential Elution from IMAC (SIMAC)

1. Redissolve the phosphopeptide sample in first 0.5 µL 100 % FA, followed by 200 µL 50 % ACN, 0.1 % TFA and adjust it to pH 1.8 with 10 % TFA, ideally measuring the pH using a micro electrode pH meter.
2. Wash 60 µL of IMAC slurry by adding 200 µL SIMAC Washing Buffer. Pellet the resin by centrifugation at 2000 × *g* for 15 s, discard the liquid and repeat the washing step. After having removed the liquid from the resin, add the phosphopeptide sample to the resin.
3. Incubate the sample under continuous shaking for 30 min at room temperature to allow the phosphopeptides to bind the IMAC resin. Prepare 200 µL gel loader tips by constricting the end of the tip to make it retain the IMAC resin. After incubation, centrifuge briefly and transfer most of the supernatant to a new 1.5 mL tube without disturbing the resin pellet and resuspend the beads in the leftover sample. Transfer the slurry to the gel loader tip. Push the liquid through the tip by applying air pressure using a plastic syringe and collect the flow-through, containing some mono-phosphorylated as well as non-phosphorylated peptides, in the same 1.5 mL tube containing the IMAC flow-through. Make sure that there are no beads in the flow-though (*see* **Note 12**).

4. Wash the IMAC column formed in the constricted end of the tip with 70 µL SIMAC Washing Buffer, washing off non-phosphorylated peptides as well as weakly binding mono-phosphorylated peptides and collect the wash in the tube containing the flow-through from **step 3**.
5. Elute the remaining mono-phosphorylated peptides slowly off the IMAC column (1 droplet/s (*see* **Note 13**)) by adding 70 µL SIMAC Acid Elution Buffer (*see* **Note 4**) to the column and applying air pressure using the syringe—pool this eluate with the flow-through/wash from **step 4** resulting in a combined mono-phosphorylated peptide sample. Make sure no IMAC resin is present in sample (*see* **Note 12**).
6. Slowly elute the multi-phosphorylated peptides off the IMAC resin into a microcentrifuge tube by adding 100 µL of SIMAC Basic Elution Buffer to the column and applying air pressure via the syringe. Make sure no IMAC beads are present in the eluate (*see* **Note 12**) and acidify the sample with 10 µL 100 % FA. Save the sample for later concentration/desalting prior to LC-MS/MS analysis.
7. Adjust the sample containing mono-phosphorylated peptides from **step 5** to 80 % ACN, 1 % TFA and incubate it under vigorous shaking for 10 min with the same amount of TiO_2 as used in the pre-enrichment procedure (*see* Subheading 3.2, **step 2**). The TiO_2 resin from Subheading 3.2, **step 9** can be reused (after regeneration) in this step (*see* **Note 10**).
8. After incubation, pellet the beads by centrifugation at 2000 × *g* for 1 min and transfer the supernatant to another tube (save the resin). Optional second enrichment (for improved recovery): The supernatant can be incubated with the same amount of TiO_2 resin as in Subheading 3.2, **step 3** for an additional 10 min under vigorous shaking. The resin used in Subheading 3.2, **step 3** can be reused (after regeneration) (*see* **Note 10**).
9. After the second incubation, pellet the resin by centrifugation at 2000 × *g* for 1 min, and discard the supernatant. Add 300 µL 50 % ACN, 0.1 % TFA to each of the resin-containing tubes, vortex briefly and combine the two slurries. Pellet the resin by centrifugation at 2000 × *g* for 1 min and discard the supernatant.
10. Dry the TiO_2 resin for 5 min in a vacuum centrifuge to remove leftover Washing Buffer and elute the mono-phosphorylated peptides by adding 100 µL TiO_2 Elution Buffer to the tube. Vortex briefly and incubate under continuous shaking for 15 min. Pellet the resin by centrifugation at 14,000 × *g* for 1 min and transfer the eluate to another tube without disturbing the resin bed. Add another 50 µL of TiO_2 Elution Buffer

to the resin, briefly vortex, centrifuge again at 14,000 × *g* for 1 min. Combine the second eluate with the first one without disturbing the resin bed. Make sure that no resin is present in the eluate as described in Subheading 3.2, **step 9**. Acidify the sample with 15 µL 100 % FA.

11. Prior to fractionation of the mono-phosphorylated peptides by HILIC and LC-MS/MS analysis of the multi-phosphorylated peptides both samples are desalted on homemade POROS R3 RP micro-columns (~1–2 cm long) packed in a 200 µL pipette tip constricted with a plug of 3 M Empore C18 disc.
12. Before applying the sample, wash the column in 100 µL RP Conditioning/Elution Buffer, followed by 100 µL RP Washing Buffer. Load the sample onto the column by applying air pressure, wash it with 100 µL RP Washing Buffer and elute off the phosphopeptides with 100 µL RP Conditioning/Elution Buffer into a new tube. Dry the phosphopeptides sample to completion in a vacuum centrifuge and store at −20 °C until time of fractionation/analysis.

3.4 Desalting of Non-phosphorylated Peptides

1. This step is optional depending on whether analysis of other post-translational modifications or the non-modified peptides is required; e.g. lysine acetylated peptides can be immunoprecipitated after desalting of the non-phosphorylated peptide sample. The Sep-Pak C18 Plus Light has a capacity of a least 1–2 mg peptide.
2. Dry the samples from Subheading 3.2, **step 2** to completeness in a vacuum centrifuge and dissolve the peptides in 3 mL 0.1 % TFA.
3. Wash the Sep-Pak cartridge with 3 mL RP Conditioning/Elution Buffer followed by washing with 5 mL of RP Washing Buffer.
4. Load the peptide solution onto the cartridge slowly (1 drop/s) and discard the flow-through. Wash the cartridge with 5 mL RP Washing Buffer. Flush the cartridge with air, removing the solvent completely.
5. Elute the peptides using 1 mL of RP Conditioning/Elution Buffer slowly (1 drop/s) into a new tube. Dry the sample by vacuum centrifugation and store at −20 °C until further use.

3.5 Packing of Hydrophilic Interaction Liquid Chromatography (HILIC) Capillary Column

1. Make a 0.5 mL 5–10 % slurry of HILIC resin in 100 % MeOH in a glass vial and sonicate it for 5 min in a sonicator bath, add the micro stir bar, and place it in the column packing device (*see* Fig. 2).
2. Cut a ~25 cm piece of 0.32 mm i.d. capillary tubing and attach the micro filter via the sleeve. Insert the other end of the capillary through the ferrule and attach it to the column packing

device (*see* Fig. 2). Connect the packing device to the compressed helium supply and adjust the pressure to 100 bars. Leave the column packing with magnetic stirring on overnight or until the column has packed. Leave to dry, as releasing the pressure from a wet column can lead to back-flow of the resin.

3. Release the pressure very slowly, remove the column from the packing device and connect it to the capillary HPLC system.

3.6 Peptide Fractionation via HILIC

The capacity of the HILIC column used in this protocol (0.32 × 200 mm) is at least 10–20 μg of peptide, corresponding to phosphopeptides originating from approximately 1–2 mg protein lysate. If larger amounts of sample are available, scale up the HILIC column and flow rate of the HPLC run.

1. Dissolve the mono-phosphorylated peptide sample in 0.2 μL 50 % DMSO (*see* **Note 14**). Add 3.6 μL H_2O, followed by 0.4 μL 10 % TFA, and finally slowly add 36 μL ACN (*see* **Note 14**). Centrifuge the sample at 14,000 × *g* for 3 min to precipitate any undissolved material.
2. Load the supernatant onto the HILIC column at a flow rate of 12 μL/min for 10 min, followed by separation of the phosphopeptides at a flow rate of 6 μL/min with an increasing aqueous gradient from 100 to 60 % B over 40 min. Collect 1 min fractions throughout the gradient into a 96-well microtiter plate.
3. Combine the fractions based on UV absorption at 214 nm to obtain a number of samples in the plate (typically 10–15—depending on time available for LC-MS/MS analysis) containing similar amounts of phosphopeptides (*see* **Note 15**). Dry the sample plate by vacuum centrifugation and dissolve the samples in 0.3 μL 100 % FA followed by 4.7 μL H_2O (for a 5 μL nanoLC-MS/MS injection).

3.7 Analysis by Mass Spectrometry

1. Analyze the samples by RP nanoLC-MS/MS. A typical nanoLC setup would include a 0.075 mm × 20 mm analytical column packed with 3 μm RP resin interfaced with a high resolution/mass accuracy mass spectrometer as described in our original paper [12]. The number of phosphopeptides identified in the analysis can be increased by maximizing the resolution of the nanoLC separation via longer columns (e.g. 50 cm) and smaller chromatographic particle sizes (e.g. 1.9 μm) using a nanoLC system capable of operating at 800–1000 bar. Depending on the speed of mass spectrometer, time available for LC-MS/MS analysis, complexity of the sample and length of nanoLC column, the samples should be analyzed using an increasing gradient of ACN from 0 to 30–35 % over 1–4 h.

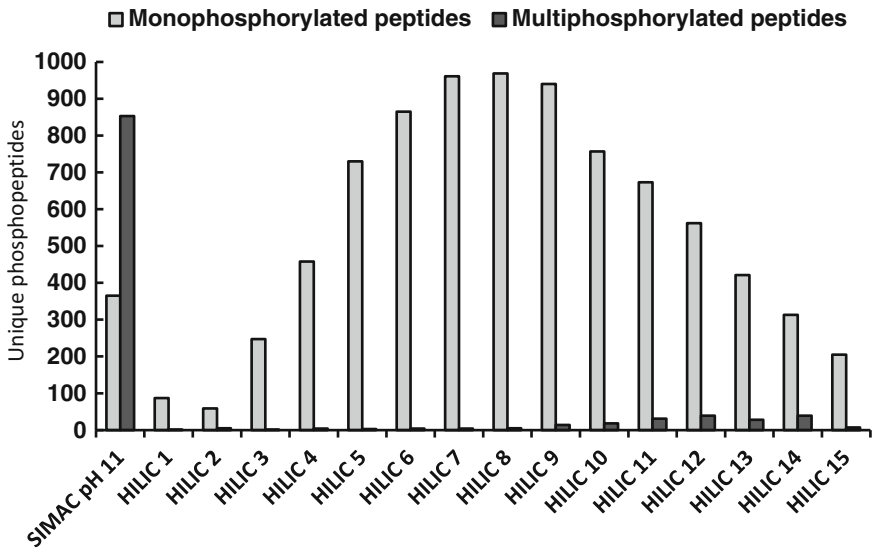

Fig. 3 Anticipated result of the TiSH enrichment-fractionation strategy after LC-MS/MS of the phosphopeptide samples. Overall, the phosphopeptide enrichment specificity is around 95 %. About 70 % of the phosphopeptides identified in the SIMAC pH 11 are multi-phosphorylated, while 77 % of the multi-phosphorylated peptides identified in the experiment originated from this fraction

2. Perform phosphopeptide identification and quantification using an appropriate software tool to generate peak lists from the raw mass spectrometry data and search it against a species-specific protein database using a database search engine (*see* Subheading 2.6, **item 2**). The anticipated results of the enrichment/fractionation strategy after LC-MS/MS analysis and data processing/database searching are shown in Fig. 3. Most of the multi-phosphorylated peptides (77 %) are identified from the SIMAC pH 11 fraction, with only 3 % of the phosphopeptides identified in the HILIC fractions being multi-phosphorylated. The multi-phosphopeptide specificity of the SIMAC pH 11 fraction is 70 %, with most mono-phosphorylated peptides identified in this fraction being very acidic, causing them to be retained to a similar degree as the multi-phosphorylated peptides. Overall, a phosphopeptide enrichment specificity of 95 % can be expected.

4 Notes

1. Triethyl ammonium bicarbonate should be employed instead of the more commonly used ammonium bicarbonate (ABC) as IMAC is sensitive to ammonium ions (Larsen MR, unpublished results), and TEAB is more volatile than ABC.

Furthermore, in contrast to TEAB, ABC will interfere with the amine-reactive chemical labeling utilized for relative quantification via stable isotope labeling.

2. Amino acid composition analysis after protein acid hydrolysis is the most accurate protein concentration measurement method [20], but requires specialized equipment and might not be available to most researchers. An alternative method requiring only a spectrophotometer is the Micro BCA Protein Assay Kit. However, it is important to ensure that the assay used is compatible with the components of the lysis buffer or at least diluted to a compatible concentration prior to the assay. Furthermore, the protein concentration reference standards should be diluted in the lysis buffer to include the same concentration of potential interfering compounds contained in the sample.
3. A recent study [21] has shown TrypZean—a recombinant bovine trypsin expressed in corn—to be a very cost-effective and specific alternative to the commonly used sequencing/mass spectrometry grade trypsin.
4. Degradation over time or batch-to-batch variation of the IMAC resin can change the affinity of the resin towards phosphopeptides. It has been reported that changing the monophosphopeptide Elution Buffer to 20 % ACN, 2 % TFA is required to achieve a specific multiphosphopeptide enrichment (unpublished results, K. Engholm-Keller (Children's Medical Research Institute, Sydney, Australia), A. Liberski and J. Graumann (Weill Cornell Medical College in Qatar, Doha, Qatar)). Optimally the enrichment should be tested using 1, 1.5 and 2 % TFA prior to performing the experiment.
5. Primary amines in proteins (N-terminals and lysines) are prone to carbamylation by isocyanate, which urea is in equilibrium with in aqueous solutions. This process is temperature dependent and while reduction and tryptic digestion is usually performed at 56 °C and 37 °C, respectively, carbamylation is reduced to low levels at room temperature [22] without significantly diminishing the efficiency of the reactions. Alternatively, to avoid carbamylation, 6 M guanidine hydrochloride can be used as a denaturing agent instead of urea [22].
6. When performing quantitative phosphoproteomics, changes in protein level due to protein expression, subcellular location etc. can affect the relative levels of phosphorylated peptides quantified by LC-MS/MS after phosphopeptide enrichment. For samples in which such protein changes occur, phospho-level normalization based on protein level changes is necessary.

7. Keep the total volume of the sample suspended in Loading Buffer at a level proportional to the sample amount (a few mL for 1–2 mg of peptide). Very diluted samples will require longer loading time than specified in this protocol.
8. The centrifugation is performed at 14,000 × *g* as the TiO_2 resin is harder to pellet in aqueous buffers due to the surface tension/viscosity.
9. Contaminating resin in the eluate can reduce phosphopeptide recovery as the phosphopeptides can re-adsorb to the resin when the eluate is acidified prior to SIMAC or RP desalting. To check for potential resin contamination of the eluate, the tube can be centrifuged for 1 min at 14,000 × *g*—if any pellet is visible, transfer the liquid to another tube.
10. The TiO_2 resin from the pre-enrichment steps (Subheading 3.2, **steps 2** and **3**) can be regenerated and reused in the later TiO_2 enrichment steps: Incubate the resin in 50 % ACN, 0.1 % TFA for 10 min after elution, centrifuge the beads at 2000 × *g* for 15 s and remove the supernatant.
11. The sample is dried to remove ammonia, which can interfere with the IMAC-based phosphopeptide enrichment in SIMAC.
12. Ensure that no IMAC resin is present in the flow-through. This can be done by loading the flow-through onto the column again, collecting the flow-through. For the eluate beads can be removed by centrifugation at 14,000 × *g* for 1 min followed by transfer of the supernatant to a new tube if an IMAC resin pellet appears.
13. Performing the 20 % ACN, 1 % TFA elution too fast will lead to mono-phosphorylated peptides in the pH 11 fraction as these species will not have been fully washed off prior to the pH 11 elution—too slowly and multiphosphorylated peptides can be eluted off the column, reducing the recovery in the pH 11 fraction.
14. In our original protocol, the phosphopeptide sample was dissolved sequentially in 10 % TFA, H_2O and finally ACN and not dissolved in DMSO prior to adjustment to HILIC solvent B conditions. However, including DMSO has later been showed to improve the solubility of large phosphopeptides and N-linked glycopeptides (unpublished results, M.R. Larsen (University of Southern Denmark)). Directly trying to dissolve the sample in 90 % ACN, 0.1 % TFA will lead to large losses of phosphopeptides not being solubilized [12].
15. The flow-trough from the column during loading most often does not contain phosphopeptides and can usually be discarded.

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