Version 2

Working

Isolation of phosphoproteins from symbiotic and aposymbiotic Aiptasia anemones for elucidation of the phosphoproteome

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reefgenomics , Aiptasia-Symbiodinium Model System


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ABSTRACT

The analysis of gene expression data via RNA sequencing (RNA-Seq) has become a standard method for model and non-model organisms to identify genes of interest associated with a specific stressor of physiological state. More recently, mass spectrometry (MS)-based proteomics started to become a standard method to assay the phenotype of an organism via means of its expressed proteins. MS has also become the method of choice for the study of protein phosphorylation. Phosphoproteomics targets proteins that are subjected to phosphorylation via means of their serine, threonine, and tyrosine residues. Phosphorylation is a reversible modification that plays a key role in controlling the activity of proteins as well as their function and subcellular localization.

To bring MS-based phosphoproteomics to the emerging coral model system Aiptasia, we developed a protocol for phosphosphproteins extraction and generation of a phosphoproteome assay (spectral) library, a comprehensive non-redundant collection of Aiptasia phosphoproteins. Based on the assay library, we then performed accurate label-free quantification (by DIA/SWATH-MS) of the Aiptasia phosphoproteome. Our method reproducibly identifies and quantifies a broad fraction of the phosphorylated proteome of Aiptasia and is comparable to methods developed for other model systems

TAGS

Aiptasia

Coral

Show tags

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

Materials

1 Laboratory Equipment

- Protein LoBind 1.5 mg tubes (Cat. 022431081; Eppendorf)
- Glass 2 ml Tenbroeck Tissue Grinder (Cat. 357421; Wheaton)
- High-pressure Barocycler 2320EXT connected to mini chiller and FT 500–MS pulse tubes (PBI Pressure Biosciences) or Ultrasonic Processor (Thomas Scientific)
- Ultrasonic cleaner
- Thermomixer
- Incubator at 37°C
- SPE vacuum manifolds for Sep-Pak (Waters Corp.)
- Centrifuges at 20°C (5415R and 5810R; Eppendorf)
- Thermo Savant ISS110 SpeedVac System (Thermo Fisher Scientific)
- NanoDrop Protein Quantification (Thermo Fisher Scientific)
- Surveyor Plus high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific) connected to an XBridge

Peptide BEH C18 column, 4.6 mm ID x 250 mm length, 3.5 µm particle size, 130Å pore size (Cat. 186003570; Waters Corp.)
- UltiMate 3000 RSLCnano UHPLC system (Thermo Fisher Scientific) connected to an Acclaim PepMap 100 C18 column, 75 µm ID x 250 mm length, 3 µm particle size, 100 Å pore size (Cat. 164261; Thermo Fisher Scientific) coupled to an Orbitrap Q-Exactive HF (Thermo Fisher Scientific)
- UltiMate 3000 RSLCnano UHPLC system (Thermo Fisher Scientific) connected to an EASY-Spray column PepMap RSLC C18, 75 µm ID x 500 mm length, 2 µm particle size, 100 Å pore size (Cat. ES803; Thermo Fisher Scientific) coupled to a Fusion Lumos Orbitrap (Thermo Fisher Scientific)

2 Tissue lysis and total protein extraction

Note: all buffers in the protocol are made with sequencing grade chemicals and ultrapure water (Milli-Q). Protein low binding tubes are used throughout the protocol. Tissue lysis is performed on ice with ice-cold lysis buffer (to avoid protein degradation)

- For reference phosphoproteome assay library generation, a pool of 20 small-sized (2 mm basal disc) Aiptasia anemones per experimental condition was used to obtain sufficient proteins. **Note: apo- and symbiotic anemones were processed separately throughout the entire protocol**
- For phosphoproteome quantification a pool of 10 small-sized (2 mm basal disc) Aiptasia anemones constituted each biological replicate
- Lysis buffer: 8 M urea in water supplemented with protease (Cat. 4693159001; Roche Applied Science) and phosphatase (Cat. 04906845001; Roche Applied Science) inhibitors
- 100% methanol
- 100% chloroform
- Pierce Micro BCA Protein Assay kit (Cat. 23235; Thermo Fisher Scientific) or comparable protein quantification assay

3 Filter-Aided Sample Preparation (FASP) (Wiśniewski et al. 2009)

- UA buffer: 8 M urea in 0.1 M Tris/HCl pH 8.5
- UB buffer: 8 M urea in 0.1 M Tris/HCl pH 8.0
- DTT solution: 0.01 M dithiothreitol in UA
- IAA solution: 0.05 M iodoacetamide in UA
- Trypsin/Lys-C mix (Cat. V5071; Promega Corp.), stock 1 µg/µl
- 0.5 M NaCl in water
- ABC buffer: 0.05 M NH_4HCO_3 in water
- 1% trifluoroacetic acid (TFA) in water
- Microcon YM-10 (Cat. 42407; Millipore)

4 Peptide desalting

- Reversed-phase C18 Sep-Pak cartridge (Cat. WAT023590; Waters Corp.)
- Oligo R3 reversed-phase resin (Cat. 1133903; Applied Biosystems)
- 100% acetonitrile (ACN)
- 100% methanol
- Washing buffer: 0.1% TFA in water
- Elution buffer 1: 75% ACN, 0.1% TFA
- Elution buffer 2: 80% ACN, 5% TFA, and 1 M of freshly added glycolic acid **(elute in elution buffer 2 if the following step is phosphopeptide enrichment)**

5 Peptide fractionation and fractions concatenation (Batth and Olsen 2016)

- Buffer A: 5 mM ammonium hydroxide
- Buffer B: 90% ACN, 5 mM ammonium hydroxide
- Buffer C: 100% ACN (for column storage)

6 Phosphopeptide enrichment (Engholm-Keller et al. 2012)

- Titanium dioxide (TiO_2) 5 µm beads (Cat. 502075000; GL Sciences)
- Loading buffer: 80% ACN, 5% TFA, and 1 M of freshly added glycolic acid
- Washing buffer 1: 80% ACN, 1% TFA

- Washing buffer 2: 20% ACN, 0.5% TFA
- Elution buffer: 4% ammonia solution in water, pH 11 (32% ammonia stock solution, Cat. 21192.323, VWR)

7 STAGE tip preparation (Rappsilber, Mann, and Ishihama 2007) and phosphopeptide desalting

- 100% Formic acid (FA)
- 10% Trifluoroacetic acid (TFA)
- Universal 200 µl micropipette tip
- 16 gauge, Kel-F Hub needle (Cat. 90516; Hamilton) or similar
- C18 extraction disks (Cat. 2215; 3M Empore)
- Oligo R3 reversed-phase resin (Cat. 1133903; Applied Biosystems)
- 100% ACN
- 100% Methanol
- Washing buffer: 0.1% TFA in water
- Elution buffer: 75% ACN 0.1% TFA

8 Phosphopeptides preparation for LC-MS

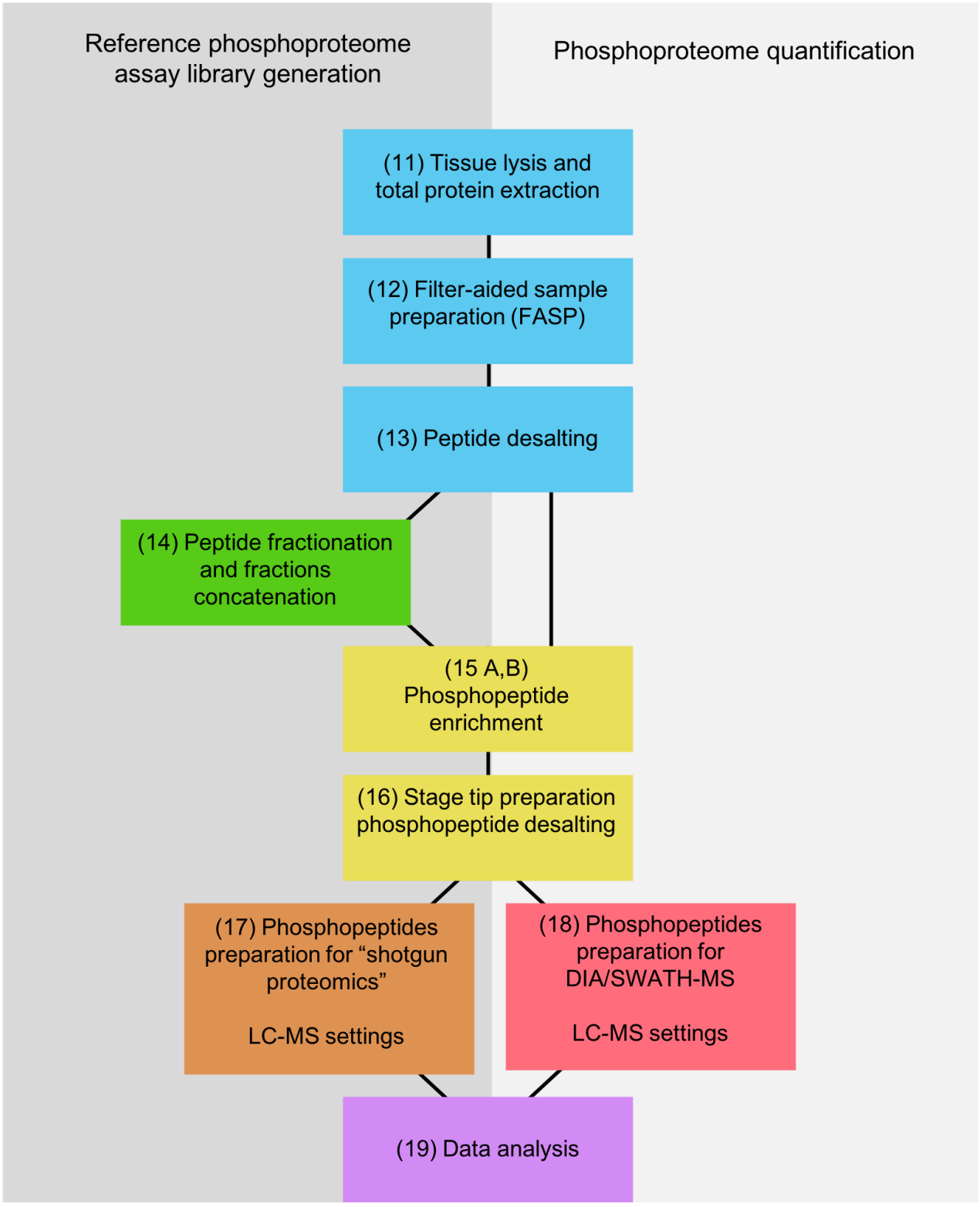
- 0.1% FA, 0.1% ACN in LC-MS grade water (Cat. 1153332500; EMD Millipore)
- Indexed retention time (iRT) standards (Cat. Ki30021; Biognosys)

9 LC-MS buffers

- Buffer A : 0.1% FA
- Buffer B: 0.1% FA in 80% ACN

Methods - Reference phosphoproteome assay library generation / Phosphoproteome quantification

10 Protocol overview



11 **Tissue lysis and total protein extraction**

- Lyse anemones in 1.5 ml of lysis buffer with a Tenbroeck Tissue Grinder. **Note: this step is critical for proteins resuspension and should therefore be thorough and consistent across samples and replicates**
- Transfer the tissue homogenate to FT 500–MS pulse tubes for further membrane disruption at high pressure with a Barocycler set at 3,000 psi (200 bar), 30 cycles (30 min), 4°C. Alternatively, sonicate the homogenate 3 x 10 sec on ice (leave the homogenate for 1 min on ice between sonication rounds to prevent heating up and protein degradation)
- Transfer the homogenate to a 15 ml collection tube and if necessary add water to equalize the final volume across homogenates. This volume (Vol) will be used in all subsequent steps of the methanol-chloroform precipitation:

- Add 4 Vol of methanol to each homogenate. Pulse vortex and quickly spin down
- Add 1 Vol of chloroform to each homogenate. Pulse vortex and quickly spin down
- Add 3 Vol of water to each homogenate. The sample should now turn cloudy and form white precipitate
- Spin down at 4,000 x g for 5 min to obtain 3 distinct layers of separation: a top aqueous layer (containing methanol, water, salts, and ionic compounds), an interphase disc (containing proteins), and a bottom hydrophobic layer (containing chloroform and lipophilic compounds)
- Remove the top aqueous and bottom hydrophobic layers, being careful not to disturb the protein interphase
- Resuspend the protein disc in 1 ml of methanol by pipetting up and down and transfer to a 1.5 ml collection tube (if necessary clean the 15 ml collection tube from protein residues with additional 200 µl of methanol). Spin down at > 20,000 x g for 1 min and carefully discard the supernatant
- Repeat the previous step
- Leave the tubes open in a SPE vacuum chamber for ~ 20 min or under the fume hood until the pellet completely dries. **Note: do not use a SpeedVac at this stage, heating up causes protein degradation and difficulty in resolubilization**
- Resuspend the protein pellet in maximum 250 µl of fresh lysis buffer. Sonicate 3 x 10 sec on ice (leave the homogenate for 1 min on ice between sonication rounds to prevent from heating up). Alternatively, the protein pellet can be stored at -80°C.
- Proceed with protein quantification by micro BCA according to manufacturer's instructions (suggested protein dilution 1:50). **Note: for reference phosphoproteome assay library generation, 1.5 mg of total protein extract (per experimental condition) are used. For phosphoproteome quantification 500-750 µg of total protein extract (per experimental condition) are sufficient**

12 Filter-Aided Sample Preparation (FASP) (Wiśniewski et al. 2009)

- Mix 250 µg of total protein extract with 200 µl of UA buffer in the filter unit (avoid touching the filter) and centrifuge at 14,000 x g for 40 min
- Discard the flow-through from the collection tube
- Add 100 µl of DTT solution and mix in a thermomixer at 600 rpm for 1 min at 37°C, then incubate without mixing for 5 min
- Centrifuge the filter units at 14,000 x g for 30 min
- Add 100 µl of IAA solution and mix in a thermomixer at 600 rpm for 1 min at 37°C, then incubate without mixing for 5 min
- Centrifuge the filter units at 14,000 x g for 30 min
- Discard the flow-through from the collection tube
- Add 100 µl of UB buffer to the filter unit and centrifuge at 14,000 x g for 40 min. Repeat once
- Add 120 µl of ABC buffer containing Trypsin/Lys-C (enzyme to protein ratio 1:50) and mix in a thermomixer at 600 rpm for 1 min at 37°C
- Incubate the units in an incubator at 37°C overnight
- Transfer the filter units to new collection tubes
- Centrifuge the filter units at 14,000 x g for 40 min
- Add 50 µl of 0.5 M NaCl and centrifuge the filter units at 14,000 x g for 20 min
- Add 50 µl of 1% TFA and centrifuge the filter units at 14,000 x g for 20 min
- Acidify the filtrate with TFA (2% by volume) and desalt the filtrate

13 Peptide desalting

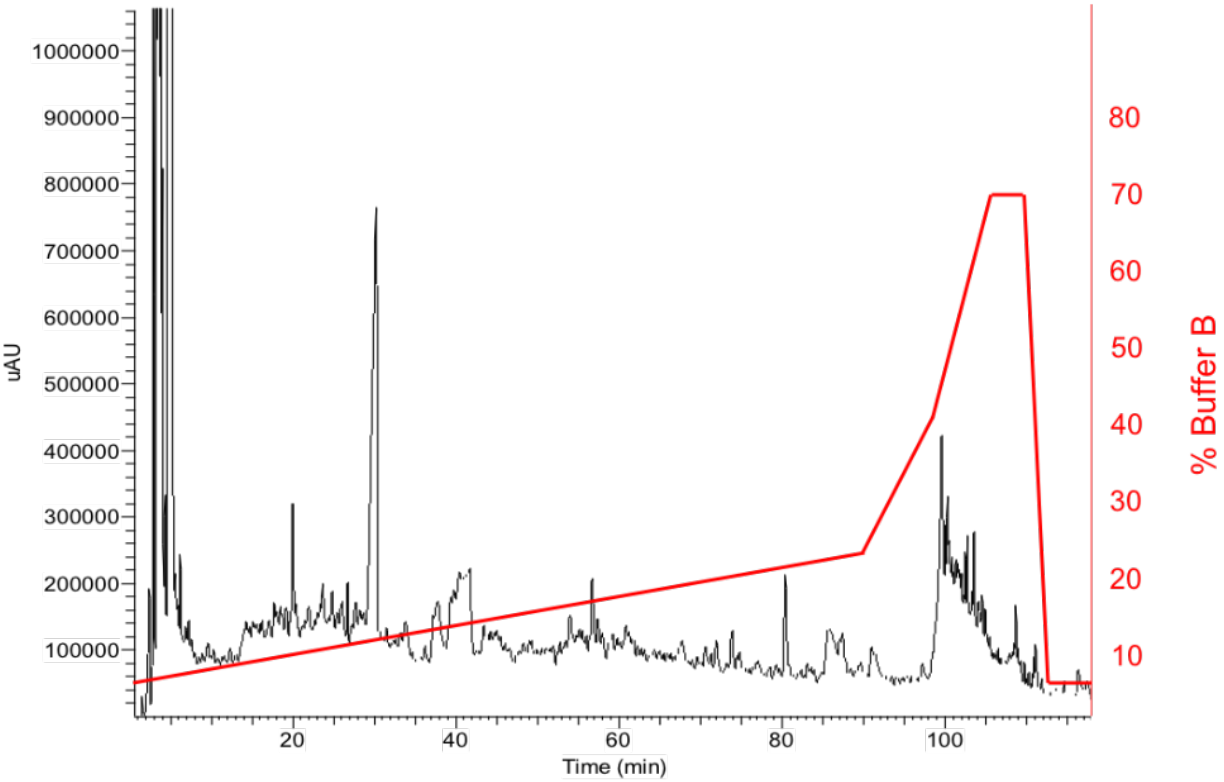
- Resuspend the R3 resin in 200 µl of 100% ACN and load in a C18 Sep-Pak cartridge. **Note: do not let the R3 resin dry at any step**
- Condition the C18 Sep-Pak cartridge containing the R3 resin twice with 100% methanol (1 cartridge volume per wash)
- Wash cartridge and resin twice with washing buffer (1 cartridge volume per wash)
- Load the acidified peptide filtrate into the cartridge (avoid touching the resin)
- Wash twice with washing buffer (1 cartridge volume per wash)
- Elute twice in protein low binding tubes with elution buffer 1 (~300-500 µl per elution). **Note: for phosphoproteome quantification no sample fractionation is performed. Therefore, elute the peptide mixture twice in elution buffer 2 (instead of elution buffer 1) and directly proceed to phosphopeptide enrichment (step 15B) without drying the elute**
- Completely dry the eluted peptides in a SpeedVac system and store the dry peptides at -80°C until fractionation

Methods - Reference phosphoproteome assay library generation (NOTE: This step is not done for phosphoproteome quantification)

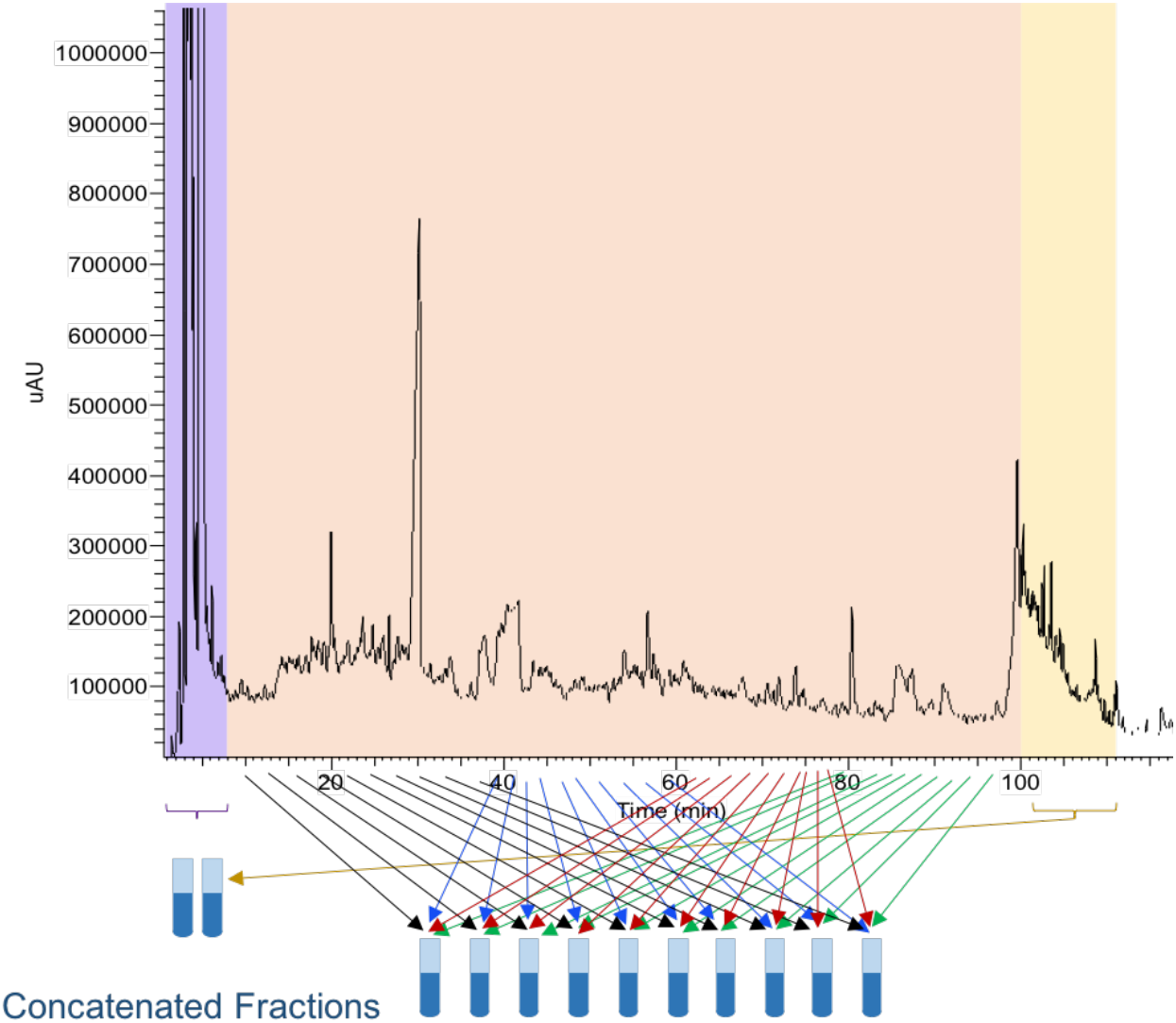
14 Peptide fractionation and fractions concatenation (Batth and Olsen 2016)

- Resuspend the peptide mixture in buffer A. The sample volume should not exceed the maximal volume of LC injection
- Connect the XBridge Peptide BEH C18 column to the HPLC system and equilibrate the column in buffer A
- Design the following 120-min gradient:

1. Step: from 0-5% buffer B to 25% in 60-90 min
2. Step: from 25 to 40% buffer B in 5-10 min
3. Step: ramp up to 60-70% buffer B in 5 min (stop the fraction collection)
4. Step: maintain 70% buffer B for 5 min before ramping down to 0-5%
5. Step: re-equilibrate column at 0-5% B for 5-10 min



- Run the program with the designed gradient (Fig. 2; adapted from Batth and Olsen, 2016) and collect fractions every 60 sec until complete peptide elution (~110 fractions)
- Store fractions at 4°C (up to 2 weeks) or decrease the fractions' volume by SpeedVac prior concatenation
- According to figure 3, concatenate the flow through (purple area) and the last fractions of the separation region (yellow area) in two fractions
- Concatenate the fractions of the separation region (orange area) in 23 fractions (Fig. 3; adapted from Batth and Olsen, 2016). Concatenate fractions in order to mix different parts of the gradient for orthogonal downstream LC-MS analysis at low pH. **Find a template for orthogonal fractions concatenation below (ref library_fraction concatenation.xlsx)**
- Decrease the volume of the concatenated fractions to ~ 50 µl by SpeedVac and store them at 4°C overnight



ref library_fraction concatenation.xlsx

Methods - Reference phosphoproteome assay library generation / Phosphoproteome quantification

15 **Phosphopeptide enrichment (Engholm-Keller et al. 2012)**

Note: the overall principle of phosphopeptide enrichment is the same for phosphoproteome assay library generation (A) and phosphoproteome quantification (B). Nevertheless, for accurate phosphopeptide quantification across samples the incubation with TiO_2 beads was performed twice in section B.

(A) Phosphopeptide enrichment for reference phosphoproteome assay library generation

- Measure the peptide concentration of each fraction at A_{280} by NanoDrop
- Bring the fractions to 1 mL with loading buffer
- Add TiO_2 beads (previously washed once in 100% ACN, once in loading buffer, and then resuspended in loading buffer) to each fraction at a 6:1 beads to peptide ratio (w/w)
- Incubate on a mixer at 1,400 rpm for 10 min
- Centrifuge for 1 min to pellet down the TiO_2 beads
- Carefully remove the supernatant. **Note: the supernatant can be SpeedVac dried and stored at -80°C as it contains TiO_2-unbound and unphosphorylated peptides**
- Resuspend the pelleted beads in 80 µl of loading buffer by pipetting up and down. Transfer the resuspended beads to a new collection tube. **Note: transferring to new tubes between washes reduces contaminations with**

unphosphorylated peptides that adsorb at the tube's plastic surface

- Centrifuge for 15 secs to pellet down the TiO_2 beads. Discard the supernatant without disturbing the beads
- Wash the beads with 80 µl of washing buffer 1, pipet up and down, and incubate on a mixer at 1,400 rpm for 2 min. Centrifuge for 15 sec and carefully discard the supernatant
- Wash the beads with 50 µl of washing buffer 2, pipet up and down, and incubate on a mixer at 1,400 rpm for 2 min. Centrifuge for 15 sec and carefully discard the supernatant
- After careful removal of the washing buffer, place the collection tubes open in a fume hood for 10 min (or in a SpeedVac for 2-5 min) to allow complete evaporation of the solvent
- The dry TiO_2-bound phosphopeptides can be stored at -20°C overnight
- Elute the phosphopeptides from the beads by adding 50 µl of elution buffer and incubate on a mixer at 1,400 rpm for 10 min. Centrifuge for 1 min to pellet down the beads and carefully transfer the phosphopeptides-containing supernatant to a new tube. Repeat the elution once. **Note: make sure the supernatant does not contain TiO_2 beads, which are a source of contamination in the LC-MS**

(B) Phosphopeptide enrichment for phosphoproteome quantification

- Measure the peptide concentration of each fraction at A_{280} by NanoDrop
- Bring the fractions to 1 mL with loading buffer
- Prepare the needed amount of TiO_2 beads for two incubations. First incubation 6:1 beads to peptides ratio (w/w), second incubation 3:1 beads to peptides ratio (w/w)
- Wash the beads once in 100% ACN, once in loading buffer, and then resuspend them in loading buffer
- Add the TiO_2 beads to the peptide mixture at a 6:1 ratio and incubate on a mixer at 1,400 rpm for 10 min (first incubation)
- Centrifuge for 1 min to pellet down the TiO_2 beads
- Transfer the supernatant to a new collection tube and reincubate it with TiO_2 beads at a 3:1 ratio on a mixer at 1,400 rpm for 10 min (second incubation)
- Centrifuge for 1 min to pellet down the TiO_2 beads
- Remove the supernatant. **Note: the supernatant can be SpeedVac dried and stored at -80°C as it contains TiO_2-unbound and unphosphorylated peptides**
- Resuspend the pelleted beads of each tube in 80 µl of loading buffer by pipetting up and down. Combine the resuspended beads in a new collection tube. **Note: transferring to new tubes between washes reduces contaminations with unphosphorylated peptides that adsorb at the tube's plastic surface**
- Centrifuge the combined beads for 15 sec and carefully discard the supernatant
- Wash the beads with 80 µl of washing buffer 1, pipet up and down, and incubate on a mixer at 1,400 rpm for 2 min. Centrifuge for 15 sec and carefully discard the supernatant
- Wash the beads with 50 µl of washing buffer 2, pipet up and down, and incubate on a mixer at 1,400 rpm for 2 min. Centrifuge for 15 sec and carefully discard the supernatant
- After careful removal of the washing buffer, place the collection tubes open in the fume hood for 10 min (or in a SpeedVac for 2-5 min) to allow complete evaporation of the solvent
- The dry TiO_2-bound phosphopeptides can be stored at -20°C overnight
- Elute the phosphopeptides from the beads by adding 50 µl of elution buffer and incubate on a mixer at 1,400 rpm for 10 min. Centrifuge for 1 min to pellet down the beads and carefully transfer the phosphopetides-containing supernatant to a new tube. Repeat the elution
- **Note: make sure the supernatant does not contain TiO_2 beads, which are a source of contamination in the LC-MS**

16 **STAGE tip preparation (Rappsilber, Mann, and Ishihama 2007) and phosphopeptide desalting**

- With a 16 gauge needle punch out holes from the C18 extraction disk
- Place the extracted C18 material inside a 200 µl tip and push it down to the extremity until it fits properly (see figure 1 from Rappsilber, Mann, and Ishihama 2007)
- Pipet ~ 2 cm of R3 resin (resuspended in 100% ACN) on top of the C18 material
- Condition and wash the STAGE tip with R3 resin as described in step 12 “Peptide desalting”:
- Condition twice in 100% methanol (1 tip volume per wash)
- Wash twice with washing buffer (1 tip volume per wash). **Note: Washes can be performed faster by applying a gentle pressure on the STAGE tip with a micropipette (avoid sudden pressure removal that destroys the R3 matrix) or by slow centrifugation**
- Acidify the phosphopeptides mixture with formic acid (14% by volume) and TFA (4% by volume)
- Load the acidified phosphopeptides mixture on the STAGE tip (avoid touching the resin)
- Wash twice with washing buffer (1 tip volume per wash)

- Elute the phosphopeptides twice with elution buffer in protein low binding tubes (50 µl per elution)

Methods - MS acquisition for reference phosphoproteome assay library generation

17 Phosphopeptides preparation for 'shotgun proteomics'

- Completely dry the eluted phosphopeptides by SpeedVac and then redissolve in 0.1% FA, 0.1% ACN, LC-MS grade water solution
- Sonicate for 2 min in an ultrasonic cleaner to better redissolve the phosphopeptides
- Quantify phosphopeptides at A_{280} by NanoDrop
- Add iRT standards to the ready to inject phosphopeptide mixture at a 1:10 ratio (v/w)

LC-MS settings (on an Orbitrap Q-Exactive coupled to a nanoLC system)

- Design a nLC method for phosphopetides separation using an Acclaim PepMap 100 C18 column (flow rate 300 nl/min) with the following 75-min gradient:

1. Step: from 5% to 40% buffer B in 55 min
2. Step: ramp up to 90% buffer B in 5 min
3. Step: maintain 90% buffer B for 5 min
4. Step: ramp down to 2% buffer B and maintain for 10 min for column conditioning

- Introduce the sample into the MS through a Nonospray Flex ion source with an electrospary potential of 1.9 kV and ion transfer tube temperature at 275 °C
- Acquire a full MS scan (350-1400 m/z range) at a resolution of 60,000 (at 200 m/z) in profile mode, maximum ion accumulation time of 100 ms and target value of $3x\,e^6$. Activate charge state screening for precursor ion
- Select the ten most intense ions above a 2e4 threshold and carrying multiple charges for fragmentation using higher energy collision dissociation (HCD). Set dd-ms2 resolution at 15,000 and dynamic exclusion for HCD fragmentation at 20 sec
- Other settings for fragment ions include maximum ion accumulation time of 100 ms, target value of $1xe^5$, normalized collision energy at 28%, and isolation width of 1.8
- Run samples with this method

Methods - MS acquisition for phosphoproteome quantification

18 Phosphopeptides preparation for DIA/SWATH-MS

- Completely dry the eluted phosphopeptides by SpeedVac and then redissolve in 0.1% FA, 0.1% ACN, LC-MS grade water solution
- Sonicate for 2 min in an ultrasonic cleaner to better redissolve the phosphopeptides
- Quantify phosphopeptides at A_{280} by NanoDrop and **normalize the phosphopeptides concentrations across samples**
- Add iRT standards to the ready to inject phosphopeptide mixture at a **3:10 ratio** (v/w) for multimplexed analysis in data-independent acquisition (DIA)

LC-MS settings (on an Orbitrap Fusion Lumos coupled to a nanoLC system)

- Design a nLC method for phosphopetides separation using an EASY-Spray column (flow rate 300 nl/min) with the same 75-min gradient used for assay library generation:

1. Step: from 5% to 40% buffer B in 55 min
2. Step: ramp up to 90% buffer B in 5 min
3. Step: maintain 90% buffer B for 5 min
4. Step: ramp down to 2% buffer B and maintain for 10 min for column conditioning

- Introduce the sample into the MS through a EASY-Spray ion source with an electrospary potential of 1.9 kV and ion transfer tube temperature at 275 °C
- Acquire a full MS scan (400-1200 m/z range) at a resolution of 60,000 in profile mode, maximum injection time of 20 ms, and target value of $5x\,e^5$
- Set the instrument in DIA mode and optimize the quadrupole settings for 32 precursor ion selection windows (each 25 Da wide) over the precursor mass range
- Set fragmentation by higher energy collision dissociation (HCD) at 30%, dd-ms2 resolution at 30,000, maximum injection time of

50 ms, and target value of $2xe^5$

- Run samples with this method

Methods - Data analysis

19 A variety of open source (TPP, OpenSWATH, DIA-Umpire) as well as commercially available software (Sprectronaut Pulsar X) can be used for assay (spectral) library generation and quantification of SWATH-MS data. It is recommended to use peptide search engines that perform analysis of post-translational modifications (PTMs). When analyzing phosphorylation, it is important to set it as a variable modification on serine, threonine, and tyrosine residues (STY+80). Refer to Schubert et al. 2015, Tsou et. 2015, and Rosenberger et al. 2017 for the open source pipelines or to Biognosys for the Sprectronaut Pulsar X integrated workflow