A Solid-Phase Extraction Capture (SPEC) workflow in nanoliter volumes for fast, robust and ultrasensitive proteomics

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Abstract

Despite great progress, sample preparation remains an area for improvement in proteomics, particularly for low-input samples where conventional protocols lead to losses and incomplete digestion. We present Solid-Phase Extraction Capture (SPEC), a modular workflow that confines protein processing to nanoliter-scale volumes within C18 or ion-exchange tips. SPEC enables highly efficient proteolysis directly on the solid phase, minimizing surface losses and reagent dilution. A two-tip configuration further allows orthogonal phase combinations for optimal digestion and on-tip manipulation. Compared to alternative protocols, SPEC improves protein recovery while accelerating protein digestion which completes in minutes. The method is compatible with a diverse set of sample types and lysis conditions, including strong detergents, FFPE tissue, and muscle fibers. SPEC's nanoliter digestion volume, high recovery, and flexible design make it a powerful tool for ultrasensitive and high-throughput proteomics, facilitating applications from spatial biology to clinical profiling. SPEC represents a universal proteome sample preparation protocol that spans all sample types, sensitivity levels, and throughput requirements.

Introduction

Sample preparation is a pivotal step in mass spectrometry (MS)–based proteomics, particularly as the field pushes toward increasingly limited sample amounts and higher throughput[1,2]. Over the years, numerous strategies have been introduced to reduce sample losses, improve digestion efficiency, and enhance compatibility with diverse biological materials. Among these, tip-based protocols have proven especially versatile, combining solid-phase cleanup with efficient enzymatic digestion in miniaturized, enclosed environments.

The STAGE tip format for solid-phase peptide extraction laid the foundation for modular proteomics workflows, while the one-pot reactor approach further consolidated cell lysis, digestion, and cleanup within a single tip-based device[3–5]. These methods emphasized ease of use, reproducibility, and compatibility with automation. Earlier, filter-aided sample preparation (FASP) became a mainstay for SDS-based lysis workflows, although its dependence on molecular weight cut-off filters limited miniaturization[6]. The in-StageTip (iST) method aimed to streamline preparation steps into a fully enclosed format, achieving improved performance at scale[7]. More recently, a similar principle termed 'One-tip protocol' has proven highly efficient in the analysis of single oocytes or even single cells[8,9].

In parallel, other groups have described powerful sample preparation strategies such as SP3, which leverages carboxyl-functionalized magnetic beads to capture and clean proteins in a single reaction device[10,11], and the related PAC protocol, which exploits aggregation-based protein capture[12]. Both methods have facilitated workflows with fewer losses and higher flexibility, although they generally operate at microliter volumes but become inefficient at very low input levels. The SISPROT protocol and its extensions (e.g., iPAC) represent an integrated approach to proteome processing and peptide fractionation in a single tip and digestion on a solid support[13]. It has recently been used in spatial proteomics[13–15]. However, SISPROT and similar systems often require additional intervening processing steps such as solvent evaporation and re-dissolving in LC-MS compatible buffers, which may limit sample recovery.

Thus, current protocols still face critical barriers when applied to ultra-low sample amounts or volumes below 1 µL. In these regimes, dilution, poor enzyme kinetics, and surface adsorption become dominant sources of variability and loss. Moreover, the lack of standardized tools for nanoliter-scale

workflows restricts broader adoption in high-throughput and spatial proteomics.

To overcome these limitations, we leveraged the Evosep One LC platform and its associated Evotip technology—pre-packed C18 tips designed for standardized, high-throughput operation—as a foundation for miniaturized, automation-ready sample processing[1,16]. We introduce Solid-Phase Extraction Capture (SPEC), a flexible and robust tip-based workflow that confines protein digestion and manipulation to volumes as low as 50–200 nL. Building on our prior work, SPEC integrates solid-phase preconcentration, efficient enzymatic digestion, and optional downstream labeling or fractionation. In the following, we describe SPEC principles in both a one-tip format (processing directly in the Evotip used for measurement) and a more versatile two-tip format (processing in a separate SPEC tip with direct elution onto the measurement Evotip), demonstrating compatibility with diverse biological samples - including muscle, and Formalin Fixed and Paraffin Embedded (FFPE) tissue. We find SPEC's compatibility with ionic detergents such as SDS and SDC—without requiring cleanup—and its short digestion time to offer compelling practical advantages.

Results

SPEC confines protein digestion to nanoliter volumes on a solid phase

A defining principle of SPEC is the confinement of proteins to nanoliter-scale volumes for digestion and sample processing directly on a solid support. This miniaturization addresses a key limitation of most existing protocols—including tip-based workflows—where microliter-scale volumes promote sample loss through surface adsorption and dilution, especially at low input levels.

We captured and concentrated proteins within a confined resin volume (typically 50–200 nL) inside a regular Evotip pipette tip packed with C18 material (**Fig. 1a**). Proteolytic enzymes diffuse into this matrix under optimal buffer conditions, resulting in highly efficient digestion despite sub-nanogram protein inputs. Compared to conventional in-tip digests that rely on reaction in the liquid phase above the tip material, SPEC minimizes void volume and maximizes enzyme and substrate concentration. Protein lysate is loaded onto a solid phase material by centrifugation , and the enzyme is pulse-spun into a small volume, allowing it to diffuse into the matrix and digest the proteins efficiently.

To confirm the spatial confinement and stability of the sample during processing, we performed fluorescence imaging of separately labeled protein samples and the LysC and trypsin proteases throughout the steps of the SPEC workflow (**Methods**). The confined

zone of the reaction volume remained sharply defined throughout, demonstrating minimal dispersion or leakage (**Fig. 1b**). The enclosed nature of the SPEC tip further allows seamless integration with automated platforms such as the Evosep One, enabling scalable sample preparation at high throughput.

Due to reduction of sample volume by one to two orders of magnitude, this design also enables excellent performance under challenging conditions such as short incubation times or ionic detergents. As a result, digestion is completed in under one hour, without requiring intermediate transfers or evaporative steps, compared to overnight digestion in most workflows (**Fig. 1c**).

The protocol proceeds through a simple sequence of steps: tip activation, protein binding, washing, on-tip digestion, and peptide elution, with optional steps for further manipulation. Digestion enzymes are applied in a convenient but small volume and diffuse into the resin, resulting in rapid and efficient proteolysis.

This basic SPEC configuration uses commercially available Evotips (C18 material), as implemented for use with the Evosep system, but can be adapted to other solid phases as needed. Depending on the chemical nature of the SPEC tip, detergents can be readily removed by adding organic solvent during the loading or washing steps or by adjusting the pH to minimize detergent binding to ionic solid phase supports. In this way proteins can be selectively retained even on C18 material due to the much stronger non-polar binding affinity of denatured proteins while detergents such as SDS or SDC are washed away. This offers a route to high-throughput, miniaturized proteomics even for SDS- or SDC-containing samples (**Fig. 1c**). Because all steps take place in a single enclosed system without transfers, the workflow inherently minimizes loss and is readily scalable.

This ‘single-tip SPEC’ format is effective for detergent containing samples, but has limitations for more advanced sample manipulations. Furthermore, C18 material can be suboptimal for enzymatic digestion, and we therefore recommend the on-tip, in solution protocol for detergent free samples. To overcome these issues, we next explored a two-tip configuration, where solid phases can be optimized independently for digestion and subsequent peptide processing.

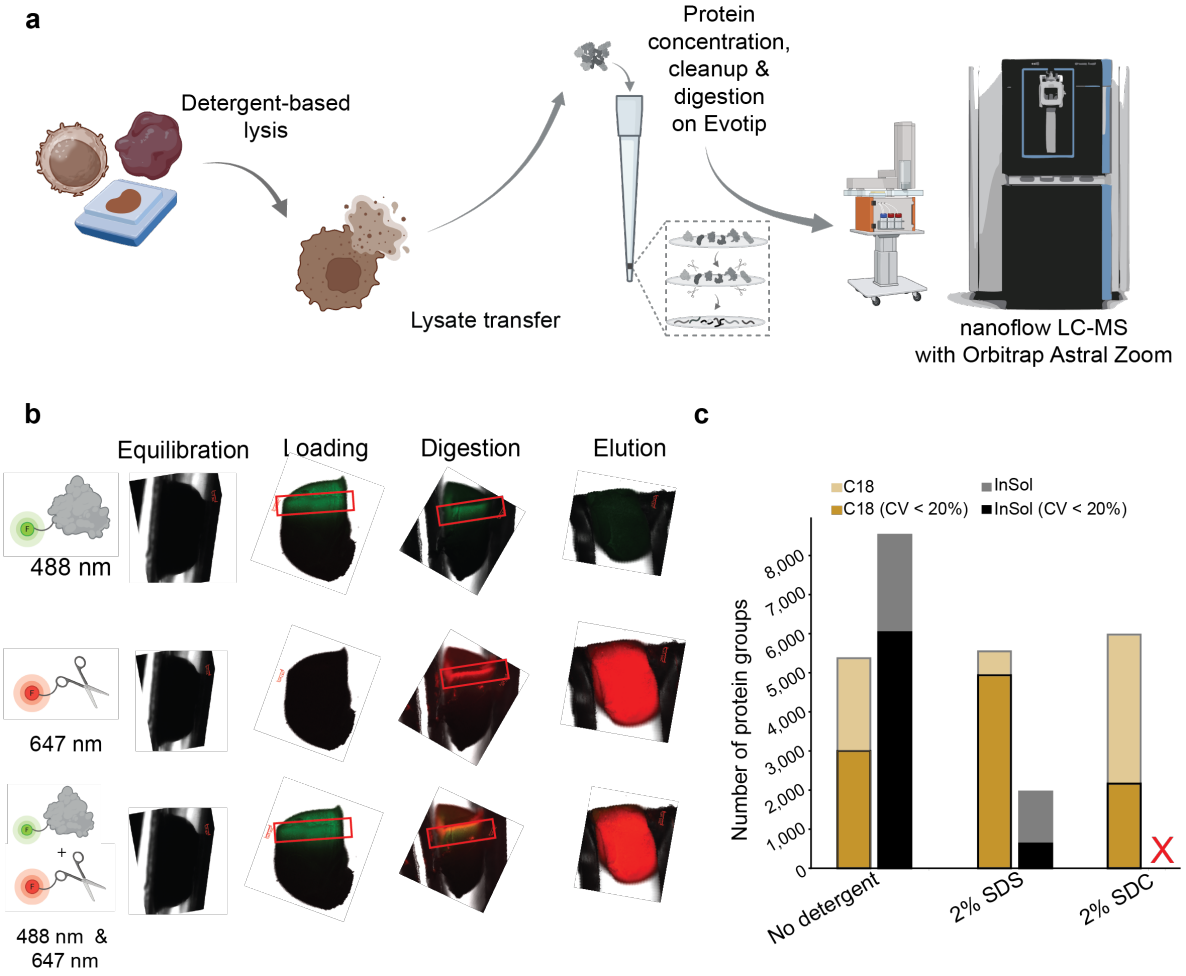

Figure 1 | SPEC confines protein digestion to nanoliter volumes for efficient and detergent-compatible proteomics. a Overview of the one-tip SPEC workflow. Protein samples from diverse sources undergo detergent-based lysis, followed by transfer to Evotips for confined digestion and cleanup in nanoliter volumes. The resulting peptides are then directly analyzed by nanoflow LC-MS. **b** Fluorescence imaging of SPEC tips during key workflow stages: equilibration, protein loading, on-tip digestion, and peptide elution. Labeled protein (488 nm, green) and fluorescently labeled trypsin (647 nm, red) demonstrate spatial confinement and co-localization throughout processing, confirming minimal sample dispersion and stable enzyme diffusion within the resin. **c** Protein group identifications from K562 lysate digested on C18-based SPEC tips under different detergent conditions. SPEC enables digestion in the presence of detergents, unlike the on-tip in solution protocol.

Two-tip SPEC configuration enhances flexibility, digestion efficiency, and detergent compatibility

To expand the capabilities of the platform, we developed a two-tip SPEC configuration, in which protein binding, digestion, and peptide cleanup are performed on distinct, independently optimized solid phases. In this format, proteins are first loaded onto a SPEC tip containing a strong ion exchange material—such as SCX or SAX resin - which offers high surface area and full enzyme access, allowing for fast and complete digestion. Peptides are subsequently eluted

and transferred directly to a second tip – the regular measurement Evotip - for desalting or other downstream manipulations. We implemented this configuration using 96- and 384-well tip-adapters compatible with standard centrifuges and automated liquid handlers, enabling parallel processing and high throughput (**Fig. 2a**).

We benchmarked the two-tip protocol on K562 lysate across three solid phases: C18, SCX, and SAX. Compared to conventional in-solution digestion, the SAX and SCX two tip formats yielded more protein group identifications and higher reproducibility (CV < 20%), with SAX tips achieving the greatest depth and quantitative precision, about 9,100 protein groups from 200ng with the Evosep Whisper Zoom 80 gradient and narrow-window data-independent acquisition (nDIA) on an Orbitrap Astral MS[17], (**Methods**, **Fig. 2b**). This establishes SAX as the preferred material, reflecting the accessibility of the protein sample to the protease.

Next, we assessed cleavage efficiency by computing the fraction of fully cleaved peptides under each condition. Again, SAX-based SPEC outperformed in-solution digestion and other tip formats, reaching up to 80% fully cleaved peptides after only 60 minutes at enzyme-to-protein ratios of 1:10 to 1:1 (**Fig. 2c**).

A key advantage of SPEC is its compatibility with strong detergents. Whereas conventional protocols often require laborious detergent removal steps, SPEC tolerates 2% SDS or 2% SDC without loss of performance. When processing K562 lysate under these lysis conditions, SCX- and SAX-based SPEC tips retained high identification numbers and reproducibility (**Fig. 2d**), in contrast to the marked drop observed with in-solution digests. Note that SDC tends to precipitate upon acidification during protein loading, making the combination of this detergent with SCX impractical. Thus, the pairing of SAX tips with SDC detergent is the most favorable one. For SDS containing samples, we also recommend SAX material – combined with dilutions to 0.5%, which is still easily loaded on the tip.

To investigate digestion kinetics, we performed a time-course experiment using SAX tips and compared it to in-solution digestion (**Methods**). Remarkably, SAX-based SPEC reached nearly 100% of protein identifications after only 5 minutes, outperforming in-solution digestion even at 2h incubation times (**Fig. 2e**). This rapid kinetics and accelerated proteolysis presumably result from the nanoliter-scale confinement and improved enzyme interaction with resin-coordinated sample. Because the number of proteins with CV better than 20% still increased at least to 30 min digestion time, we

now use a conservative 1h digestion time in our standard protocol.

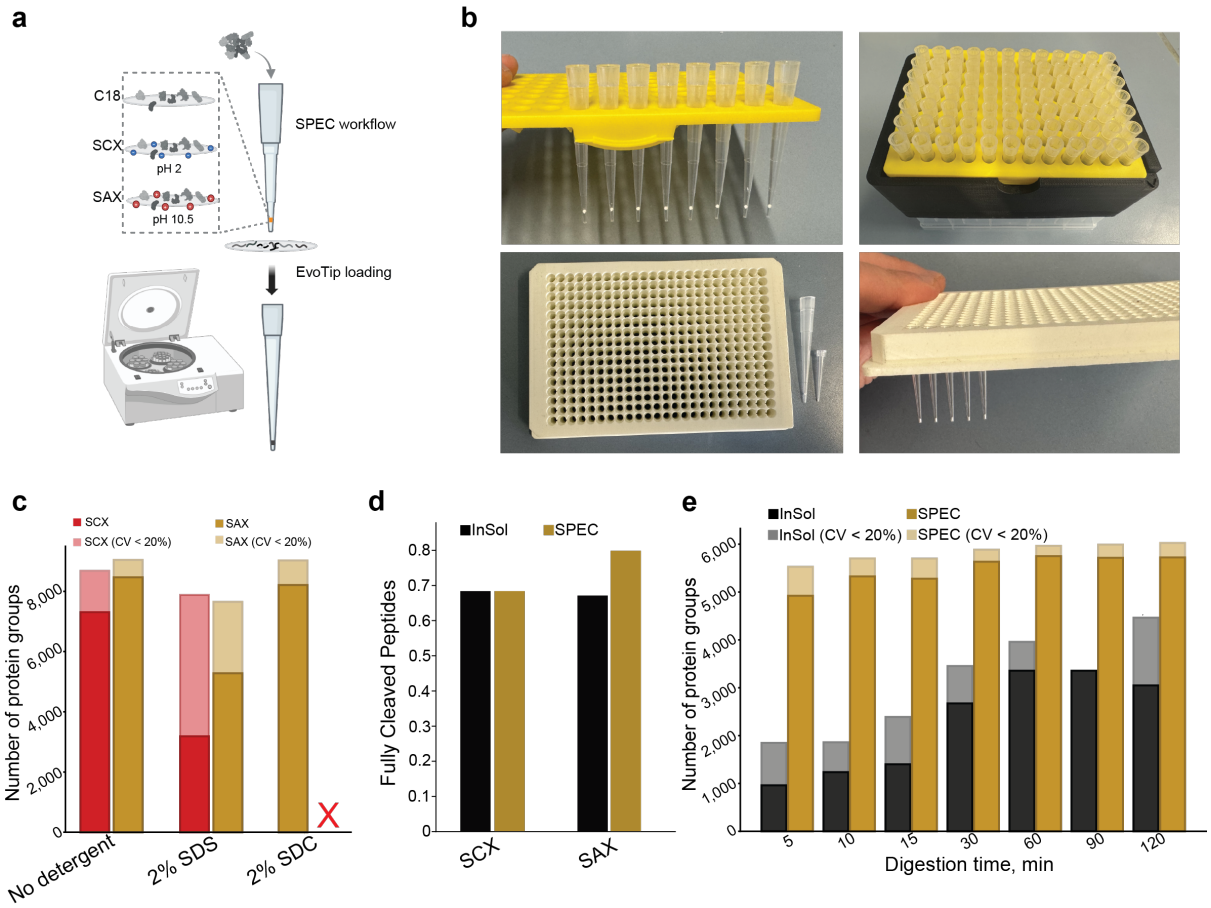

Figure 2 | Two-tip SPEC configuration enhances flexibility and performance for diverse sample types. a Schematic of the two-tip SPEC protocol. Proteins are bound and digested on customizable solid phases (C18, SCX, or SAX), followed by direct peptide transfer to Evotips for LC-MS. **b** Photographs show a 96-well and a 384-well adapter system for parallel tip processing and seamless integration into automated workflows. **c** Protein group identifications from commercial K562 lysate processed on different SPEC resins compared to in-solution digestion. **d** Fraction of fully cleaved peptides for in-tip in solution digest and SPEC, performed on SCX and SAX tips. **e** Digestion kinetics for SAX-based SPEC tips compared to in-solution digestion from 5 to 120 minutes.

SPEC enables robust proteomics across diverse sample types and lysis conditions

A major barrier in proteomic sample preparation is the need to balance effective lysis with downstream compatibility. Many common protocols, including SP3, PAC, and S-trap, either limit detergent use or require additional cleanup steps that risk sample loss. In contrast, the SPEC workflow retains full compatibility with strong detergents and diverse biological inputs without sacrificing yield or reproducibility.

To assess how broadly SPEC can be applied, we benchmarked it across various challenging sample types, including formalin-fixed paraffin-embedded (FFPE) tissue and dense, fibrous muscle tissue (**Fig. 3a**). We first tested mouse liver FFPE lysate at 200 ng input and compared it to PAC and in-solution digestion. SPEC outperformed both alternatives, yielding over 6,900 protein groups with high reproducibility (**Fig. 3b**). Notably, the number of identified proteins from 200 ng FFPE material using SPEC surpassed that of a 100 µg in-solution digest, highlighting the efficiency of structurally confining samples and maintaining small volumes.

We next examined seven distinct mouse tissues, including brain, heart, lung, kidney, liver, muscle, and spleen. Across all tissues, SPEC consistently yielded between 6000 and 8,500 protein groups at 200 ng input, with excellent quantification (**Fig. 3c–d**). This level of performance from FFPE sections opens a clear path toward spatial proteomics and retrospective analyses of archival biobank samples.

Importantly, SPEC also enables robust analysis of "digestion-resistant" tissues, such as skeletal muscle. We profiled two mouse muscle types—extensor digitorum longus (EDL) and soleus—from the same mouse and achieved >6,000 protein identifications per sample. Principal component analysis (PCA) of the proteomes showed clear separation between the two fiber types, reflecting expected biological variation (**Fig. 3e**). A rank plot of the EDL proteome further demonstrated a wide dynamic range, with consistent coverage of both high- and low-abundance proteins (**Fig. 3f**). Notably, this is the deepest reported mouse muscle proteome to date.

Finally, the ability to use detergent-rich lysis buffers such as 2% SDS or 2% SDC across all tested tissues and solid phases makes SPEC broadly applicable to difficult matrices, including highly cross-linked, lipid-rich, or insoluble samples. This removes the need for detergent removal steps, streamlining the workflow and reducing sample loss.

Together, these results establish SPEC as a highly versatile and robust method suitable for diverse sample inputs and conditions, from fresh lysate to fixed tissue and from routine lysis to challenging biochemical environments.

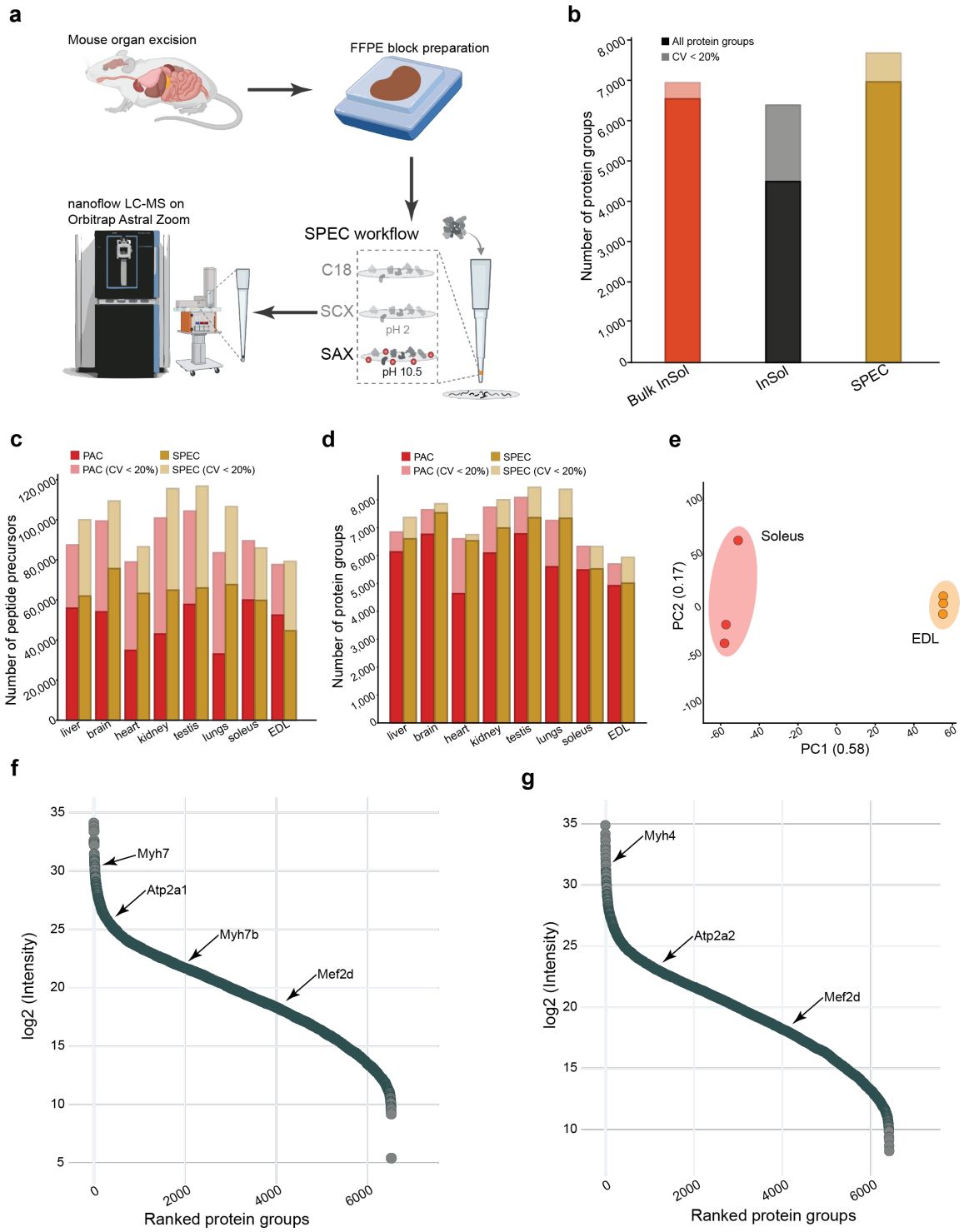

Figure 3 | SPEC enables robust proteomics across diverse sample types and lysis conditions. a Schematic of the SPEC workflow applied to tissue samples, including FFPE material and muscle. Proteins are processed on solid phase tips and analyzed by nanoflow LC-MS. **b** Protein group identifications from 200 ng FFPE liver using SPEC, or in-solution digestion (200 ng and 100 µg inputs). **c–d** Peptide precursors and protein group identifications and reproducibility (CV < 20%) across seven FFPE mouse tissues using SPEC or PAC. **e,** PCA of proteomes from EDL and soleus muscle fibers, showing clear separation between fiber types. **f-g** Protein rank plot for EDL and Soleus muscle fibers, illustrating dynamic range and depth of proteome coverage achieved with SPEC. Key muscle proteins are highlighted.

High sample recovery from minimal sample volume

To quantify the recovery of SPEC at extremely low inputs, we benchmarked it against the otherwise sensitive PAC protocol at decreasing input levels of K562 lysate (**Methods**). At a single cell equivalent (0.25 ng), SPEC yielded twofold more precursor identifications than an in-tip solution or PAC digestion, demonstrating superior retention and digestion efficiency under limiting conditions (**Fig. 4a**, **b**). This confirms that SPEC not only preserves enzyme activity in confined volumes but also enhances recovery of low-abundance proteins by reducing losses throughout the workflow compared to PAC.

To evaluate SPEC's performance on Deep Visual Proteomics[18] samples, we applied the workflow to laser-microdissected fixed HEK cells on membrane slides (Methods). Even with just 50 shapes—estimated to correspond to 2.5 ng of input—SPEC yielded over 4,000 protein groups, with >90% of those quantified at CV < 20% (**Fig. 4c**). Protein group depth scaled consistently with shape number, reaching over 7,500 identified groups from 500 shapes. This lays the groundwork for deep molecular analysis of tissue heterogeneity, cell-type zonation, and clinical pathology samples.

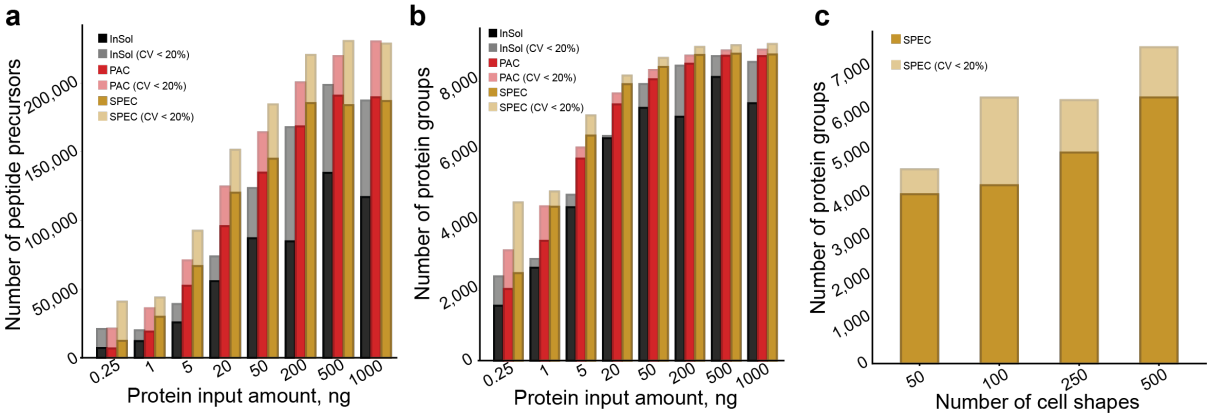

Figure 4 | SPEC maximizes recovery and sensitivity at low input levels. A Peptide precursor identification from increasing input of K562 lysate, processed with SPEC. Bars show total identifications (lighter color) and those quantified at CV < 20% (darker color). **b** Same as in **a** but for protein group identifications. **c** Protein group identifications from 50 to 500 fixed HEK cell shapes.

Discussion

SPEC advances proteomic sample preparation, offering a robust and scalable solution to long-standing challenges in the field. Its defining feature is the confinement of digestion and processing to nanoliter-scale volumes within enclosed solid-phase tips, by which SPEC overcomes critical limitations of sample loss, inefficient proteolysis, and poor recovery - while uniquely offering both fast digestion kinetics and full compatibility with strong detergents. This not only improves performance but redefines the technical limits of sensitivity, throughput, and sample compatibility.

While numerous strategies have been developed to improve proteomics sample preparation most suffer from either workflow complexity, incompatibility with detergents, or inefficiency at sub-microgram inputs. SPEC differs fundamentally by concentrating proteins within a defined resin volume of 50–200 nL – and even less for extremely small protein amounts - and enabling digestion of sample molecules reversibly coordinated on the solid phase. This minimizes void volume and surface exposure while maintaining high enzyme access, resulting in reproducible, complete digestion within minutes—even under harsh chemical conditions. We have found this versatile platform to perform equally well across a spectrum of challenging sample types and input amounts.

SPEC builds on two decades of tip-based innovations from our group and others, including STAGE tips, in-StageTip digestion, and one-tip reactors. However, by physically confining all steps to the resin phase and eliminating microliter-scale dilution, SPEC achieves a new level of miniaturization and recovery. Importantly, SPEC is highly automatable and fully compatible with many liquid handling robots and the Evosep One LC system, which will allow seamless integration and use in high-throughput workflows soon.

A further strength of SPEC lies in its modularity. The two-tip configuration—separating digestion and peptide cleanup onto independently optimized resins—enables tailored workflows. For instance, SAX-based digestion in SPEC tips offers faster and more complete cleavage than C18 or SCX tips, ideal for desalting, and enables elution directly into a regular Evotip. This modularity extends to chemical manipulations as well: on-tip multiplex labeling, digestion in isotopically labeled buffers, or enrichment steps for PTMs can all be implemented in confined volumes with minimal hands-on time. This flexibility resembles that of filter-based

protocols like FASP but without the limitations of device retention or solvent compatibility.

Beyond methodological advances, SPEC enables a new scale of proteomic experiments. The results presented here include comprehensive proteomes of FFPE tissues from multiple organs and an exceptionally deep, untargeted proteome of mouse skeletal muscle. These data were acquired in under 24 hours using a single, uniform protocol. Such throughput and robustness suggest that SPEC could serve as the default preparation method for most proteomics workflows—not only for low-input or "difficult" samples but across a broad range of biological materials. The fact that no specialized sample handling or cleanup was needed for FFPE or detergent-rich samples further simplifies pipeline development for large-scale or clinical applications.

Based on our results with single cell equivalent amounts and low numbers of FFPE shapes, we see SPEC as a central enabler for spatial and single-cell proteomics. Its sensitivity and robustness naturally lend themselves to spatially resolved workflows and we are exploring ways to directly combine laser capture microdissection (LCM) with SPEC. This approach preserves spatial context while achieving proteomic depth that is typically only possible in bulk samples. A powerful extension of SPEC is presented in our companion manuscript on nanoPhos (Oliinyk, Heymann et. al, 2025, *Biorxiv*), a phosphoproteomics workflow that combines SPEC-based digestion with zero-dead-volume enrichment and advanced DIA-MS. nanoPhos achieves exceptional sensitivity, identifying over 8,000 phosphosites from just 10 ng of input, and enables cell-type and region-specific phosphoproteomics from microdissected brain tissue. By quantifying over 17,000 phosphosites from defined neuronal cell populations, nanoPhos and phosphoDVP demonstrate how SPEC can serve as a foundation for spatially resolved, signaling-level proteomics, opening new avenues in neurobiology and tissue pathology.

In summary, SPEC offers a unified solution to many of the outstanding problems in proteomics sample preparation. It is robust, flexible, scalable, and sensitive—qualities that make it uniquely suited to meet the demands of both current and emerging applications. From plasma and FFPE to muscle and micromanipulated tissue regions, SPEC is poised to become a foundational technology in next-generation proteomics.

Acknowledgements

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Conflict of Interest

M.M. is an indirect shareholder in Evosep Biosystems. S.S. and N.B. are employees of Evosep Biosystems. All other authors declare no competing interests.

Methods

HeLa cell cultivation

Human epithelial carcinoma cells of the line HeLa (ATCC, S3 subclone) were cultured in Dulbecco's modified Eagle's medium containing 20 mM glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin. Cells were routinely tested for mycoplasma contamination. For dilution series experiments, HeLa cells were cultured until 80% confluency, harvested with 0.25% trypsin/EDTA and collected in 15 mL falcon tubes. Cells were then washed twice with cold TBS and pelleted by centrifugation at 200*g* for 10 min. Next, supernatant was aspirated, cells were snap-frozen in liquid nitrogen and stored until further use.

Cell lysate preparation

HeLa cells were lysed in 50 mM TEAB pH 8.5 containing 2 % SDS by boiling for 5 min at 98 C, followed by tip-sonication (10 pulses, 5 sec on, 5 sec off, 20% duty cycle). The samples were boiled again for 5 min at 95 °C. Protein concentration was determined using tryptophan fluorescence. Commercial K562 lysate was acquired from Promega and was used as a 10 ug/uL stock without further modification. Sample was diluted with 0.5% SDC in 50 mM TEAB to the required input concentration and transferred to preequilibrated SPEC tips.

Mouse experiments

Eight-week-old female mice of genetic background C57BL/6J were used for tissue analysis. Animals used were bred for scientific purposes, and the research in this project does not involve experiments on animals (as defined by law). All animals were sacrificed by CO_2 euthanasia prior to removal of organs in accordance with the European Commission Recommendations for the euthanasia of experimental animals (Part 1 and Part 2). Breeding, housing, and euthanasia of the animals are fully compliant with all German (i.e., German Animal Welfare Act) and EU (i.e., Directive 2010/63/EU) applicable laws and

regulations concerning care and use of laboratory animals.

Protein fluorescence labeling and high-content imaging of SPEC tips

Lysed proteins or pure enzymes trypsin and Lys-C at 0.5 ug/uL were labeled by incubating with a 1/10 (w/w) ratio of NHS-active esters linked to Alexa488 or Alexa647 respectively for 1h at room temperature. Labeled proteins were stored until use at -20 C. Imaging of SPEC tips was performed on the Axioscan 7 slide scanner (Zeiss) equipped with Colibri 7 LED light source and appropriate filter sets (for 488, 546, and 647nm channels). A 5x NA 0.8 Plan-Apochromat objective was used with the channels 488 (green) and 647 nm (red) for labeled proteins and enzymes respectively.

Organ collection and FFPE tissue preparation

After euthanasia, mice were infused through the heart with a rinsing solution (100 mM Tris pH 8.5 200 mM NaCl, and 20 mM EDTA) until blood was no longer visible. Organs except muscle fibers were removed, washed in rinsing buffer and fixed overnight in 4 % paraformaldehyde before paraffin embedding. Soleus and extensor digitorum longus muscles were removed from the mouse and freeze dried overnight to dehydration. Single fibers were pulled and pooled separately for each muscle type and stored below -65 C until further processing.

Tissue lysis for mouse organ experiments

FFPE tissue samples were deparaffinized by incubating approximately 300 µg of scraped off tissue sample in 300 µL n-Heptane for 1min at 30 °C and 700 rpm, discarding the solvent, repeating this step once more with n-Heptane, and two more times with 300 µl methanol. Deparaffinized FFPE tissues or muscle fibers were resuspended in a lysis buffer (50 mM TEAB pH 8.5, 2 % SDC) and boiled for 5 min at 95 °C, followed by tip-sonication (10 pulses, 5 sec on, 5 sec off, 20% duty cycle). The samples were then boiled again for 5 min at 95 °C. Protein concentration was then determined using tryptophan fluorescence. Sample was then diluted with 0.5% SDC in 50 mM TEAB to the required input concentration and transferred to preequilibrated SPEC tips.

Immunofluorescence Staining and Image Acquisition

HEK293 cells were cultured on 4 µm PPS frame slides (MicroDissect GmbH). Cells were fixed in 4% PFA for 5 min and washed with PBS. The cells were subsequently permeabilized with 0.1% Triton X-100 in

PBS for 5 minutes, followed by blocking in 5% BSA for 30 minutes at room temperature. Samples were then stained with Alexa Fluor 488-conjugated phalloidin for 1 hour at room temperature, followed by nuclear counterstaining with Hoechst 33342 for 5 minutes.

Fluorescence imaging was performed using a Perkin Elmer Opera Phenix high-content imaging system equipped with a 40× objective lens (numerical aperture 0.75). Images were acquired with 1×1 binning and 10% tile overlap to ensure complete coverage of the sample area.

Image Processing and Analysis

Acquired images were stitched together using SCPortrait[19] and subsequently imported into the Biological Image Analysis Software (BIAS) for further processing[18]. Cell segmentation was performed using Cellpose 2 with a custom-trained model optimized for our experimental conditions[20,21].

Laser microdissection

Contour coordinates were imported and various amounts of shapes (50, 100, 250, 500) were pooled and collected in triplicates. The shapes were cut using the LMD7 (Leica) laser microdissection system in a semi-automated mode with the following settings: power 56; aperture 1; speed 100; middle pulse count 1; final pulse 0; head current 55 – 62%; pulse frequency 2.9 and offset 190. The microscope was operated with the LMD v8.5.9136 software, and samples collected into 384-well plates, leaving the outmost rows and columns empty. Plates were then sealed, centrifuged at 2,000*g* for 10 min. The shapes were washed from the walls down to the bottom of the well using 28 µl of 100% acetonitrile (ACN) using a BRAVO pipetting robot (Agilent). The wells were then dried to completion in a SpeedVac at 45 °C. The plate was frozen at -20 °C for further processing.

Sample preparation of DVP samples

All liquid handling steps were performed on a Bravo pipetting robot. Samples were collected into 384-well TwinTec Eppendorf plates. Briefly, samples were lysed in 7 µl of lysis buffer (2% SDC, 0.1% DDM, 10 mM TCEP, 40 mM CAA, 50 mM TEAB, pH 8.5) at 95 °C for 15 min. Next, plates were centrifuged at 3000*g* for 3 min and lysates we transferred onto preequilibrated SPEC tips.

SPEC workflow

SPEC tips were prepared by placing one plug of strong-anion-exchange (SAX) material or strong-cation-exchange (SCX) material (3M Empore) in a pipette tip with

a blunt-ended syringe needle. For C18 experiments commercially available C18 Evotip Pure (Evosep Biosystems) were used instead. Before sample loading, SPEC tips were activated with 10 µl 100% dimethylsulfoxide (DMSO) and centrifugation at 700*g* for 1.5 min. Next, tips were preequilibrated with 50 µl SPEC Equilibration buffer (SAX: 20 mM CAPS, 0.01 % DDM in ddH_2O, SCX: 50% MeOH, 1 % FA, 0.01 % DDM in ddH_2O, C18: 1 % FA, 0.01 % DDM in ddH_2O,) and centrifuged at 700*g* for 2 min. Protein sample was diluted in equilibration buffer on the tip in ratio 1:10 for SAX and SCX and equilibration buffer and 75 % ACN for C18 Evotips and loaded on the solid support material by centrifugation at 200*g* for 10 min. SAX and SCX tips were washed once with 20 µl 50 mM TEAB 0.01% DDM and C18 tips with 1% FA, 0.01% DDM in ACN by centrifugation at 700xg for 1.5min. Proteins were on-tip digested by adding 5 µl of digestion buffer (0.25 µg/µl trypsin/LysC mix in 50 mM TEAB) and centrifuging for 20 sec at 100*g*. After digestion, peptides from SAX and SCX SPEC tips were directly eluted into prepared Evotips Pure by addition of 20 µl elution buffer (SAX: 0.01 % DDM, 1 % FA in ddH_2O, SCX: 0.01% DDM, 100mM CAPS in ddH_2O). C18 SPEC tips were centrifuged at 700xg for 1min to remove the digestion buffer and washed once in EvoA.

In-solution digest

For direct comparison, in-solution digestion was performed on pre-equilibrated SPEC tips. Tips were prepared through the equilibration step, then sample and digestion buffer (identical to SPEC workflows) were added and the volume adjusted to 40 µL with 50 mM TEAB, 0.01% DDM. Samples were centrifuged for 20 sec at 100xg. Digestion was performed for the same duration as standard SPEC workflows. Following digestion, peptides were loaded by centrifugation at 200g for 10 min after adding either 50 µL of 20 mM CAPS with 0.01% DDM (SAX SPEC tips) or 2% FA with 0.01% DDM (SCX and C18 tips). Peptide elution was performed according to standard SPEC protocols.

PAC digest

For PAC digestion, 5 µL MagReSyn® Hydroxyl beads (ReSyn Biosciences) were added to 40 µL ACN in a twin.tec LoBind plate (Eppendorf). Samples were added and beads briefly resuspended, then incubated for 10 min at room temperature. Following incubation, beads were washed once with 50 µL ACN. Digestion was initiated by adding 5 µL digestion buffer (identical to SPEC workflows) and adjusting the final volume to 40 µL with 50 mM TEAB, 0.01% DDM. After digestion, samples were quenched with 40 µL of 2% FA, 0.01%

DDM and subsequently loaded onto Evotips.

Peptide loading of C-18 tips

C-18 tips (Evotip Pure, Evosep) were washed once with 50 µl of buffer B (99.9% ACN, 0.1% FA), activated for 1 min in 2-propanol and equilibrated with 50 µl of buffer A. Samples were then eluted into 100 µl of buffer A in the tip, which was then centrifuged for a few seconds. After peptide binding, the disk was further washed once with 75 µl buffer A and further overlayed with 150 µl buffer A. All centrifugation steps were performed at 700*g* for 1 min, except sample loading for 2 min.

LC-MS/MS analysis

The samples were analyzed using an Evosep One LC system (Evosep) coupled to an Orbitrap Astral Zoom mass spectrometer (Thermo Fisher Scientific). Peptides were eluted from the Evotips using a 'Whisper Zoom' gradient with a throughput of 80 samples per day on an Aurora Rapid column of 5-cm length, 75-µm-internal diameter, packed with 1.7 µm C18 beads (IonOpticks). The column temperature was maintained at 60C using a column heater (IonOpticks). The Orbitrap Astral Zoom was equipped with an EASY-Spray source (Thermo Fisher Scientific). An electrospray voltage of 1,900 V was applied for ionization, and the radio frequency level was set to 40. Orbitrap MS1 spectra were acquired from 380 to 980 m/z at a resolution of 240,000 (at m/z 200) with a normalized automated gain control (AGC) target at 500% and a maximum injection time of 3 ms. For the Astral MS/MS scans in narrow-window data-independent acquisition (nDIA) mode, we used 3 Th fixed windows with a maximum injection time of 6 ms. The isolated ions were fragmented using high-energy collisional dissociation with 25% normalized collision energy.

Data analysis

Experiment-specific raw files were searched together with match-between run in library-free mode with DIA-NN v.1.9. A FASTA file containing only canonical sequences was obtained from UniProt (21,413 entries, obtained in May 2024). We allowed a missed cleavage rate of up to 2, and set mass accuracy to 19 and MS1 accuracy to 5 and the scan window to 6. Proteins were inferred on the basis of genes, and the neural network classifier was set to 'single-pass mode'. 'Report.parquet' files were analysed in python 3.11 using custom analysis scripts.

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