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Laser Capture Microdissection Assisted Spatial Proteomics

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We use this protocol and it's working

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Abstract

This protocol was used to generate spatial proteomics dataset from pediatric lung, as a part of the CZI-MPII project. For more information: **Mapping the Pediatric Inhalation Interface: Nose, Mouth, and Airways, - Chan Zuckerberg Initiative**

Materials

Please see Section 1

Safety warnings

This protocol contains potentially laser and chemical hazards. Please read carefully and work with appropriate protective equipment.

Materials and Instruments

1

Safety information

This protocol contains potential laser and chemical hazards. Please read carefully and work with appropriate protective equipment.

1.1 **For tissue sectioning**

FFPE tissue blocks
Microtome
PEN Membrane Glass Slides (Applied Biosystems, LCM0522)
Regular SuperFrost Plus slides

1.2 **For tissue staining**

Histology staining setup: xylene, gradient ethanol, hematoxylin, eosin, etc. according to the experiment design.
Microscope slides scanner and digital slide viewer for annotation

1.3 **For LCM**

Adhesive cap collection tubes (Carl Zeiss, 10138374)
Laser capture microdissection(LCM) platform, Zeiss PALM MicroBeam or equivalent
Laser safety eyewear
Gloves
70% Ethanol

1.4 **For sample preparation**

This part is adapted from Coscia et al., 2020, supplementary material and methods. All reagents and equipment are described in the original protocol.
Trypsin/Lys-C Mix, Mass Spec Grade, [M] 1 µg/µL in stock aliquots, store at -80 °C (Promega, V5071)
Nuclease-Free Water, not DEPC-Treated, (Invitrogen, AM9932)
Nuclease-free Water, Molecular Biology Grade, Ultrapure (Thermo Scientific Chemicals, J71786)
Isopropanol (2-Propanol, ISO, Merck, 33539)
Mlllex-GP Filter Unit 0.22 µm (Merck Millipore, SLGP033RS)
SDB-RPS Solid Phase Extraction Disk (Merck, 66886U)
0.2 ml PCR tubes, strip of 8, with attached caps (Eppendorf, 951010022)
0.2 ml PCR tubes, strip of 8, no cap (Thermo Scientific, AB-0452)
Compatible 0.2 ml PCR tubes caps, strip of 8 (Thermo Scientific, AB-0386)

Tissue sectioning

2 Prepare consecutive FFPE tissue sections series on PEN slides and SuperFrost Plus slides according to the experiment design.

2.1 Ideally, cut all slides from the same FFPE block in one sitting (no extra trimming in between). Sections should be 10 µm thick on PEN slides for LCM, and 4 µm thick on SuperFrost Plus slides for other staining purposes. Make sure tissues are attached to the membrane area of PEN slides.
An example of the sectioning design is below **(Fig. 1)**

> **Note**
>
> **!! Store the PEN membrane in a dry place before staining !!**

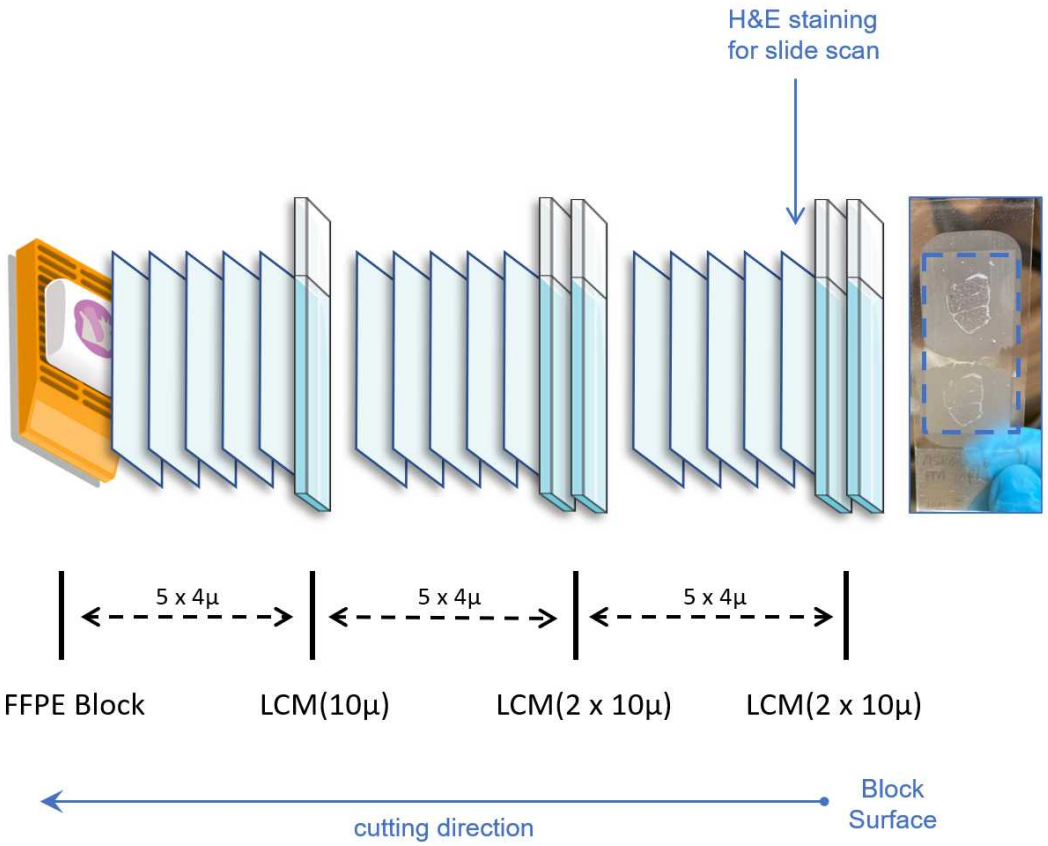

Figure 1. An example of sectioning design

Note

Notes for the floatation bath and drying the slides:
Use Milli-Q water to avoid contamination.
Set the water temperature at 37 to 40 °C

After picking up flattened sections, place the slides on the slide dryer at 40°C. When there is no visible residual water, slides can be dried vertically in an incubator at 37°C overnight.

Tissue staining

3 **For the reference slides and annotation**

Pick SuperFrost Plus slides adjacent to PEN slides as references. It can be stained with desired methods, e.g., H&E, AB-PAS, fluorescence, etc. This protocol continues with H&E staining as an example.

3.1 Scan the whole reference slide to generate a digital image of the full tissue.
Annotate histological regions of interest(ROIs) on the digital images. The step is essential since the ROIs may not be recognizable on LCM due to compromised resolution and dried tissue section.

4 **For PEN slides**
Pick PEN slides adjacent to the reference slides. Stain the slides with the desired methods.

4.1

Note

!! For PEN slides, DO NOT dehydrate with xylene after ethanol steps, DO NOT mount the slides !!

Leave the slides dry in the fume hood overnight. Keep slides as clean as possible.

4.2 It is not recommended to stain PEN slides until the annotation is completed since **step 4 to step 15.10** should be finished within one month to guarantee the sample quality.
Make sure all the apparatus and reagents are clean and freshly prepared to avoid contamination.

Laser capture microdissection

5

Please refer to the official manual and instructions of your LCM platform. This protocol only describes essential steps with ZEISS PALM MicroBeam as an example. A quick guide is available in the references.

Note

!! USE LASER SAFETY EYEWEAR !!

Instruments Setup
Turn on the instruments, microscope, computer, and software in the correct order.

1. power strip
2. Zeiss Microscope Power Supply 232
3. LCM control box, turn the key to the ON position
4. Zeiss PALM MicroBeam
5. Axiovert Microscope
6. Computer
7. PALMRobo software

6 Mount/Unmount the slides

Note

Wear gloves and clean your hands with ethanol before touching the slides and collection tube every time.

By choosing "Goto Load position" **(Fig. 2 Button A)**, move the stage to the loading position. Mount the slides properly. Return the stage to the working area. Unmount the slides in the same way.

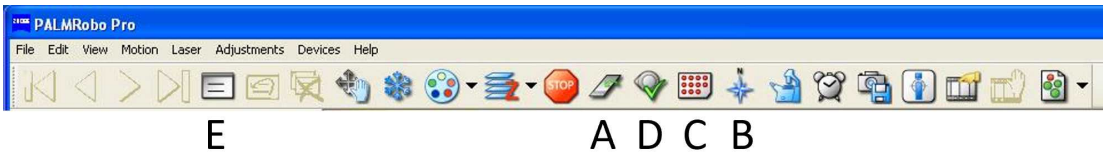

Figure 2. Top menu panel of PALMRobo software

7 Scan the slides before and after capturing

Adjust the magnification, light intensity, display gain, white balance, and focus, to be able to observe ROIs clearly. **(Fig. 3)**

Choose "Navigation Window", and "Scan" tab **(Fig. 4)**, and scan the slide in desired magnification. Save the images. Scan and save again when finish collecting ROIs from the same slide.

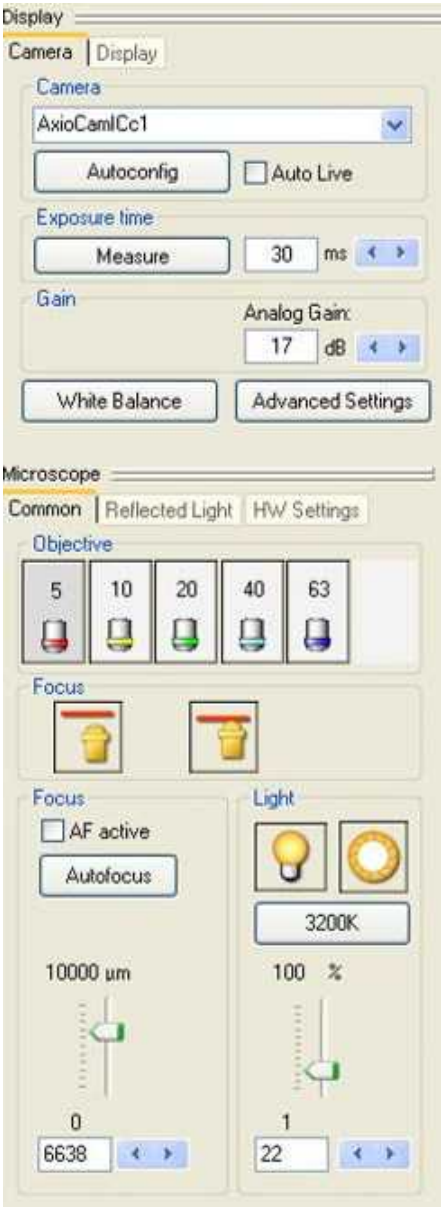

Figure 3. Settings to adjust display and microscope

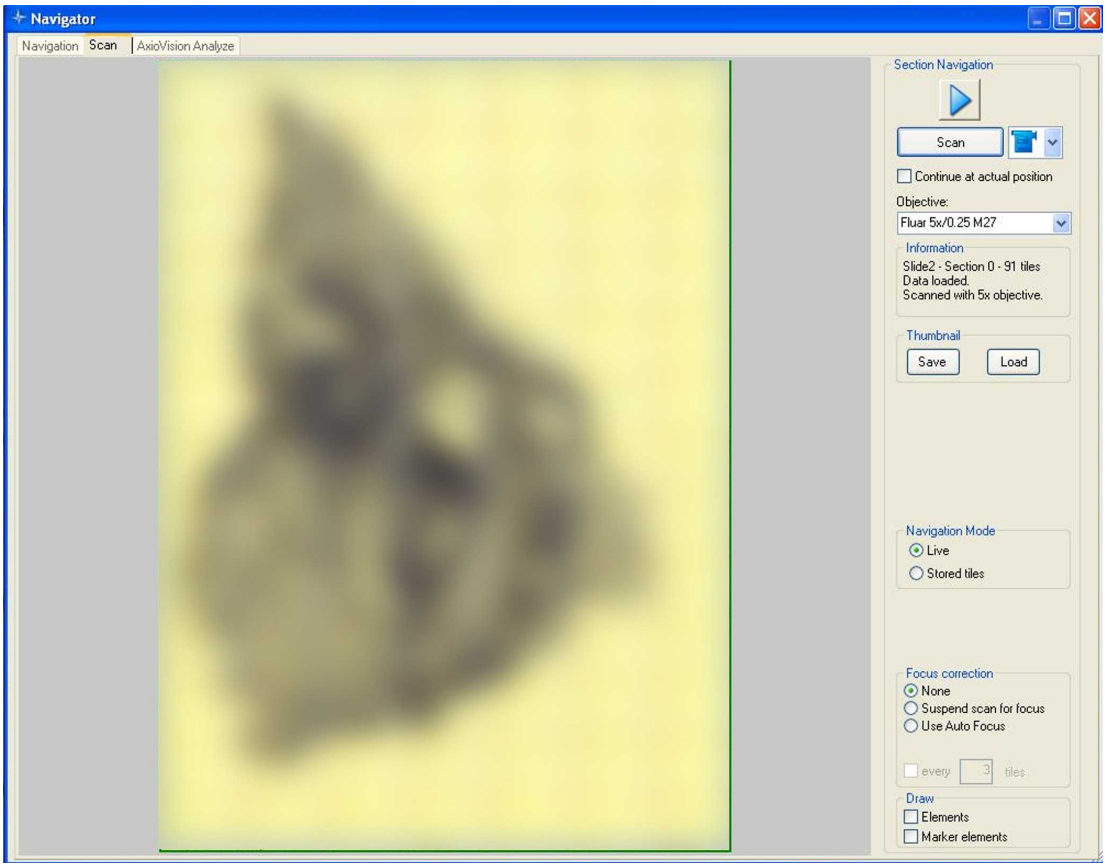

Figure 4. Navigator and Scan Window

8 **Mount/Unmount collection tubes**

Click "Capture Device"**(Fig. 2 Button C)**, and "Change Collector" **(Fig. 5, Button A)** in the pop-up window, the RoboMover will move the tube holder forward, then mount the adhesive cap tube properly. Insert the cap in the metal clip adhesive side downward. Hold the tube with the small hook.

Click "scan new collector type" or "use same collector type" **(Fig. 5, small window B)**, the tube holder will return to the default place.

Click on the center of the blue cap **(Fig. 5, Button C)**, the RoboMover will bring the cap to the the light path.

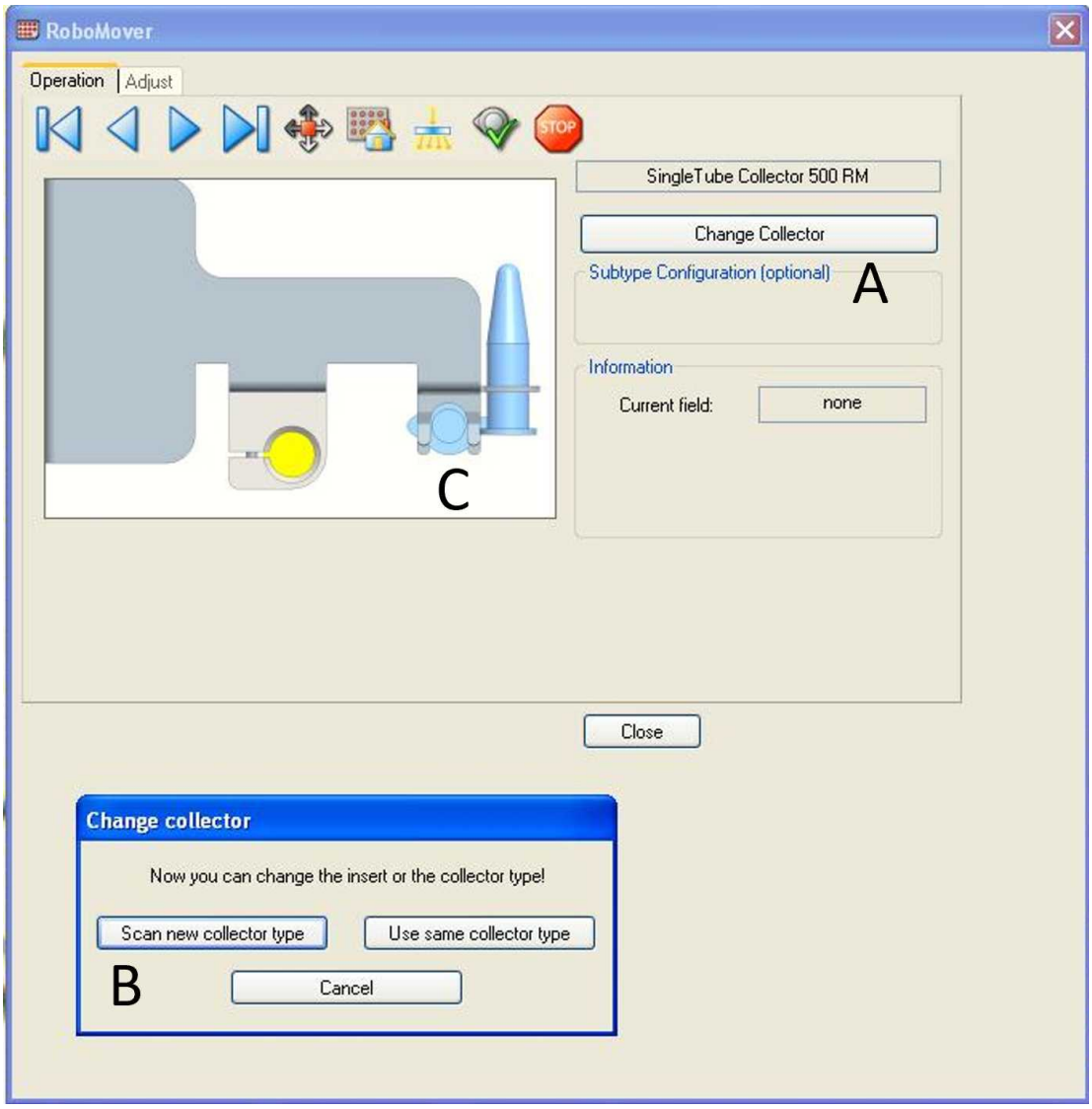

Figure 5, RoboMover window for changing the collection tube

9 **Draw ROIs and cut**

Again, adjust the display and microscope parameters to be able to see ROIs clearly. **(Fig. 3)**

Note

!! TEST THE LASER SETTING BEFORE STARTING THE REAL EXPERIMENT. Laser energy may need adjustments for different types and thicknesses of the tissue !!

Click "Freehand" **(Fig. 6 Button A)**, the mouse cursor will become a pencil, then you can draw a closed curve to select ROIs. The curve should be as close to the edge as possible. Hollow

structures should be collected without the empty space.

Click "Dot" **(Fig. 6 Button B)**, add a spot to the center or edge of the closed curve draw by freehand. It helps to detach the ROI tissue from the slides.
Click "Start laser function" **(Fig. 6 Button C)**, the laser beam will cut the selected ROI. The detached tissue should be collected in the adhesive cap.
Click "Cap Check"**(Fig. 2 Button D)** to confirm the tissue is actually captured.

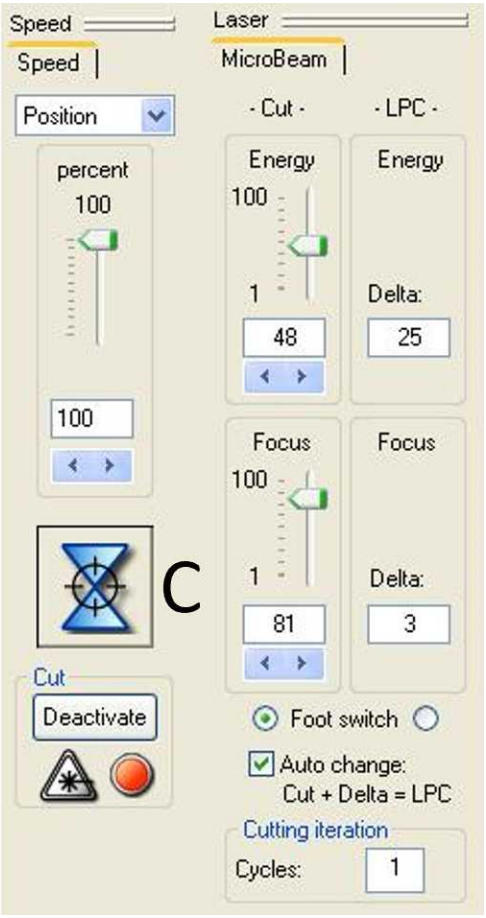

Figure 6, Drawing tools and laser parameters panel

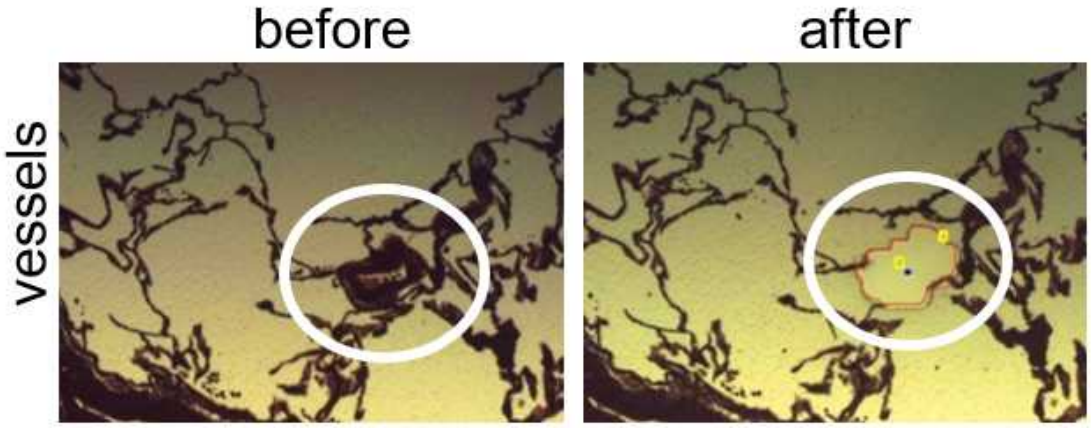

Figure 7. A region of interest before and after laser capture microdissection

10 **Experiment documentation in the Element list**

Click "Element list" **(Fig. 2 Button E)**, you will be able to see to areas of all and each element you collected.

The element list can be saved and loaded later. Together with the scanned images, it will be helpful to locate the ROIs during reviewing and thus serve as documentation of the experiment. You can also rename and recolor the elements as you wish.

Ideally should collect 600k-800k μm^2 per ROI for each biological replicate.

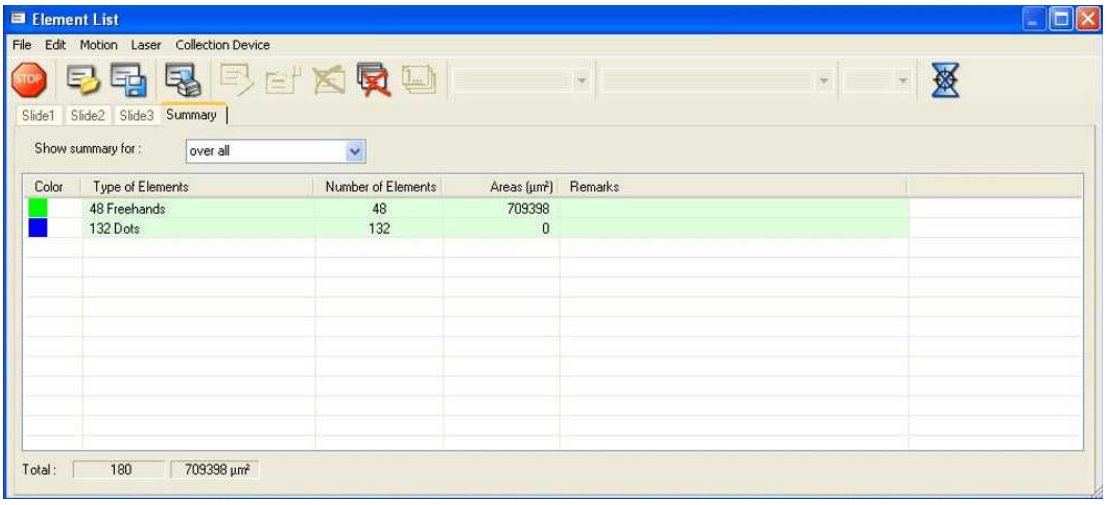

Figure 8. Element list

11 When sufficient areas of a ROI have been collected, change the collection tube and continue with the next ROI. Refrigerate the collection tube at 4 °C until sample preparation.
When all ROIs of a slide have been collected, remove the collection tube. Scan and save again in "Navigation window" **(Fig. 4)**.
After using the LCM platform, remove all slides and collection tubes, shut down the LCM platform in the reversed order indicated in step 5.

Sample preparation

3d

12 ***Please refer to the original publication in the references (Coscia et al., 2020, supplementary material and methods). Methods in this section is adapted from it and optimized for the MPII project.***

Note

!! Several chemicals in this protocol are toxic, volatile, and corrosive. PLEASE HANDLE CAREFULLY IN A FUME HOOD WITH PERSONAL PROTECTIVE EQUIPMENT !!

13 **Reagent Setup**
Prepare all stock solutions and buffers as described in the original protocol unless certain modifications are mentioned below.

13.1 **Stock solutions:**
Tris/HCl [M] 600 millimolar (mM) , pH 8 , in ddH2O, filter, store at Room temperature
Trifluoroacetic acid (TFA) [M] 25 % (v/v) , in ddH2O, filter, store at Room temperature
2-chloroacetamide (CAA) [M] 500 millimolar (mM) , in ddH2O, store at -20 °C
1,4-dithiothreitol (DTT) [M] 100 millimolar (mM) , in ddH2O, store at -20 °C
Trypsin/Lys-C Mix [M] 1 µg/µL , store at -80 °C

13.2 **Lysis buffer**

Note

!! Always prepare freshly !!

Prepare lysis buffer containing [M] 50 % (v/v) Acetonitrile (ACN), [M] 300 millimolar (mM) Tris-HCl(pH 8.0). I.e. Mix ACN and 600 mM Tris/HCl at 1:1 ratio.

13.3 **Digestion buffer (Master mix)**

[M] 10 % (v/v) TFE in ddH2O

Enzyme should be added freshly when proceed to step 15.6 at a protein:enzyme ratio of 25:1. If you add trypsin and LysC separately, keep a protein:enzyme ratio at 50:1, respectively.

13.4 **SDB-RPS StageTips wash buffer 1**

[M] 1 % (v/v) TFA in isopropanol, filter.

13.5 **SDB-RPS StageTips wash buffer 2**

[M] 0.2 % (v/v) TFA in ddH2O, filter.

13.6 **SDB-RPS StageTips elution buffer**

[M] 1 % (v/v) ammonia, [M] 80 % (v/v) ACN, in ddH2O, filter, prepare fresh

14 Tissue homogenization and formalin de-crosslinking

14.1 Open the sample collection tube carefully since microdissected ROIs are on the adhesive cap. Add 20 µL + 20 µL lysis buffer to collect **ALL COLLECTED TISSUE ROIs** from the adhesive cap to a new PCR tube in 2 moves, in total volume of 40 µL .

Prepare **Ref tube 1**: add 40 µL ddH2O in a new PCR tube for estimation, and mark at the liquid level with a water fest lab marker.

14.2 **1st Sonication**

Change the water in Bioruptor and turn on mini-chiller in advance for cooling down to 4 °C

Sonicate tissue in 15 cycles in Bioruptor: high intensity, 30s on and 30s off per cycle.
Centrifuge any condensation down

14.3 De-crosslinking with a PCR machine at 65 °C overnight (12-16 hours)

> Note
>
> **!! Test the tube before the experiment and make sure it's air- and leak-tight. We chose the Eppendorf PCR tube because it's sealed well !!**

The long heating period can lead to liquid loss.

Compare the liquid level of sample tubes to **Ref tube 1**, if necessary, adjust the volume to approximately 40 µL by adding Ultra-Pure water.

14.4 **2nd Sonication**

Turn on the mini-chiller in advance to cool down to 4 °C

Take PCR tubes out from the PCR machine, and spin down any condensation. Fill up the volume to 40 µL with water if necessary.

Sonicate tissue in 15 cycles in Bioruptor: high intensity, 30s on and 30s off per cycle.
Centrifuge any condensation down

15 **Protein reduction, alkylation, and enzymatic digestion**

15.1

Note

!! Since some of the reagents are volatile, we highly recommend preparing a reference tube, in order to control the liquid volume !!

Compare the liquid level of sample tubes to **Ref tube 1**, if necessary, adjust the volume to approximately 40 µL by adding Ultra-Pure water at any step.

15.2 Add 2 µL of DTT(stock concentration [M] 100 millimolar (mM) , working concentration [M] 5 millimolar (mM)) to the sample tube.

Incubate in a ThermoMixer at 1000 rpm, 20-25°C, 00:20:00 .

Spin down.

15.3 Add 2 µl of CAA(stock concentration [M] 500 millimolar (mM) , working concentration [M] 25 millimolar (mM)) to the sample tube.

Incubate in a ThermoMixer at 1000 rpm, 20-25°C, 00:20:00 .

Spin down.

15.4 Prepare **Ref tube 2**: add 20 µL ddH2O in a new PCR tube for estimation, and mark at the liquid level with a water fest lab marker.

15.5 Vacuum-dry the samples to a remaining volume of ca. 20 µL , at 60 °C about 45 minutes.

Keep the **Ref tube 2** closed. Prolong this step if necessary.
The long heating period can lead to liquid loss.

Compare the liquid level of sample tubes to **Ref tube 2**, if necessary, adjust the volume to approximately 20 µL by adding Ultra-Pure water.

15.6 Prepare digestion buffer Master Mix with enzyme mix.
Calculation example:

Overestimate 600k-800k µm^2 ROIS can yield about 5 µg protein. The Trypsin/Lys-C Mix at 1 µg/µl should be used at a protein:enzyme ratio of 1:25. Thus, 5 µg protein requires 0.2 µg enzyme mix.
Master Mix (MM)= 1.1 *(E+B)
Volume of enzyme(E)= 1.1*(Number of samples * required enzyme (0.2 µg) * stock concentration (1 µg/µl))
Volume of Digestion buffer(B)= 1.1*(80 µl of Digestion buffer * number of samples)

For ROIs less than 400k µm^{2}of area, recommend to use half the amount of enzyme mix, i.e. 0.1 µg per sample.

15.7 Prepare **Ref tube 3**: add 100 µL ddH2O in a new PCR tube for estimation, and mark at the liquid level with a water fest lab marker.
Add 80 µL freshly prepared Master Mix to each sample tube.

15.8 **3rd Sonication**
Turn on the mini-chiller in advance to cool down to 4 °C .

Sonicate tissue in 15 cycles in Bioruptor: high intensity, 30s on and 30s off per cycle.
Centrifuge any condensation down

15.9 Incubate in a ThermoMixer at 1000 rpm, 37°C , overnight (12-16 hours).

Note

!! Do not prolong this step in order to avoid over-digestion of samples !!

The long heating period can lead to liquid loss.
Compare the liquid level of sample tubes to **Ref tube 3**, if necessary, adjust the volume to approximately 100 µL by adding Ultra-Pure water.

15.10 Add 5 µl of TFA (stock concentration [M] 25 % (v/v) , working concentration [M] 1 % (v/v)) to acidify the solution and inactivate the enzyme mix, Mix by pipetting and spin down for 5 mins to pellet any debris.
Here is a stop point, samples can be temporarily stored at -80 °C

16 **Peptide Clean-up**

Low pH is required for peptide clean-up by SDB-RPS StageTips

In the following steps, you can choose commercial or homemade StageTips (Rappsilber et al., 2007).

In the reference, you can find more information about the compatible tip boxes, plates, and tubes.

16.1 Prepare SDB-RPS StageTips with two layers of Solid Phase Extraction Disks(SDB-RPS).

A syringe-like tool with spring, and new boxes of P200 pipette tips without filters.

Punch the disks with the syringe tip. Inject the cut piece into the tip of a P200 pipette tip. Fix the disk with 5-6 empty injections.**(Fig. 10)**

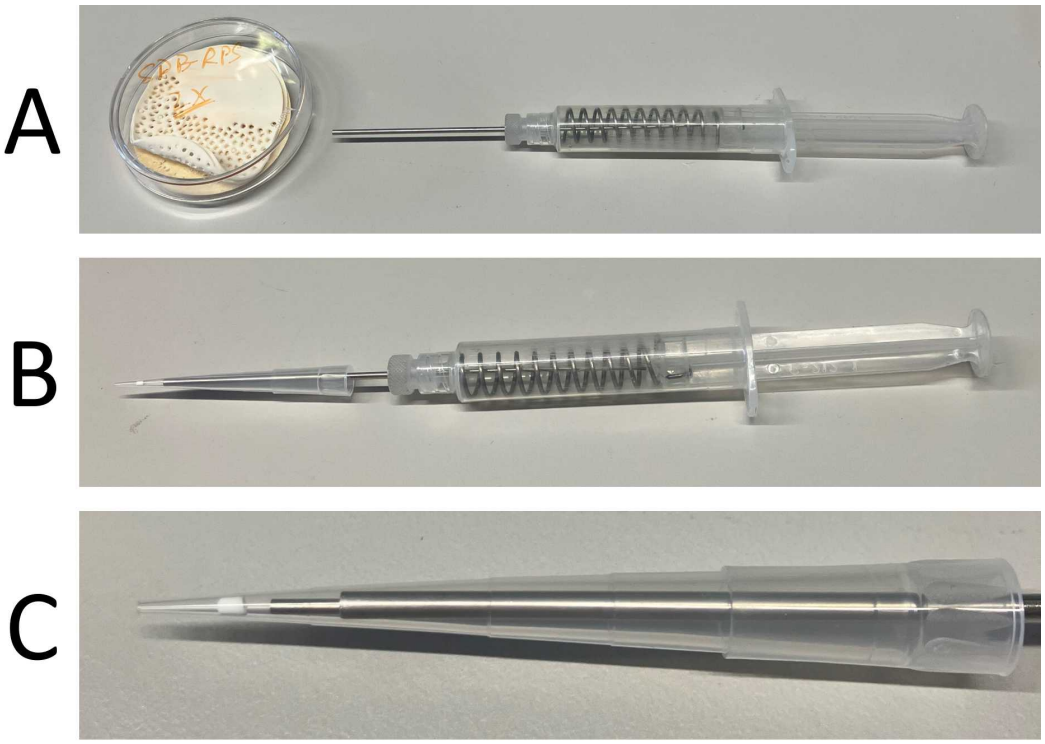

Figure 10. A: Punch the disks with the syringe-like tool. B and C: inject the cut piece into the tip of a P200 pipette tip.

16.2 Insert StageTips in the rack for holding. Label the StageTips if necessary.

Appropriately assemble the tips rack above the compatible deep-well plate, and make sure the tips are in the well **(Fig. 10, 11A)**. Use tapes to fix the racks.

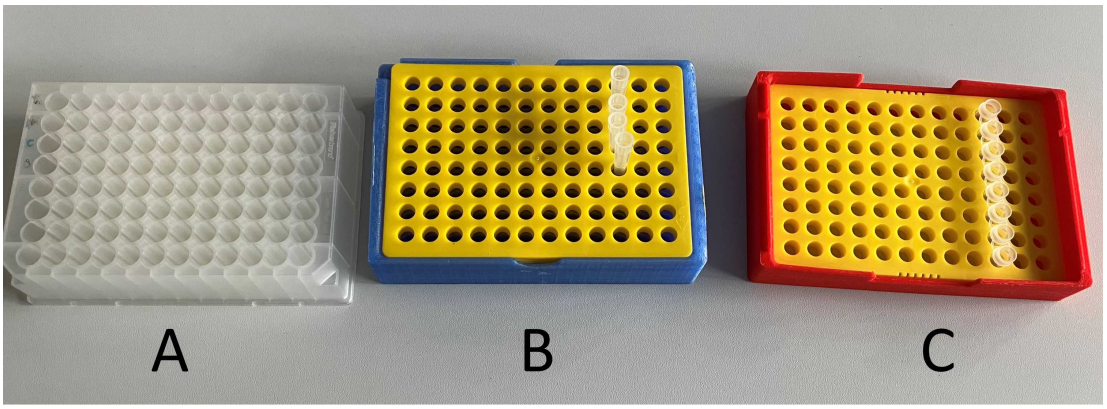

Figure 10. Plates and racks used for holding StageTips during washing and elution. A. deep-well plate to collect waste liquid; B: a rack to hold StageTips; C: a rack to hold capless PCR tubes for collecting peptides from elution.

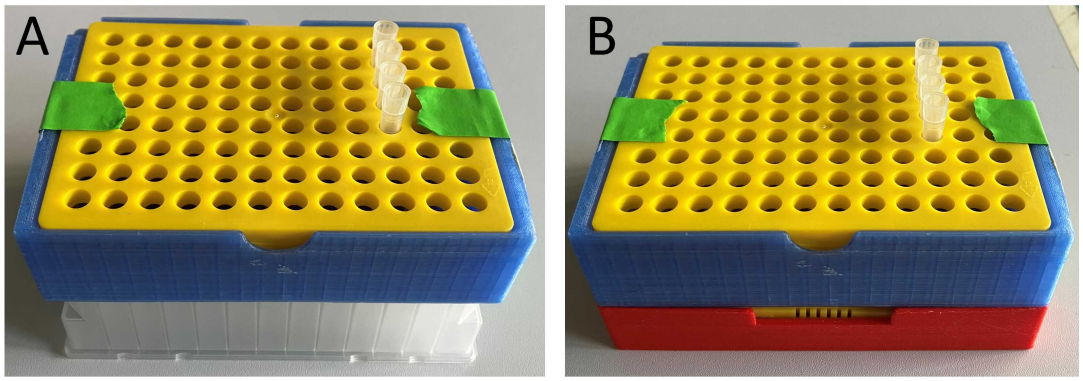

Figure 11. Assemble the plates and racks appropriately A: for washing; B: for collection.

16.3 Thaw the samples. Spin down to pellet any debris prior to sample loading.
Load ca. 90 µL of samples directly on SDB-RPS StageTips, careful not to load the bottom of the sample to avoid over-loading / clogging the StageTips.

16.4 Centrifuge at 750 x g, Room temperature, 00:08:00 . This step can be prolonged for 2-3 min if the liquid is not flowing through, but be careful not to leave SDB-RPS disks dry for too long. 8m

16.5 Discard the liquid in the deep-well plate.

Again, appropriately assemble the tips rack above the compatible deep-well plate, and make sure the tips are in the well **(Fig. 10, 11A)**. Use tapes to fix the racks. 8m

Add 200 µL of wash buffer 1 to the tip.

Centrifuge at 750 x g, Room temperature, 00:08:00 .

16.6 Discard the liquid in the deep-well plate. 8m

Again, appropriately assemble the tips rack above the compatible deep-well plate, and make sure the tips are in the well **(Fig. 10, 11A)**. Use tapes to fix the racks.

Add 200 µL of wash buffer 2 to the tip.

Centrifuge at 750 x g, Room temperature, 00:08:00 .

Discard the liquid in the deep-well plate.

16.7 **Peptide Elution** 16m

Label new capless PCR tubes for sample elution.

Insert the PCR tubes to the collection rack.

Appropriately assemble the tip rack above the compatible collection rack with capless PCR tubes. **Make sure the tips are in the PCR tubes (Fig. 10, 11B)**.

Add 50 µL of elution buffer to the tip.

Centrifuge at 750 x g, Room temperature, 00:08:00 . **DO NOT discard the flow-through.**

Again, add 50 µL of elution buffer to the tip.

Centrifuge at 750 x g, Room temperature, 00:08:00 . **DO NOT discard the flow-through.**

16.8 Carefully disassemble the racks and take out PCR tubes from the collection plate.

Vacuum-dry completely at 45 °C . It takes approximately 1 hour. If necessary, prolong the drying time until all samples are completely dried. A thin layer of white powder should be visible inside the PCR tube.

Close the PCR tubes with caps and seal the closed PCR tubes with parafilm.

17 Now the samples are ready for final preparation for mass spectrometry measurement according to experimental and instrumental setup.

Peptides can be stored at -80 °C until reconstitution for mass spectrometry measurement.

The StageTips can be kept at -20 °C until the mass spectrometry measurement result is received.

Protocol references

Coscia, F., Doll, S., Bech, J. M., Schweizer, L., Mund, A., Lengyel, E., Lindebjerg, J., Madsen, G. I., Moreira, J. M., & Mann, M. (2020). A streamlined mass spectrometry-based proteomics workflow for large-scale FFPE tissue analysis. *The Journal of pathology*, *251*(1), 100–112. https://doi.org/10.1002/path.5420 and **The supplementary materials and methods**

Rappsilber, J., Mann, M., & Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature protocols*, *2*(8), 1896–1906. https://doi.org/10.1038/nprot.2007.261

PALM RoboSoftware Quick Guide