Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips

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Mass spectrometry (MS)-based proteomics measures peptides derived from proteins by proteolytic cleavage. Before performing the analysis by matrix-assisted laser desorption/ionization–tandem mass spectrometry (MALDI–MS/MS), nanoelectrospray–MS/MS (NanoES-MS/MS) or liquid chromatography–MS/MS (LC–MS/MS), the peptide mixtures need to be cleaned, concentrated and often selectively enriched or pre-fractionated, for which we employ simple, self-made and extremely economical stop-and-go-extraction tips (StageTips). StageTips are ordinary pipette tips containing very small disks made of beads with reversed phase, cation-exchange or anion-exchange surfaces embedded in a Teflon mesh. The fixed nature of the beads allows flexible combination of disks with different surfaces to obtain multi-functional tips. Disks containing different surface functionalities and loose beads such as titania and zirconia for phosphopeptide enrichment can be combined. Incorporation into an automated workflow has also been demonstrated. Desalting and concentration takes approximately 5 min while fractionation or enrichment takes approximately 30 min.

INTRODUCTION

The mass spectrometric (MS) analysis of proteins has become a major technology in the investigation and understanding of biological processes[1,2]. In the vast majority of studies, proteins are not analyzed intact but after treatment with a protease, usually trypsin. The interface between protein digestion and MS analysis is crucial for the overall quality and sensitivity of the analysis and the robustness of the proteomic workflow. Peptides can be concentrated and cleaned in a single step through their binding to a small quantity of reversed-phase material and eluted in organic solution ready for MS analysis[3–7]. The technical details of retaining the beads in a pipette tip, which usually acts as a disposable container, differ from protocol to protocol and are mostly laborious and cumbersome. Commercial tips such as the ZipTips (Millipore), in addition to their comparably high costs, have limitations for high-sensitivity work owing to recovery problems and large elution volumes[8,9]. Furthermore, their capacity is limited and cannot be adjusted according to the need, and their functionality cannot be expanded as is the case for the stop-and-go-extraction tips (StageTips), which are the subject of this protocol. StageTips combine the flexibility and low price of self-made tools with the ease-of-use, recovery, reproducibility and robustness typical for mass-manufactured devices[7].

StageTips are made by placing a small portion of Empore material (3M) in an ordinary pipette tip (**Fig. 1**). Empore disks have chromatographic beads immobilized in a Teflon meshwork. From the commercially available large sheet, a small disk is stamped out using a blunt-ended syringe needle. The portion size is determined by the inner diameter of the needle, and can thus be adapted to the size needed. In this simple process, tips with the desired capacity and functionality can be manufactured in every laboratory[7]. The Empore material itself has a number of analytical advantages over the loose beads used in previous approaches, aside from its simpler handling. The beads are held in place ensuring equal distribution of the analyte and preventing the formation of primary-flow channels (**Fig. 1a**). As a consequence, peptides are retained and eluted with high efficiency. This allows for fast flow, high capacity and excellent recovery rates. The high reproducibility of StageTips was demonstrated in a recent application to serum analysis[11]. In our experience, one of the major advantages of using StageTips is that it makes the proteomic work flow much more robust. The mere fact of cleaning the peptides on a StageTip ensures stability of the liquid chromatography–tandem mass spectrometry (LC–MS/MS) system by removing impurities such as gel pieces and aggregates, thus preventing those from clogging the column.

As the beads are fixed, materials with different properties can easily be combined to obtain multi-functional tips[10]. Currently, Empore disks can be obtained containing reversed phase C_{18}, C_8, activated carbon and poly(styrene-divinylbenzene) copolymer (SDB), anion and cation exchange, as well as chelating beads (**Table 1**). Disks of different functionality can be combined in one tip while combination of loose beads would suffer from mixing at the interface.

This modular principle was used first for strong cation exchange (SCX) fractionation using three disks combined in a C_{18}-SCX-C_{18} StageTip (ref. 10). The first C_{18} disks allows desalting; fractionation is achieved on the SCX disk, while the final C_{18} disk again serves to desalt and directly couples the fractionation with subsequent MS analysis. This system can be expanded to achieve 2D fractionation by using a C_{18}-SCX StageTip with a separate C_{18} StageTip for desalting (**Fig. 2**). The first C_{18} disk can be used for organic solvent-based fractionation (Step C) as this does not conflict with the retention of peptides on SCX (Step D). Similarly, one can devise SDB-SAX-SDB or SDB-SAX StageTips. Note that SDB tolerates buffers with high pH whereas C_{18} material does not. The combination of functionalities in a single multi-StageTip was employed for the simple pre-fractionation of cell lysates before LC–MS/MS analysis[12]. This 'inverted' SCX-C_{18} approach was

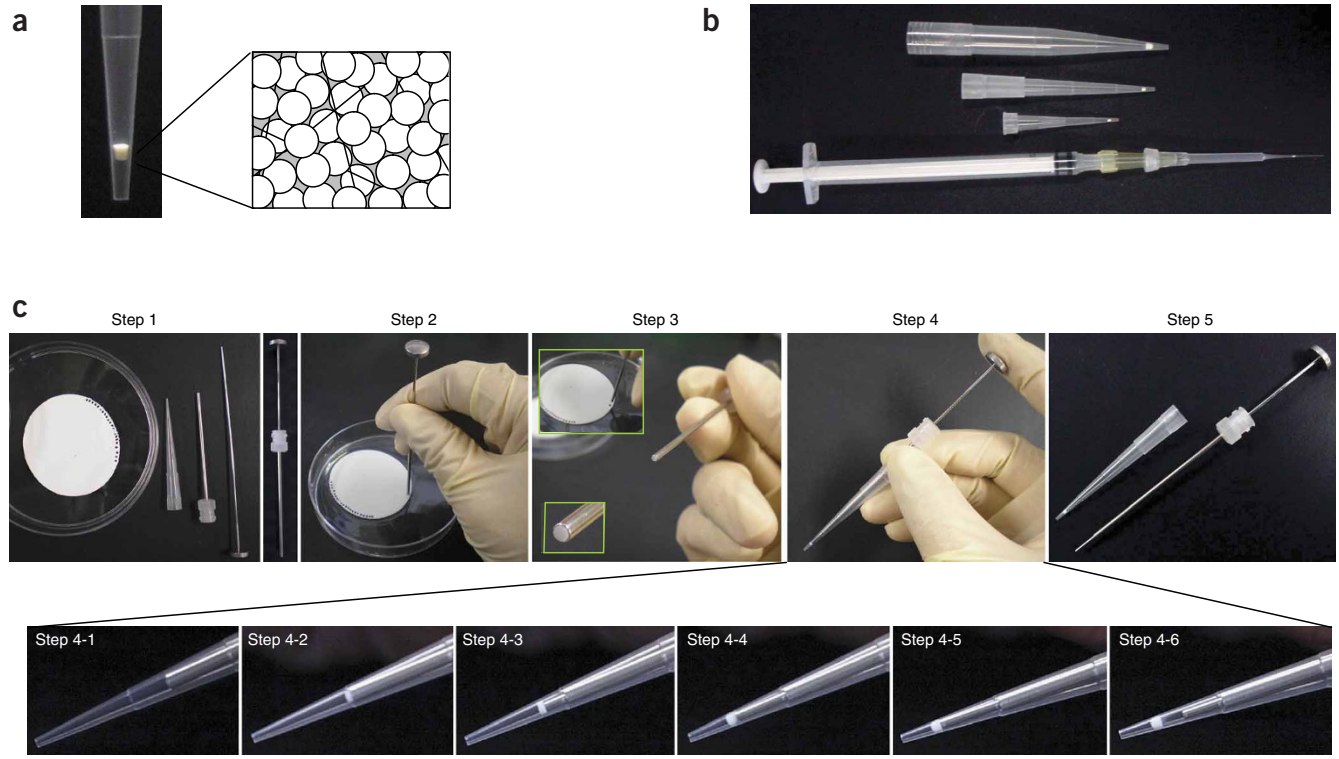

Figure 1 | Schematic representation and production of stop-and-go-extraction tips (StageTips): panel (**a**) the chromatographic beads for micro-purification are embedded in a Teflon mesh and are held in place solely by the tapering of the vessel. Liquid is added to the reservoir, that is, always from the top, and passes the column under pressure from an air-filled plastic syringe. The analyte is retained and can be washed and eluted in an analogous manner using appropriate solvent conditions. (**b**) Examples of StageTips in different sizes (1,000 µl, 200 µl, gel loader, 10-µl tips) containing disks of the appropriate diameters. The gel loader tip is mounted onto a 1-ml syringe used as pressure device. The adapter needed to fit the gel loader tip onto the syringe was made by trimming a 200-µl tip at the front end so that it matches onto the syringe and into the Gel loader tip (described in EQUIPMENT SETUP; step (1D)). Each tip size requires different adapters that can be made from other tips. (**c**) Step-by-step guide on the manufacture of a single-disk StageTip. Additional disks can just be staged onto the first disk.

expanded by further fractionating the fractions obtained by C_{18}-HPLC using SCX-StageTips before matrix-assisted laser desorption (MALDI) and nanoelectrospray (nanoES)-MS analysis[13]. C_{18}-HPLC/SCX-StageTip reduced total analysis time without affecting the number of identified proteins when compared with HPLC-based SCX-C_{18} 2D chromatography. StageTips are furthermore emerging as a central tool for the enrichment of phosphopeptides. Phosphopeptides using immobilized metal affinity materials or metal oxides chromatography (MOC), titania (titanium dioxide) and zirconia (zirconium dioxide) are packed on reversed-phase disks in pipette tips, allowing fast enrichment protocols of well below 1 h[14–16].

We here present a series of steps encompassing the production of StageTips (**Box 1**), the use of reversed-phase StageTips for desalting, clean-up and concentration of peptide mixtures

TABLE 1 | Examples of materials used in stop-and-go-extraction tips.

Empore material	Application in StageTips
C_{18}-silica	Desalting of peptides; fractionation of peptides at acidic and neutral pH
C_8-silica	Desalting of large peptides and proteins; usage as frit to retain beads in a tip
Activated carbon	No application described so far
Poly(styrene-divinylbenzene) copolymer (SDB)	Fractionation of peptides at basic pH
Anion exchange (SAX)	Fractionation of peptides by salt or pH steps
Cation exchange (SCX)	Fractionation of peptides by salt or pH steps
Chelating beads	No application described so far
Combinations by stacking of disks	
C_{18}-SCX	Desalting combined with fractionation of peptides by salt or pH steps
C_{18}-SCX-C_{18}	Desalting combined with fractionation of peptides by salt or pH steps followed by desalting again
SDB-SAX	Desalting combined with fractionation of peptides by salt or pH steps
Combination of beads and disks	
Metal oxide-C_8	Enrichment of phosphopeptides

Abbreviations: StageTips, stop-and-go-extraction tips; SAX, strong anion exchange; SCX, strong cation exchange.

(Step 1A), fractionation of peptides via reversed-phase chromatography at different pH values on StageTips (Step 1B), fractionation of peptides using multi-StageTips containing mixed functionalities including ion exchange chromatography (Step 1C) and, finally, the enrichment of phosphopeptides (Step 1D). If the aim is to obtain a small number of fractions from a complex peptide mixture, any of the alternatives provided in Steps 1B and C can be chosen. The extensive set of alternatives provided by these procedures also represents a resource for multidimensional approaches or special applications. For example, one can easily increase the number of fractions from 3–4 to 12–16 or even higher by combining two or three of the buffer-StageTip combinations provided.

StageTips containing different disks or in combination with loose beads offer a rich repertoire of chromatographic functionalities. Individual functionalities can be combined similarly to LEGO bricks to suit a wide spectrum of analytical tasks. The protocols presented here cover the currently most useful applications. We hope they will also offer insight into the construction of StageTips from building blocks to tailor them to other applications that may become important in future research.

StageTips are useful low-tech tools for pre-fractionating medium complex peptide mixtures to increase analysis depth or enrich for peptides of interest. Note that any large-scale or exhaustive analysis should make use of HPLC for pre-fractionation. The separation efficiency in StageTip is not as good as on an HPLC column, especially when a gradient is used for elution. It is also difficult to increase the number of fractions taken from a StageTip while a very large number of fractions can be collected from an HPLC run. However, the simplicity and low costs of StageTips make them ideal where an HPLC is not available or the required time and effort for an HPLC-based fractionation is not scaling to the needs of the analysis.

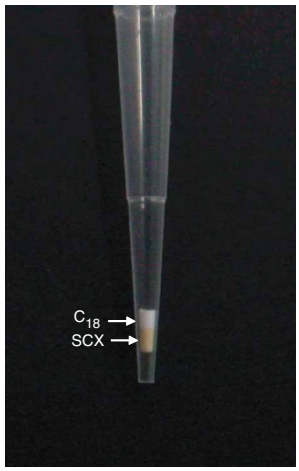

Figure 2 | Example of a multi-stop-and-go-extraction tip (StageTip) containing a C_{18} disk stacked onto a strong cation exchange (SCX) disk. The beads are held in place by a Teflon mesh and hence beads of different functionalities do not mix. It is sufficient if one of the functionalities is a disk, that is, beads and disks can be combined. However, two segments of loose beads cannot be combined without mixing.

MATERIALS

REAGENTS

- Methanol
- Acetonitrile (ACN)
- Acetic acid (AcOH)
- Ammonium hydroxide (NH_4OH)
- Trifluoroacetic acid (TFA)
- Ammonium hydrogencarbonate (NH_4HCO_3)
- Ammonium acetate (NH_4AcO)
- Dibutylammonium acetate (DBAA)
- Formic acid (optional)
- Lactic acid
- Beta Hydroxypropanoic acid (HPA)
- Nonafluorovaleric acid ▲ **CRITICAL** All reagents should be of the highest purity from Sigma except lactic acid (ACS grade, ca 90%) and HPA (from TCI, Tokyo, Japan). See **Tables 2** and **3** for buffers and stationary phase material.

EQUIPMENT

- Pipette tips of the desired size (10 µl, 200 µl, 1,000 µl, or gel loader tips for coupling StageTip with MALDI or nanoelectrospray)
- Tip box ▲ **CRITICAL** The tip box needs to be stable. We found boxes from Greiner suitable for centrifugation up to 2,000 r.p.m. (e.g. Centrifuge 5810 with rotor A-2-DWP, Eppendorf) and boxes from Eppendorf and Gilson inadequate for this purpose.
- Pressure device (Step 1A)
- One C_{18} StageTip (preparation described in EQUIPMENT SETUP, **Box 1**) (16-gauge, P200 pipette tip) (Step 1A)
- One 96-well plate for LC–MS/MS injection (Step 1A)
- Pressure device (preparation described in EQUIPMENT SETUP) (Step 1B)
- One C_{18} StageTip or one SDB StageTip (16-gauge, P200 pipette tip) (Step 1B)
- Two acidic conditioned C_{18} StageTips (16-gauge, P200 pipette tip) containing 60 µl 0.5% AcOH (Step 1B)
- One 96-well plate for LC–MS/MS injection (Step 1B)
- Pressure device (Step 1C)
- One C_{18}-SCX StageTip or one SDB-SAX StageTip (16-gauge, P200 pipette tip) (Step 1C)
- Four conditioned C_{18} StageTips (16-gauge, P200 pipette tip) containing 60 µl buffer A (Step 1C)
- One 96-well plate for LC–MS/MS injection (Step 1C)
- Pressure device (Step 1D)
- One C_8 StageTip (16-gauge, P200 pipette tip) (Step 1D)
- One desalting cartridge [e.g., 3M Empore C_{18} extraction disk cartridge, cat. no. 4215(HD)] (Step 1D) or a C_{18} StageTip
- One 96-well plate for LC–MS/MS injection (Step 1D)
- Single disk StageTips (**Box 1**)
- Concentrator/SpeedVac
- QSTAR pulsar (AB-Sciex)
- MASCOT database searching engine (Matrix Science)
- LTQ-orbitrap (ThermoFisher)

EQUIPMENT SETUP

Cutter (StageTip manufacture) Take a blunt-ended needle and a plunger or any rod that ideally just fits into the needle. For example, the following parts fit together snugly and can be ordered from Hamilton (**Table 4**).

Preparation of multi-disk StageTips Prepare a single-disk StageTip with the desired functionality as shown in **Box 1**. Add another disk onto the disk in the single-disk StageTip according to the procedure for single-disk StageTips (see **Fig. 2**).

Preparation of the integrated bead disk, MOC StageTip Add suspension of MOC beads to a C_8-StageTip such that it will result in column length of a few millimeter (3-mg titania beads, for instance). ▲ **CRITICAL** The capacity varies with the sample and needs to be established for your application. As a guide, StageTips with the mentioned amount of phospho-affinity beads can be used for a digest of 150 µg human cell lysate. Place a second C_8 disk on top of the beads if tips are shipped to different places to prevent the loss or unpacking of beads. Otherwise, the second disk is not necessary. However, it is necessary to desalt the samples before phosphopeptide enrichment.

Preparation of a pressure device Take a 1-mL syringe (or larger). Force a pipette tip onto its front end. Adjust the length of the pipette tip by trimming it at the front with a pair of scissors. The outer diameter of the trimmed pipette tip should fit into the prepared StageTip. See **Figure 1d** for an example where a gel loader tip is mounted onto a syringe with the help of an adapter. If the correct

TABLE 2 | Buffers needed for the different procedures.

Step 1A	**C_{18} (desalting)**			
Buffer A	0.5% acetic acid (AcOH)[a]			
Buffer B	0.5% AcOH, 80% ACN			
Step 1B	**C_{18} (acidic pH)**	**C_{18} (neutral pH)**	**SDB (basic pH)**	
pH-Buffer 1	0.5% AcOH	0.1% dibutylammonium acetate (DBAA), pH 7.5	0.1% ammonium hydroxide (NH_4OH)	
pH-Buffer 2	0.5% AcOH, **10% ACN**	0.1% DBAA, **10% ACN**	0.1% NH_4OH, **10% ACN**	
pH-Buffer 3	0.5% AcOH, **20% ACN**	0.1% DBAA, **20% ACN**	0.1% NH_4OH, **20% ACN**	
pH-Buffer 4	0.5% AcOH, **80% ACN**	0.1% DBAA, **80% ACN**	0.1% NH_4OH, **80% ACN**	
Step 1C	**C_{18}-SCX (salt)**	**SDB-SAX (salt)**	**C_{18}-SCX (pH)**	**SDB-SAX (pH)**
ReX-Buffer A	0.5% AcOH			
ReX-Buffer B	0.5% AcOH, 80% ACN			
ReX-Buffer 1	0.5% AcOH	0.1% NH_4OH	0.5% AcOH	0.1% NH_4OH
ReX-Buffer 2	0.5% AcOH, 80% ACN	0.1% NH_4OH, 80% ACN	0.5% AcOH, 80% ACN	0.1% NH_4OH, 80% ACN
ReX-Buffer 3	**20 mM NH_4AcO**, 0.5% AcOH, 20% ACN	**20 mM NH_4AcO**, 0.1% NH_4OH, 20% ACN	**50 mM NH_4AcO**, 20% ACN	**50 mM** ammonium hydrogen-carbonate (**NH_4HCO_3**), 20% ACN
ReX-Buffer 4	**50 mM NH_4AcO**, 0.5% AcOH, 20% ACN	**50 mM NH_4AcO**, 0.1% NH_4OH, 20% ACN	**50 mM NH_4HCO_3**, 20% ACN	**50 mM NH_4AcO**, 20% ACN
ReX-Buffer 5	**100 mM NH_4AcO**, 0.5% AcOH, 20% ACN	**100 mM NH_4AcO**, 0.1% NH_4OH, 20% ACN	**0.1% NH_4OH**, 20% ACN	**0.1% TFA**, 20% ACN
ReX-Buffer 6	**500 mM NH_4AcO**, 0.5% AcOH, 20% ACN	**500 mM NH_4AcO**, 0.1% NH_4OH, 20% ACN	—	—
Step 1D	**Titania**	**Zirconia**		
MOC-Buffer A[b]	0.1% TFA			
MOC-Buffer B	80% ACN, 0.1% TFA			
MOC-Buffer C	300 mg ml^{-1} lactic acid in MOC-Buffer B	100 mg ml^{-1} β-hydroxy-propanoic acid in MOC-Buffer B		
MOC-Buffer D	0.5% ammonium hydroxide			

[a]Note that 0.5% AcOH can be substituted in buffers A and B by other acids such as 0.5% formic acid or 0.1% trifluoroacetic acid (TFA) and [b]*Abbreviation:* MOC, metal oxide chromatography

TABLE 3 | Disks and beads as stationary phase for stop-and-go-extraction tip production.

Sorbent	**Name**	**Part number**	**Vendor**	**Comments**
Octadecyl (C_{18})-bonded silica	C_{18}	2215	3M, St.Paul, MN	Empore extraction disk
Octyl (C_8)-bonded silica	C_8	2214	3M	Empore extraction disk
Poly(styrene-divinylbenzene) (SDB) copolymer	SDB	2240	3M	Empore extraction disk
SDB copolymer modified with sulfonic acid	Strong cation exchange (SCX)	2251	3M	Empore extraction disk
SDB copolymer modified with quaternary ammonium groups	SAX	2252	3M	Empore extraction disk
Titania	Titansphere	1352B500/5020-75010	GL Sciences	10-μm bead diameter
Zirconia	Zirchrom-phase	ZR02-BULK-10 μm	Zirchrom separation	10-μm bead diameter

TABLE 4 | Equipment to use as plunger in the cutter device (stop-and-go-extraction tip manufacture).

Type of tip	**Needle part number**	**Needle name**	**Plunger part number**	**Plunger name**
Gel loader tip	90134	Kel-F Hub (KF), point style 3, gauge 22	—	Fused silica capillary (OD 360 μm)
10-μl pipette tip	90520	Kel-F Hub (KF), point style 3, gauge 20	13205	Plunger assembly N, RN, LT, LTN for model 1701 (10 μl)
200-μl pipette tip	90516	Kel-F hub (KF), point style 3, gauge 16	1122-01	Plunger assembly N, RN, LT, LTN for model 1702 (25 μl)
1-ml pipette tip	91010	Metal hub, point style 3, gauge 10	1162-02	Plunger assembly N, RN, LT, LTN for model 1710 (100 μl)

diameter cannot be achieved with one adapter, as is the case for 1,000 µl tips, multiple adapters can be combined. ▲ CRITICAL For safety it is recommended to always hold the StageTip when pressure is applied.

Preparation of a spin adaptor Take a 1.5-ml tube with lid (Eppendorf or equivalent). Use a sharp end of a pair of tweezers or scissors to puncture a hole at the centre of lid. The size of the hole should fit the pipette tip type used for the StageTip. Insert the StageTip into the hole and place it in the table centrifuge. ▲ CRITICAL It is important to confirm that the height of StageTips is short enough to close the lid of the centrifuge.

Preparation of a spin adaptor (for use with up to 4 × 96 samples) Take a tip box matching the type of pipette tip used for the StageTip. Place StageTips inside. Prepare a counter balance using an identical tip box with the same number of tips in the same positions as StageTips used. This is important for approximating the weight distribution. Then equalize the weight of the second box to that of the first on a balance by adding water to the box. ▲ CRITICAL The weight equalization has to be done anew each time when liquids are being added to the StageTips. ▲ CRITICAL When working with large numbers of StageTips, the challenge is to get the timing of the centrifugation step right so that none of the tips actually dries out. It might be better to cut the time short and use the hand-held pressure device for individual tips that have liquid left in them. Working with commercial StageTips is at this point advantageous owing to their more reproducible back pressure as a result of more controlled automated production. Alternatively, a commercially available multi-well plate vacuum manifold can be adapted for use with 96 tips, allowing to visually follow the progress of buffer running through the tips[10].

PROCEDURE

Sample pre-treatment by StageTips

1| The flow chart (**Fig. 3**) gives an overview for the different options. Conditioning, sample loading and elution are performed slightly differently depending on the stationary phase(s) in the StageTip. Use options A, B or C for clean-up and concentration using C_{18}-StageTips; StageTips with reversed-phase material for pre-fractionation in different pH modes; and Multi-StageTips (using reversed phase-ion exchange (Re-X) StageTip for fractionation and a separate C_{18} StageTip for desalting), respectively. If you wish to perform phosphopeptide enrichment, follow the steps in option D. For this you will require a StageTip containing a C_8 disk. It is worth keeping some general remarks in mind when working with StageTips (see **Box 2**).

(A) C_{18}-StageTips for clean-up and concentration

(i) *Conditioning.* Wet the disks by passing 20 µl methanol through the StageTip.

(ii) Add 20 µl buffer B to the StageTip just before the last remainder of methanol has left the tip, and pass buffer B through the tip.

BOX 1 | PREPARATION OF SINGLE-DISK STOP-AND-GO-EXTRACTION TIPS

(i) Place an Empore extraction disk of the desired functionality on a clean surface in a dust-free environment. Use a pair of flat tweezers (or wear gloves) if handling the disk. A Petri dish can be used as a container to store the disk in use for stop-and-go-extraction tip (StageTip) production, as well as serve as a clean underground for the following steps (**Fig. 1**: Step 1).

(ii) Gently press the cutter into the disk and ensure the material is penetrated at the entire circumference (**Fig. 1b**). When lifting the cutter, the small piece of disk remains inside the cutter (**Fig. 1**: Steps 2 and 3).

? TROUBLESHOOTING

Problem: Excised disk does not remain in the cutter.

Possible Reason: The disk was not properly cut.

Solution 1: The process resembles very much using a biscuit cutter on load. After a few trials it but can be performed in seconds. You may want to ensure that the disk is completely excised by rolling the cutter at an angle to the surface a couple of times around its entire rim. Then hold the cutter perpendicular to the surface and role it a bit between your fingers.

Solution 2: Methanol can be used to wet the disk material before cutting. This can facilitate the cutting and positioning of the disk

(iii) Place the cutter inside a pipette tip and release the disk using the plunger (**Fig. 1**: Step 4).

▲ CRITICAL STEP: The cutter should be long enough to reach all the way down into the pipette tip as close as possible to the final position of the disk and the plunger has to fit snugly into the cutter. If these two conditions are met, the disk automatically ends up in the right position.

? TROUBLESHOOTING

Problem: Disk placed sideways in the StageTip.

Possible reason: The cutter is too short or the plunger is of too small diameter.

Solution: Get appropriate equipment.

Note: If the disk ends up positioned sideways, discard the StageTip. As the disk is soft it is possible to use the plunger to press such a sideways placed disk into a shape that looks almost indistinguishable from a properly placed disk. However, when loading a Coomassie-containing solution, it becomes evident that the disk is not evenly sealing at its sides, resulting in unequal loading.

(iv) Press the disk gently into place using the plunger (**Fig. 1**: Steps 4–1 to 4–6).

▲ CRITICAL STEP: The disk is soft and seals very easily at its sides. Applying a larger pressure at this point results in a more densely packed disk and raises the back pressure of the StageTip unnecessarily.

(v) Remove the cutter (**Fig. 1**: Step 5). The StageTip is finished. Additional disks or bead material can be added.

Note: It is important to prepare tips with high reproducibility for constant back pressure. The softness of the disks makes it difficult to achieve this manually. To overcome this problem, well-controlled gas pressure can be used to push the disks into the pipette tips with high throughput (96 tips in 3 min)[20]. Some types of StageTips are commercially available through: Proxeon Biosystems (Denmark) and Nikkyo Technos (Japan).

(vi) For conditioning and separation, the experimenter needs to prepare devices for either pressure (EQUIPMENT SETUP D) or centrifugal elution (E and F). For parallel processing of a few samples, it is recommended to use a micro-centrifuge (E and F) instead of the pressure device (D). To increase throughput, up to 96 StageTips can be placed in an ordinary tip container or a commercial parallel treatment device (Nikkyo technos) and spun in a centrifuge (e.g., Centrifuge 5810 with rotor A-2-DWP, Eppendorf) at 1,800 r.p.m. for 2–10 min depending on buffer volume and back pressure of the tips (F).

(iii) Add 20 µl buffer A to the StageTip just before the last remainder of buffer B has left the tip, and pass buffer A through the tip. Stop before the last remainder of buffer A has left the tip to avoid the tip drying completely.
? TROUBLESHOOTING

(iv) *Sample loading*. Load the desired sample volume acidified by the addition of sufficient AcOH from a 5% stock solution onto the tip. As the C_{18} disk serves as a desalting platform, the salt concentration in the sample is not important.
▲ CRITICAL STEP The amount of AcOH needed should be tested on an equal amount of digestion buffer to that used for the sample. Do not attempt to directly measure the pH of the digest solution as this would result in the loss of peptides.
? TROUBLESHOOTING

(v) *Wash*. To wash the tip add 20 µl buffer A just before the last remainder of the sample has left the tip, and pass the buffer A through.
■ PAUSE POINT The peptide-loaded, washed C_{18} StageTips can be stored in buffer A in the fridge or at −20 or −80 °C. We have done so in our laboratories for several weeks without compromising sensitivity of subsequent MS analysis.

(vi) *Elution*. Apply 10 µl buffer B two times, collect the combined eluate in a well of a 96-well plate and prepare for LC–MS/MS (Step 2).

(B) StageTips with reversed-phase material for pre-fractionation in different pH modes

(i) *Conditioning*. Wet the disks by passing 20 µl methanol through the StageTip.
▲ CRITICAL STEP The conditioning and sample loading must be done under the same pH conditions as the separation.

(ii) Add 20 µl buffer 4 to the StageTip just before the last remainder of methanol has left the tip, and pass buffer 4 through the tip.

(iii) Add 20 µl buffer 1 to the StageTip just before the last remainder of buffer 4 has left the tip, and pass buffer 1 through the tip. Stop before the last remainder of buffer 1 has left the tip to avoid the tip drying completely.
? TROUBLESHOOTING

(iv) *Sample loading*. Load the desired sample volume under conditions of buffer 1 onto the tip.
▲ CRITICAL STEP The pH of the sample needs to be the pH of buffer 1. The concentration of organic solvent is critical and should be below 5%. To control the pH without loosing sample, use a number of aliquots of an equal volume of the buffer the sample is in, and add 10× buffer 1 to determine what volume is required to control the pH. This volume is then added to and mixed with the sample in the reservoir of the StageTip.
? TROUBLESHOOTING

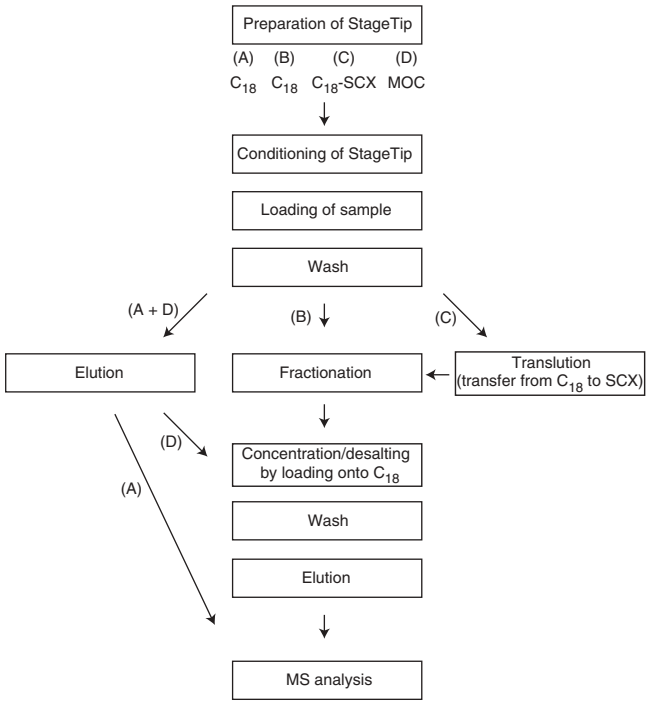

Figure 3 | Flow chart for procedure steps (A)–(D): (A) C_{18}-stop-and-go-extraction tips (StageTips) for clean-up and concentration; (B) StageTips with reversed-phase material for pre-fractionation in different pH modes; (C) Multi-StageTip: an integrated protocol using reversed-phase ion exchange (Re-X) StageTip for fractionation and a separate C_{18} StageTip for desalting; (D) Multi-StageTips with extended functionalities through loose beads: phosphopeptide enrichment using metal oxide-C_8 StageTip.

BOX 2 | GENERAL REMARKS

1. Loading of liquids: Note that all solutions are loaded from the back (like in a column) and passed through the stop-and-go-extraction tips (StageTips) a single time at moderate speed (10–30 µl min^{-1}).
2. Drying of the StageTip: A dried StageTip is a non-conditioned StageTip. No StageTip should be allowed to dry between conditioning and loading the sample. It is not problematic to let the tip run dry by passing all liquid through it. But do not let the material dry by leaving it for more than 2 min without liquid. Addition of the liquid of the next step before the liquid of the preceding step has completely left the tip minimizes the exposure of the wetted material to air and prevents drying of the disks. The air cushion between the two liquids prevents the liquids of successive steps from mixing. After conditioning, leave a small amount of the last buffer used in the tip to prevent the tip from drying out before sample loading.
3. Concentration of the final eluate: If the final sample volume needs to be identical, it has to be adjusted after concentration. The evaporation speed differs depending on the actual amount of peptides present. Hence, inequalities in volume will result. If the entire sample is to be injected, the volume variations are of importance only with respect to avoiding the injection of air into the HPLC system with small amounts of air being tolerated normally.

(v) *Wash*. To wash the tip add 20 µl buffer 1 just before the last remainder of the sample has left the tip, and pass the buffer 1 through.
(vi) *Fractionation*. Apply 20 µl buffer 2 to elute fraction 1 and collect the eluate in C_{18} StageTip 1.
(vii) Apply 20 µl buffer 3 to elute fraction 2 and collect the eluate in C_{18} StageTip 2.
(viii) Apply 20 µl buffer 4 to elute fraction 3 and collect the eluate in 96-well plate.
(ix) *Loading to C_{18}StageTip (concentrating)*. Mix the fractions in both C_{18} StageTips with the pre-deposited 60 µl buffer A using a pipette and pass them through the tips.
(x) *Wash*. To wash the tip add 20 µl buffer A just before the last remainder of the fraction has left the tip, and pass the buffer A through.
■ **PAUSE POINT** The peptide-loaded, washed C_{18} StageTips can be stored in buffer A in the fridge or at –20 or –80 °C.
(xi) *Elution*. Apply two times 10 µl buffer B to C_{18} StageTips 1 and 2, collect the eluate in different wells of a 96-well plate.
(xii) Prepare all three samples for LC–MS/MS analysis (Step 2).

(C) Multi-StageTip: an integrated protocol using Re-X StageTip for fractionation and a separate C_{18} StageTip for desalting

(i) *Conditioning*. Wet the disks by passing 20 µl methanol through the StageTip.
(ii) Add 20 µl buffer 2 to the StageTip just before the last remainder of methanol has left the tip and pass buffer 2 through the tip, leaving the last remainder of the buffer in the tip to avoid the tip drying completely.
? TROUBLESHOOTING
(iii) Add 20 µl buffer 1 to the StageTip just before the last remainder of buffer 2 has left the tip, and pass buffer 1 through the tip.
(iv) Add 20 µl buffer 6 of the salt-based fractionation to the StageTip just before the last remainder of buffer 1 has left the tip and pass buffer through the tip.
▲ **CRITICAL STEP** The ion exchange material must be at conditions suitable for using buffer 6 of the salt-based fractionation, irrespective if salt- or pH-based fractionation are intended. Omitting this step will compromise separation performance.
(v) Add 20 µl buffer 1 to the StageTip just before the last remainder of previous buffer has left the tip, and pass buffer 1 through the tip. Stop before the last remainder of buffer 1 has left the tip.
▲ **CRITICAL STEP** It is best not to flush the entire volume of buffer 1 through the tip but leave the last microliter to keep the StageTip wetted. If all of buffer 1 has left the tip, it is sufficient to add a little more buffer 1 as long as this is done immediately. If by accident the tip was allowed to dry, the entire conditioning procedure must be repeated without omitting any step.
(vi) *Sample loading*. Load the desired sample volume in the pH of buffer 1 onto the tip. As the reversed-phase disk (C_{18} or SDB) serves as a desalting platform, the salt concentration in the sample is not important.
▲ **CRITICAL STEP** The concentration of organic solvent is critical and should be below 5%. Higher concentrations of organic solvents will compromise binding of some or all peptides to the C_{18} material and will result in loss of peptides. Urea is tolerated with a concentration of 2 M.
? TROUBLESHOOTING
(vii) *Wash*. To wash the tip add 20 µl buffer 1 just before the last remainder of the sample has left the tip, and pass the buffer 1 through.
(viii) *Translution (transfer-elution)*. To elute the peptides from the C_{18} disk and allow their binding to the ion exchange disk, add 20 µl buffer 2.
▲ **CRITICAL STEP** The eluate contains the flow-through fraction of those peptides not retained by ion exchange. Collect this fraction in a well of a 96-well plate for analysis. We find this fraction to contain only few peptides normally.
Note: For the following steps you need three (or four for salt based fractionation) C_{18} StageTips that were conditioned following Step A and which contain 60 µl buffer A.
(ix) *Fractionation*. Apply 20 µl buffer 3 to the Re-X StageTip and collect the eluate in C_{18} StageTip 1 (fraction 1).
(x) Apply 20 µl buffer 4 and collect the eluate in C_{18} StageTip 2 (fraction 2).
(xi) Apply 20 µl buffer 5 and collect the eluate in C_{18} StageTip 3 (fraction 3).
(xii) Only for salt-based fractionation: apply 20 µl buffer 6 and collect the eluate in C_{18} StageTip 4 (fraction 4).
(xiii) *Loading to C_{18} StageTip (Desalting of the four fractions)*. Mix the fractions in each C_{18} StageTip with the pre-deposited 60 µl buffer A using a pipette and pass them through the tips.
▲ **CRITICAL STEP** Ensure mixing of the eluates with buffer A to lower the concentration of ACN. The concentration in the eluate—20% ACN—would be sufficient to prevent binding of some peptides to the C_{18} StageTip and would result in the loss of these peptides. At 5% ACN all except very hydrophilic peptides are retained. Alternatively, add nonafluorovaleric acid (final concentration 0.1%) to the elution buffer for increasing the hydrophobicity of peptides and allow their retention even in 20% ACN[10].
(xiv) *Wash*. To wash the tip add 20 µl buffer A just before the last remainder of the fraction has left the tip, and pass the buffer A through.
■ **PAUSE POINT** The peptide-loaded, washed C_{18} StageTips can be stored in buffer A in the fridge or at –20 or –80 °C.

(xv) *Elution*. Apply two times 10 µl buffer B and collect the eluate of each fraction in a different well of a 96-well plate. At this point one should have five peptide solutions in 20 µl buffer B for the salt fractionation: the flow-through fraction of Step 1C(viii) and fractions 1–4. For pH fractionation, one should have four peptide solutions in 20 µl buffer B: the flow-through fraction of Step 1C(viii) and fractions 1–3.

(xvi) Prepare the fractions for LC–MS/MS analysis (Step 2).

(D) Multi-StageTips with extended functionalities through loose beads: phosphopeptide enrichment using metal oxide-C_8 StageTip

(i) *Conditioning*. Wet the beads by passing 20 µl methanol through the MOC StageTip.

(ii) Add 200 µl buffer C to the MOC StageTip for pre-washing and pass the liquid through the tip.

? TROUBLESHOOTING

(iii) *Sample loading*. Desalt the sample using the desalting cartridge and elute with buffer B. For peptide quantities of 150 µg or less, use a large C_{18} StageTip instead of the desalting cartridge.

(iv) Elute in buffer C directly into the MOC StageTip.

▲ **CRITICAL STEP** In the case of titania, adding 2,5-dihydroxybenzoic acid (DHB) in the loading buffer improves the specificity for phosphopeptides[15]. However, DHB remains in the final sample solution for LC-MS, which can reduce the stability of LC-MS systems. Alternatively, aliphatic hydroxy acids such as lactic acid instead of DHB for titania and HPA for zirconia show improvement in both specificity and system stability[16].

(v) *Wash*. Add 200 µl buffer C to the MOC StageTip for first washing and pass the liquid through the tip.

(vi) Add 100 µl buffer B to the MOC StageTip for second washing and pass the liquid through the tip.

(vii) *Elution and loading to C_{18}StageTip (desalting)*. Elute with two times 50 µl buffer D and collect eluate in 100 µl 2% TFA on a C_{18} StageTip, mix well and pass through the tip. 20% ACN can be added to buffer D to ensure none of the peptides are retained by the C_8 disk used to keep the beads. For desalting on the subsequent C_{18} StageTip, however, ACN should not exceed a concentration of 5%.

▲ **CRITICAL STEP** Phosphopeptides are not stable under basic conditions, and hence the buffer D eluate should be collected as described to ensure immediate acidification.

(viii) *Wash*. To wash the tip add 100 µl buffer A just before the last remainder of the fraction has left the tip and pass the buffer A through.

■ **PAUSE POINT** The peptide-loaded, washed C_{18} StageTips can be stored in buffer A in the fridge or at –20 or –80 °C.

(ix) *Elution*. Elute phosphopeptides from C_{18} StageTip using two times 10 µl buffer B. Collect the solution in a 96-well plate for subsequent LC-MS.

? TROUBLESHOOTING

(x) Prepare the eluate for LC–MS/MS analysis (Step 2).

? TROUBLESHOOTING

Preparation of the final eluate for LC–MS/MS

2| Concentrate the final eluate of a reversed-phase StageTip using a concentrator/SpeedVac. Check the progress every 1–2 min. The final volume should not be more than 80% of the water content of the initial solution to ensure that all ACN has evaporated. The volume can be checked by comparing to a reference of known volume added to an empty well. Do not measure the volume through taking up the sample in a pipette tip, as this results in significant loss of peptides for low sample amounts. *Note*: The time taken by the organic solvent to evaporate depends on factors such as the amount and the type of peptides present. A typical time for 10 µl of 80% ACN to lose the organic solvent in a concentrator in the presence of a low amount of peptides is 2 min. For concentrated peptide solutions, this time can easily become 10 min or more. In a set of samples the concentration time might hence vary from sample to sample. Samples that are faster concentrated than others can always be diluted through the addition of 1–2 µl of 0.1% TFA to prevent them from drying to completeness.

▲ **CRITICAL STEP** Do not dry down to completion as this will result in loss of peptides.

? TROUBLESHOOTING

3| Dilute samples to desired volume by adding 0.1% TFA and analyze by MS.

▲ **CRITICAL STEP** Do not let peptide solutions stand before analysis at ambient temperature or for prolonged periods. Cool to 4 °C and analyze immediately. Storage of peptides in aqueous solutions at low concentration results in rapid loss due to adhesion of peptides to the walls of the plastic container.

? TROUBLESHOOTING

● **TIMING**

Step 1(A): 5 min

Step 1(B): 15 min

Step 1(C): 30 min
Step 1(D): 30 min

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 5**.

TABLE 5 | Troubleshooting table.

Problem	Possible reason	Solution
Conditioning stop-and-go-extraction tip (StageTip) has high back pressure when conditioning	The disk was pressed too hard into position when the StageTip was manufactured	Use less force
Sample loading StageTip has increasing back pressure when loading the sample until no liquid goes through the tip at all	The sample contains small particles that clog the StageTip	Do not continue loading the sample until the flow reaches zero. Split the sample on several StageTips and use StageTips with larger diameter
Step 1A–D No peptides are detected by mass spectrometry (MS) after prolonged storage of peptides on StageTip	Elution with buffer B can be problematic after prolonged storage of the peptides on C_{18} StageTips that have been allowed to dry excessively	In such cases, the disk is first wetted again by using 2 μl of methanol. The methanol eluate is collected and combined with the subsequent buffer B eluate. Note that methanol is more difficult to remove by concentration than ACN. It is not sufficient to concentrate the eluate to its calculated water volume; i.e., if 2 μl methanol and 20 μl buffer B were combined, this would be equivalent to 4 μl water. The sample has to be concentrated to half this volume, 2 μl, to ensure that all organic solvent is removed before injection for liquid chromatography–tandem mass spectrometry (LC–MS/MS)
Step 1A(iv) Peptides are not retained by C_{18} StageTip	Higher concentrations of organic solvents will compromise binding of some or all peptides to the C_{18} material and will result in loss of peptides	The concentration of organic solvent is critical and should be below 5%. Ensure this by dilution or concentration. Urea with a concentration of 2 M is tolerated by most peptides
Step 1A–D The StageTip ran dry during or after conditioning		It is best not to flush the entire volume of buffer through the, tip but leave the last microliters to keep the StageTip wetted. If all of liquid has left the tip, it is sufficient to add a little more as long as this is done immediately (it takes a StageTip approximately 2–5 min to dry from 80% ACN). If by accident the tip was allowed to dry completely before the peptides were loaded, the entire conditioning procedure must be repeated without omitting any step
Step 1D(ix) No phosphopeptides are detected	No or not sufficiently abundant phosphopeptides present in sample or the StageTip step failed	(1) Analyze the digest of a standard phospho-protein without enrichment to validate that the digestion worked and some phosphopeptides are present. (2) Next, enrich phosphopeptides for the standard phospho-protein alone to validate that the StageTip step worked. (3) Finally, spike your sample with the digest of a standard phospho-protein and enrich for phosphopeptides. You will see at least the standard peptides
Step 2 Peptides dried down to completion	Concentrated too long	If by accident all solvent was evaporated, take up the peptides in 0.5% acetic acid (AcOH), 80% ACN and repeat the concentration step. Adding 0.5% AcOH alone is not sufficient to recover all peptides
Step 3 Peptides stored too long before analysis	Storage of peptides in aqueous solutions at low concentration results in rapid loss due to adhesion of peptides to the walls of the plastic container	Do not let peptide solutions stand at ambient temperature before analysis or for prolonged periods. Cool to 4 °C and analyze immediately. Peptides can be recovered by raising the concentration of ACN followed by concentration to remove the organic solvent again.
No peptides are detected by MS	Amount of peptides in sample is too low, StageTip step failed or MS analysis failed	(1) Analyze a standard peptide mixture by MS without StageTip treatment. (2) Check your StageTip step by processing a standard peptide mixture

ANTICIPATED RESULTS

Pre-fractionation before lc-ms for complex mixtures

Pre-fractionation using StageTips before LC–MS/MS is a simple, easy-to-use and highly flexible approach. For illustration purposes, tryptic *Escherichia coli* cell lysate (1.5 µg) was divided into three fractions using StageTips (Steps 1B and C) and analyzed by nanoLC–MS/MS with a 60-min gradient in 0.5% AcOH using a QSTAR pulsar (AB-Sciex) followed by protein identification using MASCOT database searching engine (Matrix Science) as described in ref. 7 (**Fig. 4**). A single LC–MS/MS run with a 60-min ACN gradient in 0.5% AcOH for *E. coli* lysate without fractionation (Step 1A) resulted in the identification of approximately 1,000 unique peptides (**Fig. 4a**), matching approximately 300 unique proteins (**Fig. 4b**). Triplicate analysis increased the number of peptide and protein identifications only marginally. Four fractionation approaches were performed by StageTips: (i) Re-X SCX with salt elution (Step C, which was truncated for the purpose of comparison from four to three fractionation steps: 20, 50 and 500 mM salt), (ii) Re-X strong anion exchange (SAX) with pH-elution, (Step C), (iii) basic pH reversed-phase (Step B) and (iv) neutral pH reversed-phase (Step B). As shown in **Figure 4**, efficiency of peptide and protein identification improved considerably by initial peptide fractionation; the number of identified peptides was enhanced to approximately 140% and the number of identified unique proteins up to approximately 150% when compared with simple threefold LC–MS/MS repetition. All four StageTip fractionation methods resulted in similar improvement even though the initial starting amount was relatively low. SDB and SCX yielded just slightly more identified unique peptides and proteins than SAX and C18-DBAA.

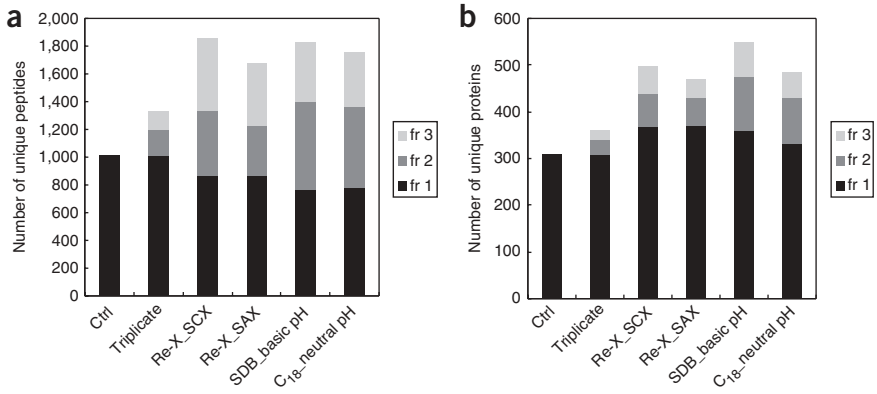

Figure 4 | Comparison of different analyses of 1.5 µg *Escherichia coli* lysate either directly injected for liquid chromatography-tandem mass spectrometry (LC–MS/MS) or after stop-and-go-extraction tip (StageTip) pre-fractionation into three fractions. Shown are the number of unique peptides (**a**) and unique proteins (**b**) identified with the different approaches. Ctrl: single analysis; Triplicate: triplicate analysis; Re-X_SCX: truncated protocol 4, salt fractionation; Re-X_SAX: protocol 4, pH fractionation; SDB_basic pH: protocol 3; C_{18}_neutral pH: protocol 3. Re-X, reversed phase-ion exchange; SAX, strong anion exchange.

Since samples need to be micro-purified in any case and StageTips are easy to use and disposable, the procedure does not add many additional tasks to the analysis while clearly increasing the depth of the analysis; and parallel sample pre-treatment facilitates the total analysis time, especially when a large number of samples should be pre-fractionated before LC–MS/MS analysis.

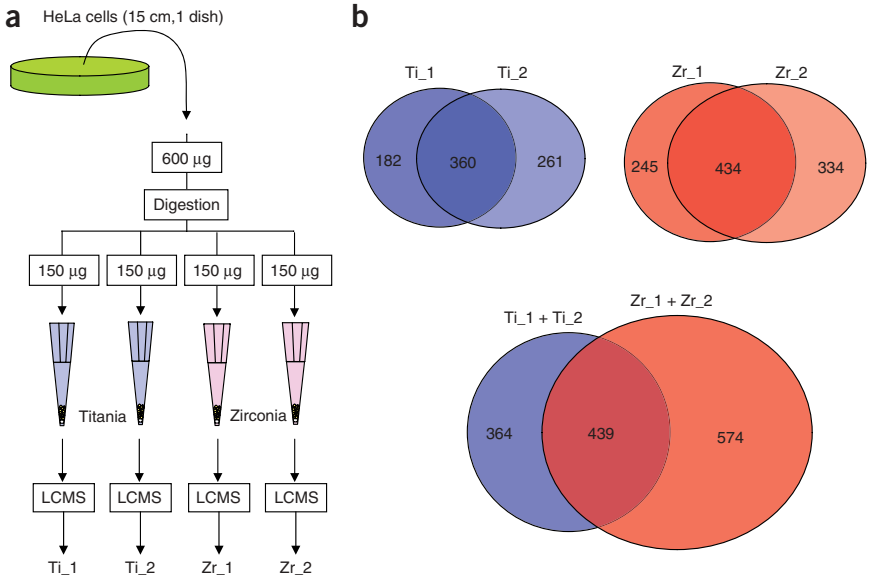

Figure 5 | Phosphopeptide analysis using titania and zirconia following Step D. Panel (**a**) shows a schematic view of the analysis. (**b**) From 600 µg HeLa extract analyzed in four portions by two titania and two zirconia metal oxides chromatography (MOC) stop-and-go-extraction tips (StageTips) resulted in 1,377 phosphopeptides. The experimental details were reported in ref. 16.

Enrichment of phosphopeptides in HeLa cytoplasmic extracts

There have been only a few reports of the application of phosphopeptide enrichment methods to real, complex samples such as crude cell extracts including very recent large-scale studies[17,18]. In those studies, approximately 10 mg of starting material was used for SCX chromatography before phosphopeptide enrichment. To our knowledge, the protocol described here is the only one for direct enrichment of phosphopeptides from cell lysates of less than 1 mg starting material. However, no current protocol is likely to give a complete account of all phosphorylation sites in a sample. This is underlined by a comparison of three very different enrichment approaches recently published[19]. We loaded 150 µg of protein digests onto each MOC tip

following Step 1D (**Fig. 5a**). HeLa cell soluble extract was prepared, two separate enrichment were conducted each for titania and zirconia, followed by LC–MS/MS analyses using an LTQ-orbitrap (ThermoFisher) to give the results shown in **Figure 5b**. The overlap between the two different materials was smaller than the overlap between simple repetitions on the same metal oxide as seen in the Venn diagrams. It is hence beneficial to use two materials in parallel (titania and zirconia, in this case).

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COMPETING INTERESTS STATEMENT The authors declare no competing financial interests.

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