

# Chapter 2

## Laser Microdissection-Based Microproteomics of Formalin-Fixed and Paraffin-Embedded (FFPE) Tissues

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### Abstract

Laser microdissection-based proteomics on formalin-fixed and paraffin-embedded tissues is usually performed from relatively large tissue areas or pools of multiple tissue pieces. However, several molecular pathology studies require working on very limited amounts of tissue. This is for example the case when very early cancer lesions have to be handled. Hereby, we present a method for the processing of very small pieces of formalin-fixed and paraffin-embedded tissues for proteomic purposes. This approach is designed in order to avoid sample loss during technical processing and to optimize the digestion of tissue areas containing as little as 2700 cells.

**Key words** Formalin-fixed and paraffin-embedded tissues, Histopathology, Cancer, Laser microdissection, Microproteomics

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### 1 Introduction

Laser microdissection (LMD) or laser capture microdissection (LCM) is the gold standard for the accurate sampling of pure bulks of cells from tissue sections. The evolution of “omics” methods allowed depicting molecular contexts in tissues for a wide panel of biomolecules. Deoxyribonucleic acids (DNA), ribonucleic acids (RNA), metabolites, and proteins can be extracted and analyzed from regions of interest of tissues containing only one cell type [1]. Proteomics has greatly evolved within the last decades owing to instrumental improvements providing optimal separation and analysis of peptides from proteins extracted and digested from laser microdissected tissue samples [2, 3]. Liquid chromatography (LC) can be performed in two separation dimensions to reduce the high complexity of proteolytic products from tissues [4]. Possibly, prefractionation can also be performed before LC, adding a further separation dimension to reduce sample complexity [5]. Liquid chromatography is then usually coupled to a mass

spectrometer in order to analyze eluted compounds. The field of mass spectrometry has also greatly evolved in terms of instrumentation. Improved mass resolution and analysis speed allow high-throughput detection and fragmentation of dozens of thousands of peptides in a few hours [6, 7]. Until recently, there was an unmet need of downscaling the amount of laser microdissected tissues for proteomic purposes. Formalin fixation combined with paraffin embedment (FFPE) tissue processing is widespread among pathology institutes [8]. FFPE tissue blocks indeed represent a gold mine for biomarker discovery studies. Recently, we developed a method for the proteomic analysis of cancerous FFPE tissue pieces containing 2700 cells [9]. The method consists in the direct digestion of formalin fixed proteins on the tissue piece itself. So far, it is the most efficient one in terms of proteolytic peptide yields, cost, and time. We present here the application of this approach for biomarker discovery purposes. The whole workflow has already successfully been applied to studies in the field of oncology [9, 10]. The biochemical procedure has also been tested for a proteomic investigation in the field of parasitology [11].

The approach also holds great promise for routine histopathological diagnosis.

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## 2 Materials

### 2.1 Reagents and Materials

1. Polyethylene naphthalate (PEN) membrane glass slides for laser microdissection (Leica Microsystems, Wetzlar, Germany).
2. Laser microdissection device: Leica LMD 7000 (Leica Microsystems, Wetzlar, Germany).
3. Milli-Q H<sub>2</sub>O.
4. Citric acid (CA) 50 mM.
5. CA 10 mM, pH 6.0.
6. NaOH 1 M.
7. Rapigest surfactant (Waters, Milford, MA) 0.1%.
8. NH<sub>4</sub>HCO<sub>3</sub> (ammonium bicarbonate) 500 mM.
9. NH<sub>4</sub>HCO<sub>3</sub> 100 mM.
10. NH<sub>4</sub>HCO<sub>3</sub> 50 mM.
11. NH<sub>4</sub>HCO<sub>3</sub> 25 mM.
12. Dithiothreitol (DTT) 500 mM.
13. DTT 131 mM.
14. DTT 171.6 mM.
15. Iodoacetamide (IAM) 500 mM.
16. IAM 194.67 mM.

17. Aluminum foils.
18. Hydrochloric acid (HCl) 10 mM.
19. Trypsin gold, mass spectrometry grade (Promega, Fitchburg, WI) 1 µg/µL.
20. Trypsin gold, mass spectrometry grade (Promega, Fitchburg, WI) 0.5 µg/µL.
21. Acetonitrile (ACN) 100%.
22. Trifluoroacetic acid (TFA) 10%.
23. Microtubes 0.6 mL.
24. KIMWIPES disposable (Kimberly-Clark, Dallas, TX).
25. Centrifuge.
26. Thermoshaker.
27. Antistatic gun Zerostat 3 (Sigma-Aldrich, St. Louis, MO).
28. Binocular microscope.
29. Sonicator.
30. Cleaning paper.
31. Speed vacuum centrifuge.
32. ZipTip (ZT) cartridge C<sub>18</sub> 2 µg (Millipore, Billerica, MA).
33. ZT C<sub>18</sub> 5 µg (Millipore, Billerica, MA).
34. NH<sub>4</sub>HCO<sub>2</sub> (ammonium hydroxide) 20 mM, pH 10.
35. Formic acid 0.1% in water.
36. Formic acid 0.1% in acetonitrile.
37. MassPREP Digestion Standard Mixtures (MPDS) Mix 1 and 2 (Waters, Milford, MA).
38. Speed vacuum (Thermo Scientific, Waltham, MA).
39. Ultra performance liquid chromatography (UPLC) Nanoacquity 2D (Waters, Milford, MA).
40. Reverse phase (RP) X-Bridge BEH C18 5 µm column (300 µm × 50 mm) (Waters, Milford, MA).
41. Trap column symmetry C18 5 µm (180 µm × 20 mm) (Waters, Milford, MA).
42. Analytical column BEH C18 1.7 µm (75 µm × 250 mm) (Waters, Milford, MA).
43. Orbitrap Mass spectrometer Q Exactive or Q Exactive plus (Thermo Fisher Scientific, Waltham, MA).

## **2.2 Citrate Buffer Solution**

Citric acid stock solution (50 mM): weigh 0.96 g of citric acid and dissolve in 99.94 mL Milli-Q water.

NaOH 1 M: Weigh 40 g of NaOH and dissolve in 1 L Milli-Q water.

Citric acid 10 mM, pH 6.0: Transfer 20 mL of the stock solution (50 mM) to a 100 mL bottle, adjust to 50 mL with Milli-Q water. Adjust the pH to 6.0 by adding 1 M NaOH. Adjust the final volume of 100 mL with Milli-Q water.

**2.3  $\text{NH}_4\text{HCO}_3$   
(Ammonium  
Bicarbonate) Solutions**

$\text{NH}_4\text{HCO}_3$  500 mM: Weigh 39.53 g of  $\text{NH}_4\text{HCO}_3$  and dilute in 1 L Milli-Q water. Store at 4 °C in a glass bottle.

$\text{NH}_4\text{HCO}_3$  100 mM: Dilute 5× the  $\text{NH}_4\text{HCO}_3$  500 mM solution:  $\text{NH}_4\text{HCO}_3$  500 mM/Milli-Q water 1:4. Store at 4 °C in a glass bottle.

$\text{NH}_4\text{HCO}_3$  50 mM: Dilute 10× the  $\text{NH}_4\text{HCO}_3$  500 mM solution:  $\text{NH}_4\text{HCO}_3$  500 mM/Milli-Q water 1:9. Store at 4 °C in a glass bottle.

$\text{NH}_4\text{HCO}_3$  25 mM: Dilute 20× the  $\text{NH}_4\text{HCO}_3$  500 mM solution:  $\text{NH}_4\text{HCO}_3$  500 mM/Milli-Q water 1:19. Store at 4 °C in a glass bottle.

**2.4 DTT 500 mM**

Weigh 0.7713 g of DTT. Solubilize with 10 mL of Milli-Q water. Aliquot in tubes with 100–500 µL and cover with aluminum foil. Freeze at –20 °C until use.

**2.5 DTT 131 mM in  
50 mM  $\text{NH}_4\text{HCO}_3$**

Mix 10 µL of DTT 500 mM with 9.1 µL of Milli-Q water and 19.1 µL of 100 mM  $\text{NH}_4\text{HCO}_3$  in a 0.6 mL microtube. Cover with an aluminum foil.

**2.6 DTT 171.6 mM in  
50 mM  $\text{NH}_4\text{HCO}_3$**

Mix 10 µL of DTT 500 mM with 4.57 µL Milli-Q water and 14.57 µL of 100 mM  $\text{NH}_4\text{HCO}_3$  in a 0.6 mL microtube. Cover with an aluminum foil.

**2.7 IAM 500 mM**

Weigh 1.85 g of IAM. Solubilize with 20 mL Milli-Q water. Aliquot tubes with 100–500 µL. Freeze at –20 °C until use.

**2.8 IAM 194.67 mM  
in 50 mM  $\text{NH}_4\text{HCO}_3$**

Mix 20 µL of IAM 500 mM with 5.68 µL Milli-Q water and 25.68 µL of 100 mM  $\text{NH}_4\text{HCO}_3$  in a 0.6 mL microtube. Cover with an aluminum foil.

**2.9 HCl 10 mM**

HCl stock solution (1 M): Commercial solutions of HCl are usually 12 M. Dilute 12× the 12 M HCl solution with Milli-Q water to obtain a 1 M stock solution: HCl 12 M/Milli-Q water 1:11.

HCl 10 mM: Dilute 100× the 1 M HCl solution with Milli-Q water: HCl 1 M/Milli-Q water 1:99.

**2.10 Trypsin Stock  
Solution (1 µg/µL)**

Reconstitute 100 µg of lyophilized trypsin in 100 µL of 10 mM HCl.

Aliquot in tubes with 1, 2, or 3 µL depending on further use, and store at –20 °C until use.

- 2.11 Trypsin 0.5 µg/µL** Dilute 2× a trypsin stock solution (1 µg/µL) in 25 mM  $\text{NH}_4\text{HCO}_3$ . Depending on the number of sample to process, the amount of stock trypsin solution should be adapted.
- 2.12 Rapigest SF Surfactant 0.1%** Reconstitute one vial (1 mg) in 1 mL  $\text{NH}_4\text{HCO}_3$  50 mM. Aliquot in tubes with 100 µL and store at  $-20^\circ\text{C}$  before use.
- 2.13 TFA 10%** Mix 10 mL of TFA with 90 mL of Milli-Q water. Store at  $4^\circ\text{C}$  in a glass bottle.
- 2.14 Zip-Tip (ZT) Solutions**  
 ZT Solution 1 (ACN + 0.1% TFA): Mix 999 µL ACN with 1 µL TFA in a tube.  
 ZT Solution 2 (ACN/ $\text{H}_2\text{O}$  1:1 + 0.1% TFA): Mix 499.5 µL ACN with 499.5 µL Milli-Q  $\text{H}_2\text{O}$  and 1 µL TFA in a tube.  
 ZT Solution 3 ( $\text{H}_2\text{O}$  + 0.1% TFA): Mix 999 µL  $\text{H}_2\text{O}$  with 1 µL TFA in a tube.  
 Larger volumes can also be prepared and stored in glass bottles at  $4^\circ\text{C}$ .
- 2.15 20 mM  $\text{NH}_4\text{HCO}_2$  (ammonium formate) solution pH 10**  
 200 mM ammonium formate pH 10 stock solution:  $\text{NH}_4\text{OH}$  (ammonium hydroxide) commercial solutions are usually 25%. For 1 L of solution, use a 1 L graduated cylinder and place 850–900 mL of Milli-Q  $\text{H}_2\text{O}$ . Add 15.4 mL of  $\text{NH}_4\text{OH}$  25%. Mix well. Add 1.62 mL  $\text{HCO}_2\text{H}$  (formic acid). Mix well. Adjust the pH to 10 with  $\text{NH}_4\text{OH}$  25% or formic acid. Adjust the volume to 1 L with Milli-Q  $\text{H}_2\text{O}$ . Store in a glass bottle at  $4^\circ\text{C}$ .  
 20 mM ammonium formate pH 10: for 1 L of solution, mix 100 mL of 200 mM ammonium formate pH 10 stock solution with 900 mL of Milli-Q  $\text{H}_2\text{O}$ . Check the pH. Store in glass bottles at  $4^\circ\text{C}$ .
- 2.16 0.1% formic acid in water** Mix 1 mL of formic acid with 999 mL of Milli-Q  $\text{H}_2\text{O}$ .
- 2.17 0.1% formic acid in acetonitrile** Mix 1 mL of formic acid with 999 mL of ACN.

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### 3 Methods

#### 3.1 Tissue Sectioning, Cell Counting, and Microdissection

1. After surgery, immerse the tissue overnight in 10% formalin, dehydrate and embed in paraffin using well-known procedures widely used among institutes of pathology [12]. Cut 5 µm-thick sections and deposit on LMD membrane slides for LMD

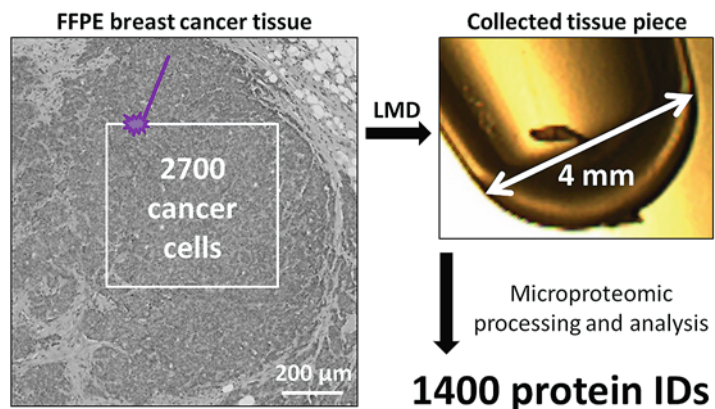
and histological glass slides for hematoxylin and eosin staining (HE). From the HE stained section, count an equivalent number of cells in tissue(s) region(s) of interest between samples. Several software products exist for cell counting from tissues such as QuPath (<https://qupath.github.io/>). Some tools such as Cytomine (<http://www.cytomine.be/>) [13] also allow sharing online tissue annotations and comments of the tissues between different institutions.

2. Perform microdissection: we used the Leica LMD 7000 (Leica Microsystems, Wetzlar, Germany) with the following laser settings: laser wavelength: 349 nm, pulse energy: 52 J, numerical aperture: 15, specimen balance: 14, laser head current: 100%, pulse frequency: 174 Hz, focus offset: 50. The settings should be adapted to the tissue type.

### 3.2 Sample Preprocessing

This step consists in collecting the tissue pieces from the cap to the bottom of the tubes to improve monitoring during processing (*see Notes 1, 2 and 3*).

1. During laser microdissection, the tissue pieces are collected in tube caps. Store the tubes at 4 °C before use, in a clean environment.
2. Use the antistatic gun to get rid of static electricity during sample preprocessing.
3. Centrifuge the tube at  $15,000 \times g$  during 5 min at room temperature (RT) and dispose the tubes in a clean holder.
4. Check for the presence of the tissue piece(s) at the bottom of the tubes using a binocular microscope. In Fig. 1 is illustrated the visualization of a LMD breast cancer tissue piece observed under the binocular microscope.



**Fig. 1** Illustration of the collection of a tissue piece by LMD and the verification of its presence at the bottom of the tube through a binocular microscope. From a breast cancer tissue piece containing 2700 cells, it was possible to retrieve 1400 protein identifications [9] using the workflow described below. Adapted from [9]

### 3.3 Antigen Retrieval

This step consists in getting rid of the methylene bridges linking proteins induced by formalin fixation.

1. Gently add 20  $\mu\text{L}$  of 10 mM pH 6.0 citrate buffer on the tissue piece at the bottom of the tube without touching it with the tip.
2. Centrifuge at  $15,000 \times g$  for 5 min at RT. If it is still visible, ensure yourself that the tissue is properly immersed in citric acid.
3. Sonicate the immersed tissue pieces for 10 min in a holder that avoids overheating of the tubes. This step allows removing air bubbles that may stay at the surface of the tissue.
4. Incubate in a thermoshaker at 99 °C for 1 h at 800 rpm. Quickly spin the tubes every 10 min in order to collect the condensed water from the cap to the bottom of the tubes.
5. Collect the tubes in a clean holder and let these cool at RT.
6. Add 2.2  $\mu\text{L}$  of Rapigest 0.1% in the samples to obtain a final 0.01% concentration and quickly spin.
7. Shake at 800 rpm for 10 min at RT.
8. Add 2  $\mu\text{L}$  of 500 mM  $\text{NH}_4\text{HCO}_3$  to all the samples. Shake 10 min at 800 rpm, quickly spin and check that the pH of the samples is 7.

### 3.4 Reduction-Alkylation-Reduction

This step consists in getting rid of disulfide bridges still existing within proteins after antigen retrieval (*see* **Notes 4** and **5**).

1. Reduction (reduction of the disulfide bridges and liberation of the thiol groups): the samples have now a volume of 24.2  $\mu\text{L}$ . Add 2  $\mu\text{L}$  of 131 mM DTT in 50 mM  $\text{NH}_4\text{HCO}_3$  to obtain a final concentration of 10 mM DTT. Incubate the samples for 40 min at 56 °C while shaking at 800 rpm and quickly spin.
2. Alkylation (blocking thiol groups with IAM): the samples have now a volume of 26.2  $\mu\text{L}$ . Let the samples cool down at RT and add 3  $\mu\text{L}$  of 194.67 mM IAM in 50 mM  $\text{NH}_4\text{HCO}_3$  to obtain a final concentration of 20 mM IAM. Incubate at RT for 30 min while shaking at 800 rpm and quickly spin.
3. Reduction (eliminate IAM in excess): The samples have a volume of 29.2  $\mu\text{L}$ . Add 2  $\mu\text{L}$  of 171.6 mM DTT in 50 mM  $\text{NH}_4\text{HCO}_3$  to add a concentration of 11 mM DTT. Incubate 10 min at RT while shaking at 800 rpm and quickly spin.

### 3.5 Digestion

This step consists in digesting the proteins from the tissue piece (*see* **Notes 6** and **7**).

1. The samples have a volume of 31.2  $\mu\text{L}$ . Add 4.26  $\mu\text{L}$  of 0.5  $\mu\text{g}/\mu\text{L}$  trypsin to obtain a final concentration of about 60  $\mu\text{g}/\text{mL}$ .
2. Incubate at 37 °C overnight while shaking at 800 rpm and quickly spin.

3. The samples have a volume of 35.46  $\mu\text{L}$ . Add 2.26  $\mu\text{L}$  of 0.5  $\mu\text{g}/\mu\text{L}$  trypsin to increase the concentration by about 30  $\mu\text{g}/\text{mL}$ .
4. The samples have now a volume of 37.72  $\mu\text{L}$ . Add 150.88  $\mu\text{L}$  of ACN to obtain a solution of 80% ACN and quickly spin.
5. Incubate at 37 °C for 3 h while shaking at 800 rpm and quickly spin.
6. The samples have a volume of 188.6  $\mu\text{L}$ . Stop digestion by adding 10  $\mu\text{L}$  of TFA 10% to obtain a final concentration of about 0.5% TFA in the tube. Shake 10 min at 800 rpm, quickly spin and check the pH of the solution (pH < 3).
7. Incubate for 45 min at 37 °C while shaking at 800 rpm in order to cleave Rapigest.
8. Centrifuge the samples at  $21,000 \times g$  for 10 min, at 4 °C.
9. Collect the supernatant and transfer in new identified 0.6 mL tubes.
10. Evaporate the samples using the Speed Vacuum. Store the samples at 4 °C if necessary for 1 week maximum. If the storage time exceeds 1 week, store the samples at -20 °C.

### **3.6 Purification/ Desalting/ Concentration of the Samples**

This step consists in purifying, desalting the sample and concentrate the proteolytic peptides by solid phase extraction (SPE).

1. Prepare the three solutions described in Subheading 2.14.
2. If the samples were dried, resuspend in 20  $\mu\text{L}$  Milli-Q  $\text{H}_2\text{O}$  with 0.1% TFA (ZT solution 3). If the SPE is performed just after the digestion, transfer the necessary volume of sample in another 0.6 mL tube and adjust to 0.1% TFA, with maximum 5% of organic solvent, in an ideal volume of 20  $\mu\text{L}$ . Depending on the expected amount of peptides resulting from the digestion process, use ZT C<sub>18</sub> 5  $\mu\text{g}$  or ZT C<sub>18</sub> 2  $\mu\text{g}$  ( $\mu\text{ZT}$ ).
3. Set the micropipette to 10  $\mu\text{L}$ . Wash the ZT with solution 1: take and withdraw in the waste. Repeat three times.
4. Activate the ZT by taking solution 2. Withdraw in the waste. Repeat three times.
5. Equilibrate the ZT: take solution 3 and withdraw in the waste. Repeat three times.
6. Load the sample. Adjust the sample to the used ZT. If the expected concentration of peptides in the sample is too high (>5  $\mu\text{g}$  when ZT 5  $\mu\text{g}$  are used, >2  $\mu\text{g}$  when ZT 2  $\mu\text{g}$  are used), dilute with a 0.1% TFA solution. Pipet ten times to load the peptides to the ZT. If the sample is diluted or if the volume of sample is higher than 20  $\mu\text{L}$ , pipet 25 times.
7. Wash the samples: take solution 3 and withdraw in the waste. Repeat five times.



8. Elute the sample: Take solution 2 and collect in a new microtube. The peptides are present in the solution in the new microtube. Repeat once. The proteolytic digest is then concentrated in 40  $\mu\text{L}$  of ACN/  $\text{H}_2\text{O}$  1:1 and 0.1% TFA.
9. Evaporate the sample completely with the speed vacuum centrifuge.
10. Proceed to the subsequent LC-MS/MS analysis or store the sample at 4  $^{\circ}\text{C}$  for 1 week maximum, at  $-20^{\circ}\text{C}$  if the analysis cannot be performed within 1 week.

### **3.7 Two Dimensions NanoLC-Orbitrap Mass Spectrometry Analysis**

1. Resuspend the samples in 10–11  $\mu\text{L}$  of appropriate buffer for 2D LC-MS and MPDS Mix in order to get a final concentration of 50 fmol alcohol dehydrogenase (ADH) per volume of injection (9  $\mu\text{L}$ ). Use different MPDS Mix solutions (1 or 2) in samples of different types. For example: if two conditions are compared (A and B), use MPDS mix 1 for samples from condition A and MPDS mix 2 for samples from condition B. This will allow us to further control that the ratio of proteins from MPDS mixes 1 and 2 is the one expected.
2. 9  $\mu\text{L}$  of the samples are injected in the 2D LC-MS system of choice (UPLC Nanoacquity 2D (Waters, Milford, MA) controlled by MassLynx in our case) coupled to a Q Exactive Plus, controlled by XCalibur. *See Notes 8 and 9.*
3. In our case, the samples are then injected in a two dimensions RP/RP system, with a first dimension in a high pH (pH 10) and a second dimension in a low pH (pH 3). The peptides are loaded on the high pH column (X-Bridge BEH C18 5  $\mu\text{m}$  (300  $\mu\text{m} \times 50 \text{ mm}$ )) at 2  $\mu\text{L}/\text{min}$  (20 mM ammonium formate solution adjusted to pH 10) and three elution steps (15 min each) with the following percentages of ACN are realized: 13.3% (fraction 1), 19% (fraction 2), and 65% (fraction 3).
4. The eluate from the “high pH” column is then diluted ten times with acidified water before being loaded on the trap column (Symmetry C18 5  $\mu\text{m}$  (180  $\mu\text{m} \times 20 \text{ mm}$ )) and separated on the “low pH” analytical column (BEH C18 1.7  $\mu\text{m}$  (75  $\mu\text{m} \times 250 \text{ mm}$ )). The gradient on the low pH column is 140 min long with the following settings: flow rate of 250 nL/min, solvent A (0.1% formic acid in water), and solvent B (0.1% formic acid in acetonitrile) with a linear gradient as follows: 0 min, 99% A; 5 min, 93% A; 140 min, 65% A. Cleaning and re-equilibration steps then take place during the following 40 min (total run of 180 min).
5. The LC eluent is then directly electrosprayed from the analytical column at 2.1 kV voltage through the liquid junction of the nanospray source. The chromatography system was coupled to a Thermo Scientific Q Exactive Plus Hybrid quadrupole-

orbitrap mass spectrometer (Thermo Fisher Scientific, USA), programmed for data-dependent acquisition mode, with the following settings:

- Top 10 (Data Dependent Acquisition).
- Parameters for MS: mass range:  $m/z$  400–1750, resolution: 70,000, AGC target:  $1 \times 10^6$ , maximum injection time: 200 ms.
- Parameters for MS/MS: isolation window:  $m/z$  2.0, stepped normalized collision energy (NCE): 21.2, 25, 28.8, Resolution: 17,500; AGC target:  $1 \times 10^5$ , maximum injection time: 200 ms, underfill ratio: 1.0%, dynamic exclusion: 10 s.

### 3.8 Data Processing for Biomarker Discovery

We propose here a method for biomarker discovery using the well-known and widely used software Maxquant and Perseus (*see Note 10*). The present settings were designed for the comparison of two different conditions (for example two cancer types from the same organ such as squamous cell carcinoma of the lung vs. adenocarcinoma of the lung).

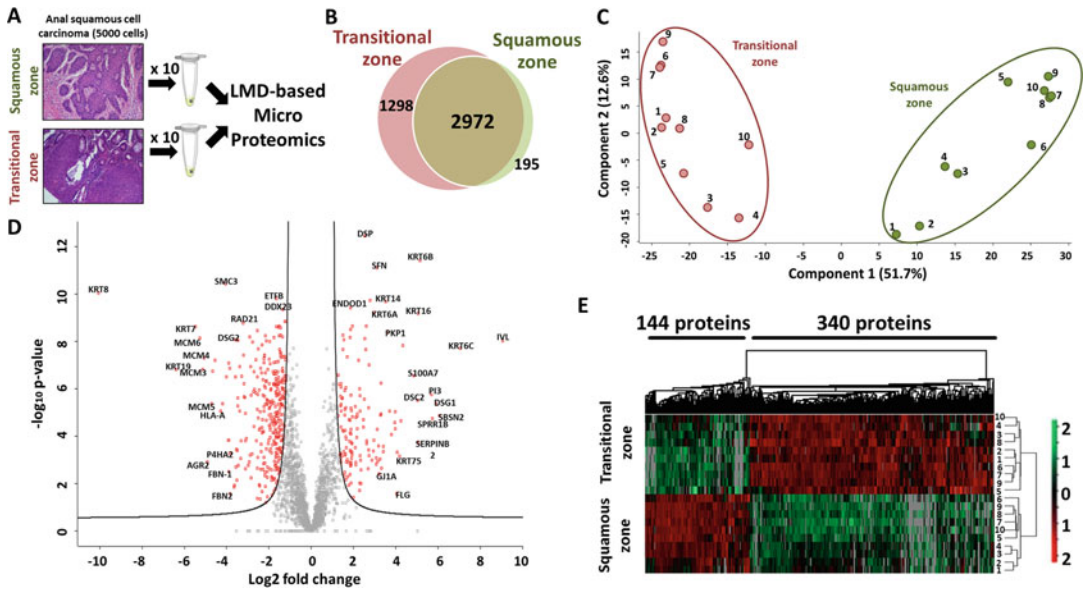
#### 1. Label-Free Quantification (LFQ)

Use the last version of Maxquant software. Load the samples from the two conditions to compare. At least five biological replicates per condition should be processed. For the identification, use Andromeda search engine with the last release of Uniprot human database for interrogation. Use the following parameters: N-ter acetylation, oxidation of methionines as variable modifications, carbamidomethylation of the cysteines as fixed modification. Set the maximum number of miscleavages at 2 and the minimal length for identification at 7 amino acids and at least two peptides required for identification, including one unique peptide. Check “LFQ” for data normalization. Set the maximum ratio count for LFQ at 2. Set the main search tolerance at 4.5 ppm. Set peptide spectrum match (PSM) and false discovery rate (FDR) at 0.01.

For detailed explanations on the different parameters for MaxQuant and an informed choice of these for data processing setup, please refer to [14].

### 3.9 Statistical Analysis

Use the latest version of Perseus. Filter rows by removing “only identified by site” and “reverse” entries. Log<sub>2</sub> transform the intensities. Perform the categorical annotation by annotating samples from each condition with the same name. Add annotation to the samples with gene ontologies (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Filter rows based on valid values by keeping 4/5 values in at least one group. Perform a two samples *t*-test with the fold change of interest, preferentially 2 ( $S_0 = 1$ ), a FDR of 0.01 and 250 randomizations. Perform a



**Fig. 2** Example of biomarker discovery study [10]. Ten biological cases from two types of anal carcinoma originating from different microanatomical regions of the anal canal were selected, each. The LMD-based microproteomics method was applied on tissue pieces containing 5000 cancer cells (a). A total of 4465 protein IDs were retrieved, including 2972 commune proteins between the two cancer types (b). The principal component analysis of the dataset clearly allowed distinguishing the samples from the two different cancer types, on a molecular basis (c). The *t*-test analysis allowed us to retrieve about 500 protein markers with at least two-fold variation between the two cancer types. These can be visualized within a Volcano plot. In this representation, every marker is highlighted in red (d). Hierarchical clustering of the dataset based on those 500 proteins also allowed deciphering the two types of cancers (e). Adapted from [10]

volcano plot with the same settings. Every significantly up or down-regulated protein stands upon the lateral lines. For PCA, filter the data from all the non-valid values and perform the PCA without category enrichment for components. For clustering, previously perform *Z*-score normalization. Clustering can be performed from the whole dataset of filtered data (for example *t*-test positive values). Euclidian distance and average linkage without *k*-mean should be used as parameters.

For detailed explanations on the different parameters for Perseus and an informed choice of these for statistical analysis, please refer to [15].

The results of a biomarker discovery study are illustrated in Fig. 2.

## 4 Notes

The overall workflow relies on a principle of on-tissue digestion. In this context, the laser microdissected tissue pieces have to remain in the tubes during the whole tissue processing, until the end of the

digestion steps. Several considerations have to be taken into account in order to avoid sample loss. Some other chemical parameters have to be adapted for the optimal digestion of the protein from the tissue samples. Finally, specific analytical settings are more adequate to the analysis of restricted amounts of proteolytic peptides from small tissues pieces.

1. The number of tissue regions to be microdissected should be reduced to the minimum in order to properly monitor the presence of the tissue piece(s) at the bottom of the tube after centrifugation.
2. The tubes should not be opened before centrifugation.
3. Under the binocular, the tissue pieces should not be confused with eventual scratches at the bottom of the tube that could be caused by the tubes holder during LMD.
4. Nothing should touch the tissue piece during the processing. Solutions should then be added by letting it flow on the boundaries of the tube.
5. The reactions have then to take place at the bottom of the tube and everything has to be set to restrict the reactions in this same exact location. Shaking is then used and not vortexing. By vortexing, the reaction buffer could indeed carry the tissue piece to the boundary of the tube where it could stay stucked.
6. The extraction process during this workflow is minimal and the digestion of the proteins occurs majorly on the tissue piece itself. In order to allow the optimal digestion of the proteins concentrated in such a limited volume, high concentrations of trypsin should be used. The digestion takes place in two steps. The first one occurs in 50 mM  $\text{NH}_4\text{HCO}_3$  buffer and represents the “classical” digestion step. The second step occurs in 80% ACN and allows the digestion of the most hydrophobic proteins.
7. The digestion is enhanced with low concentrations of RG (0.01%). This detergent can be used during the digestion, without any clean-up procedure for its removal. A cleanup before digestion indeed leads to the loss of the tissue piece [9]. RG helps for the digestion of the most hydrophobic proteins. We observed that using low concentrations of RG allowed retrieving a higher number of peptide identifications [9].
8. The analytical procedure is adapted to weakly concentrated mixtures. The processing of small laser microdissected tissues pieces gives rise to low concentrations of peptides. It was proven before that the combination of long LC elution time and long injection time for MS/MS allows retrieving a high number of peptide/protein identifications.

9. Different combinations of instrumentation can be used for LC and MS. Today, MS data from many vendors are compatible to Maxquant processing. However, a large panel of statistical software solutions exists for data normalization and processing.
10. The method presented here is set up for biomarker discovery applications. In the near future, the approach is expected to have applications in routine histopathological diagnosis.

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