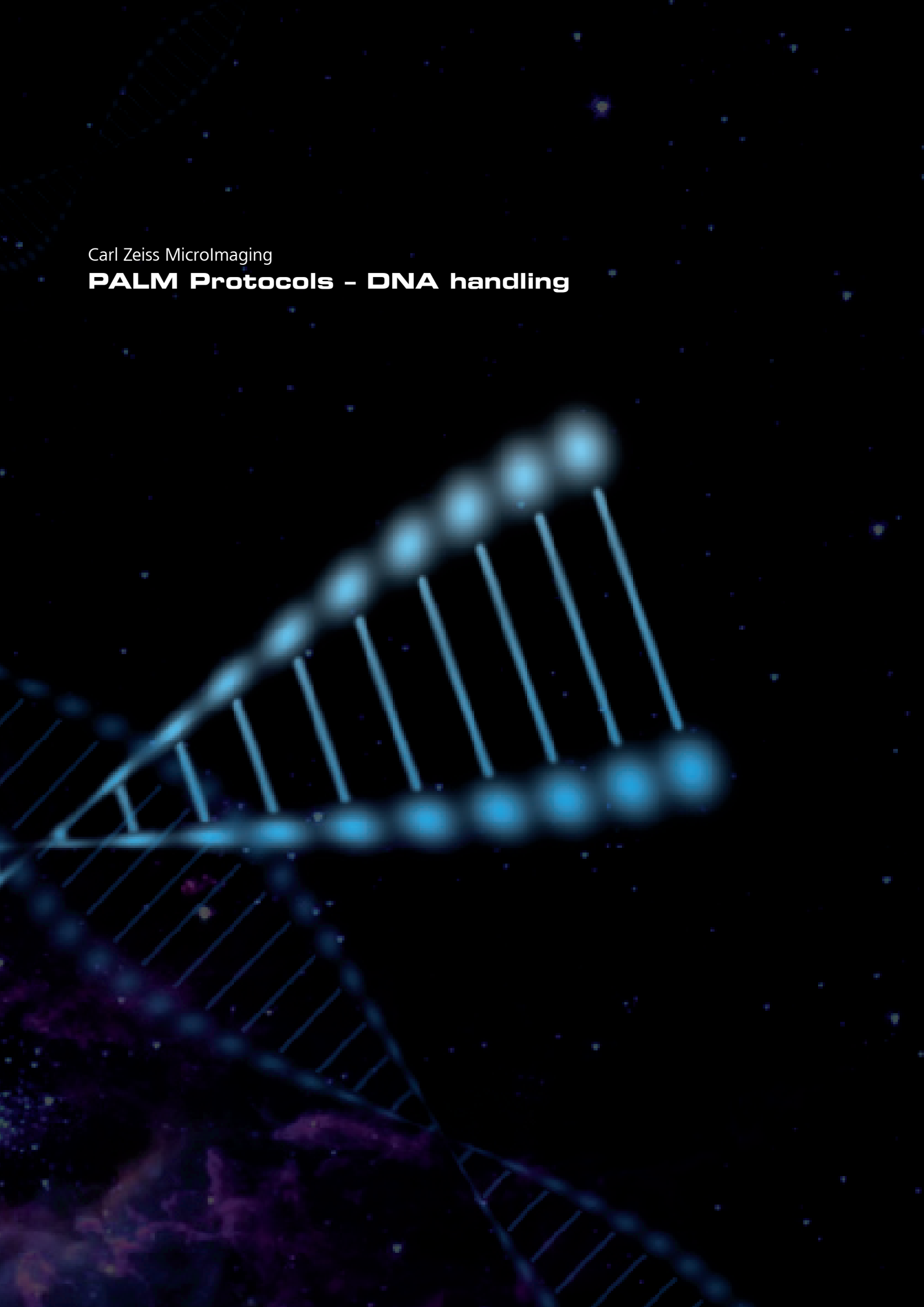
Carl Zeiss MicroImaging

PALM Protocols – DNA handling



PALM Protocols

DNA handling

Non contact Laser Capture Microdissection

Carl Zeiss MicroImaging – Location Munich – Germany

Content

Some remarks on DNA

Human Genome Project has shown new insights into poorly understood biological phenomena by providing vast DNA sequencing data. As a result of this expansion of genomics into human health applications, the field of genomic medicine was born. Genetics is playing an increasingly important role in the diagnosis, monitoring, and treatment of diseases.

Further areas that stand to benefit from DNA results include biomedical and biological research, toxicology, drug design, forensics, animal and plant genetics, and many others.

In all fields the methods for molecular testing must be able to determine and analyze DNA sequences accurately and rapidly. Whenever possible the procedures should be easy to use, highly automated, and minimized in requirement of material.

Therefore the generation of defined sample as source for the analysis is of prime importance.

Non-contact Laser Capture Microdissection (LCM) from Carl Zeiss is state of the art for precise sample preparation.

Preparation of slides – Samples on MembraneSlide

Regular glass slide (1 mm thick) => 1, thin slide (0.17 mm thick) => dot, DuplexDish and FrameSlide => between dot and 0.

MembraneSlide is a glass slide covered with a membrane on one side.
This membrane is easily cut together with the sample and acts as a stabilizing support during lifting. Therefore even large areas are lifted by a single laser pulse without affecting the morphological integrity of the tissue.
Use of MembraneSlide is especially recommended for isolating single cells or chromosomes as well as live cells or small organisms.
Carl Zeiss MicroImaging (CZMI) offers slides (1 mm, 0.17 mm) covered with Polyethylene Naphthalate (PEN)-membrane or Polyethylene Teraphthalate (PET)-membrane.

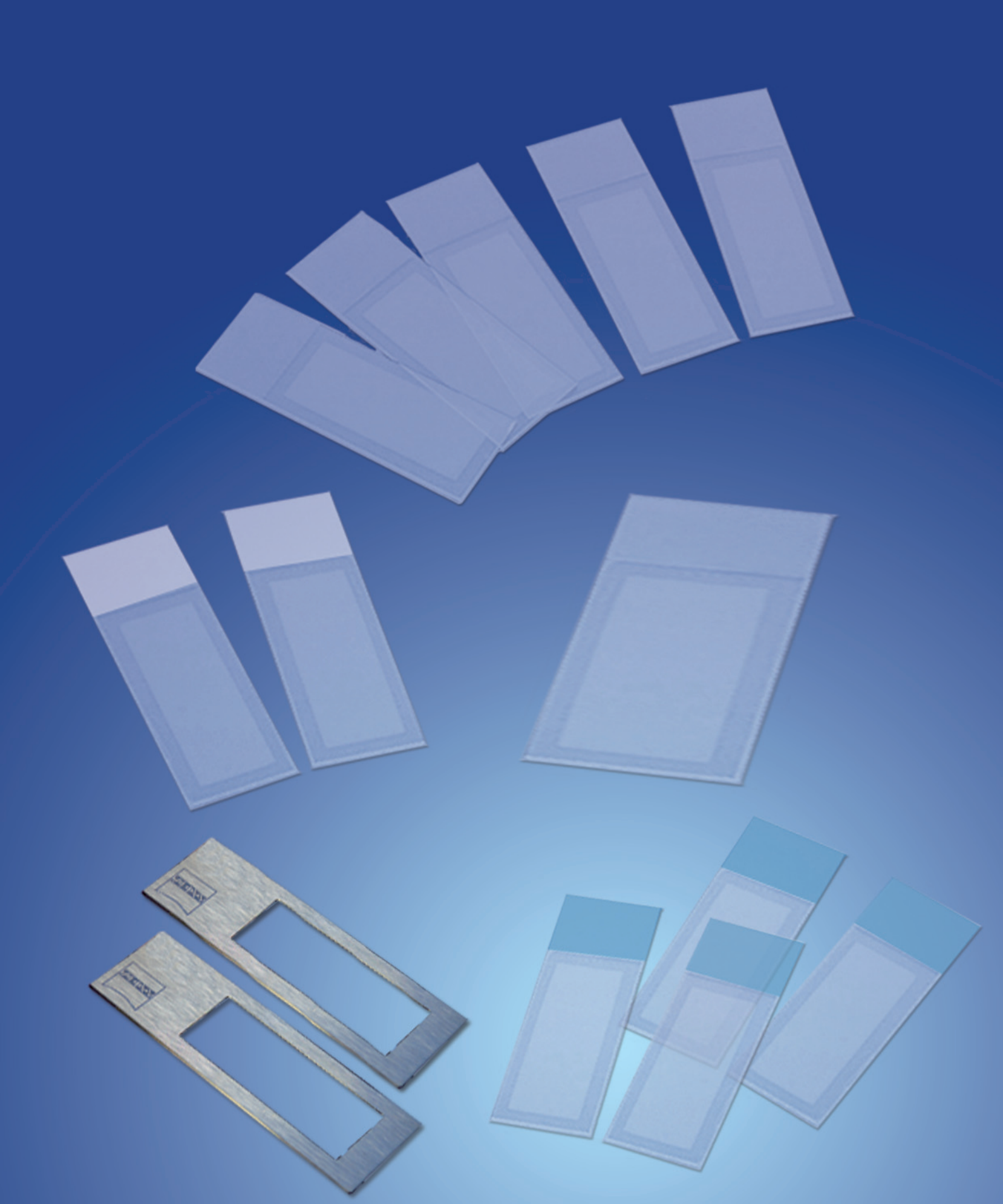
PEN-membrane is highly absorptive in the UV-A range, which facilitates laser cutting. The membrane can be used for all kind of applications.
MembraneSlide NF (nuclease free) is certified to be free of DNase, RNase and human DNA.

In addition to PEN-MembraneSlide, CZMI also offers PET-membrane covered slides. These slides are helpful for special processes, i.e. fluorescence applications. Even weak fluorescence signals can be detected with PET-slides, due to the low signal to noise ratio.

Alternatively the PET-membrane attached to a metal frame (FrameSlide PET) is also available. The structure of FrameSlide PET is resistant to microwave treatment or pressure cooking. The special bonding is inert and adapted to heat treatment even in moisture or liquid so that the membrane does not ruffle during the heating process. If you need further information about these slides, please contact:

E-Mail: labs@zeiss.de

When working with low magnifying objectives like 5x or 10x, both regular 1 mm thick glass slides and 0.17 mm glass slides can be used. To keep this flexibility for higher magnifications (20x, 40x or 63x) CZMI recommends using long distance objectives.
With those the working distance can be adapted to the different glass slides by moving the correction collar on the objective.
(see picture above)
Due to the short working distance only 0.17 mm thin cover glass slides can be used with the 100x magnifying objectives.



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)*
MembraneSlide 0.17 PET - *Order No. 415190-9071-000*
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 regular glass slides. Even archival sections can be used after removing the coverslip and the mounting medium.
To facilitate 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.

Archived samples: removing the coverslip

Depending on the applied mounting medium (whether it is soluble in xylene or water) the whole slide should be completely submerged in the respective solvent.

1. standing up in a glass jar filled with either pure xylene or warm water (30-50°C)
2. time needed for the coverslip to swim off may range from hours to days
3. gentle movement of the jar may speed up the process
4. air-dry the slide after removal

Note: It is very important NOT to use any force to push off the coverslip because this might damage the section! Wait till it falls off by itself! The necessary time depends on the age of the sample and the dryness of the mounting medium.
Fresh slides (only days old) can be decoverslipped much faster.

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

Treatment of slides

Slides are shipped *without* any pretreatment.
To remove potentially contaminating nucleases and DNA, MembraneSlides and glass slides can be treated in the same way.

MembraneSlide NF (nuclease free) is certified to be free of DNase, RNase and human DNA. Treatments to remove nucleases and contaminating DNA are therefore not necessary using these slides.

Heat treatment

To ensure nuclease-free MembraneSlides, heat slides at 180°C in a drying cabinet for 4 hours to completely inactivate nucleases.

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- as well as cryosections) 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.

Mounting samples onto slides

Frozen sections

Sectioning

Sections are mounted onto MembraneSlides the same way as routinely done using glass slides. To allow subsequent cutting and lifting by the laser 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.

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.

Removing the medium is easily done by dipping the slide 5-6 times in water. If the sections are stained in aqueous solutions, the supporting substance is normally removed "automatically" by the water containing steps.

Formalin Fixed Paraffin Embedded (FFPE) sections

Sectioning

Floating the section on warm water (40°C) as well as hot plate techniques can be applied.
After mounting the section let the slides dry *overnight* in a drying oven at 56°C to improve the adhesion of the sections to the membrane.
To allow laser cutting and lifting a coverslip and standard mounting medium must not be applied. Archival sections with mounting medium and coverslip have to be processed as described to remove the coverslip (see page 8).

Deparaffination

Residual paraffin will reduce laser efficiency, sometimes completely inhibiting cutting and lifting. If you are working with unstained sections it is therefore very important to remove the paraffin before laser cutting and lifting. MembraneSlides can be handled like normal glass slides

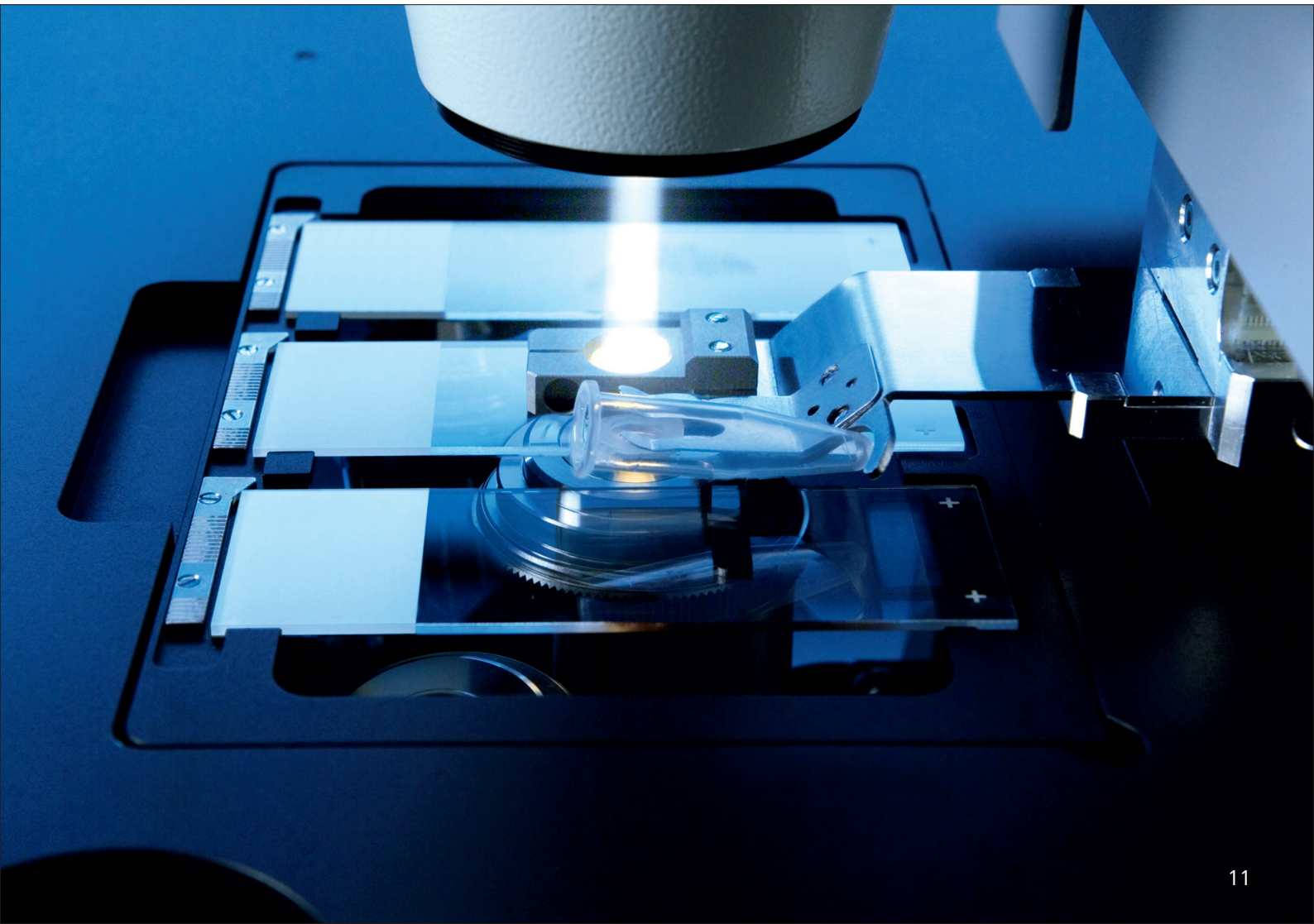
Deparaffination Procedure	
1. Xylene	5 minutes, 2 times (2 minutes minimum)
2. Ethanol 100%	1 minute
3. Ethanol 96%	1 minute
4. Ethanol 70%	1 minute

Cytospins

Cytospins can be prepared on glass slides or on MembraneSlides.
After centrifugation in a cytocentrifuge let the cells air-dry at room temperature.
Then fix for 2 minutes in 70% ethanol and air-dry again before staining.

Blood and tissue smear

Distribute a drop of blood or material of a smear over the slide.
Be careful to avoid injuries in the membrane, which would lead to leakage during fixation or washing steps and therefore would impair the Laser Capture Microdissection process.
Let smears air-dry shortly and fix them for 2 up to 5 minutes in 70% ethanol.



Staining procedures

For isolation of high quality DNA use freshly prepared, autoclaved solutions.

Formalin Fixed Paraffin Embedded (FFPE) sections

After deparaffination (see page 10) continue with the staining procedure of your choice. Most staining procedures for frozen sections can be applied for FFPE sections (for recommendations see ´Frozen sections`).

Frozen sections

Most standard histological stainings (e.g. HE, Methyl Green, Cresyl Violet, Nuclear Fast Red) are compatible with subsequent DNA isolation.
At ZEISS Labs we usually perform the Cresyl Violet or Hematoxylin/Eosin (HE) staining.

Hematoxylin/Eosin (HE)

HE-staining is used routinely in most histological laboratories. The nuclei are stained blue, the cytoplasm pink/red.

Staining Procedure
1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 1-2 minutes in Mayer's Hematoxylin solution (e.g. SIGMA, #MHS-32)
3. rinse 1-2 min in distilled water or blueing solution (e.g. BBC, #3900)
4. stain 10 seconds in Eosin Y (e.g. SIGMA, #HT110-2-32)
5. perform a quick increasing ethanol series (70%, 96%, 100%)
6. air-dry shortly (1-2 min)

Cresyl Violet

This short staining procedure colors the nuclei violet and the cytoplasm weak violet.

Staining Procedure
1. after fixation (2 min, 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
4. dip into 100 % Ethanol
5. air-dry shortly (1-2 min)

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

Note: In most cases this cresyl violet staining procedure will be sufficient for cell identification. If an enhancement of the intensity is desired, a reinforcement by two additional steps in 50 % ethanol is possible (first, before staining in cresyl violet; second, after the staining in cresyl violet).
Ambion offers the LCM Staining Kit (#1935) which also contains a cresyl violet dye.
When using this kit we strongly recommend to omit the final xylene step of the Ambion instruction manual because xylene makes the tissue very brittle and reduces the adhesion of the section to the PEN-membrane.

Toluidine Blue

The nuclei are stained dark blue, the cytoplasm lighter blue.

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 30 seconds in Toluidine Blue solution (0.1 % in water; SIGMA, #T-0394)
3. rinse in distilled water
4. perform a quick increasing ethanol series (70%, 96%, 100%)
5. air-dry shortly (1-2 min)

Methyl Green

The nuclei are stained dark green, the cytoplasm light green.

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 5 minutes in Methyl Green solution (DAKO, #S1962)
3. rinse in distilled water
4. air-dry shortly (1-2 min)

Methylene Blue

The nuclei are stained dark blue.

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 5-10 min in Methylene Blue solution (0.05 % in water; SIGMA, #31911-2)
3. rinse in distilled water
4. air-dry shortly (1-2 min)

Nuclear Fast Red

The nuclei are stained dark red, the cytoplasm lighter red.

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 5 to 10 minutes in Nuclear Fast Red solution (DAKO, #S1963)
3. rinse in distilled water
4. air-dry shortly (1-2 min)

Storage

Stained slides can be used immediately or stored dry. If the slides are stored in a freezer before LCM, the slides should be frozen in a tightly sealed container (e.g. two slides back to back in a 50 ml Falcon-tube) to avoid excess condensation of moisture during thawing.
For rethawing the container should not be opened before it is completely warmed up again to ambient temperature.

Non-contact Laser Capture Microdissection (LCM) Procedures

Please, additionally have a look into the PALM MicroBeam user manual.

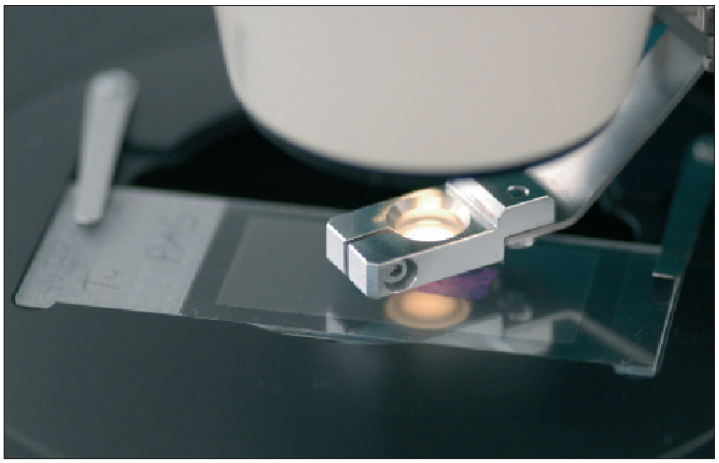
Tips to improve morphological information

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. Effects of diffusor, AdhesiveCap as well as Liquid Cover Glass are comparable to the usual coverslip for enhanced visualization.

Diffusor CM

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 Diffusor CM product information.

PALM CombiSystem



Diffusor CM - Order No. 415101-2100-320

AdhesiveCap opaque

The white/opaque filling of AdhesiveCap clearly improves visualization of morphological information of the samples due to enhanced color balance and contrast, which makes the view comparable to those of coverslipped tissue sections.
Two different microfuge tube sizes (200 µl, 500 µl) with these 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)

Liquid Cover Glass

The polymeric and low viscose Liquid Cover Glass completely embeds the tissue and smoothens the rough tissue surface, resulting in enhanced morphology after drying.
For more details and handling, please see Liquid Cover Glass product information.

Liquid Cover Glass - Order No. 415190-9020-000

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 the risk of nuclease 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 of the buffer during extended specimen harvesting.
For more details and handling, please see also AdhesiveCap product information.

AdhesiveCap opaque - Order No. 415190-9201-000 (500 µl)
AdhesiveCap opaque - Order No. 415190-9181-000 (200 µl)
AdhesiveCap clear - Order No. 415190-9211-000 (500 µl)
AdhesiveCap clear - Order No. 415190-9191-000 (200 µl)

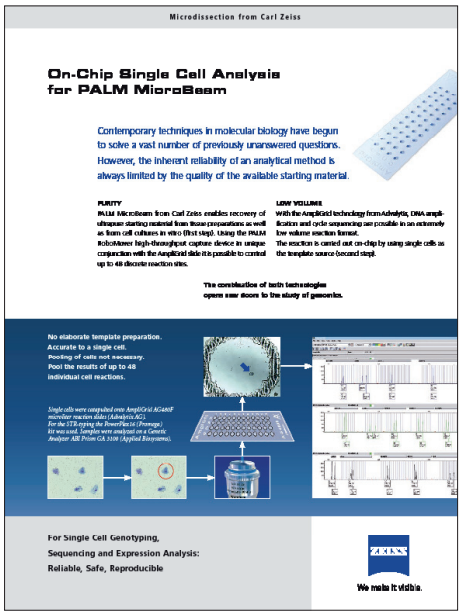
Other microfuge tubes

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

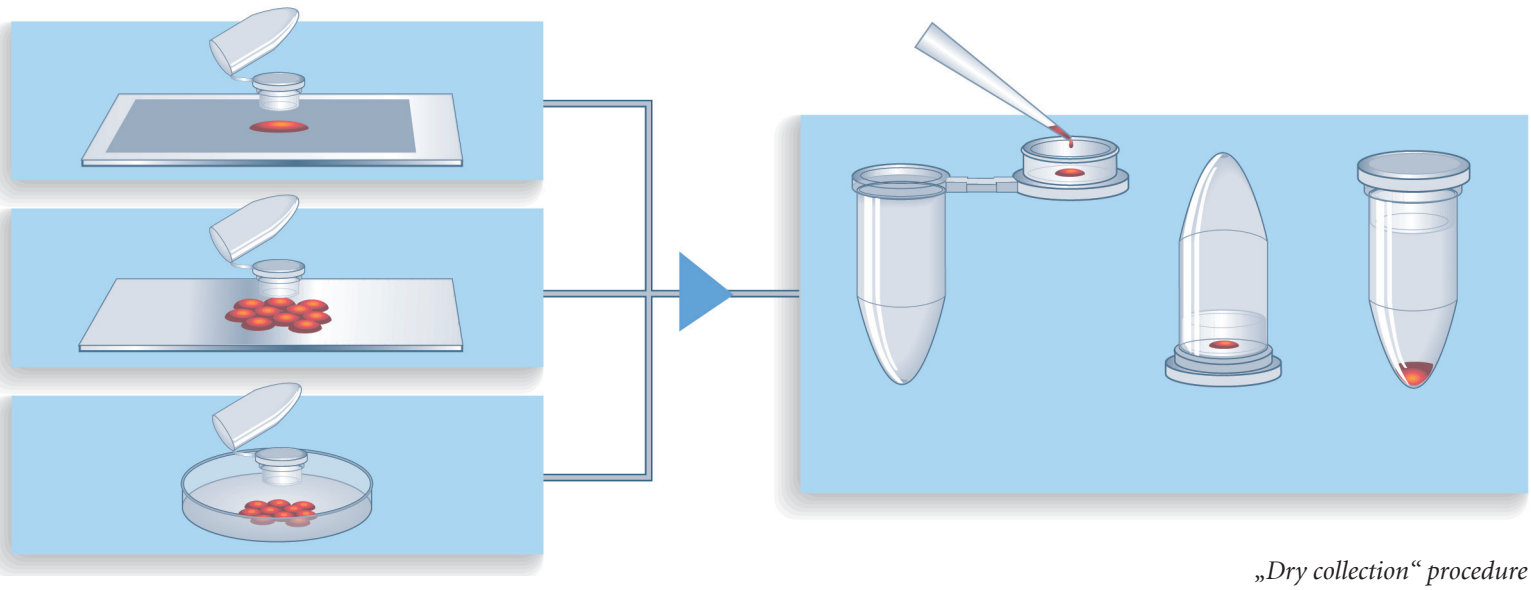
AmpliGrid AG480F

Using the SlideCollector 48 in conjunction with AmpliGrid technology from Advalytix enables working in a higher throughput LCM (48 samples simultaneously).
The AmpliGrid technology allows DNA analysis in an extremely low volume (1 µl) directly on chip.

Please, see the brochure (labs@zeiss.de)



On-chip Single Cell Analysis for PALM MicroBeam.



„Dry collection“ procedure

Collection procedures

Please have a look into the PALM MicroBeam user manual.

„Dry“ collection (AdhesiveCap)

Note: CZMI recommends AdhesiveCap as collection device for most experiments.

1. put the AdhesiveCap into the collector and check the right position of the correction collar (see page 6)
2. perform non-contact LCM of selected cells
3. after LCM add 15 µl lysis buffer to the sample inside the cap (QIAamp® DNA Micro Kit #56304)
4. add 10 µl Proteinase K (20 mg/ml) and mix by pulse-vortexing for 15 sec
5. place the tube in an “upside down” position in an incubator at 56°C for 2 - 18 h with occasional agitation
6. centrifuge the tube at 10000 rcf for 5 min (Tabletop Microcentrifuge)

If not going on immediately, store the samples at -20°C.

Note: The time necessary for complete Proteinase K digestion depends on the kind and the amount of collected material. After the Proteinase K digest the regular procedure of the QIAamp® DNA Micro Kit #56304 (page 21, step 4) can be attached.

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

Collection procedures

„Wet“ collection (other microfuge tubes)

When using “unfilled” routine microfuge tubes it is necessary to add a liquid into the cap to facilitate the adhesion of the captured cells.
The detergent Igepal CA-630 in the capturing buffer allows to smear out a small amount of liquid in the whole cap area.
Note: All kinds of aqueous solutions will run dry with extended working time.

Prearrangements - Capturing Buffer	
0.05 M EDTA pH 8.0	20 µl
1 M Tris pH 8.0	200 µl
Igepal CA-630 (SIGMA #I-3021)	50 µl
(Proteinase K)*	(100 µl)
ddH_2O	fill up to 10 ml

*Proteinase K 20 mg/ml (Qiagen #19131)

Final Concentration: 20 mM Tris, 0.1 mM EDTA, 0.5% Igepal, 1% Proteinase K

Always prepare a fresh mixture of Capturing Buffer and Proteinase K.

Note: The time necessary for complete Proteinase K digestion depends on the kind and the amount of collected material. After the Proteinase K digest and the inactivation step the routine downstream application of choice can be continued.

If not going on immediately, store the samples at -20 °C.

Collection Procedure

1. take an autoclaved microfuge tube
2. pipette 3-15 µl of Capturing Buffer without Proteinase K or DNase-free water in the middle of the cap
3. put the cap/tube into the collector check the right position of the correction collar (see page 6)
4. perform non-contact LCM of selected cells
5. centrifuge the tube at 10000 rcf for 5 min (Tabletop Microcentrifuge)
6. add 10-15 µl Capturing Buffer containing Proteinase K and mix by pulse-vortexing for 15 sec
7. incubate the tube at 56°C for 2-18h with occasional agitation
8. centrifuge the tube at 10000 rcf for 5 min (Tabletop Microcentrifuge)
9. final heating step at 90°C for 10 min to inactivate Proteinase K
10. centrifuge the tube at 10000 rcf for 5 min (Tabletop Microcentrifuge)

„Wet“ collection onto Slide48 (AmpliGrid AG480F)

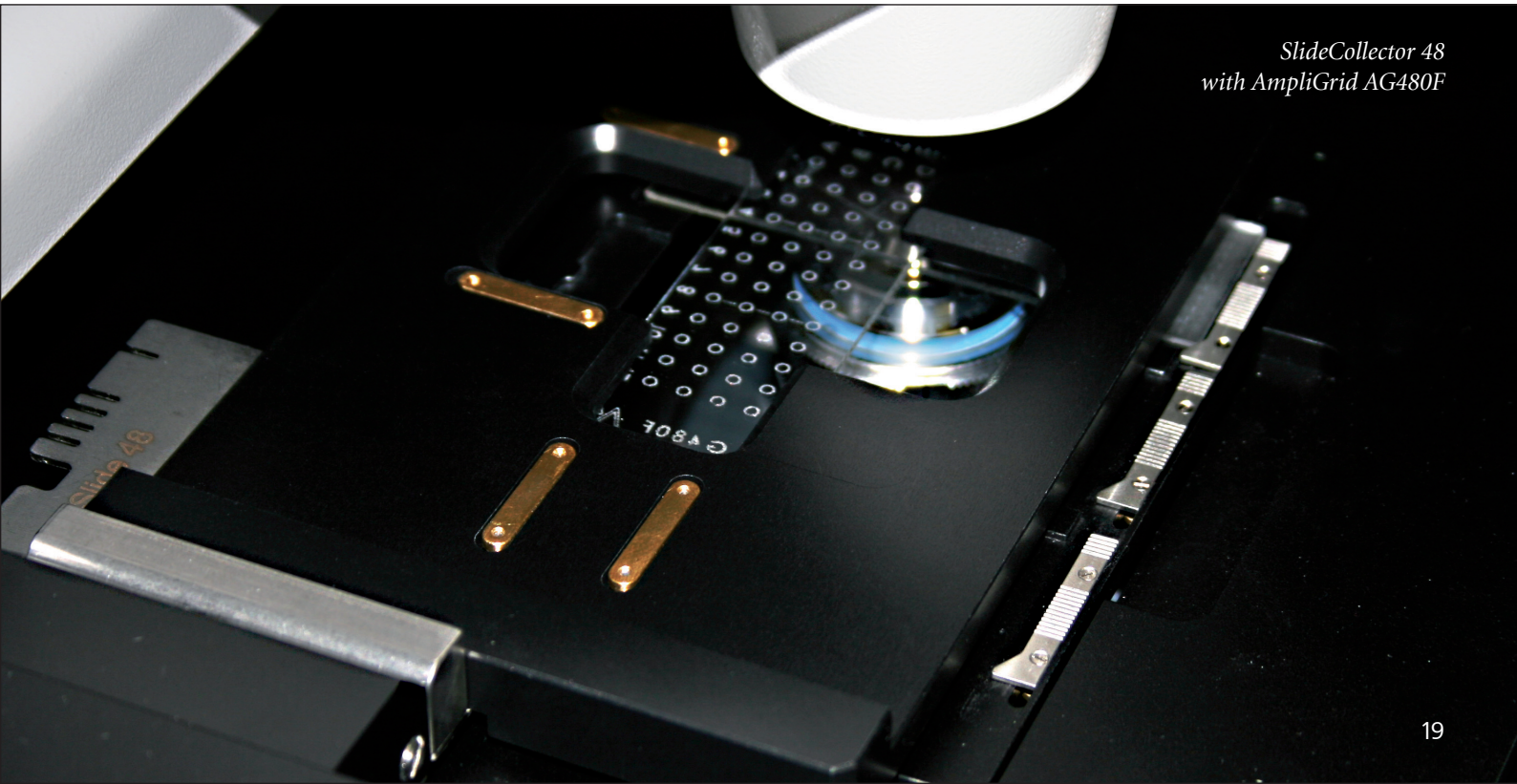
Using Slide48 technology, DNA amplification doesn’t require any template transfer and preparation. Analysis (PCR, cycle sequencing) can be performed on the same reaction site of the AmpliGrid AG480F (see page 23): Low volume PCR (1 µl) in an Eppendorf MasterCycler.

A preloading of 48 ReactionSites of the AmpliGrid with 1 µl liquid (e.g. 1% Glycerol in water) enables elongation of the working time and is necessary for adhesion of the captured samples. The LCM process onto 48 reaction sites can be operated automatically and is controlled by PALM RoboSoftware.

Capture check – looking into the cap to see the lifted samples

To control and document the efficiency of lifting it is possible to have a look into the collection device (e.g. microfuge cap) with the 5x, 10x, 20x, 40x and 63x objectives.

By using the software function “go to checkpoint” the slide is moved out of the light path and the objective lifted for looking inside.



SlideCollector 48 with AmpliGrid AG480F

Downstream Applications

DNA isolation from FFPE sections

Deparaffination and staining are done according to standard procedures for slides (please see page 10-13).

Note: Proteinase K digestion step is essential for formalin fixed samples.
The time necessary for optimal digestion depends on many factors like tissue type, fixation procedure or thickness of lifted material. An overnight digestion (12-18 hours) is a good starting point for optimization but shorter digestion times may be tested as well. To our experience at least 3 hours digestion should be applied with any extraction procedure and material.

For subsequent DNA extraction from FFPE sections ZEISS Labs prefer the QIAamp DNA Micro Kit (#56304), please see DNA isolation from frozen sections.

DNA isolation from frozen sections

To capture microdissected samples we recommend the use of AdhesiveCap.
For DNA isolation any procedure of choice can be used.

In our hands the QIAamp® DNA Micro Kit (#56304) combined with AdhesiveCap results in good yield and quality of DNA. This QIAamp® DNA Micro Kit is designed for use of small amounts of tissue.
The subsequently described protocol is suitable even for single cells.

Note: For DNA elution incubating the QIAamp MinElute Column loaded with water for 5 min at room temperature before centrifugation generally increases the final DNA yield.

Diluted solutions of nucleic acids (e.g. dilution series used as standards) should be stored in aliquots and thawed once only. We recommend storage of aliquots in siliconized tubes if possible.
This avoids adsorption of nucleic acids to the tube walls, which would reduce the concentration of nucleic acids in solution.

Applying the components of the QIAamp® DNA Micro Kit for isolation of genomic DNA from Laser Microdissected samples

1. Add 15 µl ATL to the microdissected sample in the AdhesiveCap.
2. Add 10 µl Proteinase K and mix by pulse-vortexing for 15 sec.
3. Place the 0.2 ml tube in an "upside down" position at 56°C in an incubator for 3-18h with occasional agitation.

   Note: The time necessary for complete Proteinase K digestion depends on the kind of collected material. Especially FFPE samples must be digested longer.
4. Add 25 µl Buffer ATL and 50 µl Buffer AL, close the lid and mix by pulse-vortexing for 15 sec. To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogeneous solution.
5. Add 50 µl ethanol (96-100%), close the lid, and mix thoroughly by pulse-vortexing for 15 sec. Incubate for 5 min at room temperature (15-25°C).
   If room temperature exceeds 25°C, cool the ethanol on ice before adding to the tube.
6. Briefly centrifuge the 0.2 ml tube to remove drops from the lid.
7. Carefully transfer the entire lysate to the QIAamp MinElute column without wetting the rim, close the lid, and centrifuge at 6000 x g (e.g. Eppendorf 5415D: 8000 rpm) for 1 min.
   Place the QIAamp MinElute Column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
   If the lysate has not completely passed through the column after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute Column is empty.
8. Carefully open the QIAamp MinElute Column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min.
   Place the QIAamp MinElute Column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
9. Repeat procedure of step 8 with 500 µl Buffer AW2 this time.

   Note: Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol - coming into contact with the QIAamp MinElute Column. Take care when removing the QIAamp MinElute Column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute Column.
10. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely. This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.
11. Place the QIAamp MinElute Column in a clean 1.5 ml microcentrifuge tube (not provided) and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute Column and apply 20 µl distilled water to the center of the membrane. Ensure that distilled water is equilibrated to room temperature (15-25°C). Dispense distilled water onto the center of the membrane to ensure complete elution of bound DNA.

    Note: QIAamp MinElute Columns provide flexibility in the choice of elution volume. Choose a volume according to the requirements of the downstream application. Remember that the volume of eluate will be up to 5 µl less than the volume of elution solution applied to the column.
12. Close the lid and incubate at room temperature (15-25°) for 1-5 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min.

Downstream Applications

PCR setup

Depending on the *concentration* of the isolated DNA the suitable setup for the amplification has to be selected:

The *standard volume PCR (20 µl) in a capillary cycler* is useful for highly concentrated DNA eluates, because the maximal input of target DNA in the reaction setup is limited. Only 30-50% of the eluate can be analysed.

For low concentrated DNA eluates, e.g. from a single microdissected cell, the *high volume PCR (50 µl) in a 96-well block cycler* is recommendable, as 100% of the eluate can be used for the reaction setup.

The *low volume PCR (1 µl) in an Eppendorf Mastercycler* allows a direct analysis without separate DNA isolation and transfer step. This method offers the advantage of the combination of LCM and low volume PCR on the same slide.

Standard PCR (20 µl) in a capillary cycler

QuantiFast SYBR Green PCR (QIAGEN #204052) in our hands results in exact amplification products.

PCR Procedure

1. Thaw 2x QuantiFast SYBR Green PCR Master Mix, template DNA, primers, and water. Mix the individual solutions.
2. Prepare a reaction mix according to setup. Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

Note: We recommend starting with the Mg^{2+} concentration as provided in 2x QuantiFast SYBR Green PCR Master Mix.

Reaction Setup

2x QuantiFast SYBR Green PCR Master Mix	10 µl
Primer A (10 µM)	0.5 µl
Primer B (10 µM)	0.5 µl
Template DNA	≤ 100 ng/reaction
distilled water (PCR clean)	variable
Total reaction volume	20 µl

3. Mix the reaction mix thoroughly and dispense appropriate volumes into PCR capillaries.
4. Add template DNA (≤100 ng/reaction) to the individual capillaries containing the reaction mix.
5. Program the cycler according to conditions

Capillary Cycler conditions (exemplary)

Step	*Time*	*Temp.*	*Ramp rate*
PCR initial activation	5 min	95°C	fast mode
Two-step cycling			
denaturation	10 sec	95°C	fast mode
combined annealing/extension	30 sec	60°C	fast mode
number of cycles 40			

6. Place the PCR capillaries in the cycler and start the cycling program.
7. Optional: Check the specificity of the PCR product(s) by agarose gel electrophoresis

High volume PCR (50 µl) in a 96-well block cycler

The input of the whole eluate (20 µl) to the PCR reaction mix requires an increased total reaction volume of 50 µl.

PCR Procedure

1. Thaw PCR buffer, dNTPs, template DNA, primers, and water. Mix the individual solutions. Keep samples on ice during reaction setup or while programming the cycler.
2. Prepare a reaction mix according to setup:

Reaction Setup	
10x Buffer	5 µl
dNTP-Mix (2 mM each)	5 µl
Primer A (10 µM)	1 µl
Primer B (10 µM)	1 µl
template DNA	variable
Qiagen HotStarTaq Polymerase	0.5 µl
distilled water (PCR clean)	variable
Total reaction volume	50 µl

3. Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes.
4. Add template DNA (≤100 ng/reaction) to the PCR tubes containing the reaction mix.
5. Program the cycler according to conditions.

Block Cycler conditions (exemplary)		
Step	*Time*	*Temp.*
activation step	15 min	95°C
denaturation	30 sec	95°C
annealing	30 sec	50°C
extension	30 sec	72°C
final extension	5 min	72°C
number of cycles 40		

6. Place the PCR tubes in the cycler and start the cycling program.
7. Optional: Check the specificity of the PCR product(s) by agarose gel electrophoresis

Depending on the experiment a subsequent nested PCR based on the first PCR product and internal primers can be attached.

Low volume PCR (1 µl) in an Eppendorf Mastercycler

DNA amplification and cycle sequencing, for example of a single cell, are possible in an extremely low volume reaction format (1 µl) with the Slide48/AmpliGrid technology. After lifting the cell onto the chip analysis can be performed directly on-chip without any template preparation.

PCR Procedure

1. Thaw PCR buffer, dNTPs, template DNA, primers, and water. Mix the individual solutions.
2. Prepare a reaction mix according to setup:

Reaction Setup (see Advalytix protocols *[1])	
AmpliTaq Gold	0.1 µl
10x GeneAmp Buffer I with 15mM $MgCl_2$	0.1 µl
Primer (5 pmol/µl each)	0.1 µl
dNTP-Mix (2.5 µM each)	0.1 µl
distilled water (PCR clean)	0.6 µl
Total reaction volume	1.0 µl

3. Mix the reaction mix and dispense 1 µl to each reaction site of the AmpliGrid slide.
4. Cover the PCR droplet with 5 µl of sealing solution.
5. Place the loaded AmpliGrid on the Eppendorf Mastercycler.
6. Program the cycler according to conditions.

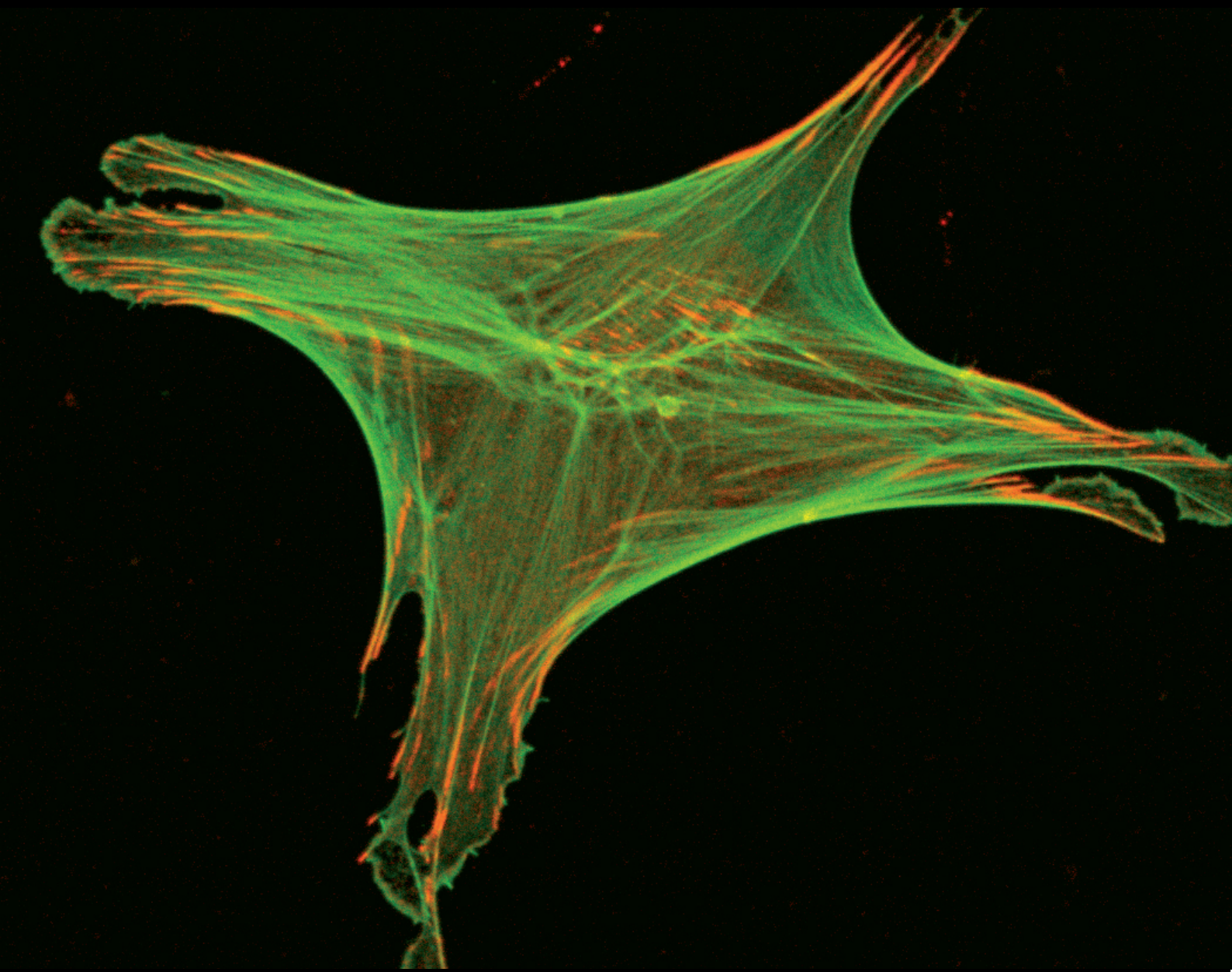
Eppendorf Mastercycler conditions (example)		
Step	*Time*	*Temp.*
PCR initial step	10 min	95°C
denaturation	40 sec	94°C
annealing	30 sec	56°C
extension	30 sec	72°C
final extension	5 min	72°C
number of cycles 40	10 min	72°C

Depending on the experiment a sequencing reaction with subsequent capillary electrophoresis analysis or a check of the specificity of the PCR product(s) by gel electrophoresis can be attached.

*[1] *Advalytix protocols: www.advalytix.com/images/download*

Carl Zeiss MicroImaging

LCM Protocols – Protein Handling for LC/MS



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

LCM Protocols

Protein Handling for LC/MS

Non contact Laser Capture Microdissection

Carl Zeiss MicroImaging – Location Munich – Germany

Content

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 teraphthalate (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:

E-Mail: 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.

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).

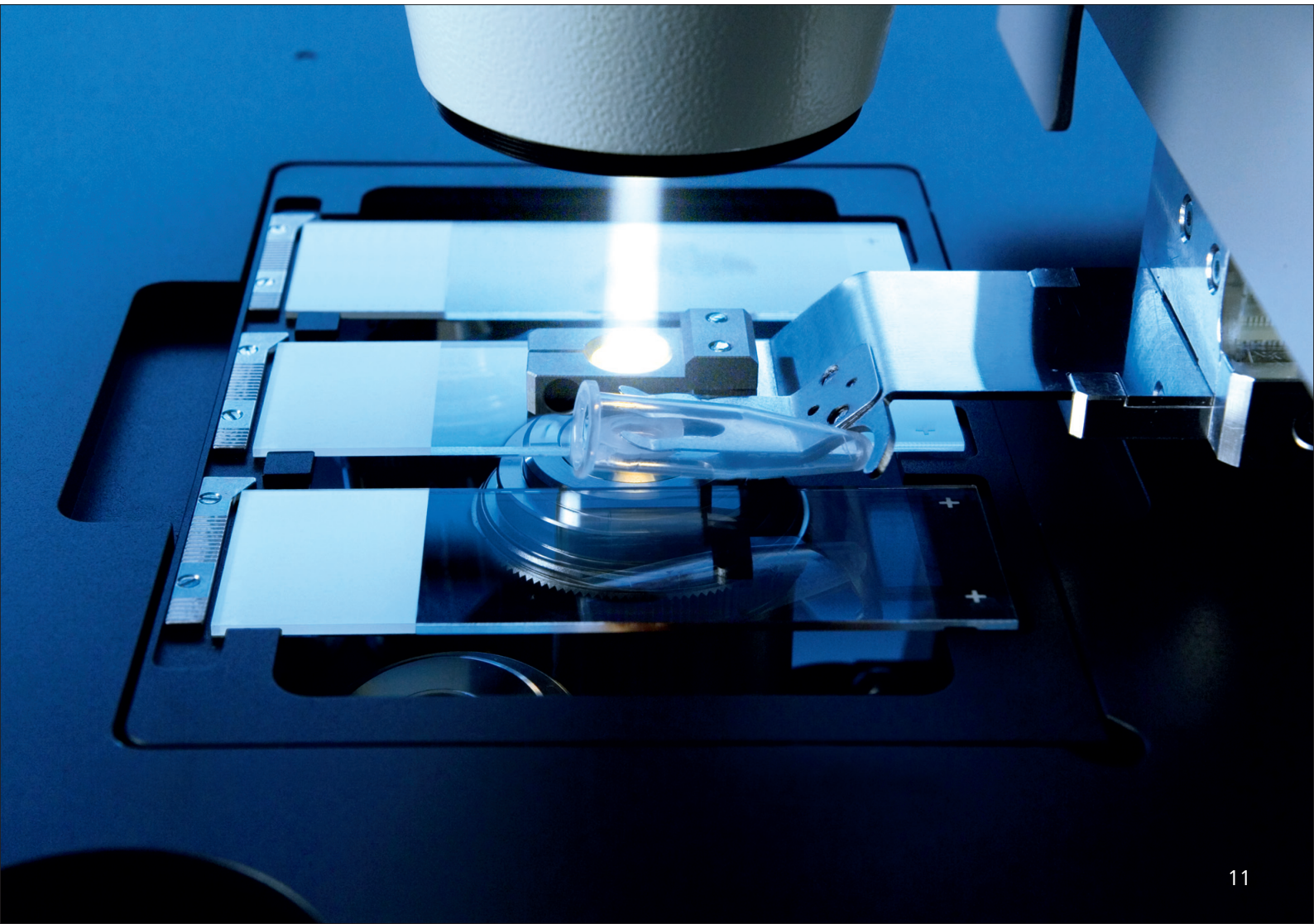
For optimal sample protection take a pre-cooled slide and touch the backside of the slide with your finger (gloves!) to warm only the region for placing the section. Now transfer section from the knife by touching with the warmed area and dry at -20°C in the cryostat for 5 minutes.

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 recommeded 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.

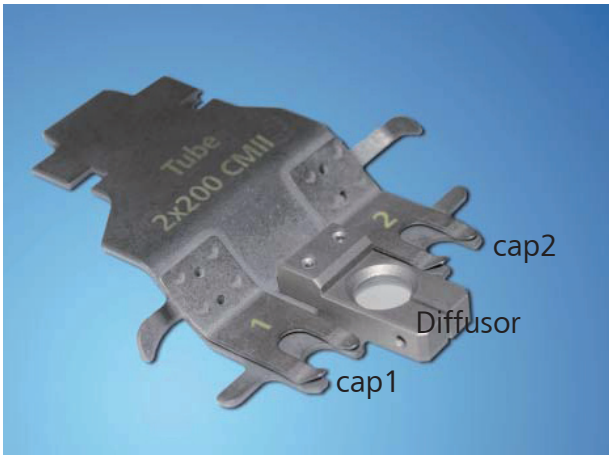
Tips to improve morphological information

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

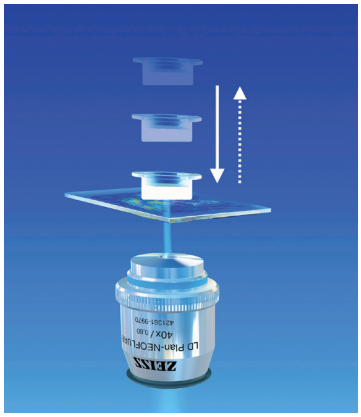
AdhesiveCap

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.

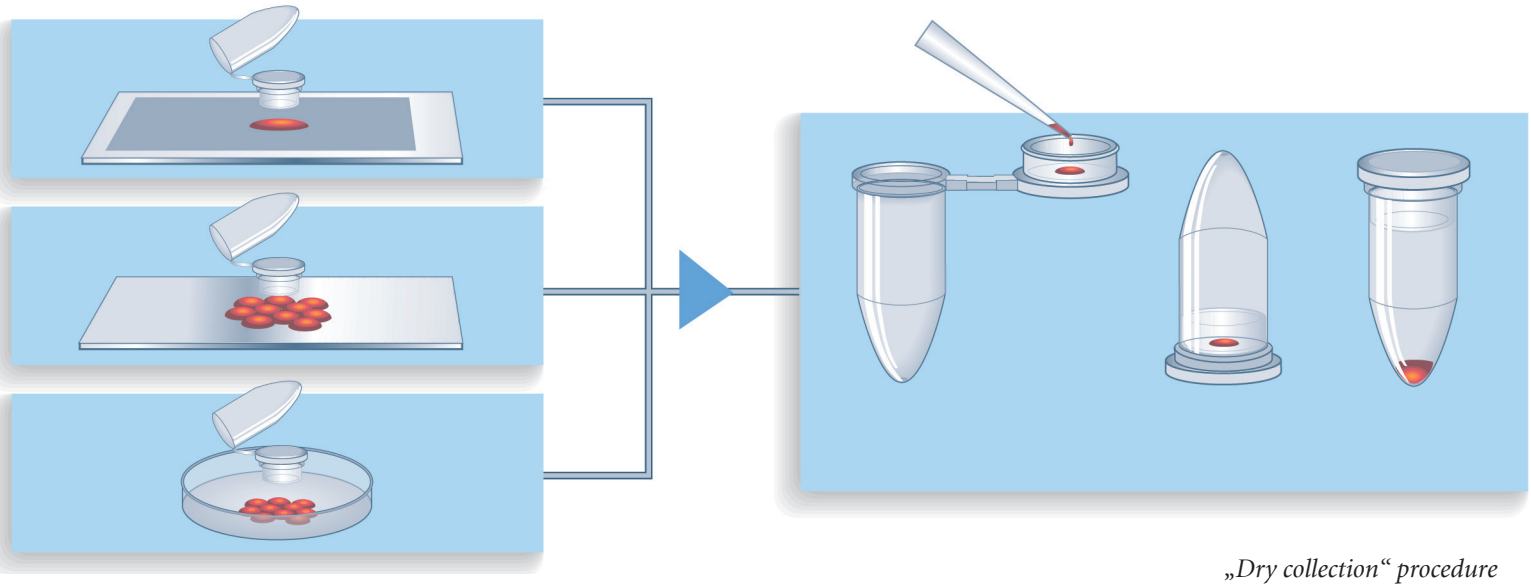


Pick-up LCM via AdhesiveCap Touch

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

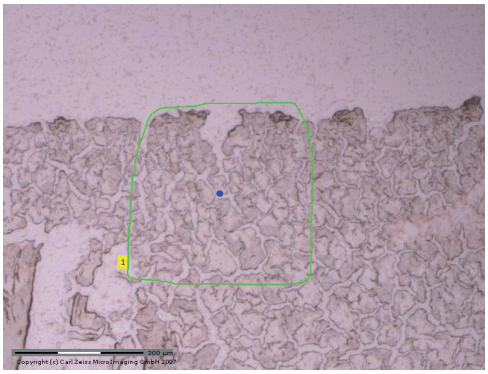
Fresh frozen mouse liver samples were cut in the cryostat at -20°C into 10-12 µm sections, mounted onto MembraneSlides 1.0 PEN and dried for approximately 5 minutes in the cryostat.

Tissue Staining

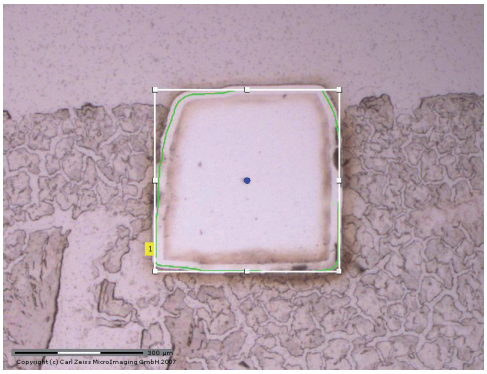
For unstained samples, sections were dehydrated first in 70% ethanol for 5 sec and then in 100% ethanol for 5 sec and subsequently air dried.
Two different stainings were performed: staining with Hematoxylin (no eosin) and with Cresyl Violet (procedures see page 12/13).
After staining and/or dehydration, LCM samples should be immediately collected.

Tissue Collection

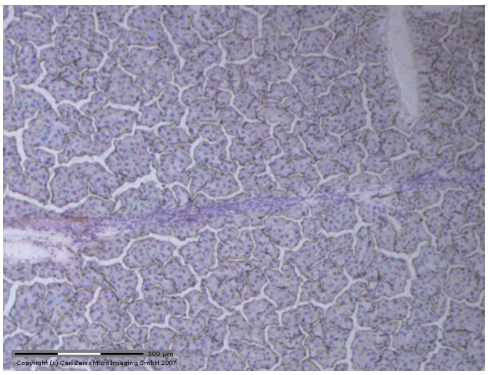
Settings for laser energy and focus on the MicroBeam system should be optimized for each type of tissue. Working with unstained samples requires a higher laser cutting energy (10-20%) due to lower absorption of laser energy. For LC/MS proteomics analysis, 50,000-100,000 cells should be collected for 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 180.000-250.000 µm^2 and collected in one AdhesiveCap; in summary about 5,2 mm^2 pooled in one cap.



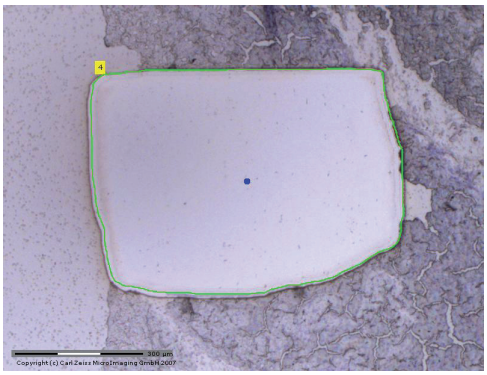
LCM of liver section: unstained tissue section



... after isolation



LCM of liver section: sample stained with Cresyl Violet



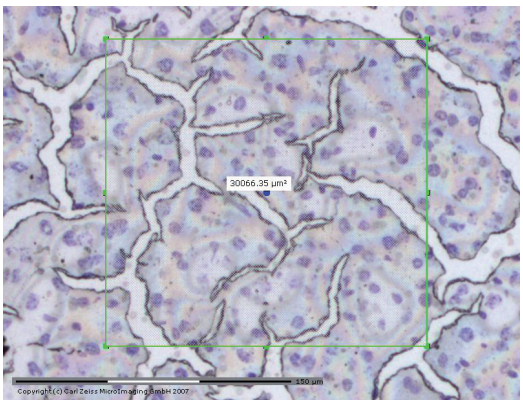
... after isolation

Downstream Applications

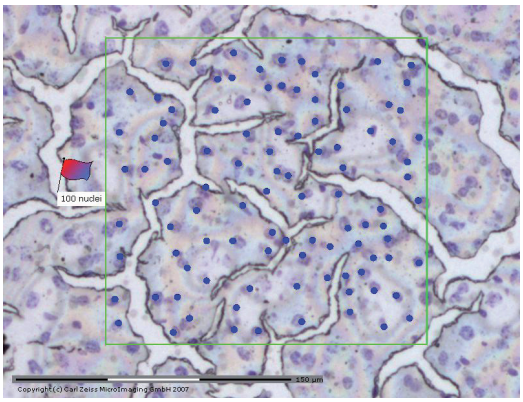
Protein Quantitation Assay (BCA) - Determine your Protein Concentration in Advance

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 µg/µl 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 µl 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 µl 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 µl for the protein quantitation assay (BCA) (page 19) and 75 µl for LC/MS (page 22).

Surfactant Cleavage for Intact Protein Analysis:

Cleave Rapigest by adding 10 µl 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:

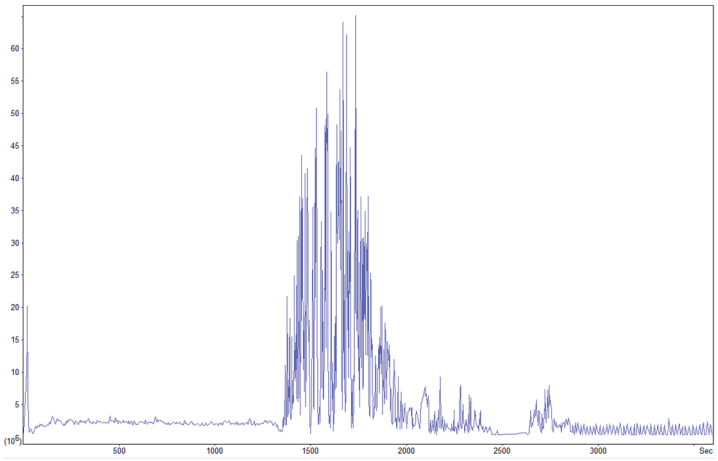
1. Transfer an aliquot (minimum: 0.1 µg/µl protein concentration) of the extracted sample into a new microcentrifuge tube.
2. Add 50 mM ammonium bicarbonate to a final volume of 30 µl.
3. Add Dithiotreitol (DTT) to the sample to make a final DTT concentration of 10 mM.
4. Heat the sample at 80°C while 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.

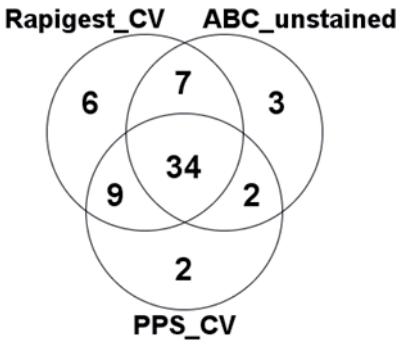
Downstream Application

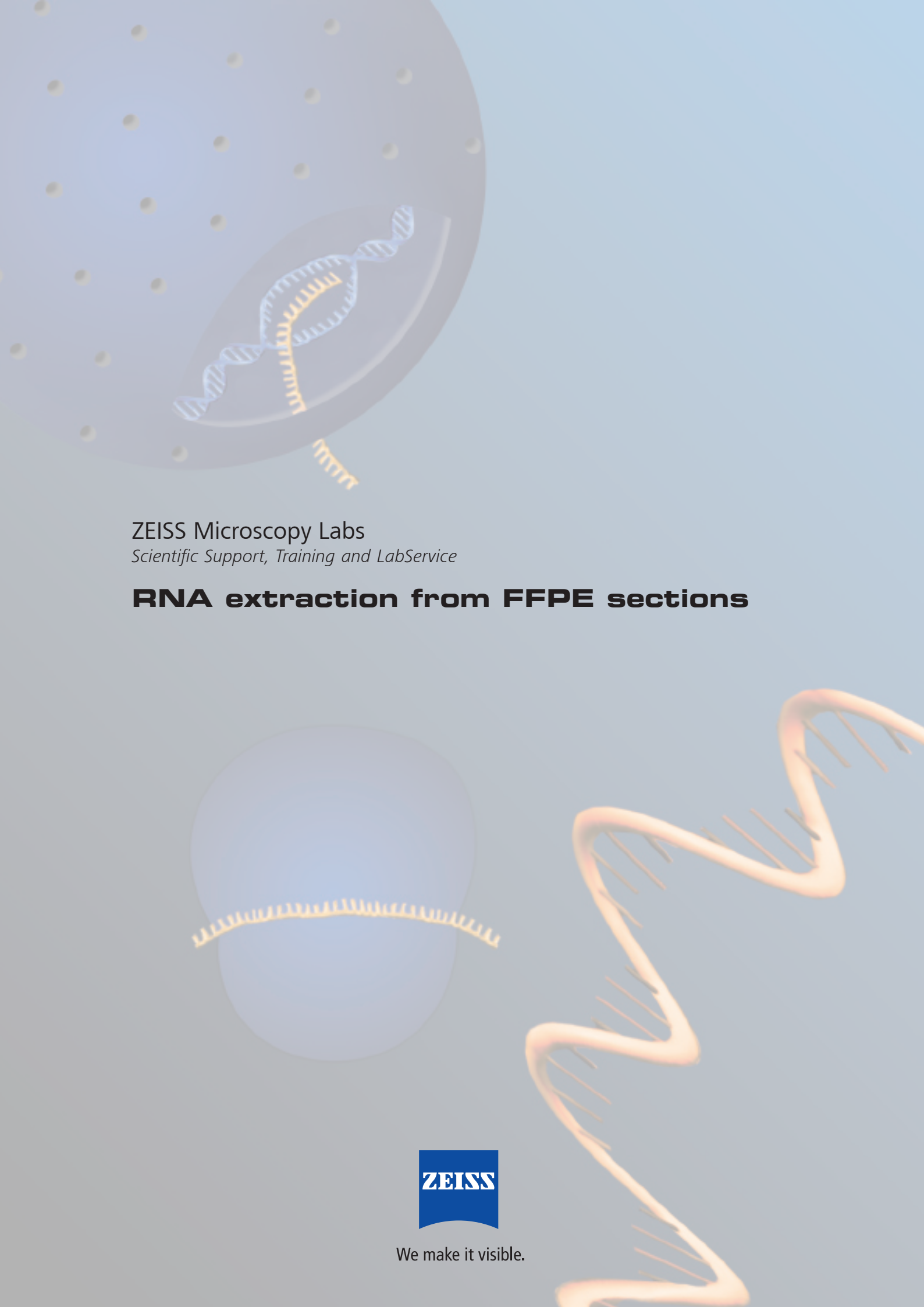
LC/MS Results after Protein Extraction and Digestion



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.





ZEISS Microscopy Labs

Scientific Support, Training and LabService

RNA extraction from FFPE sections

We make it visible.

Some helpful tips before starting:

- To find additional information around RNA work - especially for less experienced users - please consult: "**LCM Protocols - RNA Handling**" which is available at www.zeiss.de/applab.

- We recommend MembraneSlide1.0 PEN (Order No. 415190-9041-000) for routine preparation of FFPE sections. If weak fluorescence must be detected or ablation is needed before micro-dissection MembraneSlide 1.0 PET (Order No. 415190-9051-000) may be applicable.
  For other special applications please inquire.

- To ensure RNase-free conditions use only RNase-free solutions and materials.
  Incubate MembraneSlides in dry heat at 180°C for 4 hours to completely inactivate any RNases.

- Microtome cutting, transfer, deparaffination and staining can be done according to standard procedures. Longer melting/drying of the section improves the adhesion to the membrane.
  We recommend incubation at 56°C over night (≥ 16 h) before deparaffination.

- Most standard histological stains (like HE, Methyl Green, Cresyl Violet, Nuclear Fast Red) can be used for FFPE material. To our experience only Methylene Blue is not recommendable for RNA.

Note: To allow laser cutting and lifting a *coverslip* and mounting medium *must not be applied*!

- For collecting microdissected samples we recommend special AdhesiveCaps:
  AdhesiveCap 200 opaque (Order No. 415190-9181-000) or
  AdhesiveCap 200 clear (Order No. 415190-9191-000)

- The incubation with Proteinase K in our protocol is prolonged significantly compared to the original QIAGEN manual because all our tests with laser microdissected material from various tissues showed better RNA yields by applying longer digestion times.

Note: For formalin fixed samples a **Proteinase K** digestion step is essential. The time necessary for optimal Proteinase K digestion depends on many factors like tissue type, fixation procedure or element size of lifted material. An overnight digestion (12-18 hours) is a good starting point for optimization, but shorter digestion times may be tested as well. To our experience any formalin fixed material should be digested for at least 3 hours, no matter which extraction procedure is used.

- We normally use 5 to 10 µl of the final RNA solution in a RT-reaction of 20 µl (e.g., Transcriptor First Strand cDNA Synthesis Kit, ROCHE, # 04 379 012 001) using random-oligomers (instead of oligo dT) as primers for the cDNA synthesis.

Note: The use of **random or gene-specific primers** is very important since reverse transcription of formalin fixed RNA with only standard oligo dT-primers will be very inefficient and strongly 3` biased due to the numerous strand breaks and modifications inflicted by the formalin fixation and paraffin embedding procedure.

- To prognose the extractable amount of RNA from FFPE tissue is very difficult since many factors like species, cell/tissue-type, fixation, staining, fragmentation, modification and others will strongly influence the outcome. Any FFPE tissue block should therefore be tested individually.

Using components of the QIAGEN RNeasy® FFPE Kit- (Order No. 73504)

1. Add 150 µl Buffer PKD and 10 µl of Proteinase K to the tube containing the LCM elements in the AdhesiveCap and invert the tube to get contact between liquid and adhesive surface.
2. Use an incubator to digest the samples in an "upside down" position at 56°C overnight (or for at least 3 hours), then vortex and heat at 80°C for precisely 15 min in a heating block.

   Note: Please do not use any water bath for the upside down incubation.
3. Incubate on ice for 3 min.
4. Add 16 µl DNase Booster Buffer and 10 µl DNase I stock solution. Mix gently by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

   Note: DNase I is supplied lyophilized and should be reconstituted as described in "Preparing DNase I stock solution" (page 14; RNeasy FFPE handbook 09/2010).

   Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex!
5. Incubate at room temperature for 15 min.
6. Transfer the lysate to a new 1.5 ml microcentrifuge tube.
7. Add 320 µl Buffer RBC to adjust binding conditions and mix the lysate thoroughly.
8. Add 720 µl ethanol (100%) to the sample and mix well by pipetting. Do not centrifuge. Proceed immediately to step 9.
9. Transfer 700 µl of the sample to a RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge for 15 sec at ≥8000 x g (≥10000 rpm). Discard the flow-through. Reuse the collection tube in step 10.
10. Repeat step 9 until the entire sample has passed through the RNeasy MinElute spin column. Reuse the collection tube in step 11.
11. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 sec at ≥8000 x g (≥10000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 12.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.
12. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at ≥8000 x g (≥10000 rpm) to wash the spin column. After centrifugation carefully remove the spin column from the collection tube so that the column does not contact the flow-through.
13. Place the RNeasy MinElute spin column in a new 2 ml collection tube, and discard the old collection tube with the flow-through. Open the lid of the spin column and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through. It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions.
14. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 14-30 µl RNase-free water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min at full speed to elute the RNA. The dead volume of the RNeasy MinElute spin column is 2 µl: Elution with 14 µl of RNase-free water results in a 12 µl eluate.
15. The RNA solution may be stored at -20°C or used directly for reverse transcription.

    Note: Quality control by direct analysis (e.g., Agilent Bioanalyzer Pico Chip) is very limited and may only be possible with quite large microdissected samples (often some 4 mm^2 collected area from tissue sections of 5-10 µm thickness).

ZEISS Microscopy Labs

Scientific Support, Training and LabService

RNA extraction from frozen sections

ZEISS

We make it visible.

Some helpful tips before starting:

- To find additional information around RNA work - especially for less experienced users - please consult our **"LCM Protocols - RNA Handling"** which is available on request.
- To prepare sections we recommend MembraneSlide 1.0 PEN (Order No. 415190-9041-000). To ensure RNase-free conditions, incubate MembraneSlides in dry heat at 180°C for 4 hours to completely inactivate any RNases.
- MembraneSlides Nuclease Free - MembraneSlide NF 1.0 PEN (Order no. 415190-9081-000) are certified to be free of DNase, RNase and human DNA. Treatments to remove nucleases are therefore not necessary when using these slides.
- MembraneSlide 1.0 PET (Order No. 415190-9051-000) may be applicable if weak fluorescence must be detected. For other special applications please inquire.
- For collecting microdissected samples we recommend the special AdhesiveCaps:
  AdhesiveCap 500 opaque (Order No. 415190-9201-000) or
  AdhesiveCap 500 clear (Order No. 415190-9211-000).

Note: 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 for sectioning but should be kept to a minimum and have to be removed before laser cutting.

Microtome cutting and Cresyl Violet staining

The following procedure is recommended especially for RNase-rich tissue and therefore only RNase-free materials must be used and all liquids should be kept on ice.

- For optimal protection of RNA take a pre-cooled MembraneSlide (best inside the cryostat chamber at about -20°C) and touch the backside of the slide with your finger (wear gloves!) to warm only the region for placing the section on the membrane.
- Now transfer the frozen section from the blade to the slide by touching with the warmed area.
- Dry in the cryostat for 2-3 min at about -20°C. Longer drying can increase the adhesion of weakly attaching tissue sections.
- Subsequently incubate the section in ice-cold 70% ethanol for 2-3 min to reduce RNase activity by dehydration.

Note: If excess OCT or another tissue freezing medium was used, an additional washing step in ice-cold RNase-free water (1-2 min) has to be performed after this ethanol step. Dip the slide 5-6 times into the water to enhance the washing effect.

- Now incubate the section in ice-cold CV-staining solution (see below) for 30 sec to 2 min.
- Remove excess stain from the slide on an absorbant surface, then dip few times into ice-cold 70% ethanol for short washing
- Complete washing and dehydration by few dips into ice-cold 100% ethanol
- Finally air-dry at room temperature for 1-2 minutes
- After drying slides can be used immediately for LCM or may be stored at -80°C for some days.

Note: The slides should be frozen and thawed in an airtight container (e.g., two slides back-to-back in a 50 ml Falcon-Tube). To avoid condensation of moisture on the tissue do not open the container before slides are warmed up again to ambient temperature. When sections are completely dry, RNases are inhibited and RNA is remarkably stable for hours.

Preparation of CV-Staining Solution

Dissolve solid Cresyl Violet Acetate (e.g., Aldrich cat #86,098-0) at a concentration of 1% (w/v) in 50% Ethanol. Apply agitation/stirring for several hours to overnight at room temperature for thorough dissolving. Some unsolubilized powder is normal. Filter the staining solution before use.

Another possibility for Cresyl Violet staining is using the LCM Staining Kit from Ambion (#1935). We strongly recommend to omit the final xylene-step of its instruction manual because xylene makes the tissue very brittle and reduces the adhesion of the section to the PEN-membrane.

Applying components of the QIAGEN RNeasy® Micro Kit (Order No. 74004)

1. Add 350 µl Buffer RLT containing ß-Mercaptoethanol to the tube with the LCM ele ments in the AdhesiveCap, close the cap and incubate in an "upside-down" position for 30 min. Please do not use any water bath for the incubation. Thorough lysis is essential for good RNA yield.

   Note: ß-Mercaptoethanol (ß-ME) must be added to Buffer RLT before use. Add 10 µl ß-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of ß-ME.

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

   Note: Samples can now be stored at -80°C for later use or purified immediately following the original protocol of the QIAGEN RNeasy® Micro Kit (Handbook 04/2003).

3. To continue with the isolation transfer the lysate to a RNase free 1.5 ml micro-centrifuge tube.

4. Now switch to step 5 of the QIAGEN protocol "Total RNA Isolation from Micro-dissected Cryosections" (RNeasy® Micro Handbook 04/2003, pp20).

5. "Add 1 Volume (350 µl) of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge. Continue immediately with step 6."

   Note: All further steps (6-14) of the QIAGEN protocol remain unchanged and should be performed step by step as listed there.
   Please consider also the comments and tips of the QIAGEN RNeasy® manual, especially the section: "Things to do before starting"

- The final RNA solution (12 µl) may be stored at -20°C or used directly for reverse transcription.

Note: Quality control by direct analysis like the Agilent Bioanalyzer (RNA 6000 Pico LabChip® Kit) is limited to concentrations above 50 pg/µl and may only be possible with large microdis-sected samples (some 2 mm^2 of collected areas from tissue sections of 5-10 µm thickness).

- We normally use 5 to 10 µl of the final RNA solution as template in a RT-reaction of 20 µl (e.g. Transcriptor First Strand cDNA Synthesis Kit, Roche, # 04 379 012 001).

Carl Zeiss Microscopy

LCM Protocols – RNA handling

LCM Protocols

RNA handling

Non contact Laser Capture Microdissection

Carl Zeiss Microscopy – Location Munich – Germany

Content

Introduction

Some remarks on RNA

RNA is a biological macromolecule with many different functions. Messenger RNA (mRNA), transcribed from DNA, serves as template for synthesis of proteins. This protein synthesis is carried out by ribosomes, which consist of ribosomal RNA (rRNA) and proteins.

Amino acids for protein synthesis are delivered to the ribosome on transfer RNA (tRNA) molecules. RNAs are also part of riboproteins and ribozymes.

Analysis of RNA can provide a good reflection of an organism's gene expression profile. Gene expression profiling of material isolated by microdissection has become a very important method for analyzing cellular behavior in a micro scale and is used in research and clinical applications.

Therefore the isolation of high quality RNA is crucial for all subsequent steps and the success of the overall experiment.

The DOs and DON'Ts of handling RNA

RNA degradation is a common reason for failing experiments. RNA is prone to digestion by a wide variety of endogenous and exogenous RNases.

These RNases are present on almost any object that comes into contact with human skin and are difficult to inactivate. Even minute amounts are sufficient to destroy RNA. Some precautions can make the difference between an intact and degraded RNA prep (see also: www.ambion.com) and therefore between successful and unsuccessful experiments.

DOs

- designate a special area for working with RNA
- clean benches with special cleaning solutions e.g. RNaseZap (AMBION, #9780)
- wear gloves and change them frequently
- use sterile, disposable plasticware
- glassware should be treated with 0.1% DiEthylPyroCarbonate (DEPC) or oven baked at 180°C for at least 4 hours before use
- use pipette tips with filters
- aqueous solutions should be treated with 0.1% DEPC
- use only RNase-free reagents, tubes and tips
- for best results use samples that have been snap frozen on dry ice or in liquid nitrogen; all required reagents should be kept on ice
- store prepared RNA at -80°C
- to avoid condensation of moisture during thawing, the slides should be frozen at -80°C and rethawed in a tightly sealed container (e.g. 50 ml Falcon tube)
- in general use protocols (e.g. staining) with short incubation times on ice

DON'Ts

- don't touch anything with bare hands
- don't autoclave pipette tips, as water vapor may contain RNases
- don't allow frozen tissue to thaw
- don't resuspend RNA in DEPC water; residual DEPC can inhibit downstream reactions

Preparation of slides – Samples on MembraneSlide

Regular glass slide (1 mm thick) => 1, thin slide (0.17 mm thick) => dot, DuplexDish and FrameSlide => between dot and 0.

MembraneSlides are 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 are 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 Microscopy offers slides (1 mm, 0.17 mm) 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.
When working with low magnifying objectives like 5x or 10x both regular 1 mm thick glass slides and 0.17 mm glass slides can be used. To keep this flexibility for higher magnifications (20x, 40x or 63x) ZEISS recommends using long distance objectives.

With those objectives you have the possibility to adapt the working distance to the different glass slides by moving the correction collar on the objective.

Due to the short working distance of the 100x or 150x magnifying objectives only 0.17 mm thin cover glass slides can be used.

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

In addition to PEN-MembraneSlide, ZEISS also offers polyethylene teraphthalate (PET)-membrane covered slides. These slides are recommended for fluorescence applications due to a better signal to noise ratio.

Alternatively the PET-membrane is available attached to a metal frame (FrameSlide PET). This frame structure is resistant to microwave treatment. The slide should be submerged completely in buffer for heating. 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 more information about these slides, please contact:

E-Mail: labs@zeiss.de

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 using the "AutoLPC" function of PALM RoboSoftware.
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.

Archived samples: Removing the coverslip

Depending on the applied mounting medium (whether it is soluble in xylene or water) the whole slide should be completely submerged in the respective solvent.

1. standing up in a glass jar filled with either pure xylene or warm water (30-50°C)
2. time needed for the coverslip to swim off may range from hours to days
3. gentle movement of the jar may speed up the process
4. air-dry the slide after removal

Note: It is very important NOT to use any force to push off the coverslip because this might damage the section! Wait till it falls off by itself! The necessary time depends on the age of the sample and the dryness of the mounting medium.
Fresh slides (only days old) can be decoverslipped much faster.

Treatment to remove RNases

MembraneSlides are shipped *without* any pretreatment.

- To ensure RNase-free MembraneSlides or glass slides, use dry heat at 180°C for 4 hours to completely inactivate RNases.
- MembraneSlide NF (nuclease free) is certified to be free of DNase, RNase and human DNA.
  Treatments to remove nucleases are therefore not necessary using these slides.

Suggested UV treatment

To overcome the hydrophobic nature of the membrane we strongly recommend 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 cryosections) adhere better. Positive side effects are sterilization and destruction of potentially contaminating nucleic acids.

Poly-L-Lysine treatment

Additional coating of slides with Poly-L-Lysine (0.1% w/v, e.g. SIGMA, #P8920) may help for poorly adhering materials (e.g. brain sections) and should be done after UV treatment.
Distribute a drop of the solution (100µl) all over the membrane area (e.g. with a soft hair brush or a pipet tip moved horizontally along the surface.
Let air-dry at room temperature for 2-3 minutes. Avoid any injury of the membrane, as this might result in impairment of Laser Capture Microdissection (LCM).

Mounting samples onto slides

Frozen sections

Sectioning

Sections are mounted onto MembraneSlides similarly as when using glass slides by "melting" the frozen section to the warmer slide. Freezing media like OCT or similar may be used but should be kept to a minimum and must be removed before laser cutting.
To our experience massive steel knives allow better section quality than the exchangeable razor blades in the microtome.
For optimal RNA protection take a precooled slide and touch the backside of the slide with your finger (gloves!) to warm only the region for placing the section.
Now transfer the section from the knife by touching with the warmed area and dry at -20°C in the cryostat for 2-3 minutes.
Longer drying can increase the adhesion of weakly attaching tissue sections.

Fixation

As first step the dehydration in ice-cold 70% ethanol for 2-3 minutes is always recommended.

Removing the tissue freezing medium

If OCT or another tissue freezing medium is used, it is important to remove it before laser cutting because such media will interfere with laser efficiency. Removing this medium is done by dipping the slide 5-6 times into ice-cold RNase-free water. If staining is done in aqueous solutions, the supporting substance is removed "automatically" by the water containing steps.

Formalin Fixed Paraffin Embedded (FFPE) sections

Sectioning

Sections are mounted onto MembraneSlides the same way as routinely done using glass slides. Floating the section on warm water as well as hot plate techniques can be applied.
After mounting it is helpful to let the slides dry *overnight* in a drying oven at 56°C.
The longer melting/drying step will strongly improve the adhesion of the section to the membrane.
To allow laser cutting and lifting a coverslip and standard mounting medium must not be applied.

Deparaffination

Paraffin will reduce the efficiency of the laser, sometimes strongly inhibiting cutting and lifting. If you are working with unstained sections it is therefore important to remove the paraffin completely before laser cutting and lifting.
If applying standard staining procedures deparaffination is routinely included in any protocol. 1 mm MembraneSlides can be used like normal glass slides.

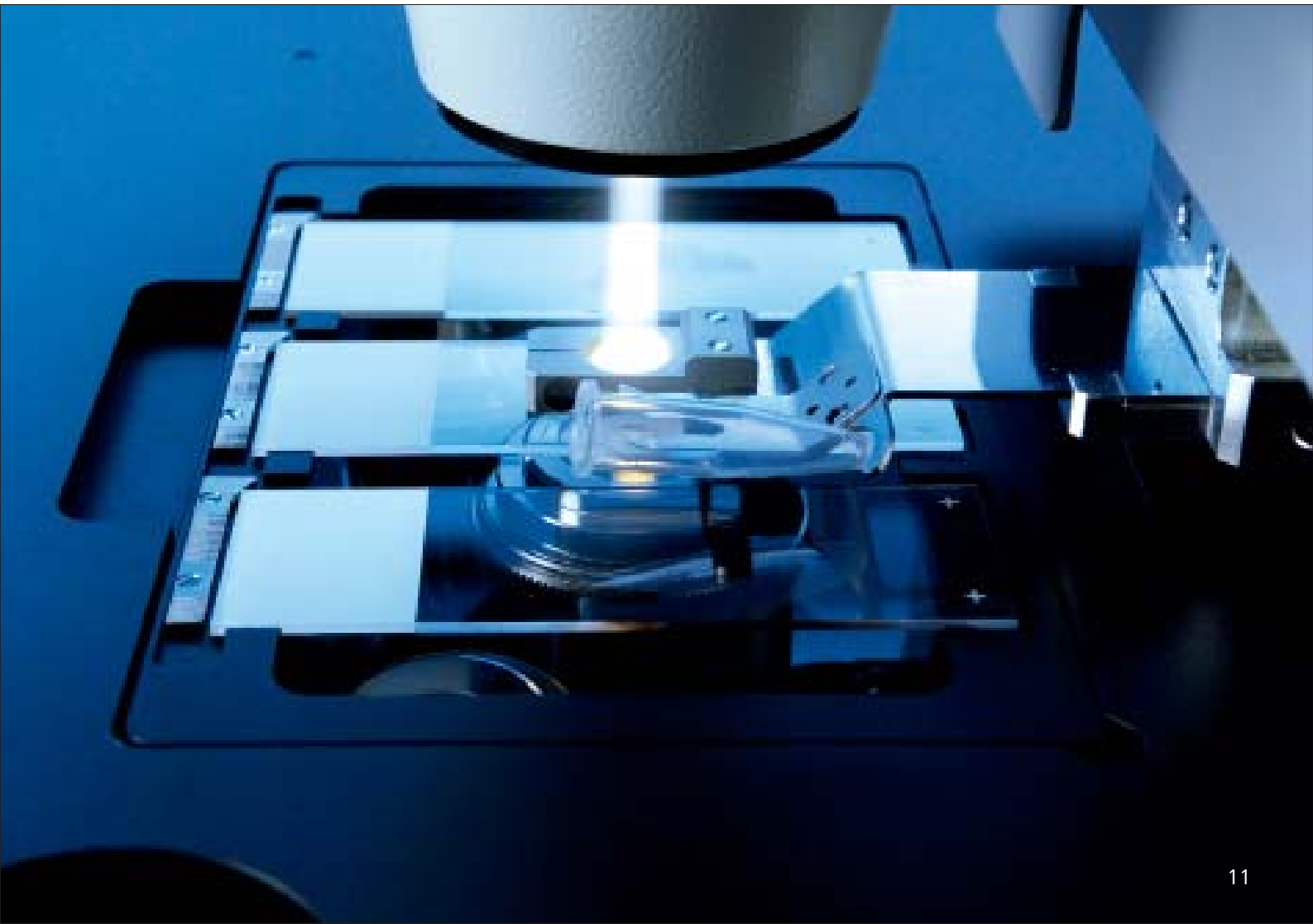
Minimal procedure	
1. Xylene	2 minutes, 2 times
2. Ethanol 100%	1 minute
3. Ethanol 96%	1 minute
4. Ethanol 70%	1 minute

Cytospins

Cytospins can be prepared on glass slides or on MembraneSlides. After centrifugation with a cytocentrifuge let the cells air-dry. Then fix for 5 minutes in 100% methanol. Allow the cytospins to dry at room temperature before staining.

Blood and tissue smear

Distribute a drop of (peripheral) blood or material of a smear over the slide. Be careful to avoid injuries in the membrane, which would lead to leakage during fixation or washing steps and therefore would impair the LMC process. Let smears air-dry shortly and fix them for 2 up to 5 minutes in 70% ethanol.



Staining procedures

For isolation of high quality RNA use only freshly prepared and precooled staining solutions and take notice of our tips on handling RNA (please see page 24).

Formalin Fixed Paraffin Embedded (FFPE) sections

After deparaffination continue with the staining procedure of your choice. Most standard staining procedures can be used for FFPE sections (for recommendations see Frozen sections).

Frozen sections

Most standard histological stainings (e.g., HE, Methyl Green, Cresyl Violet, Nuclear Fast Red) are compatible with subsequent RNA isolation.

Note: Using frozen sections endogenous RNases may still be active after the short fixation step. Therefore it is recommeded to keep all incubation steps as short as possible. Please use RNase-free water and solutions for all steps. All required reagents should be kept on ice.

At ZEISS Microscopy Labs we usually perform the Cresyl Violet or Hematoxylin staining. Skipping the Eosin staining of the HE-procedure can help additionally against RNA degradation or PCR inhibition.

Cresyl Violet

This short staining procedure colors the nuclei violet and the cytoplasm weak violet. Mucin, mastcells, amyloid or developing bone are stained red. It is recommended for RNase-rich tissues since all solutions contain high ethanol concentrations.

Procedure
1. after fixation (2 min, 70% ethanol) dip slide into 1% Cresyl Violet Acetate solution (*) for 30 sec
2. remove excess stain on absorbent surface
3. dip into 70% ethanol
4. dip into 100% ethanol
5. air-dry shortly (1-2 min)

(*) 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. (Sometimes Lot to Lot variations in the purchased Cresyl Violet powder can lead to weaker staining results if the dye content is below 75%).

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 intensification can be obtained by increasing the working temperature of all solutions to room temperature.
The endogenous RNase acitivity varies between different tissues (please see page 23). Therefore, when the short staining protocol is modified by additional steps (50% ethanol) or by increasing the working temperature we strongly recommend a quality control of the RNA (please see page 22).

Ambion offers the LCM Staining Kit (#1935) which also contains a Cresyl Violet dye.

When using this kit we recommend to omit the final xylene-step of the Ambion instruction manual because xylene makes the tissue very brittle and reduces the adhesion of the section to the PEN-membrane.

Hematoxylin/Eosin (HE)

HE-staining is used routinely in most histological laboratories and does not interfere with good RNA preparation if intrinsic RNase activity is low. The nuclei are stained blue, the cytoplasm pink/red.

Procedure
1. after fixation quickly dip slide 5-6 times in RNase-free distilled water
2. stain 1-2 minutes in Mayer's Hematoxylin solution (e.g. SIGMA, #MHS-32)
3. rinse 1 minute in DEPC-treated tap water or blueing solution (e.g. BBC Biochemical, #3900)
4. stain 10 seconds in Eosin Y (e.g. SIGMA, #HT110-2-32)
5. perform a quick increasing ethanol series (70%, 96%, 100%)
6. air-dry shortly

Storage

Stained slides can be used immediately or stored at -80°C.
To avoid excess condensation of moisture during thawing, the slides should be frozen in a tightly sealed container (e.g. two slides back to back in a 50 ml Falcon-tube).
For rethawing the container should not be opened before it is completely warmed up again to ambient temperature.

Non-contact Laser Capture Microdissection (LCM) Procedures

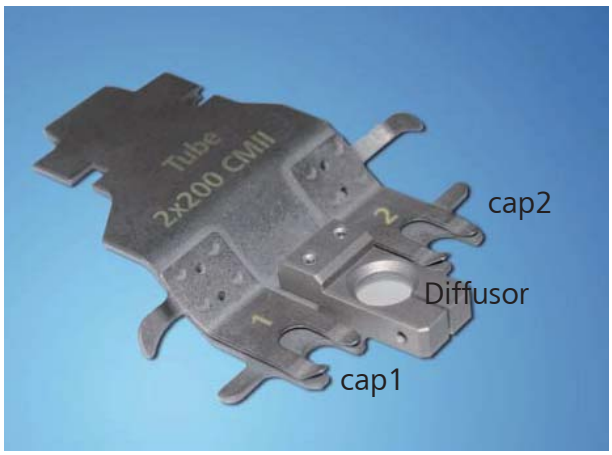
Please, additionally have a look into the PALM MicroBeam User Manual.

Tips to improve morphological information

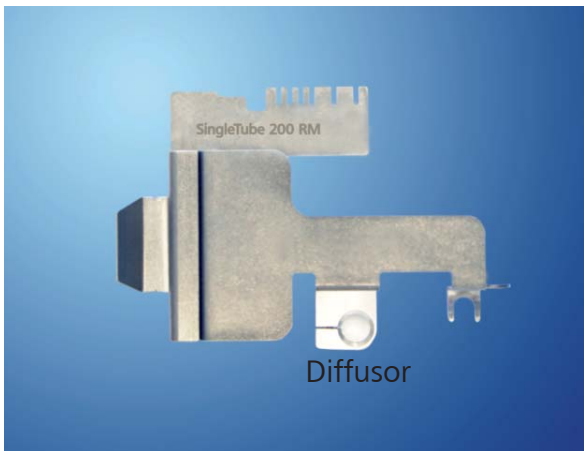
Embedding and glass covering of the specimen is inapplicable for LCM. Thus, the rough open surface of the section/material often results in impaired view of morphology.

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 Diffusor CM product information.



Tube collector 2x200 CMII - Order No. 415101-2000-410



CollectorSet SingleTube 200 RM- Order No. 415101-2000-951

AdhesiveCap opaque

The white/opaque filling of AdhesiveCap clearly improves visualization of morphological information of the samples due to enhanced color balance and contrast, which makes the view comparable to those of coverslipped tissue sections.
Two different microfuge tube sizes with these filled caps are available from ZEISS.

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)

Liquid Cover Glass

The polymeric and low viscose Liquid Cover Glass completely embeds the tissue and smoothens the rough tissue surface, resulting in enhanced morphology.

For more details and handling, please see Liquid Cover Glass product information.

Liquid Cover Glass - Order No. 415190-9020-000

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 possible RNase activities.
Beside the quick relocation of the lifted samples in the cap due to instant immo-bilization there is no risk of evaporation and crystal formation during extended specimen harvesting.
AdhesiveCap is produced with cleanroom technology and can therefore be considered as RNase-free. Autoclaving is not possible.
ZEISS recommends AdhesiveCap as a collection device for all RNA experiments.

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

AdhesiveCap opaque - Order No. 415190-9201-000 (500 µl)
AdhesiveCap opaque - Order No. 415190-9181-000 (200 µl)
AdhesiveCap clear - Order No. 415190-9211-000 (500 µl)
AdhesiveCap clear - Order No. 415190-9191-000 (200 µl)

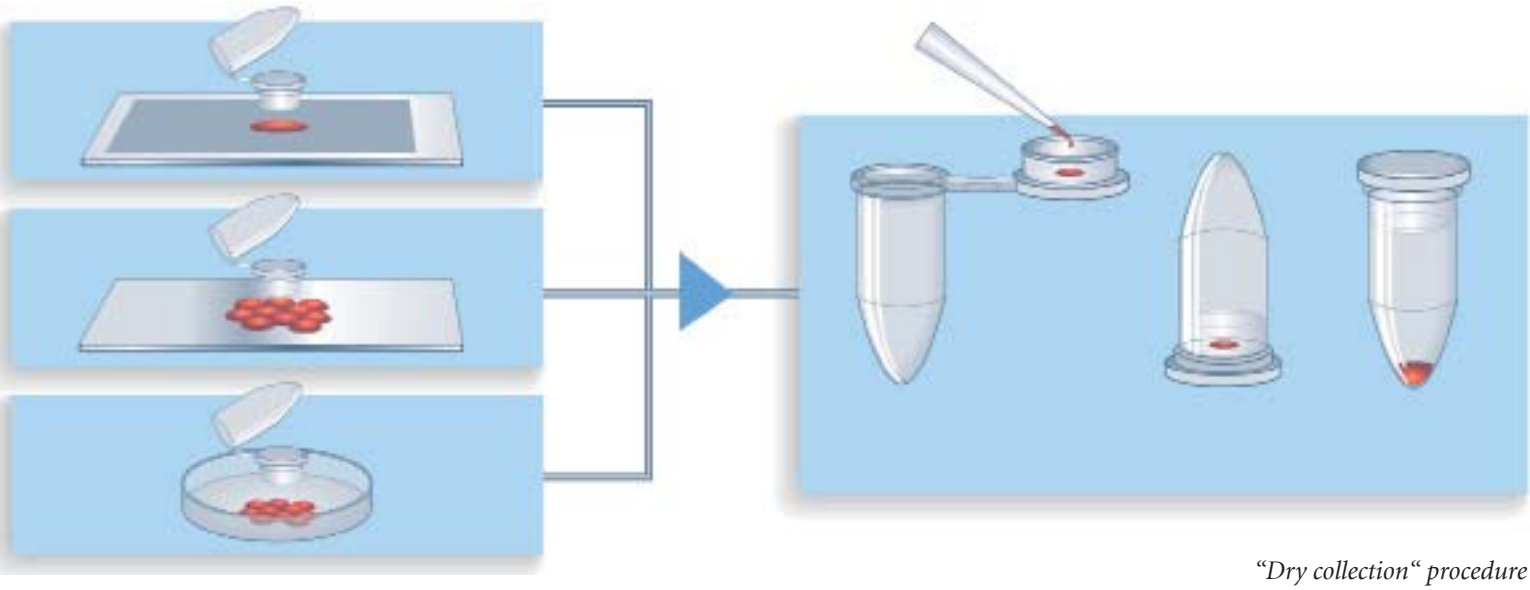
Other microfuge tubes

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

If there are no RNase-free tubes available use the following procedure to remove RNases from regular tubes.

Treatment of microfuge tubes to remove RNases
add 0.1 ml DEPC to 100 ml of double distilled water to get a 0.1% DEPC solution (DEPC: e.g. ROTH #K028.1)
1. stir for 5-6 h at room temperature to dissolve the DEPC
2. soak the reaction tubes into the DEPC solution, take care that the tubes are completely covered with liquid (not blistered!) and incubate overnight at room temperature
3. autoclave the tubes together with the solution for 20 minutes at 121°C to inactivate the DEPC
4. discard the liquid carefully and thoroughly. Dry the tubes at 50°C - 80°C
5. use the tubes as usual

Note: DEPC is toxic and should be used under a hood!



"Dry collection" procedure

Collection procedures

Please have a look into the PALM MicroBeam User Manual.

"Dry" collection (AdhesiveCap)

AdhesiveCap is the recommended collection device for all RNA experiments. Capturing without liquid minimizes RNase activity.

After LCM add a lysis buffer of your own choice (e.g. QIAGEN: 350 µl RLT buffer) and incubate "upside down" for 30 minutes.
Note: Please do not use any water bath for the upside down incubation.

Subsequently briefly centrifuge the lysate and then apply the routine RNA extraction procedure.

"Wet" collection (other microfuge tubes)

Pipette 20 µl lysis buffer 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 and AutoLPC filling the cap with liquid completely can increase the capturing efficiency.

Capture check – looking into the cap to see the lifted samples

To control the efficiency of lifting it is possible to have a look into the collection device (e.g. microfuge cap) with the 5x, 10x, LD 20x, LD 40x and LD 63x objectives. By using the software function "go to checkpoint" the slide is moved out of the light path and the cap can be lowered further towards the objectives for looking inside.

Downstream Applications

RNA from frozen sections

To capture microdissected samples from frozen sections ZEISS Microscopy Labs recommend AdhesiveCap.
For RNA extraction a procedure of choice can be used.

The RNeasy® Micro Kit (QIAGEN, #74004) combined with AdhesiveCap (500 µl) in our hands results in very good yield and quality of RNA from various tissues.
For recommended modifications to the original QIAGEN protocol please see page 19.

The final RNA solution (12 µl) may be stored at -20°C or used directly for reverse transcription.

Quality control by direct analysis like the Agilent Bioanalyzer (RNA 6000 Pico LabChip® Kit) is limited to concentrations above 50 pg/µl and may only be possible with large microdissected samples (about 2 mm^2 of collected areas from tissue sections of 5-10 µm thickness).

We normally use 5 to 10 µl of the final RNA solution as template in a RT-reaction of 20 µl (e.g., Transcriptor First Strand cDNA Synthesis Kit, ROCHE, # 04 379 012 001).

Applying components of the QIAGEN RNeasy® Micro Kit (Order No. 74004)

1. Add 350 µl Buffer RLT containing ß-Mercaptoethanol to the tube with the LCM elements in the AdhesiveCap, close the cap and incubate in an "upside down" position for 30 min. Please do not use any water bath for the incubation. Thorough lysis is essential for good RNA yield.

   Note: ß-Mercaptoethanol (ß-ME) must be added to Buffer RLT before use. Add 10 µl ß-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of ß-ME.

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

   Note: Samples can now be stored for later use at -80°C or purified immediately following the original protocol of the QIAGEN RNeasy® Micro Kit (Handbook 04/2003).

3. To continue with the isolation transfer the lysate to a RNase-free 1.5 ml microcentrifuge tube.

4. Now switch to step 5 of the QIAGEN protocol "Total RNA Isolation from Microdissected Cryosections" (RNeasy® Micro Handbook 04/2003, pp 20).

5. "Add 1 volume (350 µl) of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge. Continue immediately with step 6."

   Note: All further steps (6-14) of the QIAGEN protocol remain unchanged and should be performed step by step as listed there. Please consider also the comments and tips of the QIAGEN RNeasy® manual, especially the section: "Things to do before starting".

Downstream Applications

miRNA from frozen sections

To capture microdissected samples from frozen sections ZEISS Microscopy Labs recommend AdhesiveCap (500 µl).

The special miRNeasy® Mini Kit (QIAGEN, #217004) combined with AdhesiveCap in our hands results in very good yield and quality of total RNA including enrichment of small RNAs.
Only minor adaptations have to be made to the original QIAGEN procedure (see miRNeasy® Mini Handbook 10/2007) when applying AdhesiveCap.

Quality control by direct analysis like the Agilent Bioanalyzer (RNA 6000 Pico Lab-Chip® Kit) is limited to concentrations above 50 pg/µl and may only be possible with large microdissected samples (some 2 mm^2 of collected areas from tissue sections of 5-10 µm thickness).

Modifications for LCM samples

1. As first step add 350 µl of QIAzol Lysis Reagent to the tube of the AdhesiveCap (instead of the usual 700 µl - due to the smaller tube size).
   Close cap and invert the tube.
   The lysis should be performed upside down for 30 minutes at room temperature.

2. After short vortexing spin down briefly in a tabletop centrifuge and transfer the liquid to a 1.5 ml reaction tube.
   Add another 350 µl of QIAzol Lysis Reagent and mix by pipetting.

3. Add 140 µl Chloroform (CCl_4) and close tube securely. Shake vigorously or vortex for at least 15 seconds.

   Note: After step 3 switch directly to the QIAGEN manual at step 4 ("Place tube at room temperature for 2-3 min"). All further steps are now performed according to the normal QIAGEN procedure (steps 4 - 13).

The RNA elution in step 13 should be done with the minimal volume (30 µl) to avoid unnecessary dilution of the RNA.

The final RNA solution may be stored at -20°C or used directly for downstream reactions.

Downstream Applications

RNA from FFPE sections

For collecting microdissected samples ZEISS Microscopy Labs recommend AdhesiveCap.

ZEISS Microscopy Labs apply the QIAGEN RNeasy® FFPE Kit (#73504) with some LCM-specific modifications (please see page 22).

This procedure is very effective and allows a high final concentration of RNA due to a small elution volume (12 µl). Genomic DNA contamination is minimized by a DNase I digest on the purification column.

Deparaffination and staining is done according to standard procedures for slides (please see pages 10, 12 and 13).

The incubation with Proteinase K in our protocol is prolonged significantly compared to the QIAGEN RNeasy® FFPE protocol, because all our tests with laser microdissected material from various tissues showed higher RNA yields when applying longer digestion times.

Note: For formalin fixed samples a Proteinase K digestion step is essential.
The time necessary for optimal Proteinase K digestion depends on many factors like tissue type, fixation procedure or element size of lifted material. An overnight digestion (12-18 hours) is a good starting point for optimization but shorter digestion times may be tested as well. To our experience at least 3 hours digestion should be applied with any extraction procedure and material.

Quality control by direct analysis like the Agilent Bioanalyzer (RNA 6000 Pico LabChip® Kit) is very limited and may only be possible with quite large microdissected samples (often some 4 mm^2 collected area from tissue sections of 5-10 µm thickness).

We normally use 5 to 10 µl of the final RNA solution in a RT-reaction of 20 µl (e.g., Transcriptor First Strand cDNA Synthesis Kit, ROCHE, # 04 379 012 001) using random-oligomers (instead of oligo-dT) as primers for the cDNA synthesis.

Note: The use of random or gene-specific primers is important. Reverse transcription of formalin fixed RNA with standard oligo-dT-primers is inefficient and strongly 3-prime biased due to the numerous strand breaks and modifications inflicted by the formalin fixation and paraffin embedding procedure.

Applying components of the QIAGEN RNeasy® FFPE Kit (Order No. 73504)

1. Add 150 µl Buffer PKD and 10 µl of Proteinase K to the tube, containing the LCM elements in the AdhesiveCap and invert tube to get contact between liquid and adhesive surface.
2. Use an incubator to digest the samples in an "upside down" position at 56°C overnight (or for at least 3 hours), then vortex and heat at 80°C for precisely 15 min in a heating block.

   Note: Please do not use any water bath for the upside down incubation.
3. Incubate on ice for 3 min.
4. Add 16 µl DNase Booster Buffer and 10 µl DNase I stock solution. Mix gently by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

   Note: DNase I is supplied lyophilized and should be reconstituted as described in "Preparing DNase I stock solution" (page 14; RNeasy FFPE handbook 09/2010).

   DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex!
5. Incubate at room temperature for 15 min.
6. Transfer the lysate to a new 1.5 ml microcentrifuge tube.
7. Add 320 µl RBC to adjust binding conditions and mix the lysate thoroughly.
8. Add 720 µl ethanol (100%) to the sample and mix well by pipetting. Do not centrifuge. Proceed immediately to step 9.
9. Transfer 700 µl of the sample to a RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge for 15 sec at ≥8000 x g (≥10000 rpm). Discard the flow-through. Reuse the collection tube in step 10.
10. Repeat step 9 until the entire sample has passed through the RNeasy MinElute spin column. Reuse the collection tube in step 11.
11. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 sec at ≥8000 x g (≥10000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 12.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.
12. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at ≥8000 x g (≥10000 rpm) to wash the spin column. After centrifugation carefully remove the spin column from the collection tube so that the column does not contact the flow-through.
13. Place the RNeasy MinElute spin column in a new 2 ml collection tube, and discard the old collection tube with the flow-through. Open the lid of the spin column and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through. It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions.
14. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 14-30 µl RNase-free water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min at full speed to elute the RNA. The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl of RNase-free water results in a 12 µl eluate.
15. The RNA solution may be stored at -20°C or used directly for reverse transcription.

miRNA from FFPE sections

In contrast to mRNA and rRNA miRNAs are mostly well preserved in FFPE tissue and this allows quite good yields from LCM samples. Due to their small size the miRNAs are normally not bound efficiently by the QIAGEN columns during the regular FFPE Kit procedure. Still the miRNA fraction can be strongly enriched by simply changing one washing step of the standard protocol.
To capture microdissected samples from FFPE sections ZEISS Microscopy Labs recommend AdhesiveCap.

Modifications for miRNA samples

1. Collection and extraction is initially done according to our protocol for total RNA from FFPE (see page 22) applying components of the QIAGEN RNeasy® FFPE Kit (Order No. 73504). Step 1 till step 7 are performed as usual.
2. At step 8 an increased volume of 1120 µl ethanol (100%) is added to the tube (instead of the normal 720 µl).
   With this stronger dilution the binding conditions change in favour of small RNAs. Therefore miRNAs are no longer lost in the flow-through.
3. All further steps of the routine protocol (step 9 - 12) remain unchanged and can be done as usual.

Using other extraction methods

Apart from the QIAGEN Kits there are many other possibilities and kits to extract RNA from FFPE-material. Depending on the material and the experience of the user even simple procedures like homemade AGTC-methods or Trizol can be quite efficient. If the original extraction protocol does not contain any Proteinase K digestion step we recommend to apply a simple procedure as listed below.

Proteinase K Procedure

1. Add 20 µl digestion buffer containing Proteinase K (150 mM NaCl, 100 mM Tris pH 7.5, 0.5% Igepal, 0.5 µg/µl Proteinase K) to the tube containing the LCM elements in the AdhesiveCap.
2. Use an incubator to digest the samples in an "upside down" position at 55°C over-night. Do not use any waterbath.
3. Spin down the lysate in a microcentrifuge. (13400 rcf; e.g. Eppendorf 5415D: 12000 rpm)
4. Inactivate Proteinase K by heating to 90°C for 10 minutes.
5. Add the appropriate lysis buffer and mix by intense vortexing; if not proceeding immediately, store the digested samples at -20°C or -80°C.
6. Continue with your preferred extraction procedure.

Note: Proteinase K digestion time should be optimized for any tissue sample (at least 3 hours are recommended, but up to 18 hours may be more efficient).

Downstream Applications

One sample for DNA and RNA

The QIAGEN AllPrep® DNA/RNA Kit (#80234) is designed for simultaneous purification of genomic DNA and total RNA (including small RNAs) from FFPE-material. DNA and RNA are released sequentially by differential solubilization of the same precious FFPE sample. Pure DNA and RNA are obtained from the entire sample.

ZEISS Microscopy Labs have successfully tested this kit for its applicability for LCM samples on several FFPE materials. Both DNA and RNA can efficiently be purified simultaneously from LCM samples when some modifications to the original manual (AllPrep DNA/RNA FFPE Handbook 09/2010) are made. The first Proteinase K digestion and lysis step before separating the DNA and RNA fractions seems critical especially for the RNA yield.
Mostly we have found a time of one hour (instead of 15 minutes) to be a good compromise for both DNA and RNA yield but this may vary depending on the individual material. We strongly encourage performing pilot experiments with time courses of the first digestion time between one to three hours.
For more information please contact ZEISS Microscopy Labs directly since an universal protocol recommendation is presently not available.

Note: Proteinase K digestion time should be optimized for any tissue sample. At least 1 hour at 56°C is recommended in the first lysis step of the QIAGEN AllPrep® DNA/RNA Kit but longer times may be more efficient.

Quality control of RNA

Due to the normally very small sample size from LCM a direct quality control of the RNA requires high sensitivity technologies. Gel electrophoresis or even NanoDrop procedures are frequently not sensitive enough.

The Agilent 2100 Bioanalyzer (RNA 6000 Pico LabChip® Kit) is able to analyze RNA samples with concentrations down to 50 pg/µl and provides information about RNA quality (degradation, purity) and quantity (see also: www.chem.agilent.com).
A similar technology is available from BioRad (Experion® System, see also: www.bio-rad.com)

A prognosis of the expected amount of RNA in FFPE tissue is very difficult since many factors like species, cell/tissue-type, fixation, staining, fragmentation, extraction procedure and others will influence the outcome.
Anyway, RNA from FFPE material will frequently not show clear profiles and only rough estimations of the size distribution and the RNA amount are possible.

General remarks on RNA (distribution, content, RNase activity)

A typical mammalian cell contains 10-30 pg total RNA (mRNA, rRNA, tRNA). The majority of RNA molecules are tRNAs and rRNAs. mRNA represents only 1-5% of the total cellular RNA.

Approximately 360 000 mRNA molecules are present in a single cell, corresponding to approximately 12 000 different transcripts with a typical length of 2 kb. Some mRNAs comprise as much as 3% of the mRNA pool whereas others account for less then 0.01% (QIAGEN, Bench guide).

RNA distribution in a typical mammalian cell

Total RNA per cell	~ 10-30 pg	
	80-85%	rRNA (28S, 18S, 5S)
	15-20%	tRNAs, snRNAs, low MW species
	1-5%	mRNAs
Total RNA in nucleus	~ 14%	
DNA : RNA in nucleus	~ 2:1	
mRNA molecules	$2x10^5$ - $1x10^6$	per cell
Typical mRNA size	1900 nt	

RNA content in various cells and tissues

		Total RNA (µg)	mRNA (µg)
Cell cultures (10^7 cells)	NIH/3T3	120	3
	HeLa	150	3
	COS7	350	5
Mouse tissue (100 mg)	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

Quantitative hierarchy of RNase activity in mouse tissues (AMBION, Inc.)

Mouse tissues	Fold increase relative to brain
Pancreas	181,000
Spleen	10,600
Lung	5,300
Liver	64
Thymus	16
Kidney	8
Heart	2
Brain	1

Also the RNase activities vary dramatically across different tissues (Krosting J, Latham G, AMBION, Inc.). A comparison of total RNase activities for 8 different mouse tissues showed that total RNase activity spans a 181,000-fold range from pancreas to brain, which points out the importance of RNase control.

ZEISS Microscopy Labs: Tips for working with RNA

For best RNA quality we use fresh frozen sections on MembraneSlides. Frozen sections should not be stored for more than a few days at -80°C. After staining and drying freezing should be performed in an air tight container. Any condensation of moisture on the section can lead to reactivation of intrinsic RNase activities and therefore fast degradation of RNA.

A prognosis of the expected amount of RNA is difficult since many factors will influence the outcome (see above). From mouse liver frozen sections we usually are able to retrieve 5-20 pg RNA per cell (calculated from extractions of 1000 cells and analysis with an Agilent Bioanalyzer; Agilent Application Note 5988-EN on our website or at www.chem.agilent.com).

Archival tissues are mostly Formalin Fixed and Paraffin Embedded. RNA extraction from these tissues is often not very effective because of the crosslinking properties of aldehydes. Other methodologies for preservation of high molecular weight RNA in FFPE tissue are described by:

Vincek et al. 2005 (Diagn Mol Pathol, 14,3: 127-133) and
Olert et al. 2001 (Pathol Res Pract, 197: 823-826)

For more information see our website: www.zeiss.de/microdissection

Summarized recommendations:

- Keep attention to DOs and DON´Ts on handling RNA (page 6)
- Take AdhesiveCap as collection device for all RNA experiments (page 16)
- Choose a short staining procedure for tissues with high content of endogenous RNases (e.g. Cresyl Violet) (page 12)
- RNeasy® Micro Kit (QIAGEN, #74004) results in good RNA yield (quality and quantity) from frozen sections in our lab (page 18)
- RNeasy® FFPE Kit (QIAGEN, #73504) results in good RNA yield (quality and quantity) from FFPE tissue in our lab (page 21)