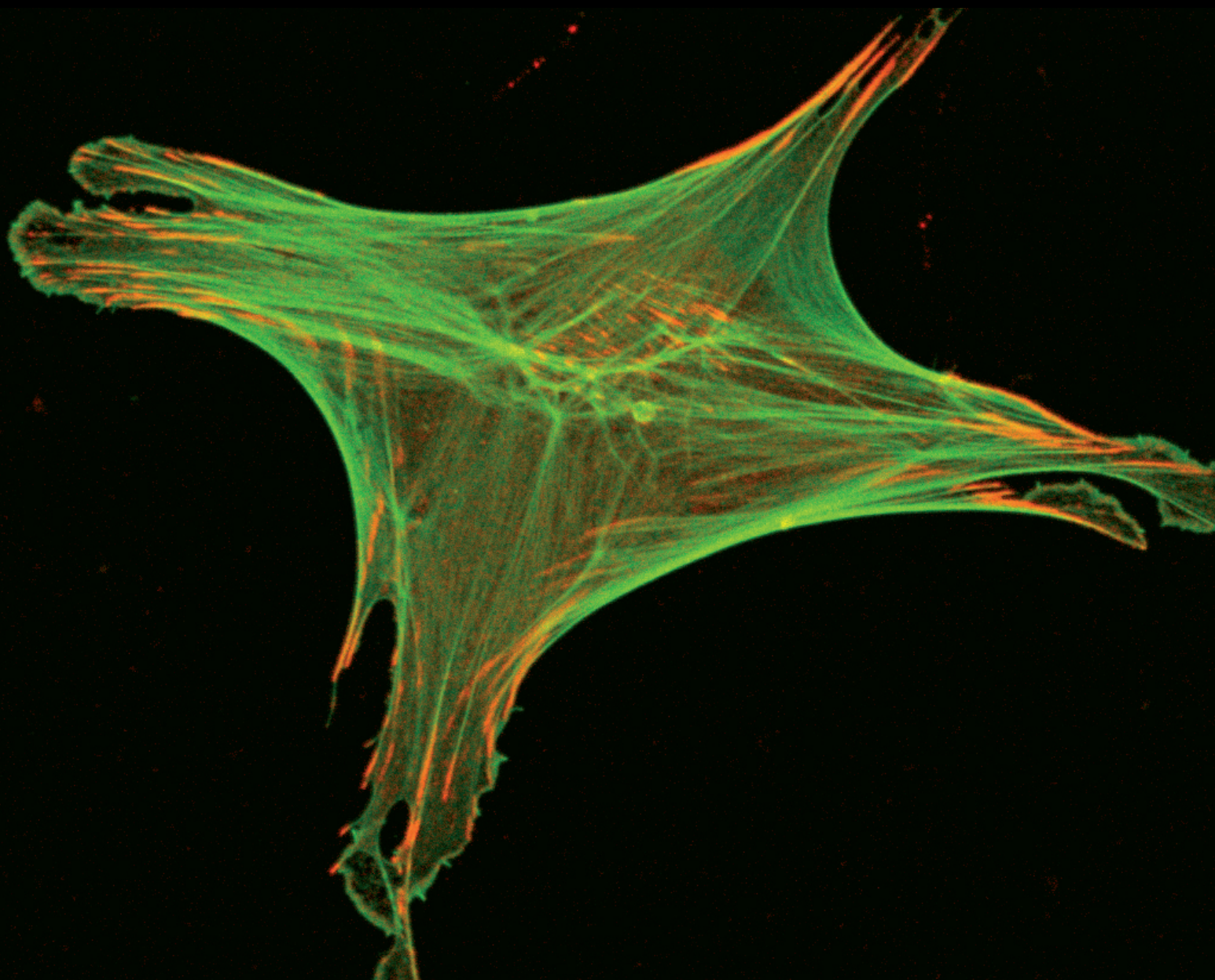


Carl Zeiss Microscopy

LCM Protocols - Protein Handling for LC/MS



human fibroblast, Alexa Fluor 488-Phalloidin & anti-Paxillin AF 647



We make it visible.

LCM Protocols

Protein Handling for LC/MS
Non contact Laser Capture Microdissection

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Introduction

Some remarks on Proteomics

Proteomics has the potential to evaluate global changes in protein expression and their post-translational modifications. But most applications face two major challenges. On the one hand they require substantial amounts of tissue for a comprehensive proteomic characterization. On the other hand the sample specificity must be guaranteed in order to obtain significant results. Especially in heterogeneous tissues this can limit the application of proteomic methodologies.

Recent developments to overcome these challenges are focusing on the improvements of sensitivity and selectivity of conventional sampling techniques.

One enabling key technology is Laser Capture Microdissection (LCM). Already proven in genomics as a precise tissue separating and collecting technology for low sample amounts, Carl Zeiss now offers a specific and fast collection method for large amounts of material to facilitate downstream proteomics. In combination with new improvements e.g. in mass spectrometric resolution and sensitivity, LCM clears the way for definite "microproteomics".

The DOs and DON'Ts of handling proteins

Contamination is a common reason for failing experiments. Keratins from the skin are prone to contaminate the sample and obscure the proteins of interest.

Proteases are present on almost any object that comes into contact with human skin and are difficult to inactivate.

Some precautions can make the difference between an intact and degraded protein prep (see also: www.ambion.com) and therefore between successful and unsuccessful experiments.

DOs

- designate a special area for working with proteins
- wear latex (not nitrile) gloves, lab coats and cover hair
- use clean tips and bottles
- only use LC/MS grade water and reagents/enzymes
- store samples frozen (-80°C preferred) to prevent sample degradation
- all sample manipulation prior to Trypsin digestion should be done in a biological safety cabinet (BSC) or a laminar flow hood

DON'Ts

- don't breath on samples; some researchers wear masks
- don't touch anything with bare hands
- don't autoclave pipette tips
- don't resuspend or use non-LC/MS grade reagents

Preparation of slides

– Samples on MembraneSlide

MembraneSlides are special glass slides covered with a membrane on one side. This membrane is easily cut together with the sample and acts as a stabilizing backbone during lifting. Therefore even large areas can be lifted by a single laser pulse without affecting the morphological integrity. Use of MembraneSlide is especially important for isolating single cells, chromosomes as well as live cells or small organisms.

Carl Zeiss MicroImaging (CZMI) offers slides covered with polyethylene naphthalate (PEN)-membrane. This PEN membrane is highly absorptive in the UV-A range, which facilitates laser cutting. The membrane can be used for all kind of applications.

MembraneSlide NF 1.0 PEN (nuclease free) is certified to be free of DNase, RNase and human DNA.

In addition to MembraneSlide 1.0 PEN, CZMI also offers polyethylene terephthalate (PET)-membrane covered slides. These slides are helpful for special processes, i.e. fluorescence applications.

In fluorescence applications even weak signals can be detected due to low signal to noise ratio. Alternatively the PET membrane is also available attached to a metal frame (FrameSlide PET). The frame structure of FrameSlide PET is resistant to microwave treatment. The special bonding is inert and adapted to heat treatment (up to 95°C) so that the membrane does not ruffle during the heating process. If you need to receive information about these slides, please contact:

labs@zeiss.de



*FrameSlide => between dot and 0,
Regular glass slide (1 mm thick) => 1.*

UV treatment

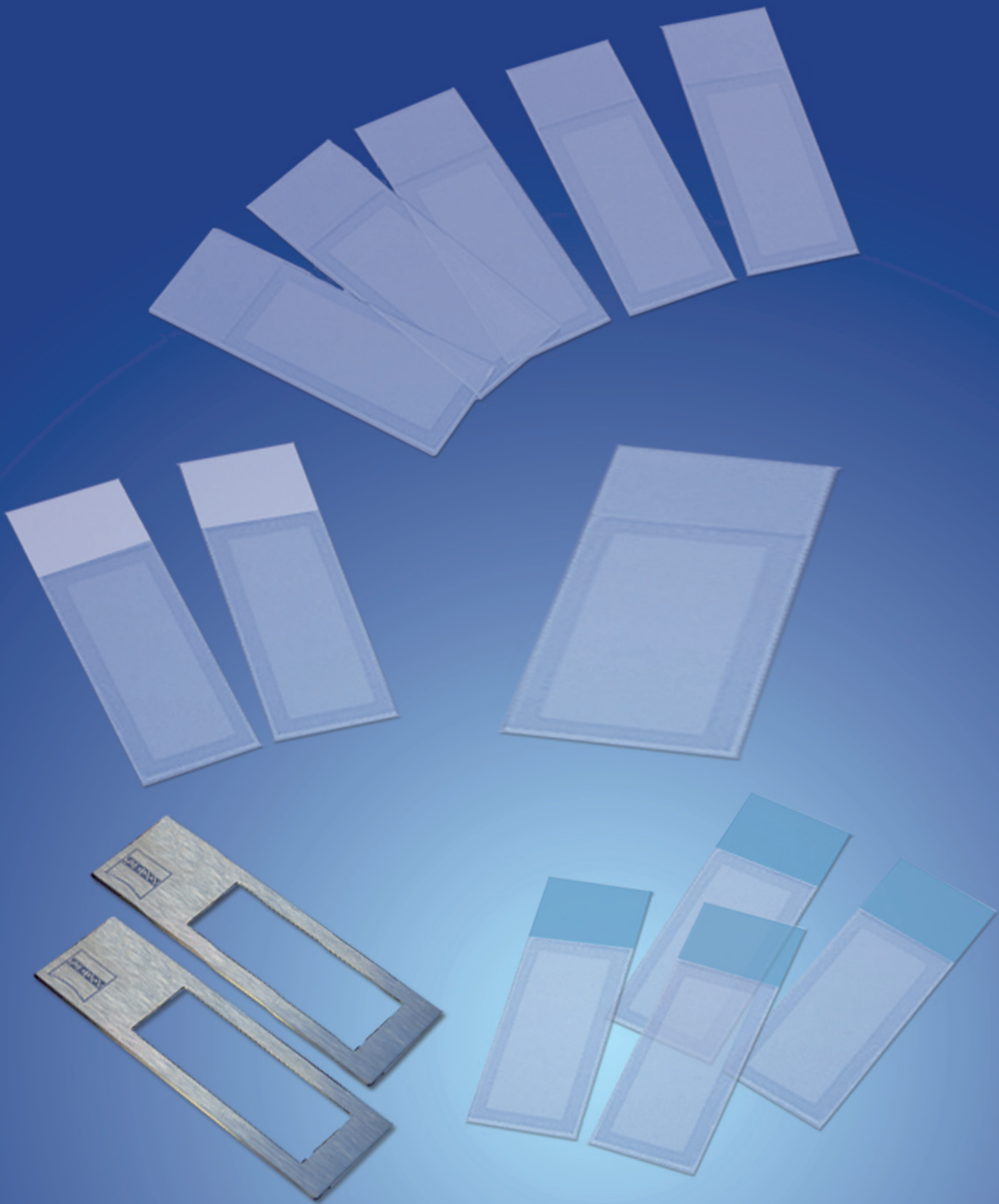
To overcome the hydrophobic nature of the membrane it is advisable to irradiate with UV light at 254 nm for 30 minutes (e.g. in a cell culture hood).

The membrane gets more hydrophilic, therefore the sections (paraffin- and cryo-sections) adhere better. Positive side effects are sterilization and destruction of potentially contaminating nucleic acids.

Poly-L-Lysine treatment

Additional coating of the slide with Poly-L-Lysine (0.1% w/v, e.g. SIGMA, #P8920) only will be necessary for poorly adhering materials (e.g. brain sections) and should be performed after UV treatment. Distribute a drop of the solution on top of the slide.

Let air-dry at room temperature for 2-3 minutes. Avoid any leakage of the membrane, as this might result in impairment of Laser Capture Microdissection.



- MembraneSlide 1.0 PEN* - Order No. 415190-9041-000 (white)
- MembraneSlide 1.0 PEN NF* - Order No. 415190-9081-000 (white)
- MembraneSlide 0.17 PEN* - Order No. 415190-9061-000 (uncolored)
- MembraneSlide 50x1.0 PEN* - Order No. 415190-9091-000 (doublewidth)
- MembraneSlide 1.0 PET* - Order No. 415190-9051-000 (blue)
- FrameSlide PET* - Order No. 415190-9101-000 (metal)

Preparation of slides

– Samples on glass slides

With PALM MicroBeam almost every kind of biological material can be microdissected and lifted directly from glass slides. Even archival pathological sections can be used after removing the cover slip and the mounting medium.

To facilitate easy lifting additional adhesive substances or “Superfrost + charged slides” should only be applied when absolutely necessary for the attachment of poorly adhering material (e.g. some brain sections or blood vessel rings). In those cases higher laser energy is needed for lifting.

From the dry glass slide sample material can be lifted directly by “AutoLPC” function of PALM RoboSoftware.

Mounting samples onto slides

Frozen sections

Sectioning

Sections are mounted onto Membrane-Slides the same way as routinely done using glass slides. To allow subsequent cutting and lifting a coverslip and standard mounting medium must not be applied. Freezing media like OCT or similar may be used but should be kept to a minimum and have to be removed before laser cutting (see topic: Removing the tissue freezing medium).

For optimal sample protection take a pre-cooled slide and touch the backside of the slide with your finger (gloves!) to warm only the region for placing the section. Now transfer section from the knife by touching with the warmed area and dry at -20°C in the cryostat for 5 minutes.

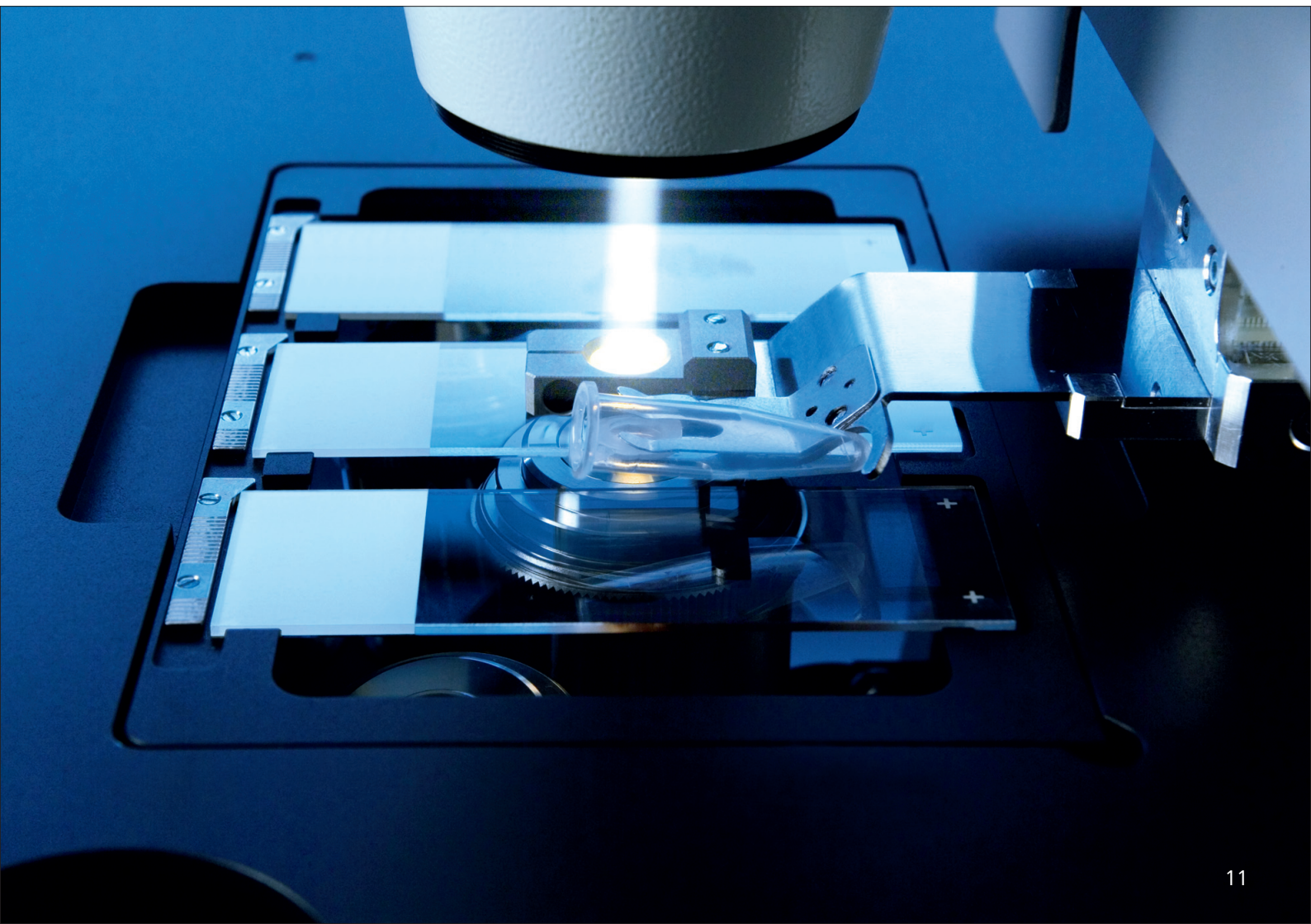
Fixation

CZMI recommends the dehydration in ice-cold 70% ethanol for 5 seconds.

Removing the tissue freezing medium

If OCT or another tissue freezing medium is used, it is important to remove it before Laser Microdissection, because these media will interfere with laser efficiency.

If the sections are stained in aqueous solutions, the supporting substance is normally removed "automatically" by the water containing steps. Separate removing of the medium is easily done by dipping the slide 5-6 times in ice-cold pyrogene-free water.



Staining procedures

For isolation of high quality proteins use only freshly prepared and precooled staining solutions.

Frozen sections

Depending on your protein analysis, some standard histological stainings may influence the experimental outcome.

At MicroImaging Labs we usually perform Cresyl Violet or Hematoxylin staining for proteomics.

Note: Using frozen sections proteases may still be active after a short fixation step. Therefore it is recommended to keep all incubation steps as short as possible. Please use ultrapure water and solutions for all steps. All required reagents should be kept on ice.

Hematoxylin

Hematoxylin staining is used routinely in most histological laboratories and does not interfere with good protein preparation if protease activity is low. Nuclei are stained in blue, cytoplasm in pink/red.

Procedure

1. after fixation (5 sec, 70% Ethanol) dip slide for 30 sec into hematoxylin solution
2. remove excess stain on absorbent surface
3. dip into distilled water or blueing solution (e.g. SIGMA, MHS-32)
4. dip into 70% Ethanol 3-5 times
5. dip into 100 % Ethanol
6. air-dry shortly (1-2 min)

Cresyl Violet

This short staining procedure colors the nuclei in violet and the cytoplasm in weak violet. It is recommended for proteinase-rich tissues since all solutions contain high ethanol concentrations.

Procedure

1. after fixation (5 sec, 70% Ethanol) dip slide for 30 sec into 1% cresyl violet acetate solution (*)
2. remove excess stain on absorbent surface
3. dip into 70% Ethanol 3-5 times
4. dip into 100 % Ethanol
5. air-dry shortly (1-2 min)

Note: In most cases this cresyl violet staining procedure will be sufficient for cell identification. If an enhancement of the staining is desired, a reinforcement by two additional steps in 50 % ethanol (first before the staining in cresyl violet; second after the staining in cresyl violet) is possible. Additional enhancement can be obtained by increasing the working temperature of all solutions to room temperature.

- (*) Dissolve solid cresyl violet acetate (e.g. ALDRICH #86,098-0) at a concentration of 1% (w/v) in 50% EtOH at room temperature with agitation/stirring for several hours to overnight. Filter the staining solution before use to remove unsolubilized powder.

Note: Sometimes Lot to Lot variations in the purchased cresyl violet powder can lead to weaker staining results if the dye content is below 75%.

Storage

After staining and dehydration, LCM samples should be immediately collected.

Non-contact Laser Capture Microdissection (LCM) Procedures

Please, also have a look into the PALM MicroBeam user and software manual.

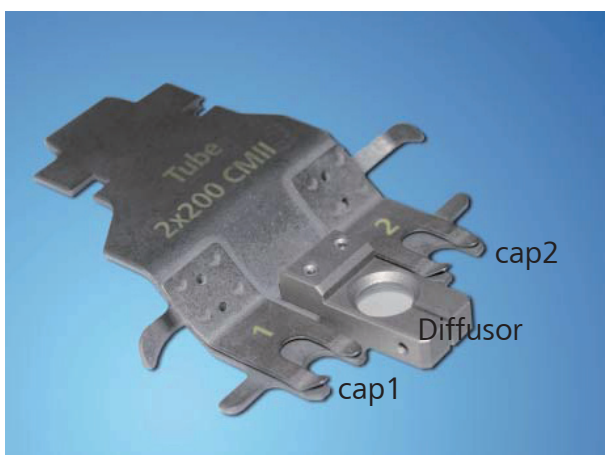
Tips to improve morphological information

Embedding and glass covering of the specimen is inapplicable for LCM. Thus, the rough open surface of the section/material often results in impaired view of morphology.

Collectors equipped with Diffusor

Holders for PALM RoboMover and PALM CapMover II are equipped with diffusors.

The opaque glass diffuses the incident microscope light, which smoothens the harshness of contrast and, depending on material and staining, even minute details as nuclei and cell boundaries show up. Even slight differences in color become visible. For more details and handling, please see product information of corresponding collectors.



TubeCollector - Order No. 415101-2000-410

AdhesiveCap opaque

The white/opaque filling of AdhesiveCap improves visualization of morphological information of the samples at the object plane due to enhanced color balance and contrast, which makes the view comparable to those of coverslipped tissue sections.

Two different microfuge tube sizes with filled caps are available from CZMI.

For more details and handling, please see AdhesiveCap product information.



*AdhesiveCap opaque - Order No. 415190-9201-000 (500 µl)
AdhesiveCap opaque - Order No. 415190-9181-000 (200 µl)*

Collection devices

AdhesiveCap

The intention of AdhesiveCap is to allow LCM (Laser Capture Microdissection) without applying any capturing liquid into the caps prior to LCM. This minimizes protease activity.

Beside the quick relocation of the lifted samples inside the cap (due to instant immobilization) there is no risk of evaporation and crystal formation during extended specimen harvesting.

For more details and handling, please see also AdhesiveCap product information.



*non-contact LCM
via AdhesiveCap*

AdhesiveCap Touch

AdhesiveCap Touch is a collection vessel completely filled with adhesive material and is also adapted for buffer-free sampling via Pick-up LCM (PALM RoboSoftware 4.5 required).

Large samples from homogeneous regions can be captured in one piece without dividing by cutting around and touching the selected area with the cap. Proteomic or metabolic profiling can be started immediately.

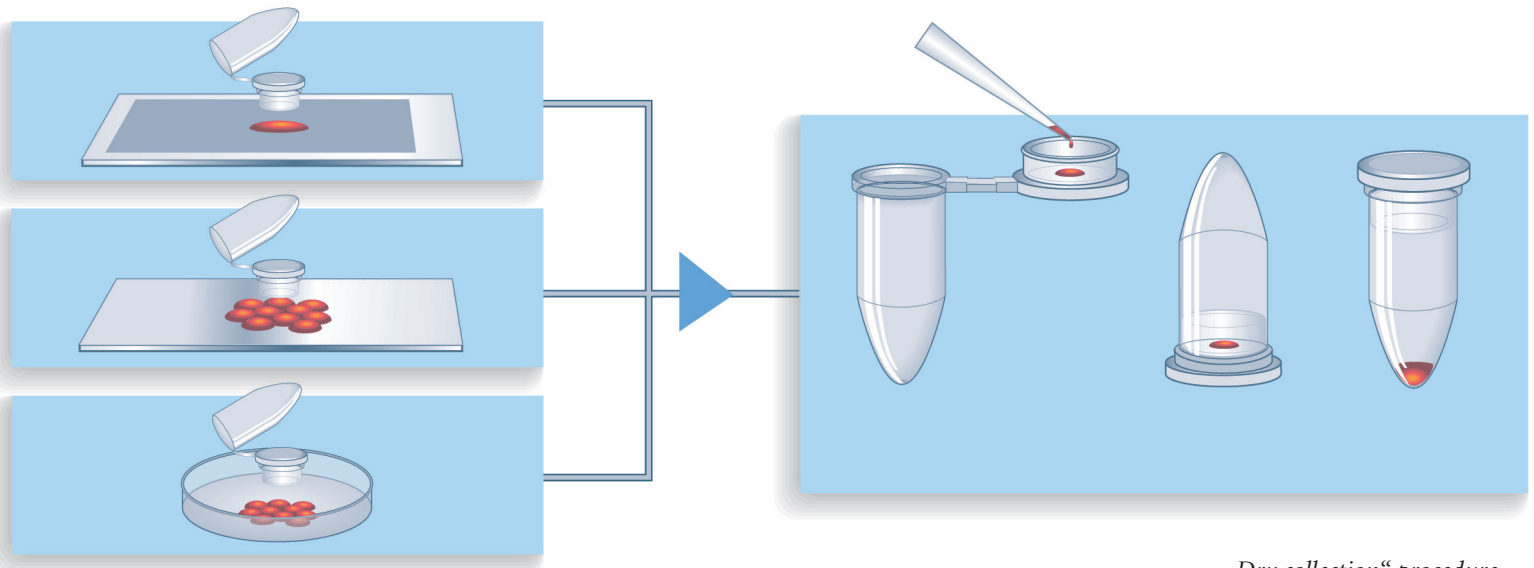


*Pick-up LCM
via AdhesiveCap Touch*

Note: CZMI recommends AdhesiveCap as a collection device for all RNA and Protein experiments.

Other microfuge tubes

Other commercially available plasticware can be used (e.g. ABgene #AB-0350; 0.5 ml tubes).



„Dry collection“ procedure

Collection procedures

Please have a look into the PALM MicroBeam user and software manual.

„Dry“ collection - AdhesiveCap, AdhesiveCap Touch

Note: CZMI recommends AdhesiveCap as collection device for all protein experiments. Capturing without liquid minimizes protease activity.

After LCM add protein extraction solution of your own choice (e.g. Rapigest SF, Waters Corporation #186001861) into the cap and incubate „upside down“.

Subsequently centrifuge the lysate and then apply the routine protein extraction procedure.

Note: Please do not use any water bath for the upside down incubation.

„Wet“ collection (other microfuge tubes)

Pipette at least 20 µl protein extraction solution into the cap.

The lifted cells or cell areas will stick onto the wet inner surface of the cap and will not fall down after the lifting procedure.

Be aware that aqueous solutions will dry out after a while.

When using glass mounted samples it may be advisory to put more liquid into the cap.

CapCheck – looking into the cap to see the lifted samples

By using the software function “go to checkpoint” the stage moves to the CapCheck-Position and the cap can be inspected. For details, please refer to PALM MicroBeam user and software manual.

Downstream Applications

Protocol Development of Microdissected Samples for Proteomics - David H. Murdock Research Institute - Kannapolis, USA under the direction of Dr. Sarah Schwartz

Proteins from frozen sections for LC/MS

In collaboration with the David H. Murdock Research Institute/Kannapolis, USA, we developed a protocol for the proteomic analysis of microdissected cells.

The Rapigest SF surfactant protein extraction solution combined with an evaluated method of digestion (reduction/alkylation) results in very good yield and quality of proteins.

Alternatively the following protein extraction solutions were also evaluated:

- 100 mM Ammonium Bicarbonate
- 100 mM Tris-HCl pH8
- 2 % PPS Silent Surfactant
- T-PER Tissue Protein Extraction Reagent

Based on results of the different extraction solutions the Rapigest method evaluated the most proteins from microdissected samples.

Downstream Applications

Sample Preparation and LCM

Tissue Sectioning

Fresh frozen mouse liver samples were cut in the cryostat at -20°C into $10\text{-}12\ \mu\text{m}$ sections, mounted onto MembraneSlides 1.0 PEN and dried for approximately 5 minutes in the cryostat.

Tissue Staining

For unstained samples, sections were dehydrated first in 70% ethanol for 5 sec and then in 100% ethanol for 5 sec and subsequently air dried.

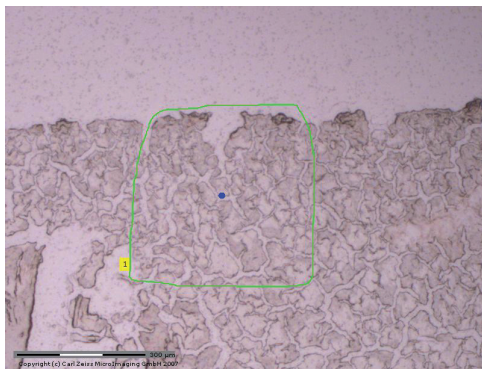
Two different stainings were performed: staining with Hematoxylin (no eosin) and with Cresyl Violet (procedures see page 12/13).

After staining and/or dehydration, LCM samples should be immediately collected.

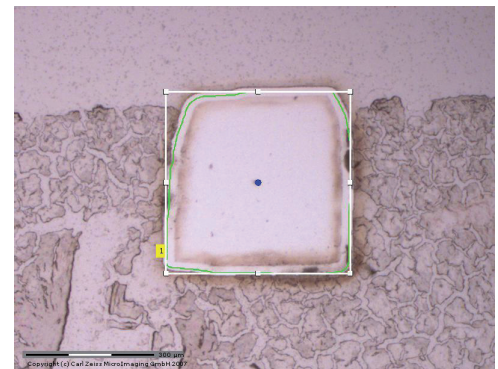
Tissue Collection

Settings for laser energy and focus on the MicroBeam system should be optimized for each type of tissue. Working with unstained samples requires a higher laser cutting energy (10-20%) due to lower absorption of laser energy. For LC/MS proteomics analysis, 50,000-100,000 cells should be collected for each biological replicate. Protein extracts can be pooled from multiple caps to achieve the necessary number of cells. Samples can be collected and either extracted immediately or stored at -80°C prior to extraction.

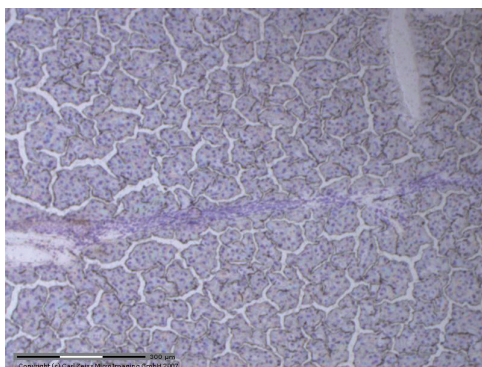
In the experiment described 20-30 large cell areas were microdissected, each about $180.000\text{-}250.000\ \mu\text{m}^2$ and collected in one AdhesiveCap; in summary about $5,2\ \text{mm}^2$ pooled in one cap.



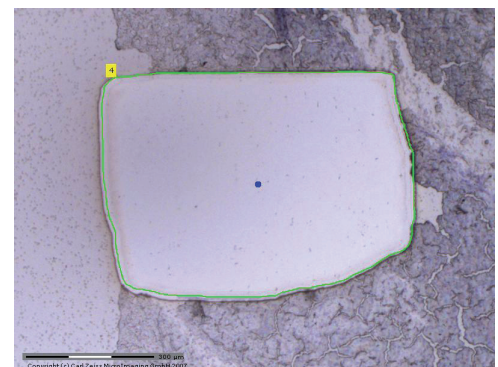
LCM of liver section: unstained tissue section



... after isolation



LCM of liver section: sample stained with Cresyl Violet



... after isolation

Downstream Applications

Protein Quantitation Assay (BCA) - Determine your Protein Concentration in Advance

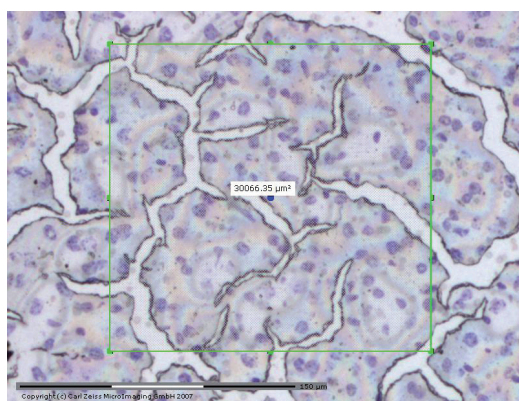
The amount of sample required for peptide analysis will depend on the number of cells collected, tissue type and the amount of protein extracted.

DHMRI Analytical Sciences highly recommended determining the protein concentration of each sample by running a protein quantitation assay (such as ThermoScientific Micro BCA Protein Assay, #23235).

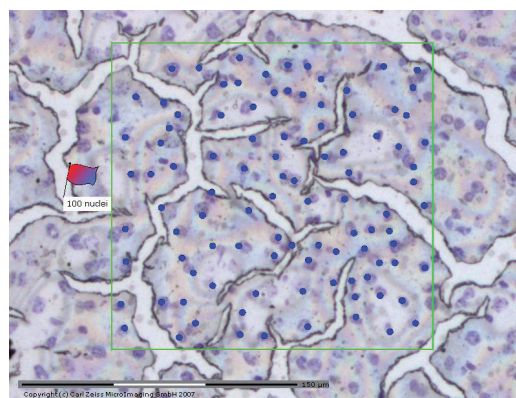
If the protein concentration is very dilute, it will be necessary to concentrate the sample (e.g. to collect multiple tubes for one sample) and then add the ammonium bicarbonate solution to reach the desired volume.

Note:

The total amount needed is 1 - 50 μg of protein. A minimum protein concentration of 0.1 $\mu\text{g}/\mu\text{l}$ is required for the following steps.



*Cresyl Violet-stained liver section,
area size 30.000 μm^2*



*Estimation of number of cells before LCM by
counting cell nuclei*

Downstream Applications

Protein Extraction from Microdissected Samples:

1. Add 50 μ l Rapigest SF surfactant protein extraction solution to the collection tube with the microdissected cells, then flip the tube and incubate in an "upside down" position for 30 min at room temperature. Accurate lysis is essential for good protein yield.

Note: We recommend using Rapigest SF surfactant solution from Waters Corporation (# 186001861) prepared at 0.1% concentration

2. After incubation heat the cap to 60°C for 1 hour.

Note: Protein extracts can be pooled from multiple caps to achieve the necessary number of cells (e.g. 50,000 liver cells, respectively 150 μ l protein extract). Caps can be collected and either extracted immediately or stored at -80°C prior to extraction

3. Spin down the lysate in the microcentrifuge for 5 minutes. (13400 rcf; e.g. Eppendorf 5415D: 12000 rpm)

4. Transfer the extracted protein solution to a clean centrifuge tube. We recommend the Eppendorf Protein LoBind tubes.

Note: Samples can be digested immediately, prepared for intact protein analysis, or stored at -80°C.

5. Depending on your experiment setup you might want to split the extracted protein solution. In our case we used 50 μ l for the protein quantitation assay (BCA) (page 19) and 75 μ l for LC/MS (page 22).

Surfactant Cleavage for Intact Protein Analysis:

Cleave Rapigest by adding 10 μ l of 10/20/70-TFA/acetonitrile/water. Heat at 60°C for 2 hours.

Note: Samples can be processed immediately or stored at -80°C.

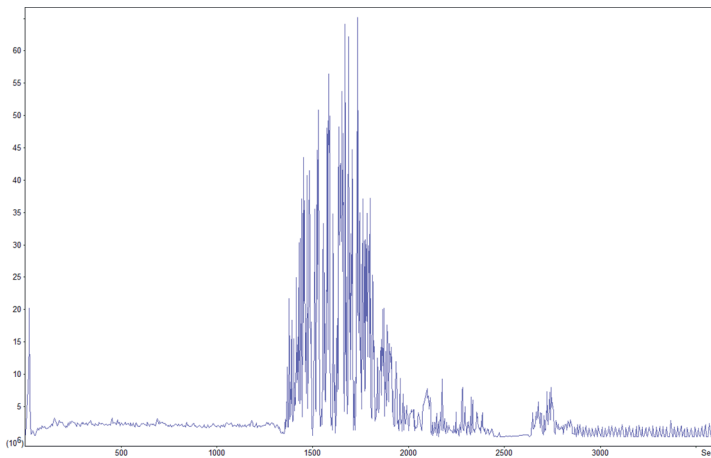
Downstream Applications

Protein Reduction/Alkylation and Digestion for LC/MS Analysis:

1. Transfer an aliquot (minimum: 0.1 µg/µl protein concentration) of the extracted sample into a new microcentrifuge tube.
2. Add 50 mM ammonium bicarbonate to a final volume of 30 µl.
3. Add Dithiothreitol (DTT) to the sample to make a final DTT concentration of 10 mM.
4. Heat the sample at 80°C while shaking for 15 minutes.
5. Add Iodoacetic acid (IAA) to the sample to make a final IAA concentration of 20 mM (2x molar excess of DTT).
6. Incubate the samples in the dark at room temperature for 30 minutes.
Note: The DTT and IAA steps are needed to reduce and alkylate disulfide protein links. This step can be eliminated if the proteins of interest do not contain disulfide links. The peptides connected by disulfide links will not be identified in the database search results if this step is skipped.
7. Add Trypsin to achieve a 1 : 50 trypsin to protein concentration. Digest for at least 4 hours at 37°C. Overnight trypsin digestion at 37°C is recommended.
8. Centrifuge the condensate to the bottom of the vial.
Note: Other enzyme digestions are possible (LYS-C, ASP-N, GLU-C,...) and will depend on the proteins present and sequence information desired.
9. Before mass spectrometry analysis, the extraction surfactant must be cleaved. The surfactants interfere with ionization resulting in very poor results if this step is not performed. Rapigest is cleaved by adding 10 µl of 10/20/70 TFA/acetonitrile/water. Heat at 60°C for 2 hours.
10. Samples were analyzed on a Thermo Orbitrap XL and the data evaluated with MASCOT.
11. Results were imported into Scaffold for comparison.

Downstream Application

LC/MS Results after Protein Extraction and Digestion

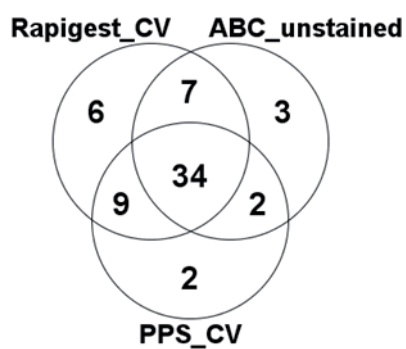


LC/MS Trace for Digest Samples

Based on the results of the BCA assay and the number of proteins identified by LC/MS, the Rapigest method evaluated extracts the most protein from LCM samples.

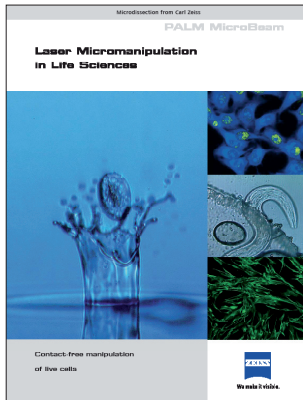
In the graphic chart the distribution of the different results calculated in Scaffold-Method is shown:

34 proteins could be identified with all extraction methods . The Rapigest method performed on CV stained tissue sections resulted in the detection of 56 proteins.

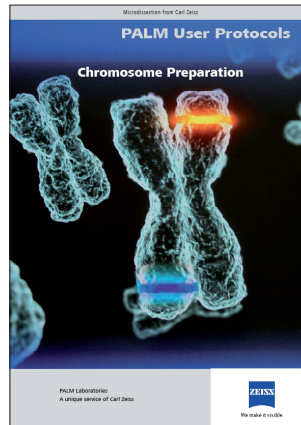


Brochures and protocols (selection)

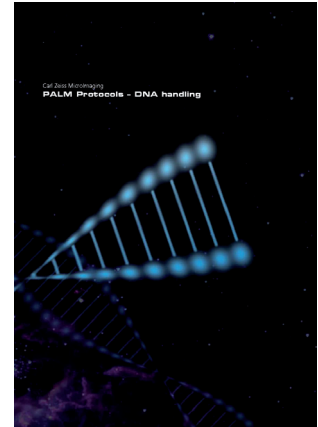
Live cells



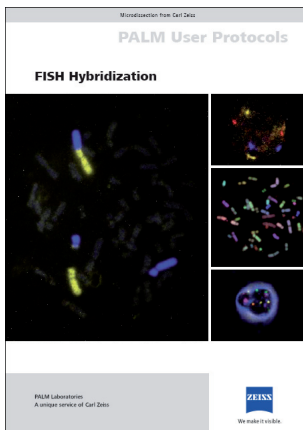
Chromosomes



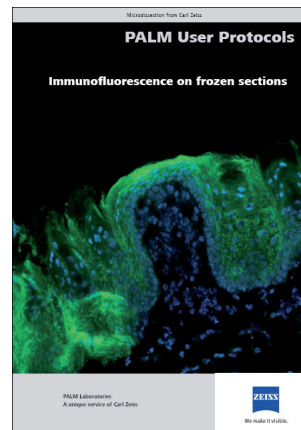
DNA



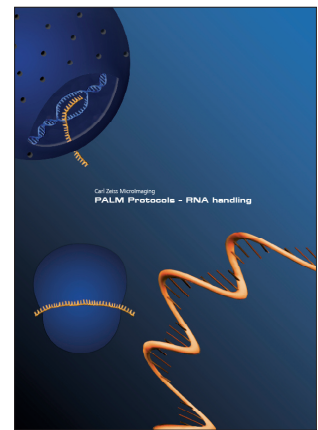
FISH



Immunofluorescence



RNA



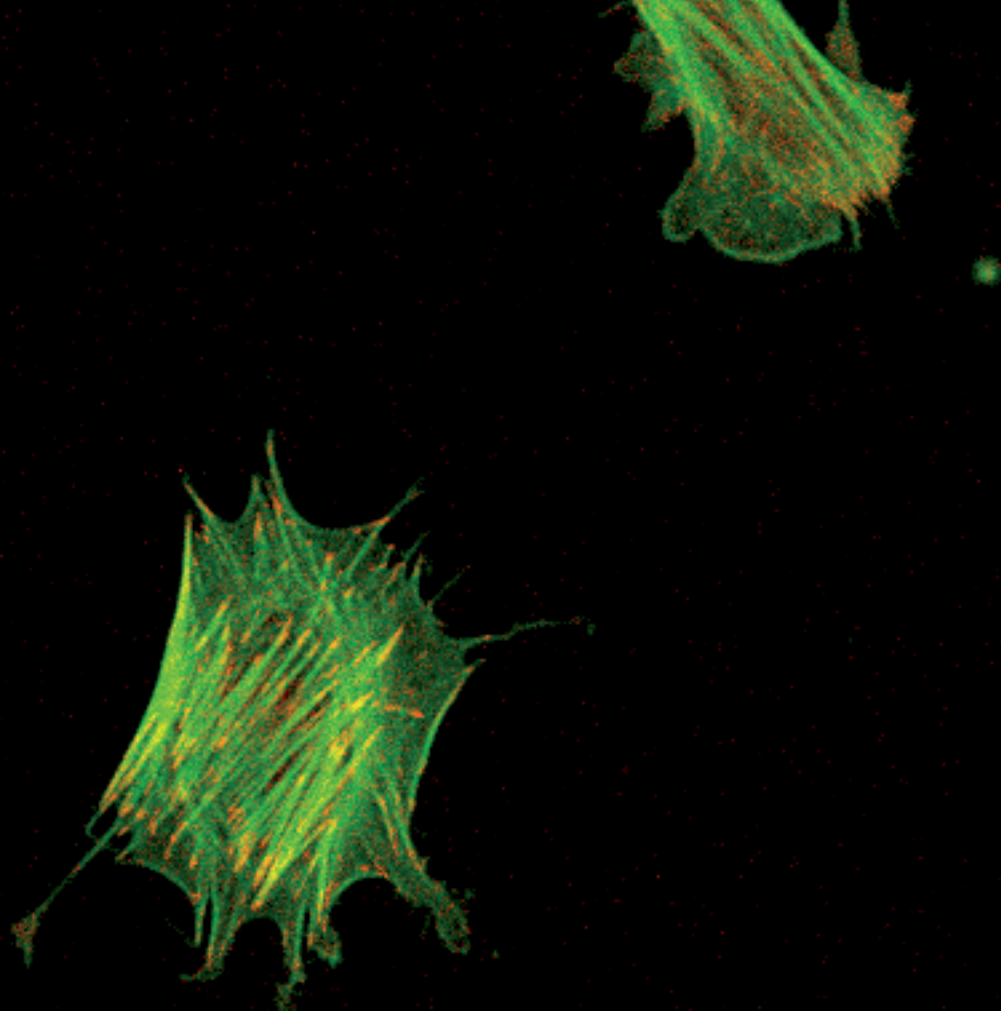
download at www.zeiss.de/labs

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We make it visible.