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Protocol

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Filter-aided expansion proteomics for the spatial analysis of single cells and organelles in FFPE tissue samples

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Abstract

Filter-aided expansion proteomics (FAXP) is a spatial proteomics approach designed for high-resolution analysis of formalin-fixed, paraffin-embedded (FFPE) tissues. Here we describe the integration of hydrogel-based tissue expansion with mass spectrometry, enabling isotropic expansion and robust protein retention while preserving spatial features. The FAXP workflow consists of several sequential steps, including tissue section dewaxing, in situ protein anchoring, hydrogel embedding, homogenization, staining, isotropic expansion, microdissection and filter-aided in-gel digestion to maximize peptide recovery. The Protocol integrates laser capture microdissection, enabling the precise isolation of single cells and subcellular components for subcellular spatial proteomics analysis. The approach achieves up to a fivefold linear expansion factor of FFPE tissue, including extracellular matrix-rich samples such as colorectal cancer, with less than 6% distortion, enabling the identification of an average of 2,368 proteins from single mouse liver nucleus shape and 3,312 proteins from single mouse liver cell shape using an Astral mass spectrometer. The method is compatible with diverse tissue types, including extracellular matrix-rich specimens, and integrates seamlessly with imaging workflows, such as immunostaining, for spatially resolved proteomic analysis. FAXP enables researchers to obtain comprehensive proteomic profiles with strong reproducibility and high sensitivity. The entire workflow takes ~27 h and requires only commercially available reagents and supplies and is thus accessible for researchers with intermediate expertise in tissue processing, microscopy and proteomics. FAXP can advance spatial proteomics-based studies, in particular of cancer heterogeneity, neurodegenerative diseases and cellular microenvironments within FFPE tissues, including archival clinical samples.

Key points

- Filter-aided expansion proteomics enables the spatial proteomics analysis of archival formalin-fixed, paraffin-embedded specimens, as well as of fresh frozen samples (following chemical fixation). It can handle a diverse range of tissue types, including liver and kidney, and extracellular matrix-rich tissues such as colorectal cancer.
- Filter-aided expansion proteomics, integrated with laser capture microdissection and image guidance, achieves bona fide single-cell shape and single-organelle shape proteomics with improved reproducibility and robustness.

Key references

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Introduction

Expansion microscopy (ExM) is a method that physically magnifies biological specimens, enabling super-resolution imaging with conventional diffraction-limited microscopes[1]. By embedding samples into a swellable hydrogel and isotropically expanding them, ExM operates with the voxel size of super-resolution microscopy and the voxel rate of diffraction-limited microscopes, revealing spatial details beyond the diffraction limit of light[2–5]. In addition, various existing ExM protocols are optimized to retain biomolecules of choice, such as nucleic acids, proteins and lipids, in their spatially accurate positions, facilitating downstream analytical workflows[5,6]. This capability has enabled advancements in spatial transcriptomics[7], lipidomics[8], matrix-assisted laser desorption/ionization mass spectrometry (MS) imaging[9], multiplexed imaging[10] and single-molecule structural visualization[11]. Integrating ExM with these 'omics' approaches has immense potential for advancing spatial biology and enabling multiomics studies.

Recent advancements in liquid chromatography–tandem MS (LC–MS/MS) have enabled the effective analysis of minute tissue samples (down to a few mammalian cells) obtained through laser capture microdissection (LCM). One such innovation is deep visual proteomics (DVP)[12–15], which integrates artificial intelligence (AI)-driven image analysis, automated LCM and high-sensitivity MS. This approach has enhanced the ability to analyze heterogeneous tissues at single-cell resolution by combining precise spatial sampling with comprehensive proteome profiling. However, despite such advancements, substantial hurdles remain in achieving robust subcellular spatial proteomics. The integration of ExM with bottom-up proteomics has emerged as a transformative approach to effectively address all of these challenges[16].

Here, we present the detailed protocol for filter-aided expansion proteomics (FAXP)[17], an ExM-based spatial proteomics protocol optimized for formalin-fixed, paraffin-embedded (FFPE) tissues (Fig. 1). This method combines hydrogel-based tissue expansion with filter-aided in-gel digestion (in-tip digestion for short) and LCM to achieve high-resolution subcellular profiling. Designed for reproducibility and scalability, FAXP integrates seamlessly into spatial proteomics workflows, offering improved peptide recovery, proteomic depth and compatibility with diverse tissue types, including extracellular matrix (ECM)-rich and FFPE specimens, such as colorectal cancer (CRC). The Protocol is streamlined to be completed in ~27 h and provides detailed step-by-step guidance for researchers, from tissue preparation to data acquisition. By enabling precise spatial analysis, FAXP represents a robust and scalable tool for spatial proteomics in basic and clinical research.

Development of the Protocol

The development of FAXP builds on advancements in ExM and spatial proteomics to address limitations in the existing workflows. Early efforts to combine ExM with proteomics demonstrated the potential for tissue expansion to enhance spatial resolution but faced challenges[18]. These included insufficient expansion factors and inefficient peptide retrieval, which limited the proteomic depth and resolution achievable with ExM-based workflows.

Around the same time as Drelich et al.[18], we developed ProteomEx[19], which combines optimized hydrogel-based tissue expansion with in-gel digestion to enhance proteomics analysis. ProteomEx achieved up to an eightfold linear (over 500-fold volumetric) expansion in paraformaldehyde-fixed mouse brain tissues and identified over 1,000 proteins from a tissue volume equivalent to ~160 HeLa cells. While ProteomEx improves spatial resolution and proteome coverage, it faces challenges with ECM-rich and FFPE samples, low throughput and restricted resolution.

Building on this foundation, we further developed FAXP[17], a refined workflow specifically designed to overcome these challenges. FAXP integrates hydrogel-based tissue expansion with in-tip digestion inspired by methods such as FASP[20], One-Tip[21] and SISPROT[22]. The optimized hydrogel composition ensures isotropic expansion and robust protein retention, accommodating a broader range of tissue types, including ECM-rich and FFPE specimens. Furthermore, FAXP is compatible with LCM and immunostaining, enabling precise spatial

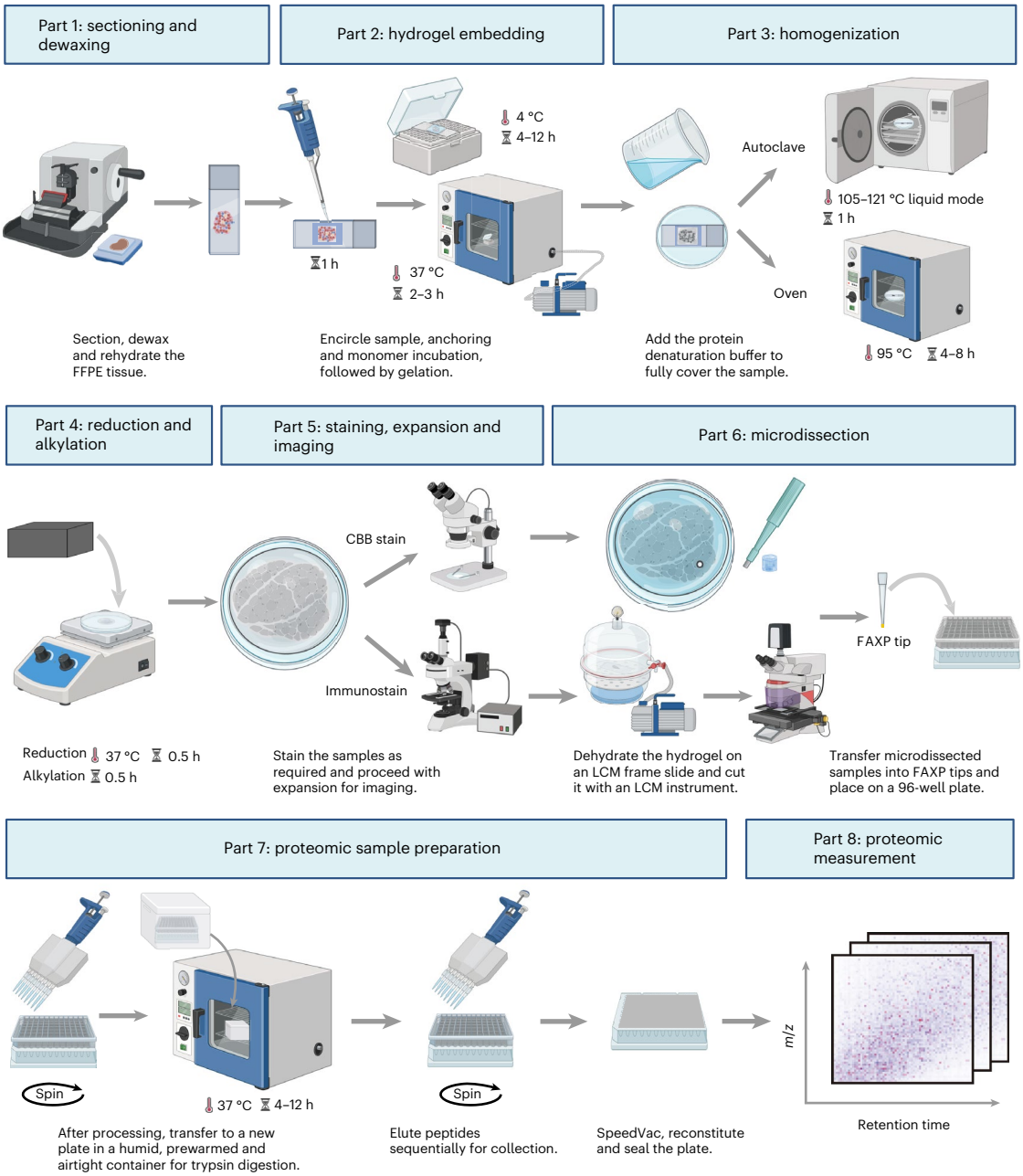

Fig. 1 | Workflow of FAXP. The FAXP protocol starts with sectioning and dewaxing of FFPE tissue blocks (Part 1), followed by chemical treatment and hydrogel embedding (Part 2). Homogenization is performed under controlled conditions, either at 105–121 °C in an autoclave or at 95 °C, based on tissue complexity and expansion needs (Part 3). After reduction and alkylation, samples proceed to staining, isotropic expansion and imaging (Parts 4 and 5). ROIs are selected using manual or LCM (Part 6). Proteomic sample preparation involves filter-aided in-gel digestion, optimized for high throughput (Part 7), followed by MS-based proteomic analysis (Part 8).

proteomics down to the single-nucleus level, identifying an average of 2,368 proteins per hepatocytic nucleus in FFPE tissues.

Overview of the FAXP protocol

The FAXP workflow consists of eight main steps designed to enable high-resolution spatial proteomics while maintaining flexibility for various sample types (Fig. 1). It begins with

sectioning and dewaxing for FFPE samples to prepare tissue slices. Hydrogel embedding follows, involving protein modification with anchor groups, hydrogel monomer incubation and in situ free-radical polymerization. Anchoring times are adjusted on the basis of sample thickness to ensure robust biomolecule retention and matched expansion factors at both micro- and macro-levels. Homogenization is then performed using vigorous denaturing buffers, either at 95 °C or via autoclaving. While autoclaving effectively homogenizes complex tissues, such as ECM-rich specimens, the high temperature and pressure may denature nuclear components, potentially compromising the efficacy of nuclear staining methods such as DAPI.

Reduction and alkylation are then applied to the entire hydrogel to enhance protein denaturation and streamline subsequent proteomic sample preparation. Alternatively, these steps can be incorporated later during proteomic workflows. The following step involves staining, where samples undergo colorimetric staining, for example, with Coomassie Brilliant Blue (CBB) staining or immunostaining, followed by isotropic expansion and optical imaging.

Microdissection is then performed on the expanded samples, either manually or using LCM. While manual dissection offers high spatial resolution, LCM achieves precision at the subcellular level. Importantly, the manual approach improves accessibility for laboratories without LCM capabilities or for studies not requiring single-cell resolution. Proteomic sample preparation follows, wherein micro-dissected samples are processed in custom-made FAXP tips using an in-tip digestion method. These tips can be manually fabricated in various formats to accommodate different sample sizes, enhancing scalability and accessibility. This flexibility makes FAXP adaptable to diverse research needs while maintaining high throughput, peptide recovery and reproducibility. Finally, the extracted peptides are analyzed by MS using data-dependent acquisition (DDA) or data-independent acquisition (DIA) modes, generating in-depth proteomic datasets. This streamlined Protocol minimizes sample loss, enhances sensitivity and integrates seamlessly into high-throughput workflows, as depicted in Fig. 1.

Applications of the method

The FAXP protocol has demonstrated its strength in high-resolution spatial proteomics, particularly in FFPE tissue sections, by enabling precise subcellular profiling and comprehensive proteomic analysis. Its robust protein retention, compatibility with imaging workflows and adaptability make it a versatile tool for diverse research fields.

In cancer research, FAXP's compatibility with FFPE tissues, a common format for clinical and archival samples, is a critical advantage[23,24]. This capability allows researchers to investigate tumor microenvironments, map protein distributions across disease progression stages and identify region-specific biomarkers. The integration of FAXP with LCM enables detailed single-cell and subcellular proteome analyses, providing insights into cellular heterogeneity and microenvironments essential for understanding cancer progression and identifying therapeutic targets.

The workflow can also be adapted for non-FFPE tissue types, including fresh-frozen and vibratome-sectioned tissues, by modifying initial preparation steps, such as omitting dewaxing and optimizing embedding protocols. These adaptations extend their applicability to developmental biology and organotypic culture studies, where preserving delicate tissue structures is essential.

FAXP's ability to physically enlarge samples while preserving their spatial distribution, along with its compatibility with LCM, makes it particularly suited for targeted analyses of subcellular structures such as nuclear compartments. This integration facilitates the precise isolation of these subcellular components, facilitating detailed studies of organelle-specific protein dynamics, metabolic processes and their roles in conditions such as neurodegenerative diseases, metabolic disorders and cancer, where organelle function is critical[25].

FAXP also holds promise for analyzing ECM-rich tissues, which pose analytical challenges due to extensive natural crosslinking, dense organization and frequent glycosylation. These features often reduce protease accessibility and peptide recovery. In FFPE samples, formalin fixation further introduces additional chemical crosslinks, exacerbating these difficulties. FAXP's physical expansion, combined with enhanced homogenization strategies such as elevated sodium dodecyl sulfate (SDS) concentrations, ethylenediaminetetraacetic acid

(EDTA) addition and autoclaving, helps loosen ECM structures and improve overall tissue homogenization. In future adaptations, incorporating a de-glycosylation step before or during homogenization could further facilitate tissue softening and expansion uniformity, especially in glycosylation-rich regions. Although glycoproteomics was not evaluated in this study, the workflow could potentially support the detection of formerly glycosylated peptides, suggesting possible compatibility with downstream glycosylation-inference pipelines. This adaptability broadens FAXP's potential application to studies of ECM remodeling and site-specific glycoproteomic features in cancer and fibrotic diseases.

Furthermore, FAXP's high protein retention and the structural stability of the expanded hydrogel after dehydration suggest its adaptability for integration with other spatial proteomics methods, such as matrix-assisted laser desorption/ionization MS imaging[9,26] and imaging mass cytometry[10]. Although the protocol is currently optimized for LC–MS/MS-based profiling, its potential for integration with molecular imaging platforms remains underexplored and may require further adaptation. The workflow may also support high-resolution immunostaining, including multiplexed staining for imaging workflows[27,28]. Although not yet fully explored, FAXP's ability to retain proteins during expansion, combined with findings from one-step nanoscale ExM showing that expansion techniques can reveal individual protein shapes[11], suggests its potential for studying protein complexes, protein-protein interactions and disease-associated aggregates. This approach could bridge structural biology and spatial proteomics, offering new opportunities to investigate cellular signaling and structural dynamics.

Comparison with other methods

The FAXP protocol builds on previous advancements in spatial proteomics, particularly ProteomEx[19], addressing key limitations while introducing improvements. Compared with ProteomEx, FAXP offers improved sample handling and processing throughput, with compatibility across a wider range of tissue types, including FFPE sections, cryopreserved tissues and vibratome-sectioned specimens. By supporting both thin (below 10 µm) and thick (up to 120 µm) sections, FAXP accommodates diverse experimental needs, making it adaptable for various research applications.

A major advancement in FAXP is its substantially improved spatial resolution. ProteomEx[19] achieves a lateral resolution of 160 µm and a volumetric resolution of 0.61 nL, whereas FAXP[17] achieves a lateral resolution of 73 µm and a volumetric resolution of 0.042 nL, representing a 14.5-fold improvement. This enhanced resolution allows FAXP to perform precise spatial proteomics manually without the need for high-end instrumentation such as LCM. However, in laboratories equipped with LCM, FAXP extends its capabilities, enabling subcellular analyses of organelles such as nuclei. In addition to subcellular analyses, the combination of LCM and FAXP enables a more accurate dissection of cellular heterogeneity.

The incorporation of in-tip digestion in FAXP further enhances peptide recovery and reproducibility compared with conventional in-gel digestion workflows. By utilizing FAXP tips, the Protocol achieves high throughput. Moreover, FAXP supports comprehensive protein identification through DIA mode using advanced instruments such as Orbitrap Astral, improving proteome coverage and quantification accuracy.

When compared with advanced spatial proteomics methods such as DVP[12–15], which integrate LCM with AI-assisted regions-of-interest (ROIs) selection, FAXP provides complementary advantages. FAXP achieves higher spatial resolution through isotropic sample expansion. Super-resolution imaging of expanded samples using conventional microscopes offers another distinct advantage over DVP, which depends on diffraction-limited microscopes for imaging. Consequently, FAXP enables the selection of ROIs based on the nanoscale features of cells in the sample. Furthermore, the integration of FAXP with LCM minimizes tissue damage and improves sampling accuracy, making it particularly effective for single-cell and organelle-specific analyses.

While FAXP introduces enhancements over ProteomEx and offers complementary strengths compared with DVP, certain practical considerations remain. The reliance on hydrogel-based expansion requires careful optimization to achieve isotropic expansion and compatibility with diverse tissue types. Furthermore, unlike DVP, which utilizes AI-guided ROIs

selection to streamline workflows, FAXP relies on manual selection and analysis. This approach may demand additional time and expertise, particularly in large-scale studies. However, these aspects are not inherent limitations of FAXP but rather reflect its current stage of development.

Experimental design

The FAXP protocol is highly versatile and adaptable to various experimental contexts, but certain key design considerations are essential for its successful implementation. Below, we outline important factors to guide researchers in customizing the Protocol for their specific needs, along with necessary controls to ensure reliability and reproducibility.

Tissue selection and preparation

For optimal results with the FAXP protocol, FFPE tissue sections should be cut onto glass slides described in this Protocol rather than adhesive-positive-charged slides. Positive-charged slides can cause strong adhesion, especially for older or poorly stored samples, making it difficult to remove the intact tissues during processing. Using the recommended glass slides and proper sample handling minimizes the risk of tissue damage or loss.

Hydrogel in situ polymerization

In situ protein anchoring time should be adjusted primarily on the basis of section thickness. For example, 4–10-µm sections typically require 1–2 h, while 10–100-µm sections may require 2–4 h. In addition, ECM-rich tissues, such as the colon, may require longer anchoring time compared with softer tissues, such as the brain; however, section thickness remains a more critical factor. For optimal hydrogel preparation, ensure that the sample does not contain any excess liquid before adding the activated monomer solution. Anaerobic conditions during polymerization are essential for achieving uniform and complete gel formation. For homogenization, lower temperatures (for example, 95 °C) require less crosslinker to achieve higher expansion factors, whereas higher temperatures, such as autoclaving at 105 °C, require more crosslinker to ensure structural stability in complex tissues.

Staining and imaging

For staining hydrogel samples, higher antibody concentrations than those used in conventional protocols are recommended. Using a primary antibody followed by a secondary antibody, rather than a directly fluorophore-conjugated primary antibody, enhances signal strength. Based on our experience, both primary and secondary antibodies are typically used at 1:100 to 1:200 dilutions, with overnight incubation to ensure adequate penetration and strong signal intensity.

During imaging, ensure that the sample side of the thick hydrogel is positioned close to the objective lens. To prevent sample drift, fix the hydrogel using either low-melting-point agarose[24,29] or a sealing film[27]. For applications proceeding to proteomics, a sealing film is preferred for better compatibility with subsequent steps.

Microdissection

For LCM collection of immunostained samples, proper drying of the hydrogel is essential. Maintaining a minimal salt concentration, such as 0.01× PBS, prevents hydrogel shrinkage during this process. Applying poly-L-lysine to the LCM steel frame slide further enhances sample adhesion. To expedite drying, the sample should be maintained at 25 °C in a vacuum desiccator.

LCM cutting is affected by factors such as temperature, humidity, airflow and static electricity. Maintaining room temperature at ~20–25 °C and relative humidity between 40% and 60%, using a windshield cover on the microscope and grounding the equipment can help minimize these issues. Hydrogel expansion improves the capture rate for regions that, after expansion, correspond to the same physical area as pre-expansion samples. However, achieving high efficiency near the single-cell level remains challenging owing to low collection rates (~25% at 0.002 mm^2, as compared with nearly 100% capture for areas above 0.05 mm^2) (ref. 30). Optimizing environmental and operational conditions is critical for maximizing LCM performance.

Proteomics sample preparation and measurement

High standards of cleanliness are essential during the assembly of FAXP tips to avoid contamination, such as keratins. The pad at the tip end must be securely tightened to create an optimal space for enzymatic digestion and other sample preparation steps. *n*-dodecyl-β-D-maltoside (DDM) is required for effective sample processing[31]; however, as the Protocol omits a desalting step, it is crucial to minimize the DDM concentration to as low as 0.015% (wt/vol, 0.15 mg/mL) to reduce potential interference with MS acquisition.

To further ensure efficient digestion and reliable proteomic performance, the design of the FAXP tip assembly was optimized for microscale applications. The self-assembled FAXP tips are designed to function primarily as reaction chambers for in-gel digestion, with the primary design consideration being the minimization of material volume to reduce enzyme consumption and improve peptide recovery. This design differs from that of commercial devices intended for peptide trapping, desalting and concentration, such as Evotip. Several commercial alternatives could potentially be suitable for similar applications. Although the FAXP approach demonstrates high reproducibility, a systematic comparison with commercial alternatives remains an important direction for future investigation. In addition to performance optimization, the use of self-made filters improves accessibility and cost-efficiency, facilitating broader adoption.

Troubleshooting and pilot testing

For researchers new to FAXP or spatial proteomics, initial experiments should be conducted on test blocks, such as mouse brain or liver FFPE sections, to become familiar with the Protocol. These trials will allow users to determine key steps, such as hydrogel embedding, staining and microdissection, before applying the Protocol to experimental samples. Pilot studies can also help refine conditions for more challenging tissue types or novel applications.

Controls

To ensure reliability, the following controls are recommended:

- Hydrogel process controls: process mouse liver or brain FFPE samples in parallel to assess the workflow consistency and distinguish tissue-specific issues from process-related ones.
- Positive controls: test the antibodies on tissue sections using conventional methods before expansion to confirm specificity.
- Negative controls: use only the corresponding secondary antibody post-expansion, without the primary antibody, to rule out nonspecific signals.
- MS quality controls: use a HeLa protein digest standard (for example, 200 pg) during MS acquisition to ensure optimal LC–MS/MS performance and reproducibility.

By considering these experimental design principles, researchers can tailor the FAXP protocol to meet their specific objectives while ensuring reproducibility and high-quality results.

Expertise needed to implement the FAXP protocol

The FAXP protocol provides detailed guidance, enabling researchers with basic molecular biology expertise to perform hydrogel-related steps, including tissue sample preparation, embedding, homogenization, reduction and alkylation, staining, expansion and imaging. Minimal prior experience is needed to begin with mouse brain or liver FFPE sections. For immunostaining, familiarity with standard techniques and access to a widefield or confocal fluorescent microscope are sufficient. While manual microdissection is straightforward, LCM expertise is required for single-cell and subcellular analyses. Proteomics sample preparation, including FAXP tip assembly, is supported by detailed instructions and supplementary videos. For MS analysis, access to advanced instruments and consultation with an MS expert is essential, as expertise in MS operation and data analysis is necessary. Moreover, troubleshooting skills are advised to adapt the Protocol for diverse tissue types and experimental setups, ensuring flexibility and reproducibility.

Limitations

While the FAXP protocol offers robust solutions for spatial proteomics, several limitations should be considered. Although it is effective in handling many ECM-rich samples, as highlighted in applications of the method, additional optimization may be required for tissues that are particularly dense or heavily crosslinked, where expansion-induced cracking or incomplete homogenization can occur. This challenge becomes more pronounced when higher linear expansion factors (LEFs) are needed for subcellular analyses, such as profiling smaller organelles such as mitochondria and peroxisomes, which require precise structural preservation and isolation. In addition, the reliance on LCM for subcellular precision enhances spatial resolution but introduces constraints related to accessibility and efficiency. LCM equipment is not universally available, and its sample collection rate is particularly low when targeting near single-cell resolution[30], potentially limiting throughput for large-scale studies.

Materials

Biological materials

Mouse liver samples were collected from C57BL/6J wild-type mice, while archival FFPE CRC tissue samples were obtained from three patients with CRC and sectioned to a thickness of 8 µm.
▲ **CAUTION** All procedures were conducted in accordance with institutional and national regulations. All animal experiments adhered to the Westlake University Animal Care Guidelines and were approved by the institutional animal care and use committee. The collection and use of human tissues were approved by the institutional ethics committee of the Second Affiliated Hospital of Zhejiang University School of Medicine and the ethics committee of Westlake University.

Reagents

For FFPE sample preparation

- Paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710)
  ▲ **CAUTION** Toxic, carcinogenic, and irritant. Handle in a fume hood while wearing gloves, goggles and a lab coat.
- 10× poly-L-lysine solution (Sangon, cat. no. E676002-0010)

(Optional) For H&E staining

- H&E staining solution (Shanghai Yuanye, cat. no. R20570-2*100mL)
- Differentiation solution, 1% (vol/vol) acid alcohol (Shanghai Yuanye, cat. no. R20778-500mL)
  ▲ **CAUTION** Flammable and irritant. Avoid inhalation or skin contact. Use gloves in a fume hood.
- Scott's solution (Shanghai Yuanye, cat. no. R20596-500mL)
- Neutral balsam (Macklin, cat. no. N861409-25mL)

For deparaffinization

- Heptane (Sigma-Aldrich, cat. no. 246654-2L)
  ▲ **CAUTION** Highly flammable and toxic. Use in a fume hood with gloves.
- Ethanol (EtOH; Sinopharm Chemical Reagent, cat. no. 10009218)
  ▲ **CAUTION** Flammable. Avoid open flames. Use in a ventilated area while wearing gloves.

For anchoring

- *N*-succinimidyl acrylate (NSA; TCI, cat. no. S0814)
  ▲ **CAUTION** Corrosive and toxic. Avoid inhalation, skin and eye contact. Handle in a fume hood while wearing gloves.
- Dimethyl sulfoxide (Sigma-Aldrich, cat. no. D5879-100mL)
  ▲ **CAUTION** May enhance absorption of harmful chemicals through the skin. Handle with care and gloves.
- 3-(*N*-morpholino)propanesulfonic acid, 0.5 M, pH 7.0 (Macklin, cat. no. M885700)
- 2-(*N*-morpholino)ethanesulfonic acid, 0.2 M, pH 6.0 (Macklin, cat. no. M885671)

For gelation

- Pentaerythritol allyl ether (PAE; Sigma-Aldrich, cat. no. 251720-100G)
- Tetrahydrofuran (Macklin, cat. no. T818769-500mL)
  ▲ **CAUTION** Highly flammable and irritant. Use gloves in a ventilated fume hood.
- *N*,*N*-dimethylacrylamide (Sigma-Aldrich, cat. no. 274135-500mL)
  ▲ **CAUTION** Toxic. Use in a fume hood with gloves to avoid exposure.
- Sodium methacrylate (Sigma-Aldrich, cat. no. 408212-50G)
  ▲ **CAUTION** Hygroscopic. Avoid inhaling dust by using gloves, a mask and a lab coat during handling. Store in a dry environment to prevent moisture absorption.
- Ammonium persulfate (APS; Sigma-Aldrich, cat. no. A3678)
  ▲ **CAUTION** Oxidizing agent and irritant. Avoid contact with skin and eyes and inhalation.
- *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED; Sigma-Aldrich, cat. no. T7024)
  ▲ **CAUTION** Toxic and irritant. Avoid contact with skin and eyes, and prevent inhalation. Handle in a fume hood with gloves and goggles.
- Repel-silane (Macklin, cat. no. R917945-200mL)

For homogenization

- SDS (Macklin, cat. no. S817790-500g)
  ▲ **CAUTION** Avoid inhalation, and contact with skin or eyes. Use gloves, a mask and handle in a well-ventilated area.
- Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2·$2H_2O$; Sigma-Aldrich, cat. no. E4884-100g)
- Tris base (Sigma-Aldrich, cat. no. T6791-500G)
- NaCl (Sigma-Aldrich, cat. no. S9888-500G)

For reduction and alkylation

- Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Adamas-beta, cat. no. 61820E)
  ▲ **CAUTION** Corrosive and toxic. Handle in a fume hood with gloves.
- Dithiothreitol (DTT; Thermo Fisher Scientific, cat. no. R0861)
  ▲ **CAUTION** Harmful if inhaled or in contact with skin. Use gloves and handle in a well-ventilated area.
- Iodoacetamide (IAA; Sigma-Aldrich, cat. no. I6125-25G)
  ▲ **CAUTION** Harmful and irritant. Handle with gloves in a well-ventilated area.

For staining

- CBB R-250 (BBI, cat. no. A610037)
- Citrate antigen retrieval solution (Leagene, cat. no. IH0305)
- Immunostaining blocking/primary antibody dilution buffer (BBI, cat. no. E674004-0100)
- ASGR1 polyclonal antibody (Proteintech, cat. no. 11739-1-AP)
- Goat anti-rabbit IgG H&L (Alexa Fluor 555) preadsorbed (Abcam, cat. no. ab150086)
- DAPI staining solution (Abcam, cat. no. ab228549)
- SYTOX deep red nucleic acid stain (Thermo Fisher Scientific, cat. no. S11380)

For enzymatic digestion and peptide elution

- Formic acid (FA; Thermo Fisher Scientific, cat. no. A117-50)
  ▲ **CAUTION** Corrosive. Handle in a fume hood with gloves, goggles and a mask.
- Methanol (General-Reagent, cat. no. G75851B)
  ▲ **CAUTION** Flammable and toxic. Handle with gloves in a fume hood.
- Trypsin (Beijing Shengxia Proteins, cat. no. HLS TRY001C-100μg)
- Ammonium bicarbonate (ABB; Sigma-Aldrich, cat. no. A6141-1kg)
- Acetonitrile (ACN; General-Reagent, cat. no. G80988B; Thermo Fisher Scientific, cat. no. A955-4)
  ▲ **CAUTION** Flammable and toxic. Avoid inhalation and use in a fume hood.
- MS-grade water (Thermo Fisher Scientific, cat. no. W6-4)

- Trifluoroacetic acid (TFA; Adamas-beta, cat. no. 81548K)
  ▲ **CAUTION** Corrosive and volatile. Handle in a fume hood with gloves and goggles.
- DDM (Aladdin, cat. no. N475300-250mg)

Equipment

- Slide, double-side and one end frosted, nonground edges (Sangon, cat. no. F518207-0001)
- Automated rotary microtome (Leica, cat. no. HistoCore AUTOCUT)
- Microtubes, 1.5 mL (Axygen, cat. no. MCT-150-C)
- Analytical balance (Mettler Toledo, cat. no. ME104E)
- Pipettes (Eppendorf, cat. nos. 3123000012, 3123000020, 3123000055 and 3123000063)
- Pipette tips (Axygen, cat. no. T-300, T-200-Y, T-1000-B)
- Thermo shaker (Allsheng, cat. no. MS-100)
- pH meter (INESA, cat. no. PHS-3C)
- Vortex mixer (Allsheng, cat. no. MTV-1)
- Anaerobic gas-producing bag (Hopebio, cat. no. HBYY001)
- Vacuum drying oven (Shanghai Yiheng, DZF-6032)
- Autoclave (TOMY, SX-700)
- Stereo zoom microscope (Zeiss, Axio Zoom.V16)
- Confocal microscope (Nikon, CSU-W1 SoRa)
- Single specimen, no. 1.5 coverslip, 98 mm × 67 mm viewing area, uncoated (MatTek, cat. no. P384G-1.5-10872-C)
- Mini vacuum pump (Kylin-Bell, GL-810)
- Laser microdissection microscope (Leica, LMD7)
- FrameSlides, polyester-membrane 0.9 µm, 50 pcs (Leica, cat. no. 11505188)
- 0.2-mL thin-wall polymerase chain reaction (PCR) tubes with flat cap, clear (Axygen, cat. no. PCR-02-C)
- Empore C18 47-mm extraction disks (Merck, cat. no. 66883-U)
- Low protein binding microcentrifuge tubes, 1.5 mL (Thermo Fisher Scientific, cat. no. 90410)
- QSP low retention pipette tips (Thermo Fisher Scientific, cat. no. TLR102-Q)
- C18 spin tips (Thermo Fisher Scientific, cat. no. 84850)
- Tips, Agilent Bravo, 250 µL, low-adsorption (BBI, cat. no. F630002-9001)
- Centrifuge (Eppendorf: 5430R, cat. no. 022620689; and 5810R, cat. no. 022625501)
- SpeedVac vacuum concentrator (Thermo Fisher Scientific, SPD2010)
- Glass inserts for 2-mL vials, 0.3 mL (Thermo Fisher Scientific, cat. no. 6PME03C1SSP)
- Trap column for nano liquid chromatography (LC) system (Thermo Fisher Scientific, cat. no. 160454)
- Nano-flow LC system (Thermo Fisher Scientific, Vanquish Neo ultra-high-performance LC system; Bruker Daltonics, nanoElute)
- Orbitrap Astral mass spectrometer (Thermo Fisher Scientific, cat. no. BRE725600)
- timsTOF pro mass spectrometer (Bruker Daltonics)

Software

- Fiji/ImageJ (https://fiji.sc/)[32,33]
- (Optional) MATLAB (https://www.mathworks.com/) or R (https://www.r-project.org/)[34]—for custom programming and advanced proteomics data analysis
- FragPipe (version 18.0)[35,36]
- DIA-NN (version 1.8.1)[37,38]

Reagent setup

NSA stock

Dissolve 100 mg of NSA in the anhydrous dimethyl sulfoxide to make the final volume at 10 mL. Store at 4 °C.

Protein-anchoring buffer
Mix 25 mL of 0.2 M 2-(*N*-morpholino)ethanesulfonic acid stock solution with 25 mL ddH_2O to make the final volume at 50 mL and test if the pH is at 6.0. Store at 4 °C.

Protein-anchoring solution
Mix 2 µL of NSA stock solution with 198 µL of protein-anchoring buffer, freshly prepared.

Anchoring termination buffer
Mix 2 mL of 0.5 M 3-(*N*-morpholino)propanesulfonic acid stock solution with 8 mL of ddH_2O to make the final volume at 10 mL and test if the pH is at 7.0. Store at 4 °C.

PAE stock solution
Dissolve 1.15 g PAE in the tetrahydrofuran to make the final volume at 10 mL and avoid light. Store at 4 °C.

Monomer stock solution

Reagents	Amount
N,*N*-dimethylacrylamide	3.137 g (~3 mL)
Sodium methacrylate	0.8624 g
PAE stock solution	269.5 µL
ddH_2O	4.380 mL
10% HCl	350 µL

Adjust pH to 6.5. Store at 4 °C. The concentration and ratio of reagents in the Protocol are fine-tunable and can be adjusted according to specific experimental requirements or operational needs. For example, increasing the proportion of PAE stock solution lowers the expansion factor but enhances resistance to vigorous homogenization procedures.

APS stock, 10% wt/vol
Dissolve 0.1 g APS in ddH_2O to make the final volume at 1 mL, freshly prepared.
▲ **CAUTION** APS is prone to inactivation over time or with improper storage, which may compromise the subsequent gelation process. Use freshly prepared APS solutions to ensure optimal performance.

TEMED stock, 10% vol/vol
Dissolve 0.1 mL TEMED in ddH_2O to make the final volume at 1 mL. Freshly prepare it and store at 4 °C.

Activated monomer solution

Reagents	Final percentage (% vol/vol)	Amount
Monomer stock solution	90	900 µL
APS stock	3	30 µL
TEMED stock	2	20 µL
ddH_2O	5	50 µL

Freshly prepare it with a pH of 6.5. Store at 4 °C before use.

Protein denaturation buffer

Reagents	Final concentration	Amount
Tris, 1 M, pH 8	50 mM	10 mL
SDS	20% wt/vol	40 g
EDTA-Na_2·$2H_2O$	25 mM	1.8612 g
NaCl, 5 M	200 mM	8 mL

Make the final volume at 200 mL, pH 7.0. Store at room temperature.

Reduction and alkylation reagent stocks
Prepare the reduction and alkylation solutions as follows: dissolve the appropriate amount of TCEP or DTT in 1× PBS (pH 7.4) to prepare 20 mM TCEP or 20 mM DTT, respectively. For alkylation, dissolve the required amount of IAA in 1× PBS (pH 7.4) to prepare 55 mM IAA, adjusting all solutions to a final volume of 25 mL. These solutions should be freshly prepared before use, and IAA must be protected from light to prevent degradation.
▲ **CAUTION** Use immediately to maintain effectiveness.

CBB solution
Mix CBB R-250 with 1× PBS to a final density of 1 mg/mL. Store at room temperature.

Trypsin stock solution
Add 200 µL of 50 mM acetic acid (pH 3) to 100 µg of trypsin, aliquot and store at −20 °C or −80 °C. The enzyme can be preserved for up to 2 months.

Digestion buffer
Mix 0.5 mL of 20% ACN with 0.5 mL of 100 mM ABB/0.03% (wt/vol, 0.3 mg/mL) DDM solution. Freshly prepare it.

Trypsin working solution
Dilute trypsin stock solution with digestion buffer to 5 ng/µL, adjust pH to 8–9 and prepare fresh.

Peptide extraction solution
Prepare the extraction buffers as follows: extraction buffer A consists of 2% ACN, 0.1% TFA and 0.015% (wt/vol, 0.15 mg/mL) DDM, while extraction buffer B contains 70% ACN, 0.1% TFA and 0.015% (wt/vol, 0.15 mg/mL) DDM. To minimize background signals in downstream MS analysis, DDM can optionally be omitted from both buffers, particularly for general in-tip digestion workflows.
▲ **CAUTION** Both buffers should be freshly prepared and stored properly before use.

MS buffer
Prepare the MS buffer with 2% ACN, 0.1% FA and 0.015% (wt/vol, 0.15 mg/mL) DDM. To minimize background signals in downstream MS analysis, DDM may be optionally omitted. Use freshly prepared buffer.

Equipment setup

Microscope
For brightfield imaging, such as CBB, samples were imaged using a stereo microscope (Zeiss Axio Zoom.V16), which provided a larger field of view suitable for visualizing stained hydrogel samples. The imaging process was controlled using ZEN v3.1 software.

For immunostained samples, both pre- and post-expansion, high-resolution imaging was performed with a confocal microscope (Nikon CSU-W1 SoRa) equipped with a 20× objective lens (0.8 NA). Image acquisition was managed using NIS-Elements AR v5.42.01 software.

Laser microdissection microscope
An LCM instrument, such as the Leica LMD7 laser microdissection microscope controlled by Leica LMD v8.4 software, was used for precise sample isolation. For visualizing nuclei, the Y5 filter cube was employed. Cutting parameters were optimized with the power set to 60, aperture between 17 and 20, speed ranging from 8 to 12, bridge size at 0, final pulse at 6, head current at 100% and pulse frequency between 120 and 150. For hepatocyte isolation, the RHOD ET filter cube was utilized under the same cutting parameters.

LC–MS/MS
For single hepatocyte and nucleus samples, proteomics data acquisition was conducted using an Orbitrap Astral mass spectrometer integrated with a Vanquish Neo ultra-high-performance LC system. Peptide separation was achieved with an Aurora Elite TS 15×75 C18

ultra-high-performance LC column. The LC method involved a 19.5-min gradient for peptide elution and a 5.5-min column washing step. The mobile phase composition included buffer A (98% MS-grade water, 2% MS-grade ACN and 0.1% FA) and buffer B (98% MS-grade ACN, 2% MS-grade water and 0.1% FA), with direct injection applied. MS was controlled using Thermo Scientific Xcalibur v4.7.69.37 and Orbitrap Astral Tune Application v1.0.100.40. Full-scan Orbitrap settings were optimized with a total carrier gas flow rate of 3.8 L/min, a resolution of 240,000, a scan-range from 400 to 800 m/z and a FAIMS (high-field asymmetric waveform ion mobility spectrometry) compensation voltage value of −42 V. The radio frequency lens was set to 45%, the normalized automatic gain control target was set to 500%, and the maximum IT was 100 ms. DIA MS2 scans were performed with a precursor mass range spanning 400–800 m/z and a scan range of 150–2,000 m/z. The DIA isolation window width was set to 20, with the maximum injection time at 40 ms. Higher-energy collisional dissociation normalized collision energy was set at 25%. The radio frequency lens was set at 45%, the normalized automatic gain control target was set to 800% and a loop control time was set at 0.6 s.

For other samples, peptide separation was performed using a custom-packed silica column coupled to a nanoElute system. The mobile phase consisted of MS-grade water with 0.1% FA (buffer A) and MS-grade ACN with 0.1% FA (buffer B), delivered at a flow rate of 300 nL/min. A 50-min linear LC gradient was applied for peptide elution. MS analysis of eluted peptides was conducted on a timsTOF Pro mass spectrometer equipped with a CaptiveSpray nano-electrospray ion source. Data acquisition was controlled using Bruker Otofcontrol v6.2 and HyStar v5.1 software. In parallel accumulation–serial fragmentation (PASEF) combined with data-dependent acquisition (ddaPASEF), each acquisition cycle included ten PASEF scans with a ramp time of 100 ms, resulting in a total cycle duration of 1.17 s. Ion mobility scanning ranged from 0.7 to 1.3 Vs/cm^2, with MS1 and MS2 scans covering an m/z range of 100 to 1,700 Th. Dynamic exclusion was applied to precursors reaching an intensity of 20,000 arbitrary units for 0.4 min. Singly charged precursors were excluded based on their position in the m/z-ion mobility plane. In PASEF combined with data-independent acquisition mode, parameters were similar to those in ddaPASEF, with an adjusted ramp time of 166 ms. The isolation windows were set as specified in Supplementary Table 1.

Procedure

Part 1: sectioning and dewaxing

● **TIMING 0.7 h**

1. Treat the glass slide with 1× poly-L-lysine solution before use.
   ▲ **CRITICAL STEP** Balance the slide's hydrophilicity: excessive hydrophilicity can cause adhesion during expansion, while insufficient hydrophilicity may lead to detachment during processing. Use treated slides within 1 week.
2. Slice the FFPE blocks with desirable thickness onto the treated glass slide.
3. Dewax the sample by immersing it in heptane or xylene for 10 min at room temperature. Discard the liquid and repeat once.
   ▲ **CAUTION** Ensure complete deparaffinization, as residual paraffin can interfere with hydrogel embedding and staining. Avoid reusing deparaffinization solutions to maintain their effectiveness.
4. Rehydrate the samples as follows.
   (A) Immerse the sample in 100% EtOH for 5 min at room temperature, then discard the liquid.
   (B) Immerse the sample in 90% EtOH for 5 min at room temperature, then discard the liquid.
   (C) Immerse the sample in 75% EtOH for 5 min at room temperature, then discard the liquid.
   (D) Immerse the sample in ddH_2O for 5 min at room temperature, then discard the liquid.
5. (Optional) Capture images using a stereo microscope in brightfield mode at large magnification. Use these images to do isotropic analysis and evaluate sample integrity.

6. (Optional) Perform H&E staining and capture images to define the target area. Alternatively, perform nuclear staining (for example, DAPI) and/or immunostaining to label specific markers. Follow the supplier's guidelines for all staining procedures.

Part 2: hydrogel embedding

● **TIMING 7 h**

7. Incubate the sample in 50–100 µL of protein-anchoring solution for 1 h at room temperature (Supplementary Video 1).
   ▲ **CRITICAL STEP** This timing is optimized for 5–10-µm FFPE samples. Adjust the incubation time proportionally for samples of different thicknesses to ensure proper anchoring.
   ▲ **CAUTION** Ensure the samples are fully covered.
8. Wash the sample with 50–100 µL of anchoring termination buffer three times. This step terminates the anchoring reaction and adjusts the system pH to 7.0. The buffer volume can be adjusted on the basis of the sample size; using an excess is acceptable and helps ensure complete quenching (Supplementary Video 1).
   ◆ **TROUBLESHOOTING**
9. Remove the buffer surrounding the sample using a pipette and absorbent paper.
10. Allow the sample to dry completely in a fume hood. Cut a square piece from fixed-thickness material (such as plastic film, coverslip or other material with defined thickness; we used plastic film of ~60 µm per layer) to create a frame that surrounds the sample. Stack two to three layers of this film around the sample to form a shallow well. Add sufficient activated monomer solution to fully cover the sample, then place the setup into a humidity chamber (with water at the bottom) and incubate at 4 °C for 4–12 h (Supplementary Video 2).
    ▲ **CAUTION** Incubation time may range from 4 h to 12 h depending on the workflow. For a faster process, 4 h is sufficient to proceed within the same day. While overnight incubation at 4 °C can be performed if necessary, this step should not be considered a pause point, as polymerization has already initiated and exposure to oxygen may compromise gelation quality.
    ◆ **TROUBLESHOOTING**
11. Cover the sample with a coverslip and secure the sandwich-like sample setup using foldback clips. Proceed with either method A or B for gelation on the basis of available equipment (Supplementary Video 2).
    ▲ **CRITICAL STEP** Treat the coverslip with repel-silane beforehand to ensure its easy removal after gelation.
    (A) **Vacuum drying oven**
        (i) Place the setup in a vacuum drying oven preheated to 37 °C.
        (ii) Purge air and inject nitrogen through the pump for three to four rounds to replace the air with nitrogen.
        (iii) Leave the setup in this anaerobic environment for 2–3 h to allow gelation.
    (B) **Airtight container with an anaerobic gas-producing bag**
        (i) Place the setup in an airtight container with an anaerobic gas-producing bag.
        (ii) Place the airtight container in an oven preheated to 37 °C and leave it for 2–3 h to allow gelation.
        ▲ **CAUTION** To prevent liquid evaporation, keep the environment moist. For example, in a vacuum drying oven, a beaker of water can be placed inside during incubation. In an airtight container, the setup can be placed alongside either damp tissue paper at the bottom or a small beaker with water.
        ◆ **TROUBLESHOOTING**

Part 3: homogenization

● **TIMING 1 h**

12. Remove the coverslip from the tissue sample. Place the slide with the sample in a 9-cm glass Petri dish. Add ~20–25 mL of protein denaturation buffer to ensure that the sample is fully submerged. Choose one of the following methods for denaturation (Supplementary Video 3).
    (A) Place the glassware containing the sample in a closed container and denature them at 95 °C for 4–8 h.

(B) Denature the sample in an autoclave under the 'STERILIZE/LIQUID' mode at 105 °C for 1 h.
▲ **CRITICAL STEP** Method A is more DNA friendly, whereas method B is more effective for complex tissues, such as ECM-rich samples. For method B, the temperature range can vary between 105 °C and 121 °C, depending on the tissue type.
▲ **CAUTION** If using an oven, note that internal surfaces may exceed 95 °C, potentially compromising nucleic acid preservation. Ensure that samples do not contact heated metal surfaces.
◆ **TROUBLESHOOTING**

13. Discard the denaturation buffer and wash the sample three times with 20–25 mL of 1× PBS (pH 7.4). The hydrogel will detach from the glass slide at this stage (Supplementary Video 4).
■ **PAUSE POINT** The sample can be preserved in 20–25 mL of 1× PBS at 4 °C for up to 1 week.
▲ **CAUTION** Ensure the denaturation buffer is thoroughly washed off to prevent interference with subsequent staining processes, such as uneven CBB staining.

Part 4: reduction and alkylation

● **TIMING 1.5 h**

14. Add 10 mL of reduction buffer (20 mM DTT or 20 mM TCEP) to the sample and incubate at 37 °C for 30 min with rotation. A smaller container is used to reduce the required volume and conserve buffer (Supplementary Video 4).
15. Discard the reduction buffer and quickly wash the sample three times with 10 mL of 1× PBS (pH 7.4).
16. Add 10 mL of alkylation buffer (55 mM IAA) to the samples and incubate at room temperature in the dark for 30 min with rotation. A smaller container can be used to reduce the required volume and conserve buffer.
17. Discard the alkylation buffer and wash the samples three times with 10 mL of 1× PBS (pH 7.4), incubating for 10 min each time at room temperature with rotation. Alternatively, transfer the samples to a larger container and wash with 20–25 mL of 1× PBS (pH 7.4) for thorough cleaning.

Part 5: staining, expansion and imaging

● **TIMING 1.5 h**

18. Method A: fluorescent staining (Supplementary Video 4).
(A) Place a suitable sample size, such as a 5-mm punch, into a 24-well plate.
(B) Add 200 µL of blocking buffer to the sample and incubate at room temperature for 1 h.
(C) Add 200 µL of the appropriate primary antibody working solution to the hydrogel sample. Incubate overnight at 4 °C.
(D) Wash the hydrogel sample three times with 1 mL of 1× PBS, with each wash lasting 30 min.
(E) Add 200 µL of secondary antibody to the sample and incubate overnight at 4 °C or for 2 h at room temperature.
▲ **CRITICAL STEP** Use a higher concentration for both primary and secondary antibodies than conventional protocols, such as a 1:200 dilution, to ensure sufficient staining.
(F) Wash the hydrogel sample three times with 1 mL of 1× PBS, with each wash lasting 30 min.
(G) (Optional) Stain the nuclei with 200 µL of DAPI (1:1000 dilution) or SYTOX deep red nucleic acid stain (1:500 dilution) for 30 min at room temperature.
▲ **CRITICAL STEP** For LCM, use SYTOX deep red nucleic acid stain to minimize background signals typically observed in the DAPI channel when using DAPI.
◆ **TROUBLESHOOTING**
(H) Wash the hydrogel sample three times with 1 mL of 1× PBS, with each wash lasting 30 min.
(I) Replace the PBS with 1 mL of ddH_2O and wash the sample twice, 30 min each. This is typically sufficient to achieve the desired expansion factor.
(J) After transferring the hydrogel sample into a confocal dish, perform imaging using a confocal microscope, such as a Nikon CSU-W1 SoRa, at the desired magnification.
▲ **CAUTION** If the fluorescent background remains high after staining, incubate the hydrogel overnight in 1 mL of 1× PBS to reduce background, and proceed with imaging the next day.

19. Method B: CBB staining (Supplementary Video 4).
    (A) Stain the sample with 25 mL of CBB solution at room temperature for 1 h or at 37 °C for 30 min with gentle rotation.
    (B) Wash the sample with 25 mL of ddH_2O, typically two rounds, 30 min each, to remove background staining and reach the desired expansion factor.
    (C) Image the sample in brightfield mode using a stereo microscope at the desired magnification. Use the image to evaluate isotropy, calculate the LEFs or select ROIs for proteomic sample preparation.
    ◆ **TROUBLESHOOTING**

Part 6: microdissection

● **TIMING 0.5 h**

20. Manual microdissection
    (A) Based on the colorimetric staining, identify the ROIs.
    (B) Collect the samples using a biopsy punch with the desired diameter.
21. LCM (Supplementary Video 5).
    (A) Expose the LCM steel frame slide to UV light for 30 min. The exposure time can be extended to improve attachment if needed.
    (B) Treat the LCM steel frame slide with enough 1× poly-L-lysine solution to cover the slide and allow it to dry completely.
    (C) Place the stained hydrogel sample at the center of the LCM steel frame slide. Transfer the slide to a vacuum desiccator, pump out as much air as possible and leave it at 25 °C until the hydrogel sample is fully dehydrated.
    ◆ **TROUBLESHOOTING**
    (D) Use a laser microdissection microscope, such as Leica LMD7, to cut the sample based on fluorescent or colorimetric signals. Collect the samples into the lids of 0.2-mL PCR tubes.
    ■ **PAUSE POINT** The dehydrated sample can be preserved inside the vacuum desiccator in the dark at room temperature for several weeks before LCM cutting.
    ▲ **CRITICAL STEP** For higher LCM sample recovery, maintain a stable environment at 20–25 °C with 40–60% humidity. Reduce airflow and static by using a windshield and grounding the equipment.
    ◆ **TROUBLESHOOTING**

Part 7: proteomic sample preparation

● **TIMING 14 h**

22. Method A: conventional in-gel digestion (This method serves as an optional alternative for larger dissected samples (for example, >3 mm) or for control comparisons).
    (A) Manually dissect Coomassie-stained tissue-hydrogels and transfer them into 1.5-mL tubes.
    (B) Destain the dissected samples in 500 µL of 50% ACN and 50% ddH_2O.
    (C) Dehydrate the samples in 500 µL of 50% ACN and 100 mM ABB.
    (D) Dry the samples using a SpeedVac for 5 min.
    (E) Add 10 µL of 12.5 ng/µL trypsin (10% ACN and 50 mM ABB) to cover the samples.
    (F) Incubate at 37 °C for 4 h.
    (G) Add an additional 10 µL of 10 mM ABB and continue incubating overnight at 37 °C.
    (H) Add 100 µL of 25 mM ABB, vortex and collect the supernatant into new 1.5-mL tubes.
    (I) Add 100 µL of 50% ACN and 2.5% FA to the sample, vortex and collect the supernatant. Repeat this step three times.
    (J) Add 100 µL of 100% ACN to the samples until the hydrogel samples shrink into small white dots.
    (K) Concentrate the peptide solutions to a final volume of 20–30 µL using a SpeedVac.
    (L) Desalt the peptides using C18 spin tips following the supplier's guidance.
    (M) Concentrate the purified peptides using SpeedVac.
    (N) Store the samples at −80 °C until ready for LC–MS/MS analysis.
    ■ **PAUSE POINT** Dried peptides can be stored at −80 °C for a couple of weeks.

23. FAXP tip assembly (Supplementary Video 6).
▲ **CRITICAL STEP** If the sample diameter exceeds 3 mm, conventional in-gel digestion should be considered.
(A) **FAXP tip-250: For hydrogel samples with diameters ranging from 1 to 3 mm**
(i) Use a 1.5-mm diameter puncher to cut one plug from the C18 disk.
(ii) Transfer the plug into the 250-µL Agilent tip and ensure the plug is placed parallelly without tilting.
(iii) Trim the bottom part of the tip.
(iv) Verify that the position and height are consistent across tips and ensure the pads are tightly contacted.
(v) (Optional) If the number of samples to be processed is small, 1.5-mL tubes can be used, but an adaptor will need to be prepared.
(B) **FAXP tip-10: for hydrogel samples with diameters of less than 1 mm**
(i) Use a 0.6 mm diameter puncher to cut one plug from the C18 disk.
(ii) Transfer the plug into the 10-µL pipette tip and push it down until it reaches the end of the tip, ensuring that the pad is securely tightened. Similar to the FAXP tip-250, an adaptor can be prepared when processing a small number of samples.
24. Method B: general in-tip digestion.
(A) Rinse and activate the FAXP tips by adding 20 µL (FAXP tip-10) or 100 µL (FAXP tip-250) of 80% ACN or MeOH. Centrifuge at 100*g* for 1 min to ensure the C18 pads are secured and stabilized.
(B) Transfer micro-dissected hydrogel samples into the FAXP tips.
(C) (Optional) For CBB-stained samples, destain by adding 20 µL (FAXP tip-10) or 100 µL (FAXP tip-250) of a solution of 50% ACN and 50% 100 mM ABB. Incubate for 30 min before proceeding to the next step.
(D) Incubate samples by adding 20 µL (FAXP tip-10) or 100 µL (FAXP tip-250) of 50% ACN and 100 mM ABB, incubating at room temperature for 10 min and centrifuging at 150*g* for 5 min.
(E) Dehydrate by adding 20 µL (FAXP tip-10) or 100 µL (FAXP tip-250) of 100% ACN, incubating at room temperature for 10 min and centrifuging at 150*g* for 5 min.
(F) Place the FAXP tips onto a 96-well plate compatible with the LC–MS/MS system.
(G) (Optional) For a small number of samples, use glass inserts in a deep-well 96-well plate or 1.5-mL tubes as alternatives.
(H) Add 1 µL or 3 µL of 12.5 ng/µL trypsin (10% ACN and 50 mM ABB) to FAXP tip-10 or FAXP tip-250, respectively.
(I) Centrifuge briefly to ensure the solution reaches the bottom of the FAXP tips.
(J) Incubate the samples in a humid, airtight container at 37 °C for 4 h, followed by overnight incubation with an additional 10 mM ABB under the same conditions.
(K) After incubation, for FAXP tip-10, sequentially add:
- 6 µL of 2% ACN and 0.1% TFA
- 6 µL of 70% ACN and 0.1% TFA (twice)
- 6 µL of 100% ACN

(L) After incubation, for FAXP tip-250, sequentially add:
- 12 µL of 2% ACN and 0.1% TFA
- 20 µL of 70% ACN and 0.1% TFA (twice)
- 20 µL of 100% ACN

(M) For each step, incubate at room temperature for 10 min and centrifuge at 150–500*g* for 5 min to ensure complete liquid flow-through. Before centrifugation, securely tape the FAXP tips plate to the 96-well plate beneath it.
(N) Concentrate peptide solutions using a SpeedVac.
■ **PAUSE POINT** Dried peptides can be stored at −80 °C for a couple of weeks.
25. Method C: single-cell and subcellular in-tip digestion (Supplementary Video 7)
(A) Rinse and activate the FAXP tip-10 by adding 20 µL of 80% ACN or MeOH. Centrifuge at 100*g* for 1 min to ensure the C18 pads are secured and stabilized.
(B) Transfer samples obtained with an LCM instrument, such as the Leica LMD7, into FAXP tip-10.

▲ **CRITICAL STEP** Perform peptide elution steps under a clean bench to minimize the risk of introducing potential contaminating proteins.

(C) Place the FAXP tips into deep-well 96-well plates for high-throughput processing. Ensure the centrifuge accommodates these plates.

(D) Add 20 µL of 100% ACN, incubate at room temperature for 10 min and centrifuge at 150*g* for 5 min to remove the supernatant.

(E) Place the FAXP tips on a 96-well plate compatible with the LC–MS/MS system.

(F) (Optional) For a small number of samples, use glass inserts in a deep-well 96-well plate or 1.5-mL tubes as alternatives.

(G) Add 0.5 µL of trypsin working solution (5 ng/µL, prepared as described in the Reagent Setup section) directly into each FAXP tip-10.

◆ **TROUBLESHOOTING**

(H) Centrifuge briefly to ensure the solution reaches the bottom of the FAXP tip.

(I) Incubate the samples in a humid and airtight container at 37 °C for 4 h.

(J) Sequentially add:

- 6 µL of 2% ACN/0.1% TFA/0.015% DDM
- 6 µL of 70% ACN/0.1% TFA/0.015% DDM (twice)
- 6 µL of 100% ACN

(K) For each step, incubate at room temperature for 10 min and centrifuge at 150–500*g* for 5 min to ensure complete liquid flow-through. Before centrifugation, securely tape the FAXP tips plate to the 96-well plate beneath it.

▲ **CRITICAL STEP** Adding DDM is recommended to enhance enzymatic digestion efficiency, though it is not essential for the elution steps.

(L) Concentrate peptide solutions using a SpeedVac.

▲ **CRITICAL STEP** Due to the minute sample amount, perform MS acquisition immediately after preparation.

Part 8: proteomic measurement

● **TIMING 1 h**

26. Redissolve the dried peptide in 7 µL of MS buffer.
27. Inject 6 µL of the peptide solution into the LC–MS/MS system and run the samples based on the equipment setup.
28. Analyse DDA-MS data using FragPipe (version 18.0) integrated with the MSFragger search engine (version 3.5). As shown in this Protocol, use the typical parameters listed below, keeping all other parameters at their default settings.
    (A) Use a SwissProt FASTA file with decoy and contaminant sequences (generated using FragPipe).
    (B) Set the MS data type to 'IM-MS' for Bruker trapped ion mobility separation data.
    (C) Set fragment mass tolerance to 0.05 Da.
    (D) Use trypsin for digestion, cleaving after 'KR' residues, except when followed by 'P'.
    (E) Treat the carbamidomethylation of cysteine as a fixed modification.
29. Analyse DIA-MS data using DIA-NN (version 1.8.1) with the typical parameters outlined below. Use a suitable library that matches the samples.
    (A) False discovery rate is 1% for precursors.
    (B) Fixed modifications include C carbamidomethylation.
    (C) Variable modifications are N-term M excision and Methionine oxidation.
    (D) Peptide length range is 7–50 amino acids.
    (E) Precursor charge range is two to four.
    (F) *m/z* ranges include precursor ions (100–1,700) and fragment ions (100–1,700).
    (G) Enable 'Unrelated runs', 'Use isotopologues' and 'MBR'.
    (H) Apply heuristic protein inference.
    (I) Use 'Robust LC (high precision)' as the quantification strategy.
    (J) Keep all other settings as default.

    ◆ **TROUBLESHOOTING**

30. For single-cell and subcellular DIA-MS data analysis:
    (A) Use Orbitrap Astral to acquire MS data.
    (B) Use a library-free mode with a FASTA file downloaded from SwissProt.
    (C) Set precursor ranges to 400–800 *m/z* and fragment ion ranges to 150–2,000 *m/z*.
    (D) Add '--relaxed-prot-inf' under 'Additional Options'.
    (E) Configure protein inference as 'Genes' and quantification strategy as 'Robust LC (high precision)'.
    (F) Keep all other settings as default.

Troubleshooting

Troubleshooting information can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
8	FFPE tissue section detachment during anchoring termination buffer wash	The sample fails to adhere to the slide	Apply poly-L-lysine solution evenly to the slide surface and allow it to dry completely before mounting the FFPE section
10	The activated monomer solution is lost during the incubation	The plastic film fails to adhere securely to the glass slide	Ensure that the solution fully covers the sample. Lay the sample as flat as possible to minimize leakage. If the solution is lost or appears insufficient during incubation, add more to ensure complete coverage
11	Hydrogel fails in gelation or has a soft texture	Anaerobic conditions are not adequately maintained during the gelation process	Place an anaerobic indicator inside the vacuum drying oven or airtight container to monitor the oxygen level
12	The tissue-hydrogel composite remains attached to the glass slide after homogenization	The denaturation buffer pH is incorrect (should be pH 7.0) The slide surface is highly hydrophilic	Replace the buffer with one adjusted to pH 7.0 and repeat homogenization Prepare the glass slide as suggested in the Protocol Incubate the sample in 1× PBS at 37 °C for 30 min to facilitate detachment
18G	DAPI/deep red fails to stain	Overly aggressive homogenization conditions Incorrect buffer usage	Adjust homogenization settings to avoid excessive denaturation temperatures that may compromise nuclear integrity Ensure the use of the recommended buffer, as outlined in this Protocol
19	Tissue within hydrogel breaks after expansion	Sample quality issues Poor gelation due to ineffective reagents or improper anaerobic conditions Inadequate denaturation	Collect fresh tissue with normal morphology and strictly control fixation and dehydration Use effective reagents and follow the Protocol carefully Ensure anaerobic conditions during gelation If denaturation at 95 °C is insufficient, consider autoclaving the samples
21C	Hydrogel shrinkage or deformation during drying onto the LCM steel frame slide	Rapid water evaporation Insufficient adherence to the LCM steel frame slide	Add a small amount of salt to slow evaporation Control temperature at 25 °C within a vacuum desiccator to manage evaporation rates Treat LCM steel frame slide with poly-L-lysine
21D	Low collection rate during LCM cutting of expanded hydrogel samples	Environmental factors such as high humidity	Keep room temperature at 20–25 °C and humidity at 40–60%. Use a windshield cover and grounding to reduce airflow and static interference
25G	The solution in the FAXP tip has completely evaporated after enzymatic digestion	Insufficient preheating Inadequate humidity	Preheat the incubation container to 37 °C Maintain humidity within the container using a wet paper towel
	Failure to obtain peptides from single-cell or subcellular samples	Insufficient enzymatic digestion	Note to add DDM to the digestion buffer to enhance enzymatic efficiency and improve peptide recovery
29	Insufficient proteome coverage	Unsuitable acquisition mode Limited library	Employ DIA for comprehensive coverage Choose library-free mode

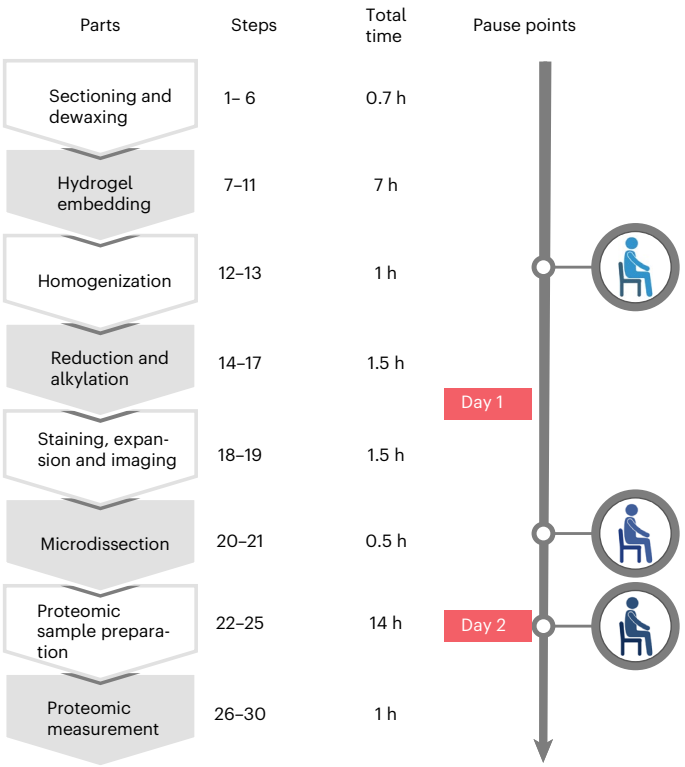

Fig. 2 | Estimated timing and recommended workflow for each part of the Protocol. Graphical workflow of the Protocol, where steps are shown on a daily basis, and pause points indicate where the sample can be stably stored before proceeding.

Timing

The timing is estimated based on processing a 5-µm-thick mouse liver FFPE tissue slide using autoclave-based homogenization and CBB staining (Fig. 2). The durations listed below reflect the total time for each part. Hands-on time may vary, particularly for proteomics sample preparation, depending on the number of samples processed. Note that the estimated durations do not include downstream proteomics data analysis.

Part 1 (Steps 1–6), sectioning and dewaxing: 0.7 h

Part 2 (Steps 7–11), hydrogel embedding, including anchoring, activated monomer incubation and gelation: 7 h

Part 3 (Steps 12–13), homogenization: 1 h

■ **PAUSE POINT** The sample can be preserved in PBS at 4 °C for up to 1 week.

Part 4 (Steps 14–17), reduction and alkylation: 1.5 h

Part 5 (Steps 18–19), staining, expansion and imaging: 1.5 h

Part 6 (Steps 20–21), microdissection: 0.5 h

■ **PAUSE POINT** The dehydrated sample can be preserved inside a vacuum desiccator in the dark at room temperature for several weeks before LCM cutting.

Part 7 (Steps 22–25), proteomic sample preparation: 14 h

■ **PAUSE POINT** Dried peptides can be stored at −80 °C for a couple of weeks.

Part 8 (Steps 26–30), proteomic measurement: 1 h

Anticipated results

This Protocol facilitates reliable tissue expansion and proteomic analyses, delivering high-resolution and reproducible outcomes for spatial proteomics. Using a 1-h protein-anchoring

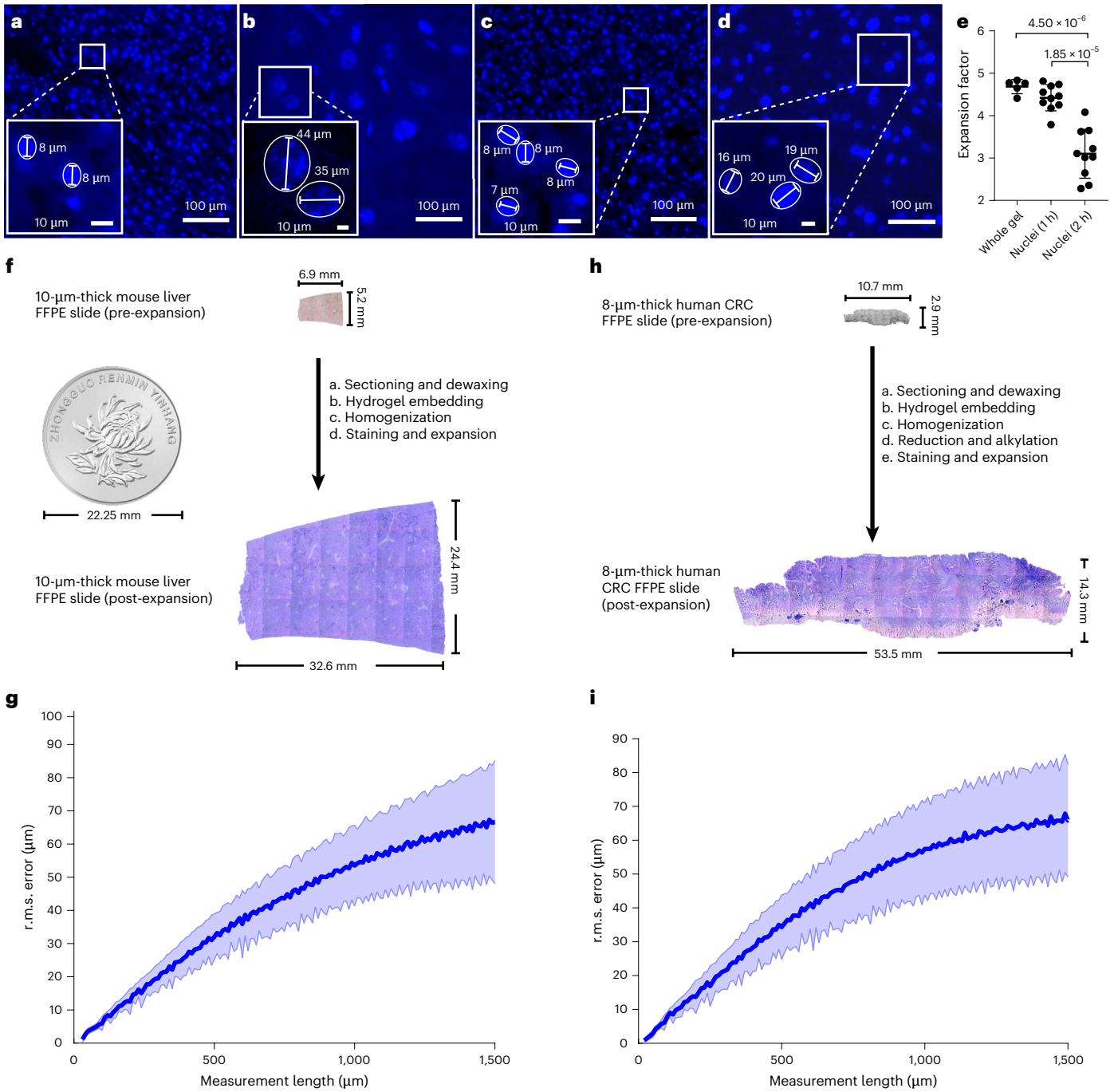

Fig. 3 | Validation of tissue expansion using FAXP. a,**b**, The DAPI staining of mouse liver FFPE slides following 1-h anchoring (**a**) and after expansion (**b**). **c**,**d**, The DAPI staining of mouse liver FFPE slides following 2-h anchoring (**c**) and after expansion (**d**). The insets display nuclei with measured diameters. Scale bars are included. **e**, Comparisons of colorimetric staining of whole-hydrogel samples ($n = 5$) and DAPI nuclear staining under 1-h and 2-h anchoring conditions ($n = 10$). *P* values were determined using Welch's *t*-test (two-sided). The data are presented as mean ± standard deviation (s.d.). **f**, Brightfield images of mouse liver FFPE slides before (top) and after (bottom) expansion, stained with CBB. A Chinese 1-yuan coin (22.25 mm in diameter) is shown for size reference only. **g**, r.m.s. error in length measurements between pre- and post-expansion liver slides ($n = 4$), with an example provided in **f**. The blue line represents the mean, and the shaded area indicates the s.d. **h**, Brightfield images of human CRC FFPE slides before (top) and after (bottom) expansion, stained with CBB ($n = 3$). **i**, r.m.s. length error comparison between pre- and post-expansion human CRC slides ($n = 3$), with an example shown in **h**. The blue line indicates the mean, and the shaded area represents s.d. Figure reprinted from ref. 17, Springer Nature Limited.

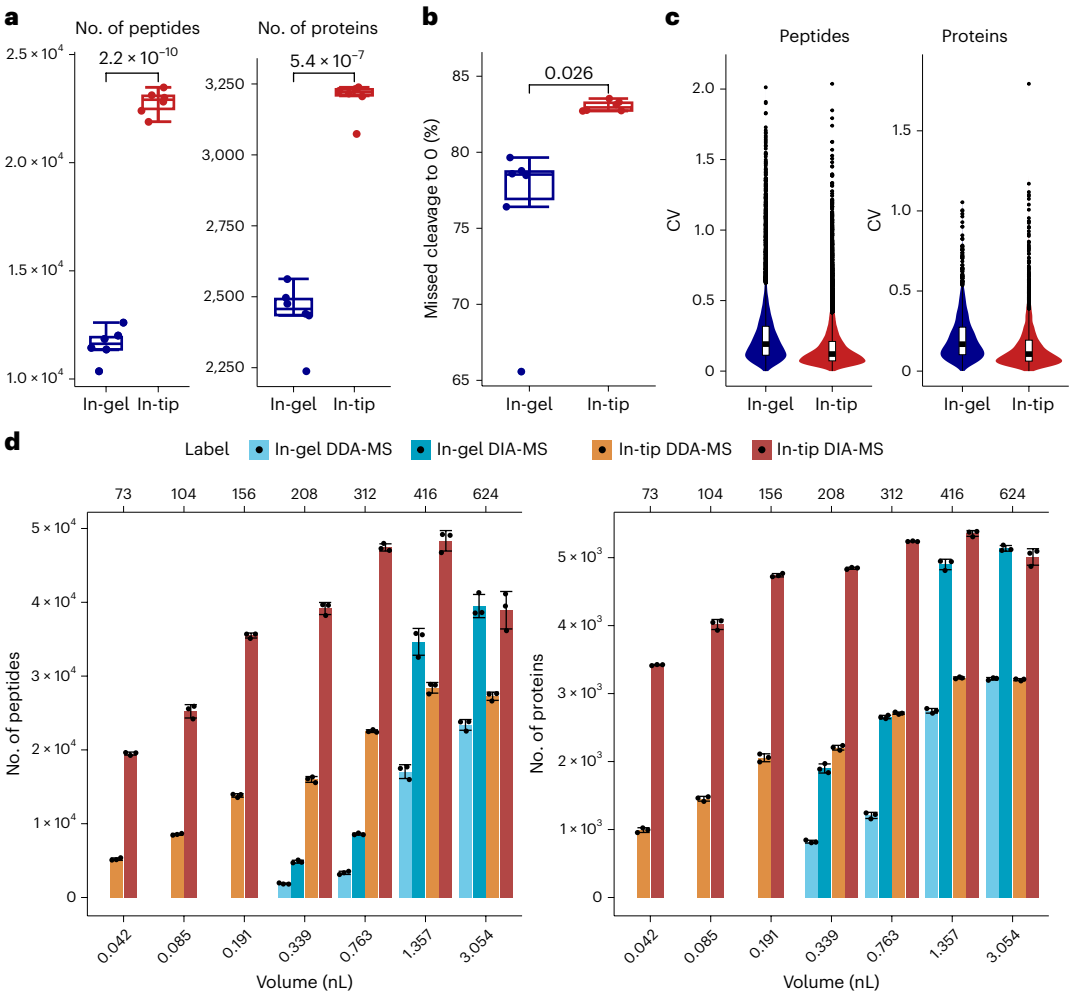

Fig. 4 | Performance and detection range of filter-aided in-gel digestion. **a**, A comparison of peptide and protein identifications between conventional in-gel digestion (shortened as In-gel) and filter-aided in-gel digestion (shortened as In-tip). Statistical significance was calculated using Welch's two-sided *t*-test ($n = 6$). **b**, The proportion of peptides without missed cleavage sites identified via in-gel and in-tip digestion methods. Statistical analysis was performed using Welch's two-sided *t*-test ($n = 6$). The boxplots depict median values (center lines), interquartile ranges (box limits) and whiskers extending to data points within 1.5 times the interquartile range. Individual data points are plotted as dots, with outliers displayed beyond the whiskers. **c**, The coefficient of variation (CV) illustrating peptide and protein abundance reproducibility across digestion methods. **d**, The number of peptides and proteins identified across varying sample volumes, evaluated using in-gel and in-tip digestions. The sample volumes (*x* axis) are shown below, with the corresponding pre-expansion sampling diameters (μm) indicated above. The methods include in-gel DDA-MS (light blue), in-gel DIA-MS (blue), in-tip DDA-MS (orange) and in-tip DIA-MS (red). The data represent means ± s.d., based on $n = 3$ technical replicates for each method and sample volume. Figure reprinted from ref. 17, Springer Nature Limited.

condition, the LEFs of cell nuclei, visualized via DAPI staining, align closely with those of whole tissues (LEFs: 4.42 ± 0.31 versus 4.69 ± 0.17, respectively), confirming sample expansion at both the micro- and macro-level (Fig. 3a–e). Extended anchoring time decreases LEFs (3.10 ± 0.58), emphasizing the importance of precise timing. Root mean square (r.m.s.) measurement errors of ~5.5% of feature-length distances (Fig. 3f–g) validate the Protocol's ability to achieve isotropic expansion in FFPE mouse liver tissue. This level of performance also extends to FFPE CRC tissue, which exhibits an r.m.s. error of ~6% (Fig. 3h,i).

The in-tip digestion enhances proteomic sensitivity and reliability compared with conventional in-gel digestion[19]. Peptide and protein detections increase substantially (by 96.6% and 31.1%, respectively; Fig. 4a), with missed cleavage rates improving to ~17%, compared with ~23.8% for in-gel digestion (Fig. 4b). The coefficient of variation for both peptides and proteins remain below 0.2 (Fig. 4c), underscoring reproducible quantification. For tissue samples as

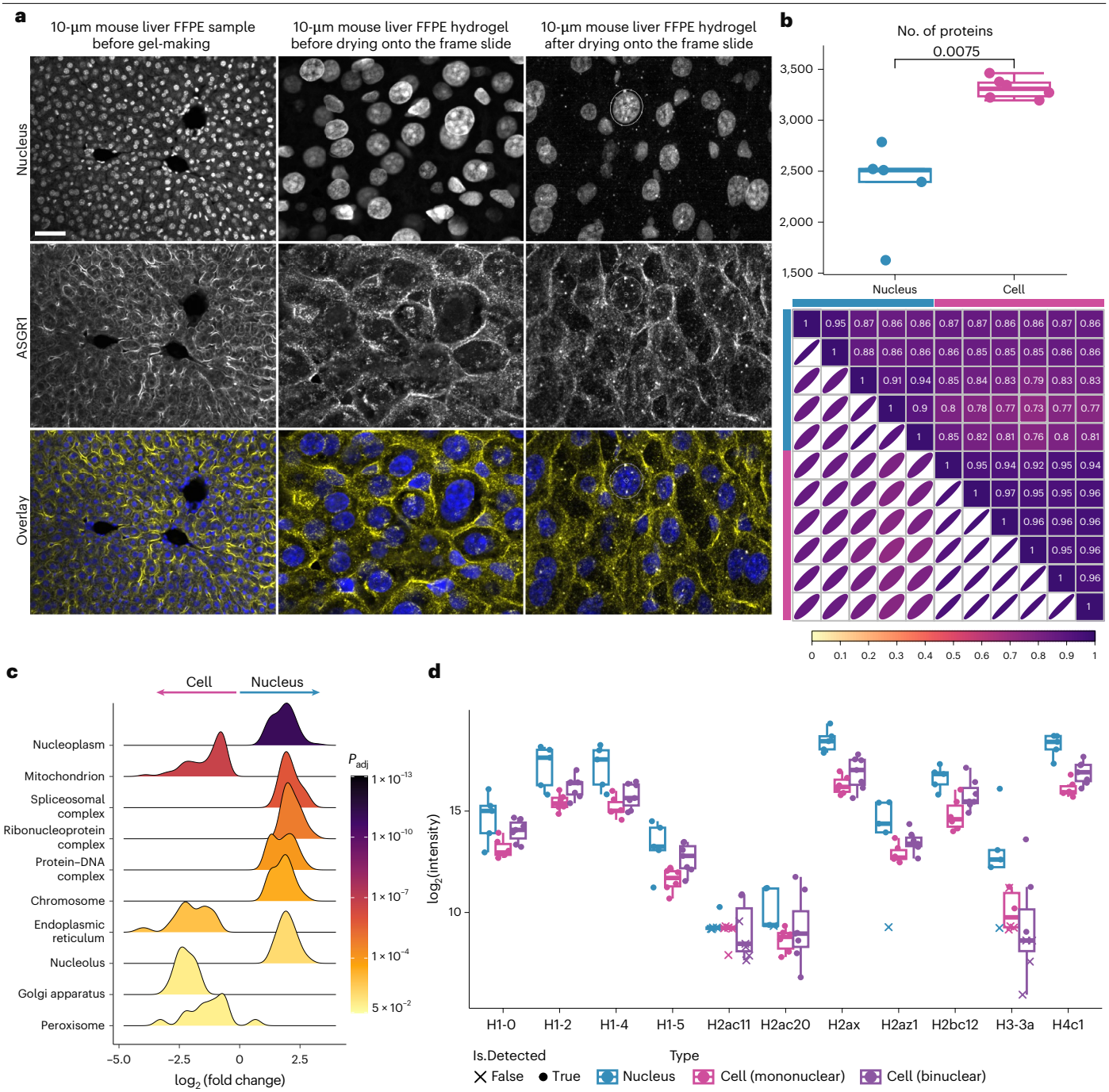

Fig. 5 | Analysis of mouse liver single-cell and single-nucleus samples. **a**, Representative images of 10-µm mouse liver FFPE samples showing nuclear staining and ASGR1 immunostaining at different preparation stages: before gel-making (left), after gel-making but before drying onto the steel frame slide (middle) and after drying onto the steel frame slide (right). The top row displays nuclear staining, the middle row shows ASGR1 immunostaining and the bottom row presents the merged signals. Scale bar, 50 µm. **b**, A comparison of protein identifications between single nucleus ($n = 5$; blue) and single mononuclear hepatocyte ($n = 6$; magenta). The P values were calculated using Welch's t-test (two-sided). Boxplots show the medians (center lines) and interquartile ranges (box limits), and whiskers extend to 1.5 times the interquartile range. Outliers are shown as individual points beyond the whiskers. Bottom: a heat map showing Pearson's correlation coefficients for protein quantification between single mouse nuclei and mononuclear hepatocytes. **c**, A ridge plot showing protein enrichment in cellular compartments for single mononuclear hepatocytes and nuclei, including only categories with false discovery rate <0.05. P_{adj}, adjusted P value. **d**, The histone distribution across nuclei ($n = 5$; blue), mononuclear cells ($n = 6$; pink) and binuclear cells ($n = 7$; purple). The dots represent quantified proteins (Is.Detected = true), while crosses indicate proteins not detected and subsequently imputed (Is.Detected = false). Figure reprinted from ref. 17, Springer Nature Limited.

small as 0.042 nL (equivalent to a lateral resolution of 73 µm), in-tip digestion outperforms in-gel digestion in identifying both peptides and proteins across DDA and DIA modes (Fig. 4d). Peak efficiency is achieved for tissue volumes ranging from 0.763 nL to 1.357 nL, with no additional gains beyond this range. Compared with in-gel digestion, the in-tip method improves lateral and volumetric resolution by 2.85-fold and 8.07-fold, respectively, while achieving markedly enhanced peptide and protein identifications from ultra-low tissue volumes. These results emphasize the method's capability for high-resolution proteomic analysis in microscale tissue applications.

Integrating FAXP with LCM enables the precise isolation and proteomic analysis of subcellular structures while addressing limitations of conventional LCM[39]. Without tissue expansion, LCM often generates prominent laser burn marks around nuclei, which may lead to sample contamination and compromise isolation integrity (Supplementary Fig. 1a). FAXP, however, effectively alleviates these issues. With FAXP, nuclei expand more than fourfold in diameter (from ~8 µm to ~35 µm), resulting in a 64-fold increase in volume (Supplementary Fig. 1b). The expansion reduces contamination, simplifies isolation and enhances downstream proteomic analysis by allowing intact nuclei collection.

Using mouse liver FFPE sections, FAXP also demonstrates excellent compatibility with immunostaining workflows (Fig. 5a). Nuclei and hepatocytes are isolated and identified using the ASGR1 plasma membrane marker. Importantly, even after hydrogel dehydration onto the LCM steel frame slide (right panel in Fig. 5a), immunostaining signals remain preserved and largely unchanged in size, underscoring the robustness of the method and its compatibility with LCM workflows. Subsequent proteomic analysis identifies an average of 2,368 proteins from single nuclei and 3,312 proteins from single mononuclear hepatocytes (Fig. 5b). Strong correlations are observed among proteomes, with Pearson's correlation exceeding 0.86 for nuclei and 0.92 for hepatocytes (Fig. 5b).

Functional analysis highlights distinct proteomic profiles: nuclear-specific proteins are enriched in categories such as nucleoplasm, chromosome and nucleolus, while cellular proteomes associate with organelles such as mitochondrion and endoplasmic reticulum (Fig. 5c). This demonstrates the method's ability to effectively resolve detailed subcellular proteomes.

Histone protein expression exhibits a gradient, with the highest levels observed in nuclei, intermediate levels in binuclear hepatocytes and the lowest levels in mononuclear hepatocytes (Fig. 5d). This trend reflects variations in chromatin organization and nuclear content, demonstrating the method's capability to resolve proteomic differences across hepatocyte subtypes and nuclear configurations.

In summary, FAXP enables the robust isotropic expansion and high-sensitivity proteomic analysis of FFPE specimens. Its integration with advanced imaging and subcellular isolation workflows offers a versatile and scalable solution for spatial proteomics, making it a powerful tool for studying complex biological systems.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The main data discussed in this Protocol are available in the supporting primary research paper and its source data[17]. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[40] partner repository with the dataset identifier PXD041412.

Code availability

The code used for data analysis in this Protocol is available via GitHub at https://github.com/guomics-lab/FAXP and via Zenodo at https://doi.org/10.5281/zenodo.13843661 (ref. 41).

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Author contributions
T.G., Y.Z. and K.D.P. conceptualized and supervised the project. Z.D., Y.Z., K.D.P. and C.W. developed and validated the method. Z.D., C.W. and J.C. carried out the proteomic experiments, while W.J. and Z.D. conducted the proteomic data analysis, with the isotropic analysis performed by Z.D. C.W. and J.C. created the video tutorial. Z.D. drafted the manuscript, with revisions contributed by all authors.

Competing interests
T.G. and Y.Z. are shareholders of Westlake Omics Biotechnology. T.G., Z.D., Y.Z., C.W. and K.D.P. are listed as inventors on a granted patent (Chinese patent no. ZL 202310889444.4) submitted by the Westlake Laboratory of Life Sciences and Biomedicine, which pertains to the FAXP methods. All other authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-025-01256-3.

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Reporting Summary

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n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
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☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Images were acquired using Zeiss ZEN v3.1, NIS-Elements AR v5.42.01, and Leica LMD v8.4 software. MS data was acquired using Bruker OtofControl v6.2 and HyStar v5.1 for the timsTOF Pro, as well as Thermo Scientific Xcalibur v4.7.69.37 and Orbitrap Astral Tune Application v1.0.100.40 for the Orbitrap Astral MS.
Data analysis	MS data were analyzed using the FragPipe platform (version 18.0) with the MSFragger search engine (version 3.5) and the DIA-NN software (version 1.8.1). For all numerical comparisons, Welch's t-test was performed, and statistical visualizations were generated using PRISM (version 10.0.3) and R (version 4.4.0). MATLAB (version R2021a) was used for isotropic analysis, following a previously established protocol. The Pearson's correlation coefficient was calculated after excluding missing values. Gene ID conversion was conducted using clusterProfiler (version 4.11.0). Gene Ontology (GO) term annotations were performed using org.Mm.eg.db (version 3.18.0), and redundant GO terms were manually simplified. Fast gene set enrichment analysis (GSEA) was conducted using clusterProfiler to identify Cellular Component Ontology terms ranging from 10 to 300. Gene sets with an adjusted p-value < 0.05, as reported by GSEA, were considered statistically significant.

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Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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The main data discussed in this protocol are available in the supporting primary research paper (https://doi.org/10.1038/s41467-024-53683-7) and its source data. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD041412.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	Tissue regions from both male and female patients were randomly selected from FFPE slides, with no analysis of sex or gender differences.
Reporting on race, ethnicity, or other socially relevant groupings	Not applicable
Population characteristics	We included three colorectal tissue samples from patients with histologically confirmed cancer, representing various subtype regions, including normal, low-grade dysplasia, high-grade dysplasia, and carcinoma. No specific demographic factors, such as age, were analyzed.
Recruitment	Samples were retrospectively collected from archived FFPE material acquired at the Second Affiliated Hospital of Zhejiang University School of Medicine.
Ethics oversight	This study complies with all relevant regulations for human study participants and was conducted in accordance with the Declaration of Helsinki. The collection and use of human tissue were approved by the Institutional Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine and the Ethics Committee of Westlake University.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical methods were used to estimate sample size. Sample sizes for each experiment are provided in the figure legends, and they were selected to ensure they are sufficient for statistical comparisons between groups. The sample sizes were determined based on similar published studies (Nat Commun. https://doi.org/10.1038/s41467-022-34824-2).
Data exclusions	No data were excluded in this protocol study.
Replication	All attempts for replication were included in the manuscript.
Randomization	Randomization was applied where appropriate, including during sample processing and data acquisition, to reduce technical bias in the benchmarking experiments.
Blinding	Blinding was not applied in this study, as the experimental design did not necessitate it due to the specific objectives of the research.

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Materials & experimental systems

n/a	Involved in the study
[ ]	[x] Antibodies
[x]	[ ] Eukaryotic cell lines
[x]	[ ] Palaeontology and archaeology
[ ]	[x] Animals and other organisms
[x]	[ ] Clinical data
[x]	[ ] Dual use research of concern
[x]	[ ] Plants

Methods

n/a	Involved in the study
[x]	[ ] ChIP-seq
[x]	[ ] Flow cytometry
[x]	[ ] MRI-based neuroimaging

Antibodies

Antibodies used

1. Anti-ASGR1 (Proteintech, 11739-1-AP, 1:200 dilution for both pre- and post-expansion)
2. Goat anti-Rabbit IgG (Abcam, ab150086, 1:1000 dilution for pre-expansion and 1:200 for post-expansion)

Validation

All antibodies are commercially available and validated in the literature as cited on the manufacturer's websites as well as by the datasheets they provide. Antibody validation and validation criteria are available on the following websites:
1. Anti-ASGR1 (Proteintech, 11739-1-AP), https://www.ptglab.com/products/ASGR1-Antibody-11739-1-AP.htm
2. Goat anti-Rabbit IgG (Abcam, ab150086), https://www.abcam.com/en-us/products/secondary-antibodies/goat-rabbit-igg-h-l-alexa-fluor-555-preadsorbed-ab150086

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals

Four male C57BL/6J wild-type mice (Mus musculus; four months old) and one female C57BL/6J wild-type mouse (Mus musculus; two months old) were used. All mice were obtained from the Laboratory Animal Resources Center of Westlake University and housed in controlled barrier facilities, with macroenvironmental temperatures maintained between 20 and 26°C and humidity levels between 40% and 70%. Food and water were provided ad libitum, and the mice were kept on a 12-hour light/dark cycle. Housing conditions were closely monitored and maintained.

Wild animals

N/A

Reporting on sex

Male and female mice were used in separate benchmarking experiments, without comparing sexes, so results are not disaggregated by sex.

Field-collected samples

N/A

Ethics oversight

All animal maintenance and experimental procedures were conducted according to the Westlake University Animal care guidelines, and all animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) under protocol #22-083-GTN.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks

N/A

Novel plant genotypes

N/A

Authentication

N/A