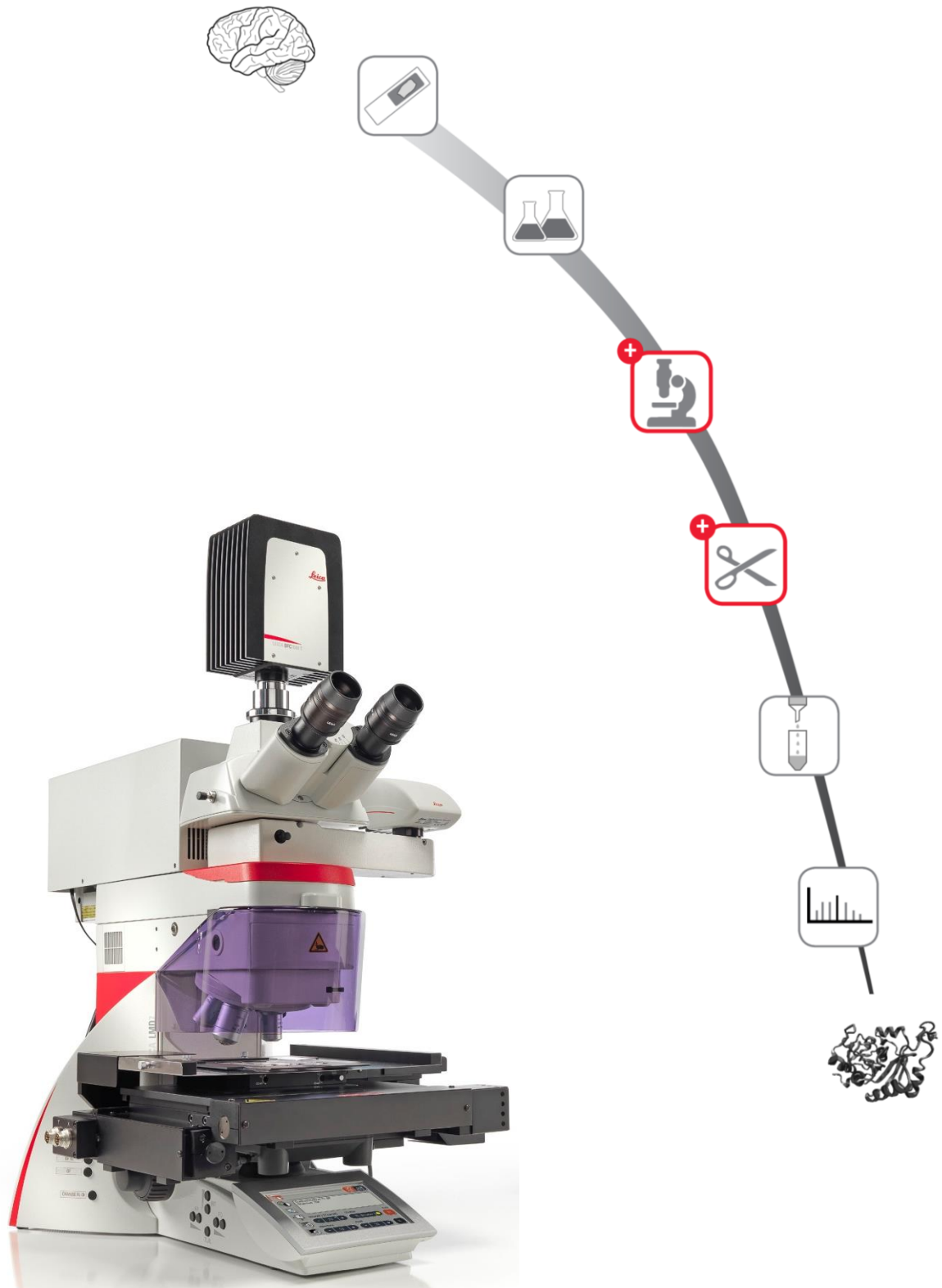
From Eye to Insight

Protocol: Laser Microdissection of FFPE Brain Tissue for Proteomics



INTRODUCTION

The development of diseases can have manifold reasons. On the one hand, there are infectious diseases which can be traced back to the invasion of bacteria, viruses, fungi, or parasites. On the other hand, there are also body's own dysfunctions commonly linked to the absence, dysregulation or malfunction of cellular components.
For example, during cancer development the cell cycle gets out of control, resulting in the unrestricted proliferation of cells and the failure of affected organs. Other diseases such as Parkinson's disease go along with the cell dead of dopaminergic neurons leading to typical motoric deficiencies. Alzheimer's disease in turn is characterized by protein aggregates called Amyloid Plaques and an associated dementia.

In any case, diseases have reasons and symptoms which can be directly connected to the underlying cellular components. Researchers have the ability e.g. to search for mutations, investigate gene activity, or study the fate of gene products or their effectors. In other words, from a cellular view, diseases can be explored on the level of DNA, RNA, proteins, carbohydrates, lipids, or small molecules. To study these cellular components with the help of modern lab methods such as DNA sequencing, NGS, qPCR, or mass spectrometry their purification is recommended if not mandatory.

Laser microdissection (LMD) is a method to dissect defined areas from microscopy samples. Compared to other purification methods such as FACS or immuno-precipitation, LMD enables the user to visually control the selection process and directly define the sample area to dissect within its morphological context. This area can be as small as sub-cellular compartments e.g. single chromosomes. Researchers can also dissect tumors, single neurons or any kind of microscopic structure they are interested in for downstream analysis. Thereby LMD helps to exclusively purify the structure of interest and exclude contamination with unwanted tissue areas or cells.

This protocol is dedicated to our **Proteomics Workflow** to get an overview of the required work steps starting from the choice of LMD consumables through sample preparation, sectioning, fixation, staining, to laser microdissection and protein purification. Specifically, it describes how to collect pure Alzheimer's plaques from brain tissue for proteome analysis.

Please keep in mind that each sample is different and might need some special treatment. In case you have any questions concerning your individual protocol or you want to contribute, please do not hesitate to contact us via the link at the end of this document.

PREARRANGEMENTS

Choice of consumable

Membrane based slides are the best choice for reliable Laser Microdissection. For downstream proteomics standard PET frame slides (#11505151) have the best price performance, or PET RNAse-free slides (#11505190) or EP-DIRECTOR™ slides (#11600249 for membrane-free ablation) are recommended.

Sterilizing Membrane Slides

Several methods to sterilize LMD membrane slides are available: A) autoclaving, B) chemical treatment or C) UV-C (254 nm) irradiation.

Autoclaving

1. Place slides into a steel basket such as a slide holder for paraffinization
2. Place the basket into a beaker or jar and cover tightly
3. Autoklave at 121 °C for 20 minutes

UV-Treatment

Incubate PET-slides in a UV-C (254 nm) crosslink chamber and deliver at least 1 joule of energy (maximum power at 30 – 45 minutes, for #11505151 or max. 15 minutes for #11505190). Please refer to the manufacturer's information when using other UV-light sources.

UV-light sources from sterile flow hoods can also be used for sterilization instead of a cross linker.

Note: UV-treatment should be done shortly before membrane slides are used. The UV-treatment also activates the membrane surface for better section adherence.

Caps

The caps may be used dry, but can also be pre-filled with LC-MS grade water or suitable buffer.

Attention: The kind of tube can influence the MS downstream results. Tubes from Axygene are recommended for downstream proteomics analysis. Thermo Scientific tubes cannot be recommended.

FIXATION & SECTIONING

In general, for downstream mass spectrometry proteomics cryo-sectioned or Formalin Fixed Paraffin Embedded (FFPE) samples can be used. For both kinds of samples protocols are published [for frozen samples see Hondius et al., for FFPE samples see Drummond/Nayak et al.]. To test your established downstream protocol, start with dissected areas of 1.5 - 2 mm^2 (10 µm section thickness).

FFPE Samples

Fixation

You can use your standard fixation protocol or use tissue from biobanks.
Fixatives should penetrate the tissue quickly and thoroughly. Incomplete fixation causes many artifacts and can cause shrinkage. The most common fixative is 10% formalin buffered with PBS (phosphate buffered saline) to neutral pH (10% formalin corresponds to 4% formaldehyde, see below).

Paraffin Embedding and Sectioning

After fixation, water must be removed from the tissue and replaced by an organic solvent before paraffin infiltration.

1. Wash the tissue with ascending concentrations of alcohol (such as ethanol) as follows:

   - 70% ethanol 30 minutes 2x
   - 95% ethanol 30 minutes 2x
   - 100% ethanol 30 minutes 2x

   During these steps the tissue will harden.

2. The alcohol is then replaced by an organic solvent such as xylene (100%), which is miscible with alcohol and paraffin wax. The tissue is cleared twice for 1 hour each time.

3. Paraffin infiltration is carried out two to four times for 2–4 hours. Incubation time depends on the size of the tissue. Normally, embedding larger tissue pieces takes 22 hours, smaller tissue samples such as biopsies are incubated for 6 hours, and tissues for quick sections may require only 1 1/2 hours. The temperature of the liquid paraffin is 55–60°C, which corresponds to 2–4°C above the melting point.

   The entire procedure of fixation and embedding can be done in a tissue processor, which is standard equipment in a histopathology lab. Recommended is the Leica ASP300 S automated tissue processor (contact your local Leica Microsystems representative for more information).

4. Paraffin infiltrated tissues are embedded in a mold with liquid paraffin to form a block for better handling during microtome sectioning. This is easily done using a paraffin embedding station such as the Leica EG1150 H.

5. The tissue block can be stored at room temperature or below. For better cutting results with the microtome, cooling the paraffin block down to –20°C is recommended.

6. Section the block

   - Clean the microtome with sterile solutions before sectioning to avoid contamination
   - Mount the tissue block onto the microtome
   - Trim the sample to obtain a plane surface and access to the tissue
   - Cut the tissue into 5–15 µm sections (approx. plaque diameter)
   - Place the paraffin ribbons on the surface of the water in a waterbath at 37°C– 0°C; in this way, the floating ribbons are easier to stretch and subsequently can be placed without folds on the slides. Please note that the water must have LC-MS grade for mass spec analysis

7. The paraffin must be removed prior to staining the paraffin-embedded sections. This is achieved by washing the slides with xylene followed by a series of descending concentrations of ethanol as follows:

• 100% xylene	20 seconds	3x (3 separate containers)
• 100% ethanol	30 seconds	2x (2 separate containers)
• 95% ethanol	30 seconds	2x
• 70% ethanol	30 seconds	2x

STAINING

In general, most stains are compatible with downstream proteomics. Most common stains for Alzheimer plaques are Congo Red, DAB (3,3'-diaminobenzidine), IHC, or Cresyl Violet (CV). Exception: Toluidine blue is not recommended for direct mass spec downstream analysis.

Cresyl Violet Staining

Cresyl violet stain is commonly used for neuronal tissue. It is a basic stain that binds to acidic molecules of neuronal cytoplasm, such as RNA-rich ribosomes. Cresyl violet permanently stains a section and is suitable for paraffin sections and subsequent LC-MS/MS analysis.

Reagents:

- Cresyl violet staining solution
- Xylene
- Ethanol
- LC-MS grade water

Procedure

Use CV staining solution and LC-MS grade water according manufacturer's instruction. All steps should be done in a fume hood.

1. Wash slides with sections in xylene (100%) for 30 seconds (2-times) and agitate the slide by dipping during the washing step

2. Wash slides with sections in 100%, 95%, 70% ethanol for 30 seconds each step and agitate the slide by dipping during each washing step

3. Wash slides in LC-MS grade water for 30 seconds and agitate the slide by dipping during the washing step

4. Incubate with CV solution for 3 minutes

5. Rinse in LC-MS grade water 5 times

6. Wash slides with sections in 95% ethanol for 2 minutes and agitate the slide by dipping during the washing step

7. Wash slides with sections in 100% ethanol for 5 minutes and agitate the slide by dipping during the washing step

8. Air dry the slide for at least 24 hours in a clean drying chamber

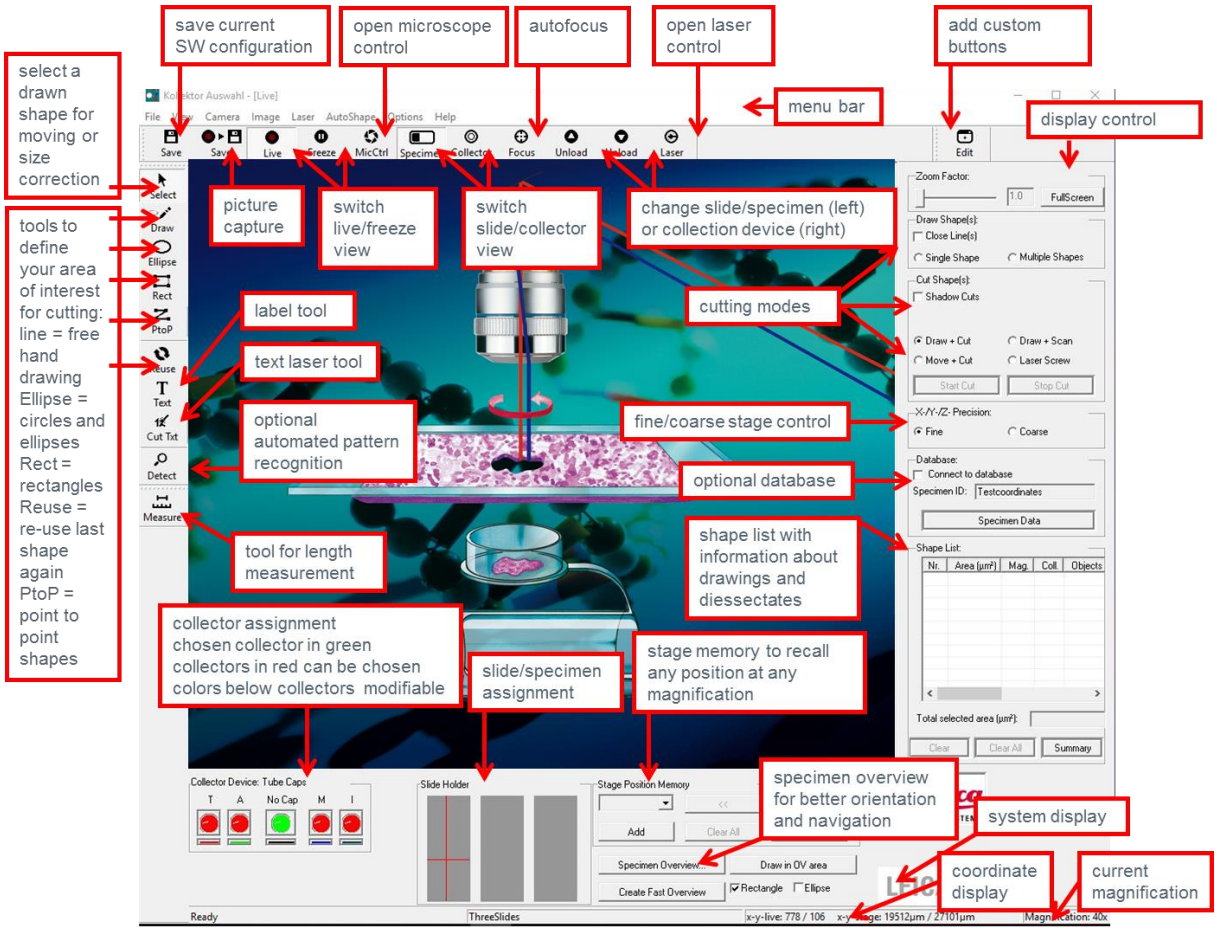
Attention: Working with human samples requires extreme care to avoid contaminations. The outlined methods are very sensitive and will detect smallest amounts of contaminating proteins from non-clean environments. In addition, polymers from plastic (e.g. Thermo Scientific tubes) might have a bad influence on LMD samples in downstream processing.

LASER MICRODISSECTION

Proteomics is an emerging application for laser microdissection. The challenge of retrieving a sufficient amount of proteins for downstream experiments can be overcome in several ways:

- The entire area of interest can be microdissected (or ablated using “Draw+Scan”) using low magnification objectives such as the 2.5x, 5x, 6.3x or 10x.
- Cut regions or cells of interest from several consecutive tissue sections.
- Hundreds of single cells can be cut out from the tissue automatically using the Leica AVC module

To dissect sufficient samples use the Leica LMD Software:



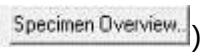
Mount the prepared LMD slide and load at least one collection vessel using the unload buttons (

unloads the specimen holder for slides; 

unloads the collection device for collection vessels). Almost unlimited amounts of dissectates can be easily pooled into the same collection vessel or separated into individual collection vessels.

The tube cap (collection device) can be pre-filled with buffer (usually 5 – 20 µl) for direct digestion of isolated material and/or for stabilization of proteins. Dry tube caps allow a cap inspection (

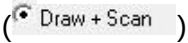
) before and after cutting for visualization of the dissected and collected material.

First you need to identify your areas of interest (e.g. by morphology, size, anatomical context or orientation). The specimen overview (

) or a lower magnification can help here to identify your region of interest. Mark your area of interest for dissection using the free-hand line tool (

), or if large areas, exceeding a field of view, are desired, use the PtoP tool (

), make sure to leave some space between the line and the target cell border to take the laser width into account.

If DIRECTOR™ slides are used for membrane-free ablation, make sure to activate the ablation mode Draw + Scan (

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Hind: Set up the laser for perfect cutting or ablation and falling properties prior collecting valuable materials in a tissue area outside the area of interest.

Activate the laser for dissection and enjoy the system extracting pure starting material ready for downstream analysis.

Hind: The optional pattern recognition module AVC Professional can save your valuable time: Train the system to automatically detect and collect your areas of interest. AVC Settings can easily be stored and recalled for following experiments.

PREPARATION OF PROTEINS

The following techniques for protein detection have been successfully tested with proteins gained from LMD dissectates:

- LC-MS/MS
- SDS-PAGE followed by Silver staining or Western Blotting
- 2D-DIGE (two-dimensional differential fluorescence gel electrophoresis with labeling of proteins with Cy2, Cy3 and Cy5 dyes)
- HPLC-ESI/MS
- MALDI

This paragraph goes into detail concerning LC-MS/MS analysis of Alzheimers Plaques.

The general procedure for protein analysis via LC-MS/MS of Alzheimers plaques requires solubilization of the plaques (e.g. in formic acid), breakdown of bisulfide bridges and capping those to prevent reconnecting, proteolytic digestion of proteins and clean-up of peptides for LC-MS/MS analysis.

Depending on your tissue fixation there are different approaches. In general, according to the low sample amounts, common viscosity or milky appearance of intermediate solutions will not occur. Instead samples appear rather “empty”. However, you can expect good results as confirmed by several publications (see citations at the end of the document).

Dissectates from FFPE samples can be collected in LC-MS grade water and stored at -80°C, if necessary. Subsequently samples are brought into a buffer (ammonium bicarbonate + acetonitrile solution) and boiled for de-paraffinzation. Reverse cross-linking of proteins is achieved by formic acid incubation overnight. Sulfide bridges of protein are removed with the help of DTT and binding sides are capped afterwards by iodoacetamide.

Mass spectrometry facilities will have their own best practice protocols for such analysis. Established protocols for LMD dissectates can be found in the literature (e.g. Drummond & Nayak et al. for FFPE samples and Hondius et al. for cryo sections).

REFERENCES

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FEEDBACK

Do you miss anything in the protocol or do you have questions or suggestions?
Please contact us.

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