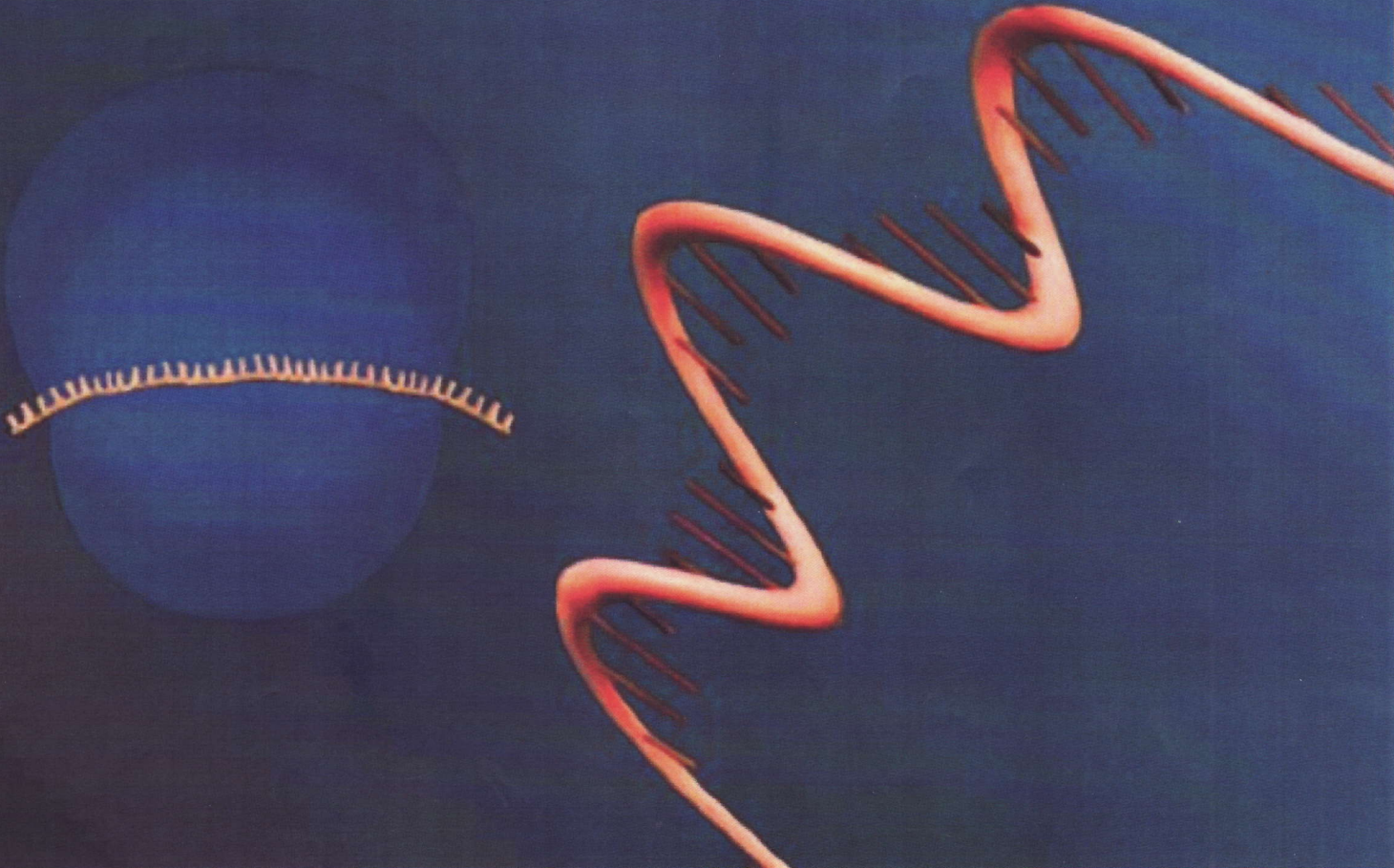


Carl Zeiss Microscopy

LCM Protocols - RNA handling



②

RNA handling
Non contact Laser Capture Microdissection

Content

5	Introduction - Some remarks on RNA
6	The DOs and DON'Ts on handling RNA
7	Preparation of slides
7	Samples on MembraneSlide
9	Samples on glass slides
9	Archived samples: Removing the coverslip
9	Treatment to remove RNases
9	Suggested UV treatment
9	Poly-L-Lysine treatment
10	Mounting samples onto slides
10	Frozen sections
10	Formalin Fixed Paraffin Embedded (FFPE) sections
11	Cytospins
11	Blood and tissue smear
12	Staining procedures
12	Formalin Fixed Paraffin Embedded (FFPE) sections
12	Frozen sections
12	Cresyl Violet
13	Hematoxylin/Eosin (HE)
13	Storage
14	Non-contact Laser Capture Microdissection (LCM) Procedures
14	Tips to improve morphological information
14	Diffusor
15	AdhesiveCap opaque
15	LiquidCover Glass
16	Collection devices
16	AdhesiveCap
16	Other microfuge tubes
17	Collection procedures
17	"Dry" collection (AdhesiveCap)
17	"Wet" collection (other microfuge tubes)
17	Capture check - looking into the cap to see the lifted samples
18	Downstream Applications
18	RNA from frozen sections
20	miRNA from frozen sections
21	RNA from FFPE sections
23	miRNA from FFPE sections
23	Using other extraction methods
24	One sample for DNA and RNA
24	Quality control of RNA
25	General remarks on RNA (distribution, content, RNase activity)
26	ZEISS Microscopy Labs: Tips for working with RNA
27	Other protocols (DNA, Chromosomes, Live Cells,...)

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.

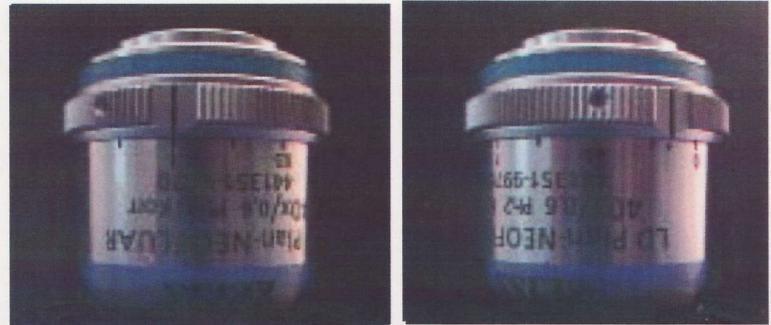
DOs

- designate a special area for working with RNA
- clean benches with special cleaning solutions e.g. RNaseZap (AMBIION, #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.

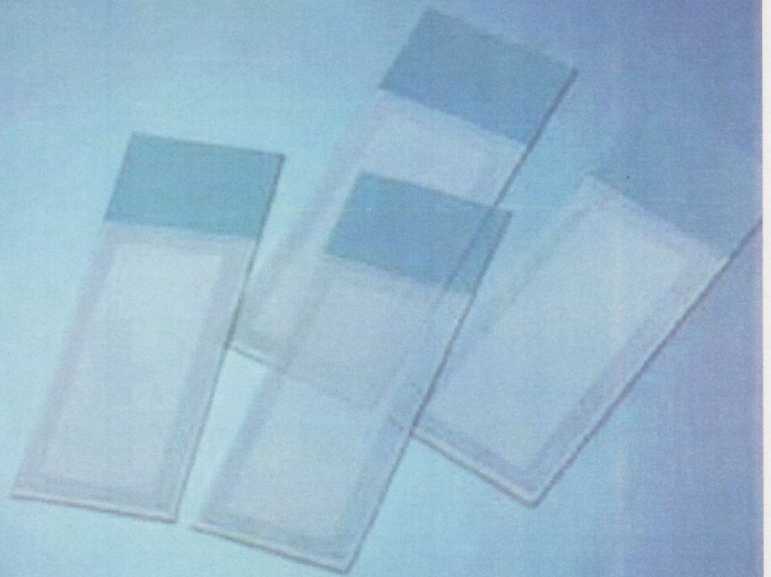
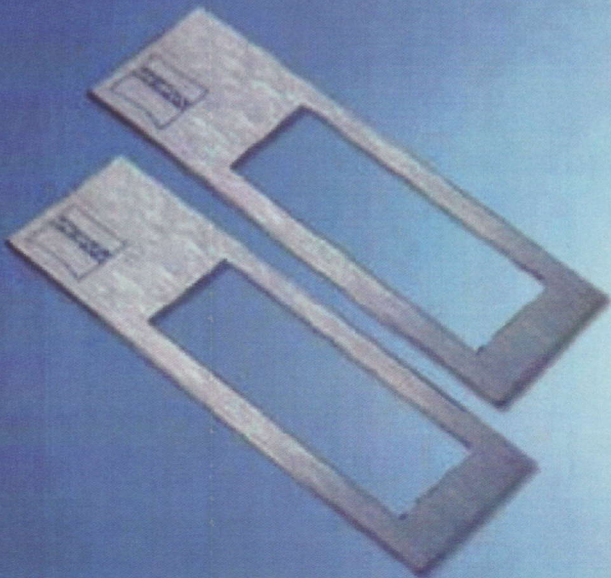
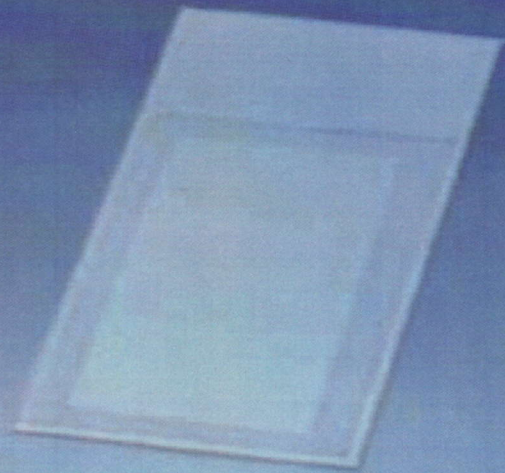
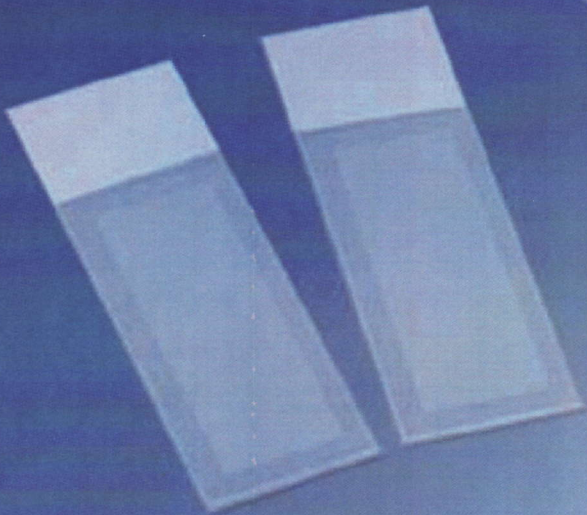
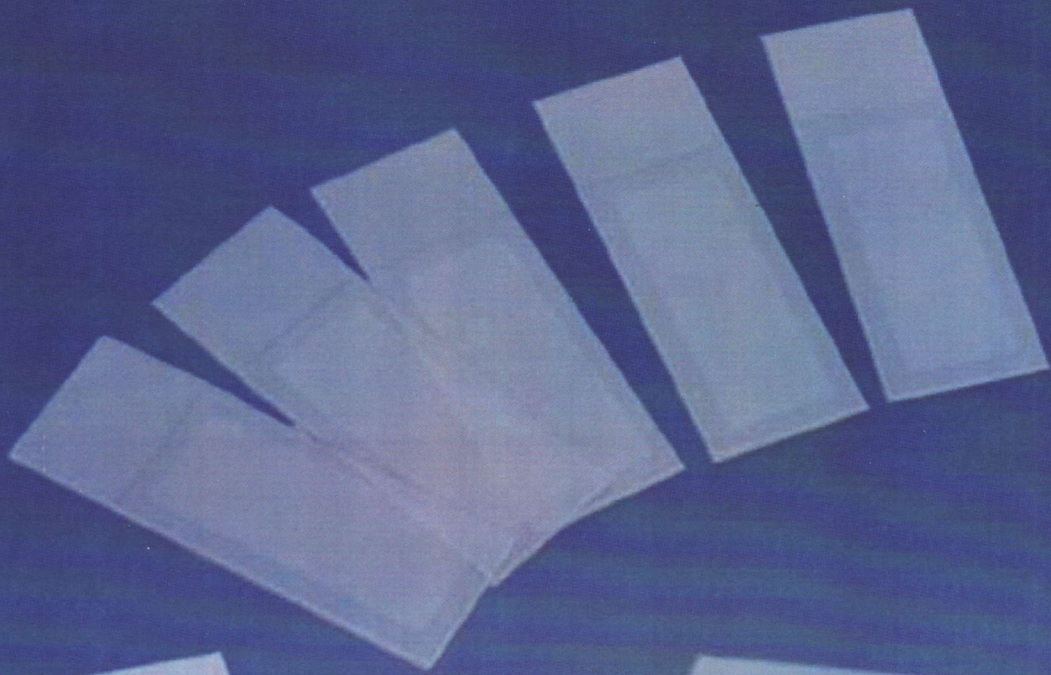
MembraneSlide NF (nuclease free) is certified to be free of DNase, RNase and human DNA.

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

PET-Fluorescence

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



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 (double width)

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.

not Superfrost Slides

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.

Can't heat oven

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.

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 decover-slipped much faster

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 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 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 |

ice cold
70%
eth

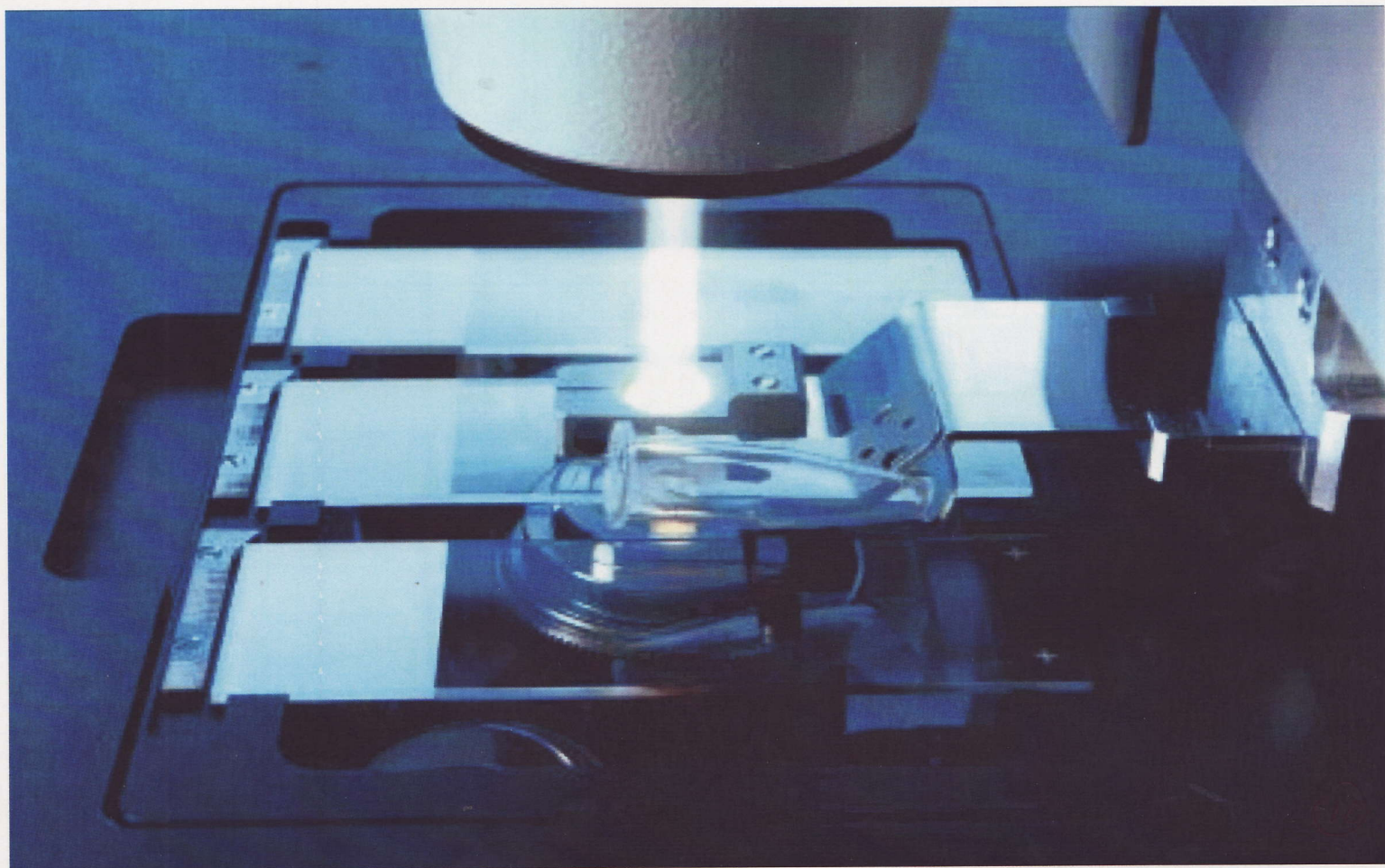
ice
cold
H₂O
dips

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 **Cresyl Violet**

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).

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.

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 recommended 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.

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)

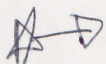
- (*) Dissolve solid Cresyl Violet Acetate (e.g. ALDRICH #86,098-0) at a concentration of 1% (w/v) in 50% EtOH at room temperature with agitation/stirring for several hours to overnight. Filter the staining solution before use to remove unsolubilized powder. (Sometimes Lot to Lot variations in the purchased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

→ skip if not needed

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

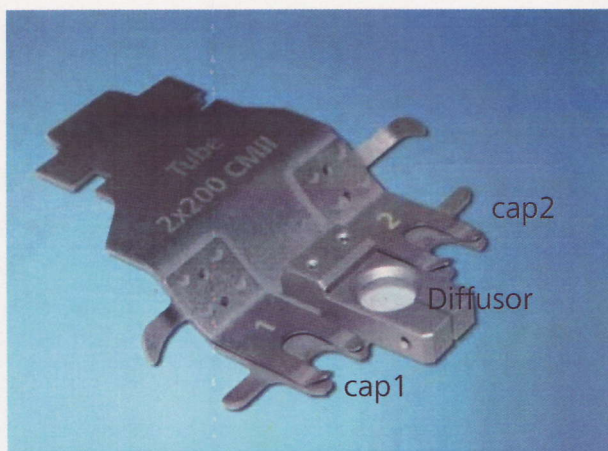
Please, additionally have a look into the PALM MicroBeam User Manual.

Tips to improve morphological information

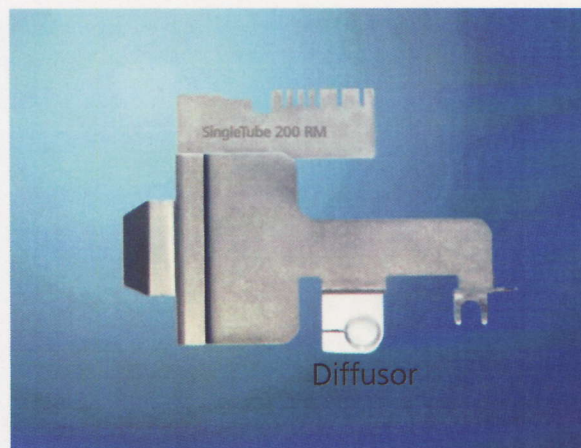
Embedding and glass covering of the specimen is inapplicable for LCM. Thus, the rough open surface of the section/material often results in impaired view of morphology.

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



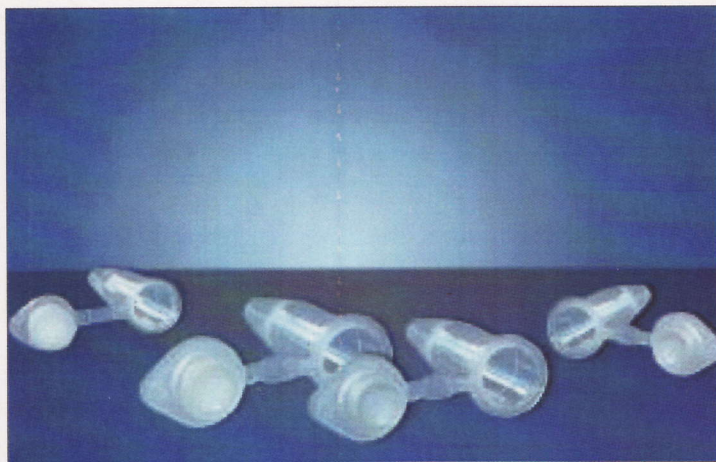
CollectorSet SingleTube 200 RM- Order No.
415101-2000-951

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 μ l)
AdhesiveCap opaque - Order No. 415190-9181-000 (200 μ l)

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

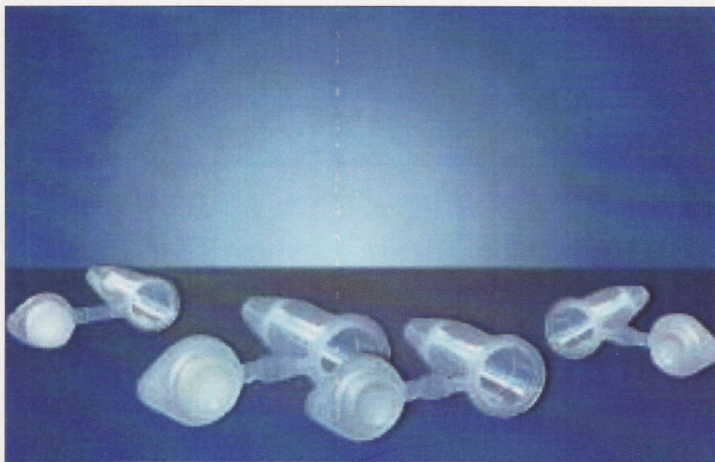
The intention of AdhesiveCap is to allow LCM (Laser Capture Microdissection) without applying any capturing liquid into the caps prior to LCM. This minimizes possible RNase activities.

Beside the quick relocation of the lifted samples in the cap due to instant immobilization 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)

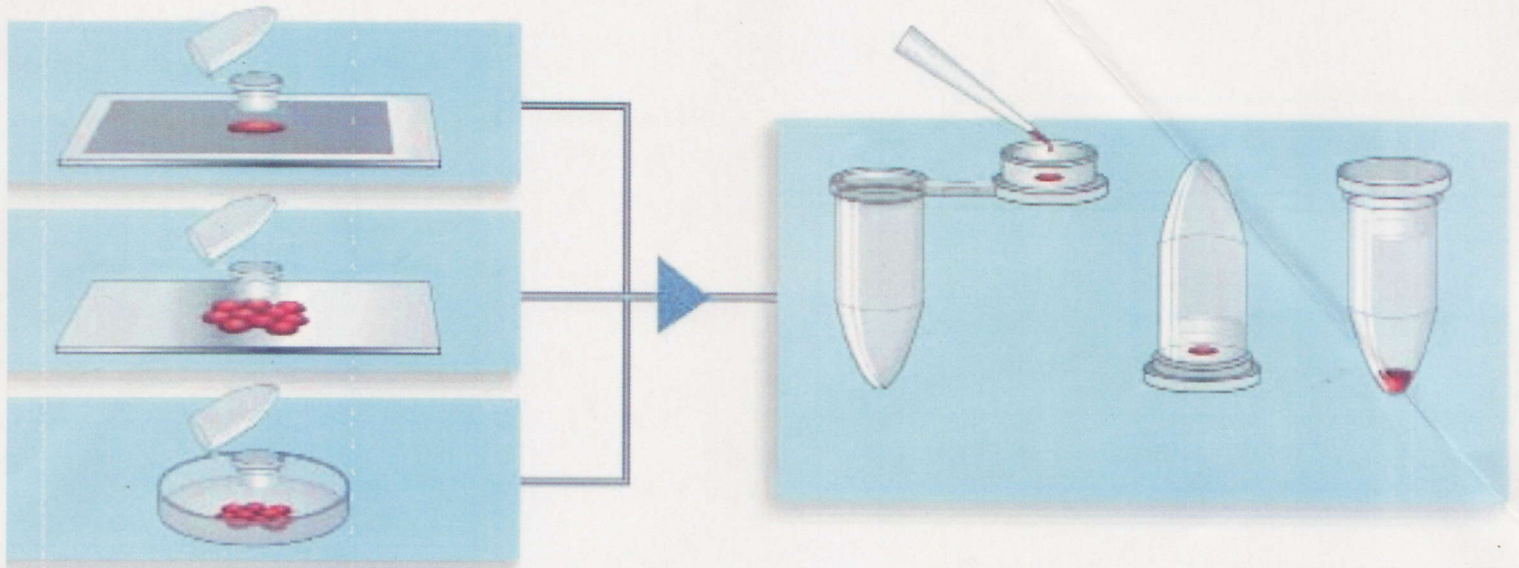
If there are no RNase-free tubes available use the following procedure to remove RNases from regular tubes.

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 μ l 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 μ l 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 check-point" 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 Lab-Chip® Kit) is limited to concentrations above 50 pg/µl and may only be possible with large microdissected samples (about 2 mm² 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 β-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: β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-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 β-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 mm² 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 (CCl₄) 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 mm² 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, ^{close cap!} 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 $\geq 8000 \times g$ (≥ 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 $\geq 8000 \times g$ (≥ 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 $\geq 8000 \times g$ (≥ 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.

cap has drying effect

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 than 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	2×10^5 - 1×10^6 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 (Krostring 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.

-50 ml
conical
tube

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)

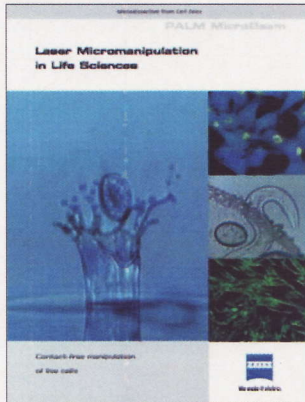
For more information see our website: www.zeiss.de/microdissection

Summarized recommendations:

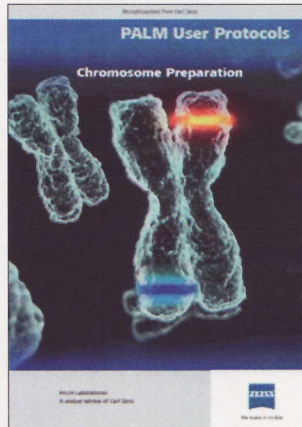
- Keep attention to DOs and DON'Ts on handling RNA (page 6)
- Take AdhesiveCap as collection device for all RNA experiments (page 16)
- Choose a short staining procedure for tissues with high content of endogenous RNases (e.g. Cresyl Violet) (page 12)
- RNeasy® Micro Kit (QIAGEN, #74004) results in good 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)

Brochures and Protocols

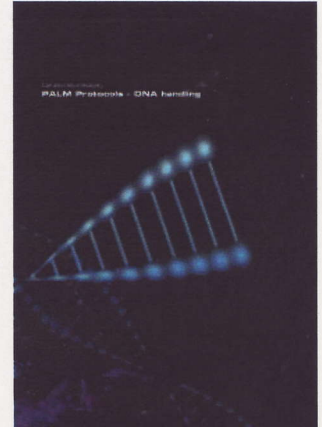
Live cells



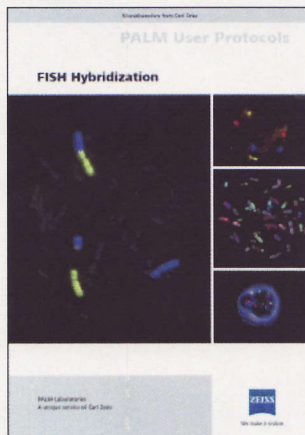
Chromosomes



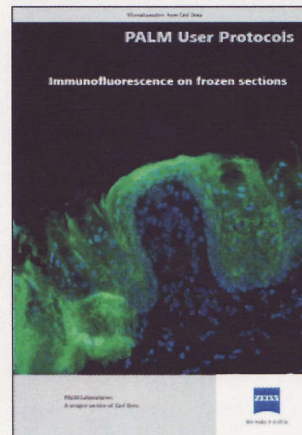
DNA



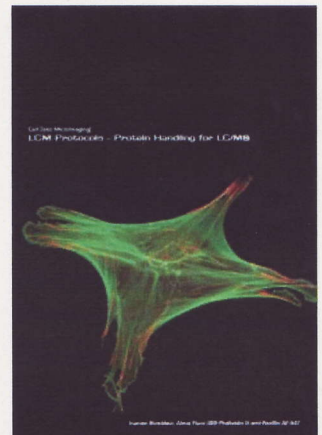
FISH



Immunofluorescence



Proteins

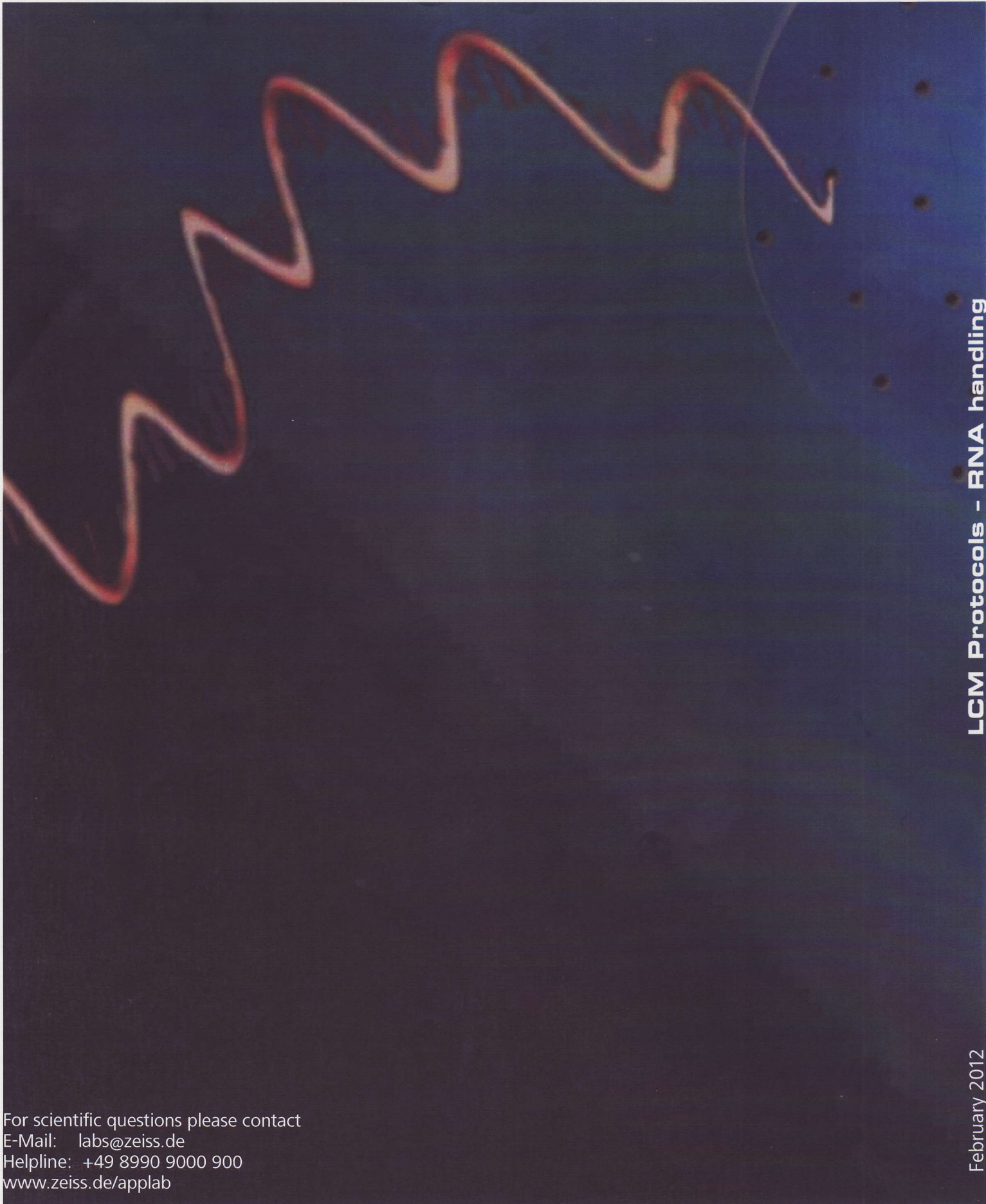


For questions, comments or information requests please contact:

ZEISS Microscopy Labs Application Lab

E-Mail: labs@zeiss.de
Helpline: +49 8990 9000 900

Download of protocols: www.zeiss.de/applab



For scientific questions please contact
E-Mail: labs@zeiss.de
Helpline: +49 8990 9000 900
www.zeiss.de/applab

Carl Zeiss MicroImaging GmbH
07740 Jena, Germany

BioSciences | Location Munich
Phone : +49 8990 9000 800

