

## Proteomic Analysis of Human Bladder Tissue Using SELDI<sup>®</sup> Approach Following Microdissection Techniques

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### Summary

Lysing of a complete biopsy sample results in a mixture of desired and undesired proteins, reflecting the originating cell types. Therefore microdissecting tissue material is mandatory prior to sample lysis and all downstream applications of protein analysis (proteomics). The two most important dissecting methods for bladder tissue specimens are manual microdissection and laser microdissection. Sample transfer can further be separated into manual laser pressure catapulting (LPC) and laser capture microdissection (LCM). One of the possible downstream applications of protein analysis is surface-enhanced laser desorption ionization (SELDI) time-of-flight mass spectrometry. The small quantities of tissue obtained by microdissection are sufficient for use in the SELDI technique.

**Key Words:** Proteomics; SELDI; mass spectrometry; lysis; microdissection.

### 1. Introduction

Following completion of the human genome project (1,2) the study of proteins is assuming much greater importance. The proteome is by definition (3) the totality of all proteins expressed in a cell; its analysis is called proteomics. Progress in sophisticated, sensitive, and high-throughput analytical methods is impressive, but should not distract from the importance of primary sample preparation. The inherent cellular heterogeneity of tissues is a major problem when dealing with sample preparation for proteomics (4). A tumor biopsy, for example, will contain—in addition to the diseased tissue—surrounding normal tissue including epithelium, endothelium, connective tissue, and infiltrating lymphocytes. Microdissection is the method of choice for addressing this problem. Regarding the complex tissue architecture, it is necessary to use consecu-

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tive sections with every 10th section hematoxylin and eosin (H&E) stained for diagnostic purposes. From two consecutive H&E-stained sections the tissue architecture of the nine unstained sections in between can be estimated without generating unacceptable artifacts. Microdissection itself is a well-known tool from genomics and has been adapted slightly for proteomics. For large tissue areas manual microdissection using syringe needles for dissecting and transferring tissue is still the method of choice, while observing under a low-magnifying stereo microscope. As soon as the areas of interest become smaller, a higher-magnifying microscope is required to observe the manipulations; therefore, laser-assisted microdissection then becomes the method of choice. The two most important laser-based microdissection methods are laser pressure catapulting (LPC) (5,6) and laser capture microdissection (LCM) (7,8). Since LCM allows for direct procurement isolation of the cells of interest, LCM seems to have become the preferred tool for proteomics.

SELDI<sup>®</sup> mass spectrometry has established itself as a sensitive, high-throughput method for protein analysis. As the obtained protein patterns are highly reproducible, this method is nowadays one of the leading technologies in screening samples for the presence of a specific protein, e.g., a tumor marker. Finding such a specific protein will enable enhancement of current diagnosis and may even lead to new therapeutic approaches.

## 2. Materials

1. Snap-frozen normal urothelium and pTaG1 papillary bladder tumor biopsies.
2. Storage equipment: dry ice,  $-80^{\circ}\text{C}$  freezer.
3. KILLIK frozen section medium (Bio-optica, Milano, Italy).
4. Cryostat.
5. Glass slides (Engelbrecht, Edermuende, Germany).
6. PEN-polyethylene-membrane, 1.35 mm thick (P.A.L.M. Microlaser Technologies, Bernried, Germany).
7. Adhesive tape (Tesa AG, Hamburg, Germany).
8. 50-mL polypropylene centrifuge tubes.
9. Ethanol 100%, 90%, 70%.
10. Ultrapure water ("Millipore water").
11. Mayer's hematoxylin solution (Sigma Diagnostics, St. Louis, MO).
12.  $\text{NaHCO}_3$ .
13. Xylene.
14. Complete<sup>™</sup> Protease inhibitors cocktail tablets (Roche Diagnostics).
15. Nonidet NP40 10% aqueous solution (Roche Diagnostics).
16. Dulbecco's Phosphate-Buffered Saline (PBS, GIBCO<sup>™</sup> Invitrogen Corporation, Grand Island, NY).
17. Stereo microscope 40X.
18. Microlancets (Microlance3, Becton Dickinson).
19. Arcturus<sup>®</sup> PixCell II (Arcturus, Mountain View, CA, USA).

20. CapSure™ LCM caps (Arcturus).
21. Safe-Lock-Tubes 2,0 mL (Eppendorf AG, Hamburg, Germany).
22. PALM® MicroBeam (P.A.L.M.).
23. PCR tubes PCR-02D-C 0.2 mL (Axygen Scientific, Inc., Union City, CA).
24. AdhesiveCaps (P.A.L.M.).
25. Proteinchip Biology System II (Ciphergen, Fremont, CA).
26. SAX2 Proteinchip Array (Ciphergen).
27. WCX2 Proteinchip Array (Ciphergen).
28. IMAC3 Proteinchip Array (Ciphergen).
29. Bioprocessor 96-Well (Ciphergen).
30. Bioprocessor accessory, 96-well disposable cartridge and gasket (Ciphergen).
31. 200- $\mu$ L 8-channel pipettor (e.g., Eppendorf, Eppendorf AG, Hamburg, Germany).
32. 10 mM HCl.
33. Autoclaved ultrapure water (“Millipore water”).
34. 10 mM ammonium acetate.
35. 50 mM nickel sulfate.
36. 0.1% Triton X100 in PBS.
37. Plastic lid of a pipet tip tray.
38. Vacuum device.
39. Trifluoroacetic acid.
40. Acetonitril (Sigma, HPLC grade).
41. Cinnamic acid (Sigma).

### 3. Methods

The subsequent descriptions are divided into (1) sample acquisition, (2) sample preparation and treatment, (3) performing microdissection, (4) sample lysis, and (5) SELDI analysis.

#### 3.1. Sample Acquisition

All biopsies are taken within an ethically approved prospective study on photodynamic diagnosis with 5-aminolevulinic acid (**9,10**) for bladder cancer. The study involves consequent molecular analysis of biopsy material. Visibly manifest tumors, suspicious areas, and normal urothelium were collected endoscopically with biopsy forceps and snap-frozen in liquid nitrogen immediately thereafter. For subsequent sample treatment the tissue was stored at  $-80^{\circ}\text{C}$  (long-term) or on dry ice (short-term).

#### 3.2. Sample Preparation and Treatment

##### 3.2.1. Sectioning

The frozen tissue pieces were embedded with KILLIK frozen section medium and cut into 5- $\mu\text{m}$  sections with a routine cryostat, using the recommended D-shaped knife.

### 3.2.2. Tissue Preparation

For (1) manual microdissection, (2) laser capture microdissection (LCM) with Arcturus PixCell II, and (3) laser microdissection with PALM using software function “auto laser pressure catapulting (autoLPC),” the frozen sections can be applied directly onto common glass slides using routine handling procedures.

If using PALM software function “LPC,” the slides must be prepared in a special way (*see Note 1*):

1. Trim the PEN-membrane to cover 75% of the clear area of a slide with a sterile scalpel.
2. Immerse the slides into 100% ethanol for 2–3 min to clean and degrease the slides.
3. Apply the PEN-membrane onto the wet slides.
4. Fix the film by gluing with adhesive tape at the edges.
5. Apply the frozen section onto the film.

### 3.2.3. Staining

Every tissue was sectioned in a consecutive sequence. The first section was H&E-stained, embedded with a mounting medium, and furnished with a cover slip using routine protocols (*11,12*) to ensure diagnosis by a pathologist. The regions of interest (ROIs) were then marked under microscopic surveillance using a permanent marker with a fine tip. These slides were used as a template to enhance orientation on the slides for actual microdissection.

The slides for actual microdissection were fitted with the consecutive sections and stained according to this protocol (*see Note 2*):

1. 70% Ethanol (30 s).
2. Millipore water (10 s).
3. Mayer’s hematoxylin solution (30 s).
4. Millipore water (10 s).
5. Aqueous solution of NaHCO<sub>3</sub> (0.5%; 10 s).
6. 70% Ethanol (30 s).

To all of the solutions above the Complete protease inhibitor was added into every other solution with the concentration according to the manufacturer’s recommendation (*see Note 3*).

The prepared slides are now ready for performing manual microdissection and microdissection with PALM. Until microdissection the slides are stored in tap water (with Complete protease inhibitor) to prevent desiccation. On the other hand for LCM with Arcturus PixCell II the slides have to be drained, dried, and desiccated after the 70% ethanol step according to the following protocol (*see Note 4*):

1. 90% Ethanol (30 s).
2. 100% Ethanol (30 s).

3. Xylene (30 s).
4. Xylene (30 s).

### 3.3. Microdissection

Lysis buffer for immediate SELDI application is a 1% solution of Nonidet NP40 in PBS (*see Note 5*).

#### 3.3.1. Manual Microdissection

The hematoxylin-stained slide is removed from the storage water and processed immediately. Under the stereomicroscope with overall 40× magnification the ROIs are microdissected using careful scraping movements with a sterile needle (*see Note 6*). With this needle the small tissue pieces are transferred into a 200- $\mu$ L PCR tube, which contains 12  $\mu$ L lysis buffer (*see Note 7*). Samples are then stored at  $-80^{\circ}\text{C}$ .

#### 3.3.2. LCM with PixCell II, Arcturus

##### 3.3.2.1. DESIGN OF PIXCELL II-LCM DEVICE

1. Inverted microscope (Olympus IX50, modified by Arcturus).
2. X-Y stage with manual joystick control.
3. Pulsed IR laser, solid-state laser diode (wavelength 810 nm, power output max 100 mW) with three manual interchangeable predefined laser spot sizes (7.5, 15, 30  $\mu\text{m}$ ).
4. LCM unit with cap insertion tool.
5. Interface for complete control over laser functions (pulse duration, output power).
6. Color CCD camera (0.5" chip), attached to microscope.
7. PC for software-driven control and performance of the LCM.

##### 3.3.2.2. PRINCIPLE OF LCM WITH PIXCELL II

The pulsed IR laser is attached and collimated with an inverted microscope, replacing its regular transmitted light source. The laser beam is focused by a three-step optic to a manual interchangeable spot size between 7.5 and 30  $\mu\text{m}$ . A special cap (CapSure™ LCM Cap) is positioned directly onto the stained section. This cap is covered with a thin ethylene vinyl acetate polymer sheet with low melting temperature capabilities. The cap is designed to fit as a lid for a routine Eppendorf centrifuge cup. The ROIs are focused and irradiated with the low-power IR laser beam. Each pulse of the laser melts the thermoplastic film within the predefined laser spot size (7.5, 15, or 30  $\mu\text{m}$ ) and glues the tissue onto the film. When lifting the cap from the slide, the selected tissue remains attached and is so separated from surrounding connective tissue and captured for further analysis. The whole setup is software-controlled, allowing the adjustment of the laser parameters.

### 3.3.2.3. PERFORMING LCM WITH PIXCELL II

The slide is put onto the precentered manual stage and is moved manually until the ROI is visible. The slide is then fixed to the stage by applying a vacuum. Subsequently fine movements to center the ROI and to perform the LCM movements are carried out using the X-Y stage. The CapSure caps are loaded into their dovetail assembly unit and one of them is picked up, moved to the work area on the slide, and lowered onto the section. The laser is turned on and the typical settings as needed to achieve melting (power: 40 mW; duration: 6.00 ms; repeat: 0.2 ms; spot size: 30  $\mu\text{m}$ ; target: 300 mV; current: 4.7/4.8 mA; temperature: 21.5°C) are adjusted. After location of the ROI the laser is fired, moved slightly and fired again until the whole work area is processed (*see Note 8*). When complete, the cap is removed from the cryostat section, transferred to an Eppendorf tube, and stored on dry ice or at  $-80^{\circ}\text{C}$  (*see Note 9*).

### 3.3.3. Laser Microbeam Microdissection With PALM

#### 3.3.3.1. DESIGN OF THE PALM-LMM DEVICE

1. Inverted microscope (Axiovert 135, Zeiss).
2. PALM Robot-Stage (computer-controlled X-Y stage).
3. Pulsed air-cooled UV-nitrogen laser (wavelength 337 nm, pulse energy 270 nJ/pulse).
4. Interface for complete control over laser functions (continuously adjustable focus, pulse adjustment, output power).
5. LPC capture unit.
6. Color CCD camera (0.5" chip), attached to microscope.
7. PC for software-driven control and performance of the LMM.
8. Unit mounted on a vibration-isolated stone tabletop.

#### 3.3.3.2. PRINCIPLE OF LMM WITH PALM

A pulsed UV laser is attached and collimated with an inverted microscope at its port of the fluorescence excitation light source. The laser beam is focused using the original microscope's objective to a minimal beam spot size of less than 1  $\mu\text{m}$ . The high photon density of the laser enables tissue dissection by means of locally restricted ablative photodecomposition without heating. After an internal defocusing of the laser the power is too low to cut tissue, but the pure pressure of photons is high enough to move ("shoot") small tissue pieces against gravity. This phenomenon (laser pressure catapulting [LPC]) is used for transferring ready-cut samples into a collecting cap. The whole setup is coupled with software (PALM RoboSoftware) combining drawing software and a control unit, which allows the use of different laser software features.

### 3.3.3.3. LMM WITH MANUAL TRANSFER

For this kind of LMM only the feature “CUT” is used. ROIs are encircled with one of the line-drawing tools. When using the “CUT” feature the laser cuts along predefined lines. The cutting itself is dependent on predefined laser focus and laser energy. The cut pieces are then manually picked up with a sterile needle and transferred into a 200- $\mu$ L PCR tube containing 12  $\mu$ L lysis buffer as above.

### 3.3.3.4. AUTOLPC INTO BUFFER OR OIL

The ROIs are encircled with a line drawing tool, and using the “autoLPC” feature the whole predefined area is covered by laser shots. The density of laser shots (number of shots per  $\mu\text{m}^2$ ) can be predefined in the setup menu. Each shot transfers a small piece of tissue solely by photon pressure rather than by cutting. In order to harvest the material a capture cap is filled with 3  $\mu$ L lysis buffer or mineral oil and is inserted into the LPC capture unit above the specimen.

### 3.3.3.5. LPC INTO OIL/ADHESIVECAP AFTER LMM

For this feature the section has to be mounted onto a PEN-membrane-coated slide. After encircling, the “CUT” function is used to cut the outer border of the ROI, while leaving a tiny bridge between the ROI and the surrounding tissue, between the start and stop positions of the initial laser cut. A few single laser shots are then positioned at this catwalk for catapulting the whole area, with the membrane attached, into a prepared cap located in the capture unit. The capture caps are filled with 3  $\mu$ L mineral oil or lysis buffer, respectively. In addition, special AdhesiveCaps (P.A.L.M.) with a self-adhesive surface can be used; therefore the usage of oil can be omitted.

### 3.3.3.6. PERFORMING LMM WITH PALM

The slide is positioned in the holding device, specimen facing upward. With the graphic tools all the ROIs are marked. Depending on the type of application, a capture cap is prepared, inserted into the capture unit, and positioned just above the slide. The desired laser feature is chosen and applied. When the LCM is completed, the capture cap is removed from the capture unit, transferred to an Eppendorf cup, and stored on dry ice or at  $-80^\circ\text{C}$  (*see Note 10*).

## 3.4. Sample Lysis

The samples are put on wet ice and thawed. Over a period of 1 h they are vortexed several times and spun down to ensure maximum protein recovery. Pipetting the whole small volume several times is a good alternative but has to be

undertaken carefully to minimize loss of material and volume (*see Note 11*). Finally the sample is spun down, to separate supernatant from the remaining tissue-debris (*see Note 12*).

### 3.5. SELDI Analysis

#### 3.5.1. Design of the Ciphergen SELDI Device

1. Time-of-flight mass spectrometer (TOF MS), tube length 0.8 m.
2. Pulsed UV-nitrogen laser (337 nm, max 150 mJ).
3. High vacuum supply.
4. Chip loading interface.
5. PC for software-driven control of the laser parameters and performing the TOF MS.
6. Unit mounted in an under-the-bench steel case.

#### 3.5.2. Principle of SELDI

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry allows the analysis of high molecular (“biologic”) molecules by mass spectrometry. MALDI deals with a “soft” UV laser irradiation following cocrystallization with an energy-absorbing molecule (EAM), called the matrix. Therefore the high-energy ionization with an electron beam is avoided. Combining MALDI with retentive surface chromatography of the initial carrier material itself gives the highly versatile surface-enhanced laser desorption ionization (SELDI) method. Miniaturizing the whole experiment down to 1  $\mu\text{L}$  sample volume per experiment results in the protein chip arrays with chromatographic active surfaces, thus reproducibly capturing proteins according to their specific chemistry out of the whole lysate. Only a fraction of all proteins is analyzed by mass spectrometry, thus minimizing overloading effects as well as ion signal suppression and possibly enhancing proton ionization efficiency. The resulting peaks, according to protein-specific mass-over-charge values, represent a characteristic fingerprint of the original lysate and can be used successfully to spot disease-related protein patterns by means of heuristic self-learning pattern detection algorithms (*13*).

#### 3.5.3. Chip Preparation

According to the desired chip surface chemistry a different chip preparation protocol is required. All steps are performed in 50-mL tubes on an appropriate orbital shaker.

For WCX2:

1. Wash the chips in 10 mM HCl for 5 min.
2. Wash the chips two times with autoclaved water for 1 min each.
3. Wash the chips two times with 10 mM ammonium acetate for 5 min each.

For SAX2:

Wash the chips two times with 10 mM ammonium acetate for 5 min each.

For IMAC3:

1. Wash the chips two times with 50 mM nickel sulfate for 5 min each.
2. Wash the chips two times with autoclaved water for 1 min each.
3. Wash the chips two times with 0.1% Triton X100 in PBS for 5 min each.
4. Let all chips dry and place them into the bioprocessor. Allow the chips to dry for about 2 min, until no more liquid is connecting the single spots.

#### 3.5.4. Sample Application

Apply 1  $\mu$ L of sample onto each spot and attach the disposable cartridge and gasket. Cover the whole unit with an appropriate lid (e.g., plastic lid of a pipet tip tray). Place a precisely fitting damp paper tissue into the lid first. Set the whole unit onto the orbital shaker and let incubate for 20 min. Using the 8-channel pipettor, wash every well three times with PBS and three times with autoclaved water using 200  $\mu$ L, each. Use a vacuum device (*see Note 13*) to dry each well completely before removing the cartridge and gasket from the bioprocessor. Allow the chips to air dry completely. In the meantime prepare the EAM solution (*see Note 14*):

1. 2500  $\mu$ L autoclaved water.
2. Add 25  $\mu$ L trifluoroacetic acid.
3. Vortex briefly.
4. Add 2475  $\mu$ L acetonitrile.
5. Vortex briefly.

and the matrix solution (*see Note 15*):

1. Measure 7 mg of cinnamic acid.
2. Add 200  $\mu$ L of EAM solution.
3. Vortex.
4. Centrifuge at 15,000g for 1 min.
5. Supernatant is the readymade matrix solution.

Then apply 0.8  $\mu$ L of matrix solution to each spot twice. Allow the spots to dry before performing the second round (*see Note 16*).

#### 3.5.5. Performing SELDI Analysis

Insert the chip into the Proteinchip Biology System II, following the manufacturer's instructions. Using the controlling software, measure the chip. Instrument settings have to be optimized for each sample. A good starting point is the following spot protocol (screen shot):

1. Set high mass to 20,000 Dalton, optimized from 1000 Dalton to 20,000 Dalton.

2. Starting laser intensity 220.
3. Detector sensitivity 8.
4. Focus mass at 6000 Dalton.
5. Set SELDI acquisition parameters 20 delta to 5 transients per to 15 ending position to 80.
6. Set warming positions with 2 shots at intensity 155 and do not include warming shots.

Analysis of spectra is performed with the CIPHERGEN software features “Comparison Wizard” and “Biomarker Wizard” (see **Note 17**).

#### 4. Notes

1. P.A.L.M. does offer pre-prepared slides. These slides are already covered with the plastic film described; therefore, handling is simplified. In addition the reproducibility is enhanced, as the tension of the sheet is crucial for successful microdissection.
2. Hematoxylin is the best staining technique for this purpose. As hematoxylin is a dye specific for chromatin it stains nuclei exclusively and no proteins. The last detail is important as a dye stains proteins by modification or attachment. In both cases the protein itself is altered or shows at least a different mass-over-charge value in the mass spectrum. For this purpose the routine counterstaining with eosin is omitted. We have tried several other microscopic dyes (e.g., methylene blue, toluidine blue) vs no staining at all (native tissue) (data not shown), but according to efficiency of protein recovery, protein integrity, and visibility during microdissection, hematoxylin is the first choice.
3. Even if a protease inhibitor is present, a time frame of 30 min for the overall procedure (first thawing of the cryostat section until final sample freezing) should not be exceeded in order to maintain sufficient protein integrity. Due to autolysis and protein degradation, peak intensities will decrease and new peaks (degradation products) will occur (data not shown).
4. Complete sample dehydration is mandatory to obtain good microdissection results for LCM, especially regarding sample transfer. For this reason the two steps with xylene follow 100% ethanol. To prevent the 100% ethanol stock bottle from absorbing moisture add a little dehydrated copper sulfate into the bottle. If the copper sulfate turns color from gray to blue the ethanol is no longer absolute.
5. The lysis buffer contains Nonidet NP40 (10% stock solution) and phosphate-buffered saline (PBS) 1:10, giving a final concentration of 1% Nonidet NP40 in PBS. The stock solution must be refrigerated. The ready-to-use dilution should be stored on ice until use and should be discarded after 12 h. Previous experiments have shown the 1% solution to be best for lysis. CIPHERGEN recommends a different lysing buffer containing 8 M urea. This particular buffer has given an insufficient lysing efficiency for our samples. In addition one has to work at room temperature, as the buffer crystallizes on wet ice.
6. A minimum of 15,000 cells is strongly recommended.

7. We have tried other cups prior to the ones now listed in the materials section. We once favored the use of 500- $\mu$ L Sarstedt screw cups. However, cup geometry is not perfect for handling the smallest volumes (compare to **Note 12**) and the plastic material used by the manufacturer tends to gain electrostatic voltage, thus complicating sample transfer into the 12- $\mu$ L buffer in the bottom of the cup. The 12- $\mu$ L buffer acts as a collecting main (*see Note 12*), thus enabling an easier transfer from the dissecting needle into the cup. In addition the immediate sample lysis improves protein recovery and protein integrity as proven in own experiments (data not shown).
8. A minimum of 5000 shots is strongly recommended.
9. After removing the cap from the section not only the microdissected (“glued”) areas remain, but in addition some unwanted material may stick to the transfer film. Getting rid of these cells is important for obtaining pure fractions. Thus we recommend pressing the transfer film side of the cap onto the gluing area of a regular Post-it note. The microdissected areas will remain unaffected, whereas all other material is removed.
10. For the biopsy material as described we recommend manual microdissection. Tissue architecture (papillary tumors, exophytic papillary tumors) with comparative large ROIs shows that a manual technique is best regarding labor time vs cell numbers dissected. In addition, the manual methods demonstrate the highest protein recovery during lysis. The PALM autoLPC function seems to suffer from material loss while catapulting into the collecting cap. Perhaps grounding of the cap (electrostatic voltage) can significantly improve transfer efficiency. P.A.L.M. has detected this problem already and using the company’s pre-prepared slides (*see Note 1*) increases transfer efficiency. If using the special AdhesiveCaps, these problems are significantly minimized.
11. Some tissue material seems to be hard to lyse; thus vortexing alone is not successful. We then recommend rigid pipetting of the whole volume. It is important to avoid the buildup of foam, as this will lead to a loss of material. In addition we emphasize the use of “high recovery pipet tips” (e.g., sterile aerosol pipet tip, Labcon, San Rafael, CA).
12. Most of the microdissected sections seem to remain unaffected by lysing. This debris should not find its way onto the chip surface. Therefore centrifugation is mandatory. For this step it is necessary to use a really small cup with a spiky cone end, as there is very little supernatant, which needs to be transferred by a pipet tip afterward. This dictates the amount of collecting volume as a too little volume will result in an insufficient supernatant. Trying to reduce the number of cells, which are necessary for downstream application, is possible by reducing the volume of lysing buffer/collecting gain. But one reaches very soon the limits of proper manageability.
13. We connect a regular tubing to the vacuum faucet at the bench. We insert the large end of a 1000- $\mu$ L pipet tip into the tubing. The small end is cut so that the resulting diameter is small enough to be inserted into a single well but too large to touch the spot surface.

14. The ready-made EAM solution can be stored for 1 wk at room temperature in the dark.
15. Store cinnamic acid in the dark at  $-20^{\circ}\text{C}$ . The matrix solution must be used the same day.
16. After complete drying the chips can be measured. However it may be necessary to store them until final analysis. We recommend vacuum sealing into aluminized bags and adding a capsule with desiccant. Our own experience proved this storage to be good for at least 5 d. For longer storage until measurement or a repeated measurement, one might consider the reapplication of matrix solution. It should be borne in mind that the ratio of protein to matrix cannot be significantly altered without influencing the mass spectrum.
17. These tools are the next level of spectra analysis after observation with the naked eye. For a high-resolution approach and especially when analyzing large case numbers we strongly recommend the use of a more sophisticated detection algorithm.

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## References

- 1 Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., SuHon, G. G., et al. (2001) The sequence of the human genome. *Science* **291**, 1304–1351.
- 2 McPherson, J. D., Marra, M., Hillier, L., Waterston, R. H., Chinwalla, A., et al. (2001) A physical map of the human genome. *Nature* **409**, 934–941.
- 3 Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J.X., Gooley, A.A., Wilkins, M.R., et al. (1995) progress with gene-product mapping of the mollicutes: *mycoplasma genitalium*. *Electrophoresis* **16**, 1090–1094.
- 4 Krieg, R. C., Paweletz, C. P., Liotta, L. A., and Petricoin, E. F. (2002) Clinical proteomics for cancer biomarker discovery and therapeutic targeting technology. *Cancer Research and Treatment* **1**, 1–10.
- 5 Srinivasan, R. (1986) Ablation of polymers and biological tissue by ultraviolet lasers. *Science* **234**, 559–565.
- 6 Schutze, K. and Clement-Sengewald, A. (1994) Catch and move—cut or fuse. *Nature* **368**, 667–669.
- 7 Emmert-Buck, M. R., Bonner, R. F., Smith, P. D., Chuaqui, R. F., Zhuang, Z., Goldstein, S. R., et al. (1996) Laser capture microdissection. *Science* **274**, 998–1001.
- 8 Bonner, R. F., Emmert-Buck, M. R., Cole, K., Pohida, T., Chuaqui, R., Goldstein, S., and Liotta, L. A. (1997) Laser capture microdissection: molecular analysis of tissue. *Science* **278**, 1481–1483.
- 9 Zaak, D., Kriegmair, M., Stepp, H., Stepp, H., Baumgartner, R., Oberneder, R., et al. (2001) Endoscopic detection of transitional cell carcinoma with 5-amino-levulinic acid: results of 1012 fluorescence endoscopies. *Urology* **57**, 690–694.

- 10 Kriegmair, M., Baumgartner, R., Lumper, W., Waidelich, R., and Hofstetter, A. (1996) Early clinical experience with 5-aminolevulinic acid for the photodynamic therapy of superficial bladder cancer. *Br. J. Urol.* **77**, 667–671.
- 11 Messmann, H., Knüchel, R., Bäumlner, W., Holstege, A., and Schölmerich, J. (1999) Endoscopic fluorescence detection of dysplasia in patients with Barrett's esophagus, ulcerative colitis, or adenomatous polyps after 5-aminolevulinic acid-induced protoporphyrin IX sensitization. *Gastrointest. Endosc.* **49**, 97–101.
12. Burck, H. C. (ed.) (1988) *Histologische Technik*. Georg Thieme Verlag, Stuttgart.
13. Romeis, B. and Böck, P. (ed.) (1989) *Mikroskopische Technik*. Urban & Schwarzenberg, München.
14. Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M., et al. (2002) Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* **359**, 572–577.