



Sample Preparation For Leica Laser Microdissection

Protocol Guide for Leica Microsystems Laser Microdissection Systems

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At the forefront of laser microdissection technology, only the Leica LMD6500 and Leica LMD7000 offer:

- Laser beam movement via optics – fast and precise cuts
- Specimen collection by gravity – contact- and contamination-free
- Dedicated objectives for LMD – highest possible laser power
- Adjustable laser – for thick, thin, soft, and hard tissue

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<http://www.leica-microsystems.com/contact-support/local-addresses/>

Introduction

Laser microdissection (LMD) is the ultimate tool for perfection. LMD makes it possible to obtain homogenous, ultrapure samples from heterogenous starting material. A researcher can selectively and routinely analyze regions of interest down to single cells and chromosomes to obtain results that are reproducible, and specific.

LMD is commonly used for the following investigations:

- Absolute identification of mutations within cells of interest
- Gene expression profiling of a specific cell type within a tissue
- Identification of cell proteins during a specific physiological event
- Isolation and further cultivation of transgenic cells from a culture

The application of the laser for cell microdissection was introduced commercially in the mid-nineties. The new technology quickly became a routine application in:

- pathology
- cancer research
- neuroscience
- cell biology
- forensics
- prenatal diagnostics
- plant science, and
- many more.

During the last decade the application of the laser microdissection technology expanded. Today the laser microdissection technology is used e.g. for manipulation of living cell culture (LCC), climate research and micro engraving.

This manual is designed to guide researchers through the current specimen preparation methods for laser microdissection and offer helpful hints on nucleic acids and protein preparation for downstream analyses. All protocols discussed in this guide serve as a starting point to establish and perfect your own protocols.

Beside this general protocol guide many published protocols can be found at the Leica LMD website:

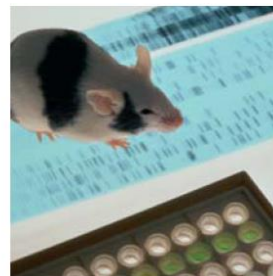
<http://www.leica-microsystems.com/products/light-microscopes/life-science-research/laser-microdissection/details/product/leica-lmd7000/publications/>

Important: Read the Instruction Manual for your laser microdissection system and for all other products used in your work before following a protocol. Your lab may have specific protocols that differ from those in the guide. Leica Microsystems' protocols do not supersede your protocols. This guide does not replace the Instruction Manual. Leica Microsystems does not guarantee safety or performance of another company's products that it recommends, nor does it guarantee the safety or successful outcome of experiments using the suggested protocols for your specific work.

You are welcome to contribute to future editions of this guide by sharing your protocols with Leica Microsystems. To do this, please go to Section 13 where you will find the form for submitting the protocol or contact us:

<http://www.leica-microsystems.com/contact-support/local-addresses/>

We would also appreciate if you could provide us with your peer-reviewed publications. We will reward your input with a small gift.



Preparing Biological Specimens for Laser Microdissection

A fundamental step for LMD is to develop a specimen preparation technique that perfectly balances the dissection of specimens and downstream analysis, e.g. mRNA quantitation.

Generally, fresh, frozen tissues are preferred for isolating RNA and proteins. Frozen sections can be obtained quickly on a cryostat. High quality RNA is achieved from tissues frozen immediately after surgery, which minimizes RNA degradation over time by ubiquitous RNases or heat.

DNA can be prepared from both frozen or formalin-fixed, paraffin-embedded (FFPE) material. The advantage of working with paraffin-embedded sections is superior morphology preservation as compared to frozen sections. Preserving morphology can be critical for diagnostics and for final selection of specific cells for further analyses. However, preparing RNA from fixed paraffin sections is difficult due to RNA fragmentation by formalin, which is the common fixative used for preparing paraffin sections.

Recently, alternative fixatives such as methacarn, DSP or HOPE were described as successfully enabling reproducible isolation of high molecular weight DNA and RNA, as well as native proteins, from paraffin-embedded sections (see page 18).

Samples for LMD can also be obtained from plain glass slides or archived slides. Protocols for preparation are given on page 21.

More information regarding detailed published application protocols can be found at the Leica Application Guide.

Important: All protocols serve only as a starting point and may need modification to obtain the best results from your microdissected specimens. Once successfully established, protocols must be strictly followed to assure reproducibility of experiments. Any changes in tissue handling, such as different fixation times or storage, may lead to different laser cutting parameters. For example, longer fixation times can change the hardness of the tissue.

Section 1. Accessories and Consumables for Leica Microsystems LMD Systems

Objectives

The objectives described on the following page are specifically intended for laser microdissection. They are designed for high UV-light transmission upon laser cutting, and to provide the best image quality. The Leica Microsystems SmartCut Series comprises the following objectives for LMD: UVI 6.3x and HCX PL FLUOTAR family (5x, 20x, 40x, 63x, and 150x). Improved optics provides high performance for laser microdissection of single cells as well as larger tissue areas. See Table 1 for a quick objective overview.

Table 1. Leica Microsystems Objectives for Laser Microdissection

Order no.	Objective	Description
11506215	HCX PL FLUOTAR 1.25X/0.04*	A "general" objective with the lowest magnification for overview of entire tissue sections and generation of overview images. It is used with a diffusion plate for illumination of full view field.
11518146	UVI 5x/0.12 Microdissection	Low range magnification for cutting large areas
11518145	UVI 6.3X/0.13 Microdissection	Especially designed for laser microdissection with high UV-light transmission; perfectly suited for cutting larger areas e.g. for proteomics
11506505 (PH obj. 11506507)	HC PL FL 10x/0.30	Medium range magnification with acceptable cutting; frequently used in pathology for microdissection of big surfaces such as tumor areas; suitable for live cell cutting (LCC)
11518147	UVI 10x/0.25 Microdissection	
11506242 (PH obj. 11506243)	HC PL FL L 20x/0.40 XT CORR**	Preferred objective for live cell cutting (LCC); also frequently used in pathology
11506208 (PH obj. 11506209)	HC PL FL L 40x/0.60 XT CORR**	The standard objective, mostly used when dissecting single cells
11506222 (PH obj. 11506217)	HCX PL FL L 63x/0.70 XT CORR**	High range magnification for cutting single cells with higher precision
11506214	HCX PL FL L 150x/0.90 Microdissection	Dry objective, recommended for cutting of chromosome spreads in combination with POL FrameSlides; for microdissection only. Not suitable for MembraneSlides.

*for fast specimen overview images, not suitable for laser microdissection

**L – long working distance which enables L objectives to image through the glass slide.

CORR – correction ring for optimal spherical aberration correction when working with glass slides, MembraneSlides or Petri dishes that have different thicknesses.

PH obj. – phase contrast objective.

Specimen Holders and Collection Devices

Leica Microsystems LMD systems support the following holders for mounting samples onto the microscope stage. These are:

1. Standard holder for a single slide (motor stage configurations)
2. The holder for three regular size slides for parallel working with serial sections (available for scanning stage only)
3. Holder for a single large neuroscience slide or Petri dish (for all configurations)

Table 2. Order numbers of specimen holders for slides & Petri dish.

Holder	Motor Stage	Scanning Stage
Slide holder	11 532 732 (included in the entire LMD system)	11 505 226 (included in the entire LMD system)
Double slide holder	11 505 214	11 505 227
Petri dish (Ø 5cm) holder	11 505 257	11 505 227
Holder for 18-well double stack ibidi slide	none	11 505 255

For laser microdissection Leica Microsystems LMD systems support following devices for sample collection:

1. Standard collection device for 0.2 ml or 0.5 ml tubes
2. Collection device for 8-well strips
3. Adjustable collection device for multi-well slides, 8-well strip caps, chamber slides and self-made devices (available for scanning stage only)

Table 3. Order number of collection devices for dissectates.

Collector	Motor Stage	Scanning Stage
for 0.2 ml PCR tubes	11 505 131 (set of 4 levers; (included in the entire LMD system)	11 505 229 (included in the entire LMD system)
for 0.5 ml PCR tubes	11 505 130 (set of 4 levers; (included in the entire LMD system)	11 505 228 (included in the entire LMD system)
for 8-well strips	11 505 258	11 505 276
for multi-well slides and ibidi slides, 8-well caps		11 505 276
48 (6x8)-well caps		included in holder package
for petri dish (Ø 5cm)	none (can be applied manually)	included

Specimen microdissected out of tissue slides are regularly collected into the PCR tube caps of either 0.2 or 0.5 ml tubes, however collection direct into 8-well caps, multi-well slides like ibidi and chamber slides is possible as well. Dissected cultured cells are collected into 8-well

strips, in Petri dishes or in 18-well ibidi slides, respectively. The latter option is available with scanning stage only. Table 2 summarizes the holder devices and Table 3 summarizes the collection devices.

Please refer to the Instruction Manual or contact your local Leica Microsystems representative for more detailed information.

Slides

LMD applications require specifically designed slides to free the dissectate from the section. The tissue sections are placed on a UV-absorbing membrane. Upon the laser action, the membrane and the tissue in the cut line are vaporized. Since the membrane is not adherent to the slide, the dissectate (with the attached specimen) drops into the cap of a standard microcentrifuge tube (thin-walled PCR tube with flat cap, 0.2 or 0.5 ml).

Leica Microsystems offers high-quality MembraneSlides that provide high collection rates for laser microdissection. There are five different MembraneSlides to choose from:

- PEN (polyethylene naphthalate)
- PPS (polyphenylene sulfide)
- PET (polyethylene terephthalate)
- POL (polyester)
- FLUO (fluoropolymer)

Membranes are mounted on regular glass slides (PPS/PEN), on steel frames (PEN/PPS/PET/POL/FLUO) (Fig. 1), or on the bottom of special Petri dishes (Fig. 2) or μ -Slides (Fig. 3) for cell cultivation. Depending on tissue type, thickness, and inspection method, one slide type may work better for a certain application. Therefore it is advisable to test different combinations of membranes and slide supports for the best results. Membrane combinations are summarized in Table 4. To test and to find best choice fitting the experimental design needs prior the start of a new project, Leica offers a suitable LMD DemoSlide Kit (11600292) containing all membrane slides.

Tissue sections should be mounted onto the membrane prior to laser microdissection to achieve best results. Mounting of tissue sections onto glass slides is relatively easy since the glass provides solid support. Only difference between handling of glass MembraneSlides and common glass slides is that any membrane damage must be avoid. However, mounting tissue sections on the membrane of a steel FrameSlide can be more challenging. To simplify this procedure, a Plexiglass support (11532325) can be used to provide a rigid mounting surface (Fig. 3).

Slides may be sterilized by autoclaving or UV-treatment. However, RNase-free certified slides are also available (see Table 4).

Beside the MembraneSlides plain glass slides or DIRECTOR™ slides can be used in combination with the Draw+Scan mode for membrane-free sample ablation.

Fig 1. Glass MembraneSlide (on top) and the FrameSlide (bottom).

The membrane is mounted on the upper side of the slide (the side with the frosted end for marking). The membrane is glued on the glass at the edges in the central part of the slide. There is a space of 1 μm between the glass and membrane. The slides must be handled with care to prevent damage to the membrane. If moisture collects between the glass and the membrane, this will impede the laser and collection action. See the description of the FrameSlide (Fig. 3).

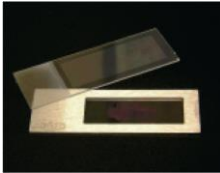


Fig 2. Petri Dish and ibidi 18-well slides for Live Cell Cultivation (LCC) for LMD.

The Petri dish is placed on the holder for firm mounting on the microscope stage. Since the PEN membrane is perforated, gas exchange occurs. For better attachment and growth of cells, the membrane can be coated with poly-L-lysine or collagen prior to seeding (see more information on page 32).



Fig 3. Sterile 18-well μ Slide for cell cultivation for LMD.

Fig 4. FrameSlide (on Top) and the FrameSupport (Bottom).
FrameSlide: The front side of the slide contains the engraved Leica Microsystems logo. Please note that only the back side of the slide (the side opposite the Leica Microsystems logo) is foil mounted, and only this side can be used for mounting tissue sections.

FrameSupport: The Plexiglass FrameSupport contains a lifted surface (clear area) that fits exactly into the cavity or the front side of the FrameSlide. The MembraneSlide must be turned over to be mounted onto the FrameSupport (not shown).



More detailed information on LMD consumables can be found at the Leica Science Lab:
<http://www.leica-microsystems.com/science-lab/laser-microdissection/application-specific-consumables-for-laser-microdissection/>

Table 4. MembraneSlides and Petri Dishes for Laser Microdissection - Comparison of cutting performance, depending on membrane type, membrane support, and objective used.

Order No. (*RNase-free Slides)	Product Description/ Quantity	Usable Area for LMD (approx.)	Membrane Thickness	Objectives ^a Recommended Objective CORR Settings for high-quality Images							Fluorescence Applicability	Remarks
				5x	6.3x ^a	10x ^a	L20x CORR ^a	L40x CORR ^a	L63x CORR ^a	150x		
11505273 50 slides/box	Glass, PPS-membrane (25 x 76 mm)	19 mm x 41 mm	1.2 µm	++	++	+++	+++ D = 1.0	+++ D = 1.0	+++ D = 1.0	-	+	Suitable for almost all applications (except DIC); PPS is semi-permeable for water, do not dip into water/water-containing solution
11505268 50 slides/box	Steel frames, PPS-membrane (25 x 76 mm)	16 mm x 45 mm	1.2 µm	++	++	+++	+++ D = 0	+++ D = 0	+++ D = 0	+++	++	Suitable for almost all applications (except DIC)
11505158 *11505189 50 slides/box	Glass, PEN-membrane (25 x 76 mm)	19 mm x 41 mm	2 µm	++	++	+++	+++ D = 1.0	+++ D = 1.0	+++ D = 1.0	-	+	The most popular slides; standardly used in 95% of all applications
11600288 50 slides/box	Glass, PEN-membrane (25 x 76 mm)	19 mm x 41 mm	4 µm	++	++	+++	+++ D = 1.0	+++ D = 1.0	+++ D = 1.0	-	+	The most popular slides improved for Leica LMD applications
11600289 50 slides/box	Steel frames, PEN-membrane (25 x 76 mm)	16 mm x 45 mm	4 µm	++	++	+++	+++ D = 0	+++ D = 0	+++ D = 0	+	+	
11505151 *11505190 50 slides/box	Steel frames, PET-membrane (25 x 76 mm)	16 mm x 45 mm	1.4 µm	++	++	+++	+++ D = 0	+++ D = 0	+++ D = 0	++	+	Recommended for MALDI-TOF
11505188 *11505191 50 slides/box	Steel frames, POL-membrane (25 x 76 mm)	16 mm x 45 mm	0.9 µm	++	++	+++	+++ D = 0	+++ D = 0	+++ D = 0	+++	++	Recommended for chromosome spreads
11505215 (sterile) 100 slides/box	LARGE Glass PEN-membrane (50 x 76 mm)	39 mm x 45 mm	2 µm	++	++	+++	+++ D = 1.0	+++ D = 1.0	+++ D = 1.0	-	+	For large tissue sections (e.g. in neuro-biology for brain slides), requires optional slide holder (see page 8)
11532860 (sterile) 100 slides/box	LARGE Glass PPS-membrane (50 x 76 mm)	39 mm x 45 mm	1.2 µm	++	++	+++	+++ D = 1.0	+++ D = 1.0	+++ D = 1.0	-	+	
11600250 5 slides/box	Steel frames, FLUO-Membrane (25 mm x 76mm)	16 mm x 45 mm		-	+	+++	+++ D = 0	+++ D = 0	+++ D = 0	+	+++	For Fluorescence and DIC-contrast
11505172 20 dishes/ box	50 mm Petri dish with PEN-membrane	30 mm diameter	2 µm	+	+	+++	+++ D = 0	+++ D = 0	- D = 0	-	+	For live cell cutting; direct LMD of adherent cells for recultivation or analysis (see page 32)
11505240 (sterile) 15 slides/box	µ-Slide, 18-well with PEN-membrane	30 µl vol. per well	2 µm	+	+	+++	+++ D = 0	+++ D = 0	- D = 0	-	+	
11600249 (10 slides/box)	EP-DIRECTOR® slides	50 mm x 20 mm	membrane free	++	++	+++	+++ D = 1.0	+++ D = 1.0	+++ D = 1.0	-	+	

Laser Microdissection Performance: – not suitable; + satisfactory; ++ good; +++ very good

^aThese objectives possess improved optics for UV-light transmission in conjunction with the best image quality. Please refer to page 7 for details on objectives for laser microdissection. D stands for the position of the correction ring.

Sterilizing MembraneSlides

Important: Please note that sterilizing slides by autoclaving or UV-treatment does not guarantee complete destruction of RNases. For RNA preparation, especially from a single cell (corresponds in average to 10–15 pg of total RNA) or from a small number of cells, RNase-free certified slides are recommended (see Table 4). Alternatively, regular MembraneSlides can be pretreated with reagents such as RNaseZap® (Ambion).

Autoclaving

1. Place slides into a steel basket such as a slide holder for paraffinization
2. Place the basket into a beaker or jar and cover tightly
3. Autoclave at 121°C for 20 minutes. These conditions correspond to a standard autoclaving set-up.

Chemical Treatment

Treat Membrane Slide with RNase decontamination solution according manufacturer's instructions. Always rinse membranes and slides in RNase-free water after chemical treatment to remove remaining chemicals.

UV-Treatment

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

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

Note: Sterilizing methods can be combined and should be done shortly before MembraneSlides are used.

Microcentrifuge Tubes for Specimen Collection

During LMD, the specimen falls via gravity into the cap of the selected microcentrifuge tube. The cap may be used dry, but can also be filled with buffer. In this way, degradation of nucleic acids can be prevented, or proteins can be directly rescued for electrophoresis or chromatography. Live cells can be captured directly into culture. This might be crucial when cutting out hundreds or thousands of individual cells.

Regular 0.2 ml and 0.5 ml microcentrifuge tubes (thin walled PCR tubes with flat cap) are mounted into the collection devices. Please note that microcentrifuge tubes of some suppliers may not fit into the collection device because of the shape or the size of a particular cap.

Suitable tubes are supplied by Axygen. In addition, it is possible to accommodate particular types or brands of tubes with specially fabricated tube holders. The specimen might also be collected directly into 8-well strips. This option is regularly used for the Live Cell Cutting (LCC, see page 32). Compatible 8-well strips are from Greiner Bio-One (e.g. 762071).

For more detailed information on collection devices, please refer to the Instruction Manual or contact your local Leica Microsystems representative.

Axygen PCR Tubes		
Leica Microsystems Ref. No.	Size	Order no. Axygen
11 090 615 027 006	0.5 ml	732-0675
11 090 615 028 006	0.2 ml	732-0674

Section 2. How to Prepare Tissue Sections

Handling tissues and preparing slides for laser microdissection does not differ much from standard procedures for regular glass slides. However, some protocol deviations and certain precautions are necessary to obtain intact nucleic acids and proteins for downstream analyses. A workflow comparison for preparing frozen and paraffin sections is summarized in Table 5.

Table 5. Workflows for Preparing Frozen and Paraffin Sections

Frozen sections – fast method preserving DNA, RNA & proteins, but the morphology might be diminished	Paraffin sections – time consuming, but morphology is superb; limitations concerning high molecular weight RNA, DNA, and native proteins may occur
surgery ↓	surgery ↓
embed tissue in freezing medium ↓	tissue fixation ↓
flash-freeze ↓	paraffin infiltration and preparation of paraffin block ↓
section in cryostat ↓	section with microtome ↓
stain ↓	stain ↓
LMD ↓	LMD ↓
isolate DNA, RNA or proteins ↓	isolate DNA, RNA or proteins ↓
downstream analysis	downstream analysis

Preparing Frozen Sections

Precautions for working with RNA

Frozen sections are the first choice when analyzing DNA, RNA or proteins. When working with RNA, precautions must be taken to avoid RNA cleavage by the ubiquitous RNases. RNases are also present on fingertips and are easily transferred to glassware and consumables, so gloves must be worn at all stages of work with RNA. Typically, labs designate specific areas for RNA work.

Special pretreatment of water or solutions is strongly recommended since RNases survive autoclaving. Water or solutions should be treated with 0.1% solution of DEPC (diethylpyrocarbonate) for at least 12 hours at 37°C and then either heated to 100°C for 15 minutes or autoclaved for 15 minutes to break down the DEPC into carbon dioxide and ethanol. Trace amounts of DEPC can modify purine residues in the RNA by carboxymethylation. Carboxymethylated RNA is an inefficient template in *in vitro* translation systems, but is not a problem for hybridization experiments.

Important: DEPC is toxic and a suspected carcinogen and must be handled with caution. DEPC cannot be used to treat Tris buffers since it reacts with amino groups present in Tris base and is inactivated. Typically Tris buffers are prepared in DEPC pretreated water.

Glassware is typically baked at high temperatures, such as 180°C for 8 hours or more.

Reusable plasticware and electrophoresis tanks melt at high temperatures and do not tolerate organic chemicals such as chloroform that can remove RNases. Instead, treat plasticware as follows:

1. Dip in 3% hydrogen peroxide (H₂O₂) for 10 minutes
2. Rinse in RNase-free water or dip in a 0.1% solution of DEPC for 2 hours at 37°C
3. Rinse in RNase-free water
4. Heat to 100°C for 15 minutes or autoclave for 15 minutes (traces of DEPC)

Important: Unopened bags of disposable plastic microcentrifuge pipettes are considered not to be contaminated by RNases without further treatment. One caveat is that trace amounts have been reported to still exist even in these sources of plasticware.

Alternatively, reagents for RNases decontamination such as RNaseZap[®] (Ambion) can be used to clean the gel tray, spatulas, slides, and other surfaces.

For more hints on safe work with RNA, please refer to Ausubel et al., Sambrook et al. or RNeasy Micro Kit Handbook (Qiagen).

Note: Most reagents and plasticware can be obtained certified RNase- and DNase-free.

Workflow for Preparation of Frozen Sections

In addition to RNase-free conditions when working with RNA, the whole procedure of tissue handling should possibly be carried out cold and quickly. Therefore, tissue or organs should be flash-frozen (snap-frozen) after surgery followed by cryo-sectioning. Sections should be stained within a very short time, not exceeding in total 30 minutes. The stained tissues should be used directly for laser microdissection, or alternatively, the dried sections can be stored in tightly closed bags, slide boxes or other containers containing desiccant at -80°C even for a few months or longer. Just before laser microdissection, the container with the slides should be taken out of the freezer and slowly adjusted to room temperature to avoid formation of water condensation inside the container, e.g. allow for at least 15 min each step to equilibrate at -20°C , 4°C and room temperature. This precaution is critical for RNA quality, especially when working with small numbers of cells, since water (moisture) activates RNases.

Note: All listed methods are exemplary suggestions. Other methods (e.g. for direct tissue freezing in the cryotome) can be established as well.

Flash-freezing Methods

Tissues are embedded in a freezing medium such as Jung Tissue Freezing Medium™ (Leica Microsystems) or OCT (Tissue-Tek®) and immediately flash-frozen.

Important: Tissue freezing medium can interfere with laser microdissection and must be completely removed with 70% alcohol (e.g. ethanol). For thicker sections, keep the tissue in 70% alcohol longer.

Method 1: Flash-freezing using 2-methyl-butane (synonym: isopentane)

1. Precool 2-methyl-butane in a beaker surrounded by dry ice. This prevents the 2-methyl-butane from bubbling over when the dry ice is added.
2. In a beaker or specimen container, add crushed dry ice to the 2-methyl-butane to make a slurry mixture (work in a hood).
3. When bubbling stops, the 2-methyl-butane is at the correct freezing temperature of approximately -90°C .
4. Immerse the embedded tissue slowly; eventually it will sink to the bottom of the 2-methyl-butane.

Safety Note: Be sure to fully evaporate the 2-methyl-butane after freezing to prevent the possibility of explosion in the freezer.

Method 2: Flash-freezing in liquid nitrogen

1. Place liquid nitrogen in a styrofoam container.
2. Place the styrofoam container inside a Petri dish lid; a support rack may be needed to hold the Petri dish lid.
3. Place the tissue into a disposable mold and embed it in the tissue freezing medium; or alternatively, place the tissue (embedded in the tissue freezing medium) on a coverslip and place into the liquid nitrogen.

Method 3: Flash-freezing using 2-methyl-butane and liquid nitrogen

1. Place a beaker with 2-methyl-butane into liquid nitrogen and wait until the 2-methyl-butane cools down to -80°C . This is the point when the wall of the beaker turns white, the 2-methyl-butane is now solid.
2. Insert the tissue into 2-methyl-butane and let it freeze. Alternatively, the tissue can be put onto a floating platform made of cork.

Tissues that are flash frozen without embedding medium can be post embedded in cryostat embedding media. However, the tissue will thaw somewhat in the process. This can compromise RNA preservation and introduce undesirable histological artifacts.

Sectioning

Preparing Cryo Sections

Cryo blocks can be directly sectioned (recommended for RNA preparation) or stored at -80°C . The temperature of the cryo blocks should be adjusted to -20°C in the cryostat before sectioning.

1. Clean the cryostat before sectioning to avoid contamination
2. Mount the cryo block onto the specimen clamp
3. Trim the sample to get a plane surface and an approach to the tissue
4. Cut the block into 5–25 μm sections and immediately place them on the slides for LMD

Important: If you are using FrameSlides, the FrameSupport is strongly recommended. Sections can be fixed with ice-cold acetone for 2–3 minutes, 70% or 100% ethanol for 20 seconds or mixture of ethanol : acetic acid (19:1) to increase the adhesion of the tissue to the PPS-, PEN-, PET-, POL- or FLUO-membranes.

Typical sectioning temperatures are shown in Table 6, page 18. Section thickness depends on the tissue type and the size of the dissected area. Keep the slides in the cryostat or on dry ice, if LMD is performed on the same day. Alternatively, they may be stored in sealed slide boxes with a desiccant at -80°C until needed.

Important: Dry slides at room temperature for 15 minutes using a drying chamber with desiccant or at 40°C for 10 minutes. This is a critical step for successful LMD. Sample slides should be stained just prior to microdissection. Some staining protocols are given in Section 4.

For routine research and frequent cryo sectioning, the Leica CM3050 S cryostat is recommended. Versatility, such as the ability to cool down to –40°C, is an important advantage of this system. This is important for sectioning tissues such as mamma. For less frequent usage a cryostat such as the Leica CM1850 UV with UVC disinfection and the Precision Cryoembedding System can be used (contact your local Leica Microsystems representative for more information).

Table 6. Knife Edge Temperature for Cryo Sectioning

Knife Temperature	Tissues
–10°C to –15°C	adrenals, bone marrow, brain, cartilage, spleen or bloody tissue, testicular
–15°C to –17°C	bladder
–15°C to –18°C	breast - less fatty, cervix, intestine, liver, thyroid
–15°C to –20°C	kidney, lung, pancreas, prostate, ovary, rectal, skin without fat, uterus
–18°C to –22°C	heart and vessel
–20°C to –25°C	muscular
–25°C to –30°C	skin with fat, breast - fatty

Preparing Paraffin Sections

Paraffin embedding is a process in which fixed tissue (utilizing neutral buffered formalin or other fixatives) is infiltrated by paraffin to stabilize it for long term storage, easy sectioning and for histological examination, it also affects macromolecules such as RNA. However, other fixatives have been reported to be gentle enough for RNA, DNA or protein downstream analyses (see below).

Please be aware that the methods, temperatures and protocols may need to be optimized depending on the investigated tissue and research objectives.

Tissue Fixation

Fixatives should penetrate the tissue quickly and thoroughly. Incomplete fixation causes many artifacts and can cause shrinkage. The most common fixative is 10% formalin buffered with PBS (phosphate buffered saline) to neutral pH (10% formalin corresponds to 4% formaldehyde). However, formalin breaks nucleic acids and therefore is not suitable for subsequent RNA and DNA analyses, if high molecular weight of nucleic acids are needed or

quantitative analyses are planned. Other well-known fixatives are:

- 5% acetic acid
- 4–6% glutaraldehyde
- 100% ethanol
- 100% ice-cold acetone
- Bouin's solution (contains picric acid and it is not compatible with RNA analysis)

Paraffin sections are advantageous over frozen sections in preserving morphology. However, typical fixatives such as formalin degrade RNA. Methacam can also be used as a fixative for paraffin-embedded sections. Methacam has been reported to provide better RNA quality enabling quantitative PCR. Methacam is a mixture of 60% (v/v) absolute methanol, 30% chloroform and 10% glacial acetic acid. Tissue was fixed at 4°C for two hours. (Takagi, et al. *J Histochem.* 2004 Jul; 52(7):903; Shibutani, et al., *Lab Invest.* 2000 Feb; 80(2):199–208)

Another successfully used fixative in conjunction with RNA, DNA, and protein analyses is HOPE (HEPES-glutamic acid buffer mediated organic solvent protection effect).

This patented solution is commercially available from Innovative Diagnostik Systeme GmbH (www.dcs-diagnostics.de).

Paraffin embedding and Sectioning

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

1. Wash the tissue with ascending concentrations of alcohol (such as ethanol) as follows:
 - 70% ethanol 30 minutes 2x
 - 95% ethanol 30 minutes 2x
 - 100% ethanol 30 minutes 2x

During these steps the tissue will harden.

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

Important: In general, paraffin hardness corresponds to the hardness of the tissue to be embedded. The best microtome sectioning results are obtained from paraffin blocks of the same or nearly the same hardness. There are two types of paraffin:

- Low melting point paraffin is softer. Sectioning can be more difficult, but ribboning is easier. Tissue embedded in this type of paraffin may give better results in PCR.
- High melting point paraffin is harder. It gives better support for hard tissues. Cutting thinner sections is possible, but ribboning is more difficult.

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

4. Paraffin infiltrated tissues are embedded in a mold with liquid paraffin to form a block for better handling during microtome sectioning. This is easily done using a paraffin embedding station such as the Leica EG1150 H.
5. The tissue block can be stored at room temperature or below. For better cutting results with the microtome, cooling the paraffin block down to –20°C is recommended.

6. Section the block
 - Clean the cryostat before sectioning to avoid contamination
 - Mount the tissue block onto the microtome
 - Trim the sample to obtain a plane surface and an approach to the tissue
 - Cut the tissue into 5–15 μm sections
 - Place the paraffin ribbons on the surface of the water in a waterbath at 37°C–0°C; in this way, the floating ribbons are easier to stretch and subsequently can be placed without folds on the slides. Please note that the water must be pure for PCR experiments or RNase-free, if RNA analysis is intended.

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

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

For typical staining protocols, see Section 3.

Dissectate collection from plain glass slides via LMD

If necessary it is possible to collect material directly from plain glass slides, coverslips or archived slides. Therefore the sample must be uncovered (no coverslip!). Place the slide upside down to the LMD system and mark your regions of interest and select the “Draw+Scan” mode instead of the common “Draw+Cut” mode. The “Draw+Scan” mode will dot dissect the material by scanning the area of interest with the laser. The laser settings should be adjusted to the minimal power needed for ablation of the desired material.

Note: The direct ablation of the material includes a direct laser force to the material which might influence the yield and quality. We recommend to use membrane slides if possible to guarantee best yields and no alteration of the quality.

Preparation of archived slides for LMD

As written above, regular glass slides for tissue sections can be used for laser microdissection. However, there is an additional possibility to process the regular archived slides to obtain the tissue for the microdissection. The protocol describes all steps of the procedure.

a. Removal of the cover slip:

1. Archived slide is placed in a vertical position in a closed container with a solvent such as xylene to dissolve the cover slip seal and the mounting medium. Heating the container from 40°C up to 70°C speeds up the process.
2. Wait until the cover slip falls off. This may take hours to days.
3. Do not use a tool to pry off the cover slip.
4. The tissue section has to be rehydrated in xylene and washed through alcohol series 95%, 75%, 50% and finally rinsed with PBS or pure water, and air-dried.

b. Removal of the tissue from the slide:

1. Place "Quick Mount" mounting medium on the archived slide. Thickness of the medium should be about 1–2 mm.
2. Allow the "Quick Mount" to cure overnight at 37°C or for 2 h at 70°C. "Quick Mount" must remain pliable.
3. Trim excess "Quick Mount" around tissue section.
4. Place in a distilled water bath at 50–60°C for around 1 h.
5. Use a fine razor to lift the section and peel off.

c. Transfer tissue onto the Leica Microsystems MembraneSlide

1. Lay "Quick Mount" with tissue onto membrane.
2. Flatten lightly with finger tip pressure.
3. Place in oven in a horizontal position at 56°C for 3 h or until "Quick Mount" is completely cured.

d. Removal of "Quick Mount" from the Leica Microsystems slide

1. Place slide in xylene until "Quick Mount" dissolves.
2. Rehydrate with xylene, and then dehydrate with the series of 100%, 95%, 75% alcohol, then wash with PBS water and air dry.
3. The slide is ready for the laser microdissection.

Please note: Depending on the conditions under which archived slides have been stored, and duration of storage, the macromolecules (DNA) may be of poor quality and quantity. It is recommended to utilize the archived paraffin block to cut new sections onto MembraneSlides for laser microdissection, than working with archived slides. However, direct ablation of tissue without coverslip from glass is also possible (draw and scan mode), but not comparable regarding the much better results of downstream analysis with laser microdissected samples derived from MembraneSlides.

Section 3. Preparation of Chromosome Spreads

Preparation of chromosomes of white blood cells from human peripheral blood.

Cells were cultured for 72 to 96 hours, then treated with colcemid (to arrest cells in metaphase) for 1 hour. After this step cells are treated with a hypotonic solution (KCl in Milli Q water). The white blood cells will swell and the erythrocytes become lysed. This step will take 10–12 minutes.

After a washing step to remove lysed erythrocytes the cells are fixed in methanol/acetic acid (3 + 1, v/v). Fixed cells were dropped on the MembraneSlides and were air dried overnight and stained with Giemsa staining solution (1 : 20 diluted in Gurr buffer, which is a standard buffer used for Giemsa staining made by dissolving commercially available tablets) for 3 minutes. After washing in water they were air dried.

This protocol was kindly provided by Dr. Henry Dijkman, Department of Pathology, Radboud University Nijmegen Medical Center, The Netherlands

Section 4. Typical Staining Protocols

Before staining, the paraffin should be removed from the tissue section. Please see page 20 for the typical protocol. For all types of sections, both frozen and paraffin-embedded, tissue can be fixed with various organic solvents to ensure better adhesion to the foil slide (see Page 16). This is the last step prior to staining. In this section, typical staining procedures are described. You will also find some “quick” procedures that may be useful when investigating RNA. The rough rule of thumb says that the entire staining procedure for subsequent RNA isolation should not exceed a total of 30 minutes.

Please be aware that protocols may need adjustment depending on the investigated tissue and your research objectives.

Quick Toluidine Blue Staining

Toluidine blue is a basic stain, which can also be used as a quick stain for light microscopy. Sections are permanently stained bluish-purple, similar to H&E. Toluidine blue may be used to stain both frozen and paraffin sections. It can stain mast cells and it is successfully used for staining frozen brain sections. Suitable for subsegment RNA preparation.

You will need

- Toluidine blue
- DEPC-treated water is used for subsequent RNA preparation, otherwise regular distilled water can be used (see page 14)
- 75% ethanol
- 0.22 µm sterile syringe filter

1% toluidine blue solution: Dissolve 0.1 g toluidine blue in 10 ml DEPC water and filter through a sterile filter.

Procedure

1. Apply toluidine blue solution and incubate for 3 minutes
2. Rinse twice in DEPC water for 15 seconds
3. Rinse once in 75% ethanol in DEPC water for 3 minutes
4. Air-dry or dry on a heater at 40°C for 10 minutes

H&E (Hematoxylin and Eosin) Staining (method 1)

H&E is a common histology stain. Hematoxylin stains the nuclei, and eosin stains the cytoplasm. H&E is suitable for both paraffin and frozen sections. Ready-to-use reagents are also commercially available.

Reagents

- 70% ethanol
- 95% ethanol
- 100% ethanol
- Hematoxylin (Hematoxylin Solution, Harris modified, Sigma Diagnostics, Order no. HHS-16)
- Eosin (Eosin Y solution, alcoholic with phloxine B, Sigma Diagnostics, Order no. HT110-3-32)
- 0,1 % NH₄OH (blueing reagent): 200 µl NH₄OH 100% + 200 ml H₂O or Scot's tape water substitute

Procedure

Place the slide in the following solutions for the designated time frames:

1. Distilled H₂O 30 seconds
2. Hematoxylin 1 minute
3. H₂O 30 seconds
4. Blueing reagent 30 seconds
5. Eosin solution 10 seconds
6. 70% ethanol 30 seconds
7. 95% ethanol 30 seconds
8. 100% ethanol 30 seconds or air-dry at room temperature

Results

- Nuclei - blue-black
- Cytoplasm - varying shades of pink
- Muscle fibers - magenta
- Fibrin - deep pink
- Red blood cells - orange/red

H&E Staining (method 2)

See the above application range and typical results of staining.

Reagents

- Gills Hematoxylin
 - Hematoxylin 6.0 g
 - Aluminium Sulphate 4.2 g
 - Citric Acid 1.4 g
 - Sodium Iodate 0.6 g
 - Ethylene Glycol 269 ml
 - Distilled Water 680 ml
- Eosin
 - Eosin Yellowish 1.0 g
 - Distilled Water 100 ml
- Lithium Carbonate 1 %
 - Lithium Carbonate 1 g
 - Distilled Water 100 g
- Acid Alcohol 1 %
 - 70% Alcohol 99 ml
 - conc. Hydrochloric Acid 1 ml
- Scott's tap water substitute

Before you begin, add 20 g sodium bicarbonate and 3.5 g magnesium sulphate to a beaker containing 1 L distilled water. Mix thoroughly with a magnetic stirrer to dissolve the salts, then pour into a storage container.

Procedure

1. Wash the slide with distilled water for 30 seconds
2. Place sections in the hematoxylin solution for 30 seconds
3. Wash in tap water
4. "Blue" sections in Scott's tap water
5. Wash in tap water
6. Place sections in 1% acid alcohol for a few seconds
7. Wash in tap water
8. Place sections in eosin for 15 ~ 30 seconds
9. Wash in tap water
10. Dehydrate with 95% ethanol for 30 seconds, 100% ethanol for 30 seconds, and air-dry 5 ~ 10 minutes

Quick Cresyl Violet Staining

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

Cresyl violet staining solution

1. Add 1 g Cresyl violet into 200 ml distilled water
2. Add 1 ml 10% acetic acid
3. Boil until completely dissolved
4. Filter the solution through filter paper
5. Add H₂O up to 250 ml

Procedure

1. Wash once in PBS for 30 seconds
2. Apply Cresyl violet staining solution for 30 seconds
3. Rinse in PBS for 30 seconds
4. Rinse twice in DEPC water for 15 seconds each time
5. Rinse once in 75% ethanol for 1 minute, 95% ethanol for 30 seconds, and 100% ethanol for 30 seconds
6. Air-dry for 10 minutes

Modified Cresyl Violet Staining for RNA-Research

For RNA protection Cresyl violet can be prepared and applied without PBS or water steps, preventing degradation of RNA by humidity activated RNases.

Modified Cresyl violet staining solution

1. Prepare Cresyl violet staining solution at least one week prior usage
2. Add 0.5 g Cresyl violet into 50 ml 100% ethanol
3. Mix solution and store at 4°C sealed air-tight and dark

Procedure

1. Mount section and fix in cold (-20°C) 75% ethanol for 2 minutes
2. Apply Cresyl violet staining solution directly with syringe and sterile filter to the section and incubate for 1 minute, swivel gently
3. Dip for 5 seconds in 75%, 90% and 100% ethanol
4. Final fixation is done in fresh 100% ethanol for 1 minute
5. Dry sample in drying chamber with desiccant

Adapted from Gründemann et al., NAR, 2008

Quick Thionin Staining

This stain is specific for DNA and Nissl substance, which is primarily ribosomal RNA. Thionin binds to acidic proteins and nucleic acids with a specificity determined by the pH of the final staining solution. This stain is suitable for both paraffin and frozen sections and subsequent RNA preparation.

Reagents

- Thionin (acetate)
- Sodium Acetate
- DEPC-treated water is used for subsequent RNA preparation, otherwise regular distilled water can be used (see page 14)
- 75%, 95%, 100% ethanol
- 0.22 µm sterile syringe filter

0.5% Thionin staining solution

1. Dissolve 1.36 g of sodium acetate (anhydrous) or alternatively 2.26 g sodium acetate (trihydrate) in 100 ml H₂O and fill up to 250 ml
2. Add 0.5 g thionin to 100 ml of the sodium acetate solution.
3. Stir and heat gently for 1 hour. Filter the solution after the dye has dissolved. Store in a closed bottle.

Procedure

1. Apply thionin staining solution and incubate for 1 minute
2. Rinse twice in DEPC water for 15 seconds
3. Rinse once in 75% ethanol for 1 minute, 95% ethanol for 30 seconds, and 100% ethanol 30 seconds
4. Air-dry for 10 minutes

Direct Visualization of GFP

GFP is an abbreviation for green fluorescent protein. Following exemplary protocol needs to be optimized for the investigated tissue.

Reagents

- Formaldehyde based fixative (4% PAF, 10% NBF, PLP)
- 1x PBS
- 18% Sucrose in 1x PBS
- Freezing medium

Procedure

1. Harvest tissue as quickly as possible and trim for correct fixation (4 mm thick slices).
2. Immediately place into fixative (use a tissue to fixative ratio of at least 1:10).
3. Incubate for 8–24 hours; keep out of direct light to avoid bleaching the GFP.
4. Place tissues in 1x PBS for 10–15 minutes. Use a larger volume of buffer to completely remove fixative from the tissue.
5. Repeat this washing step once.
6. Transfer tissues into 18%–20% sucrose (dissolved in 1x PBS). Use a larger volume to allow tissue to float freely.
7. Place in the refrigerator at 4°C overnight.
8. The tissue will sink to the bottom when completely infiltrated with sucrose; if the tissue is still floating after 24 hours, incubate for one more night.
9. Remove the tissue from the sucrose and dab off any excess sucrose.
10. Prepare a standard frozen block.
11. Store the block at –70 °C until needed.
12. Cut the sections on a cryostat (usually 4–10 µm) and thaw the mountant.
13. Wash away the mountant for LMD.

Rapid Immunohistochemistry using Vectastain® ABC-AP Kit and Vector® Blue

In this protocol an unlabelled primary antibody is used, followed by biotinylated secondary antibody. The secondary antibody is conjugated to the alkaline phosphatase (AP) complex. The detection reaction is triggered by Vector®Blue substrate (Vector Laboratories).

You will need to prepare

- 10x PBS: add 80 g NaCl, 2 g KCl, 18 g Na₂HPO₄ x 2 H₂O, 2.4 g KH₂PO₄ to 800 g distilled H₂O. Adjust to pH 7. Fill up with H₂O up to 1 L and autoclave.

Other reagents:

- Acetone
- Normal Goat Serum
- Vector Blue Substrate Kit (Vector Laboratories, Alexis)
- Vectastain ABC-AP-Kit (Vector Laboratories, Alexis)

Procedure

1. Prepare frozen sections of 8 µm on PET or PEN-slides.
2. Fix the sections with cold acetone at 4°C for 10 minutes.
3. Dry the sections at room temperature for 10 minutes.
4. Wash briefly in 1x PBS.
5. Apply 500 µl of primary antibody dilution onto the section and incubate at room temperature for 10 minutes. The dilution must be adjusted individually for each antibody.

Important: A good starting point may be a dilution of 1:100. In this case, prepare the dilution as follows: Add 20 µl of normal goat serum as the blocking reagent and 5 µl of primary antibody to 500 µl 1x PBS. Depending on the species in which the antibody was raised and the tissue used, it may be necessary to use a different blocking reagent.

6. Wash briefly once in PBS
7. Apply 500 µl of secondary antibody dilution onto the section and incubate for 15 minutes at room temperature. The dilution has to be adjusted individually for each antibody.

Important: A good starting point may be a dilution of 1:100. Add 4 µl normal goat serum as the blocking reagent and 5 µl biotinylated secondary antibody to 500 µl 1x PBS. Depending on the species in which the antibody was raised and the tissue used, it may be necessary to use a different blocking reagent.

8. Wash briefly once in PBS at room temperature.
9. The staining reaction is carried out with the Vectastain® ABC-AP Kit and Vector® Blue from Vector Laboratories (see manufacturer's instructions).
10. Incubate with Avidin-Alkaline phosphatase complex.
11. Apply ABC reagents from the Vectastain ABC-AP-Kit for 10 minutes.
12. Wash briefly once in PBS.
13. Incubate with Vector® Blue AP substrate solution for 10 minutes.
14. Wash briefly with RNase-free water.
15. Leave MembraneSlides to dry in the dark.

Rapid immunohistochemistry using Streptavidin-HRP Conjugate

In this protocol an unlabelled primary antibody is used, followed by a biotinylated secondary antibody. The secondary antibody is conjugated to horseradish peroxidase (HRP or POD). The detection reaction is triggered by diaminobenzidine (DAB)/H₂O₂ as a substrate.

Prepare solutions as stated in the previous protocol

Other reagents:

- Acetone
- Normal Goat Serum
- DAB plus substrate solution (DAKO Cytomation)
- Streptavidin HRP (DAKO Cytomation)

Procedure

Follow steps 1 to 8 from the protocol above.

9. Apply 500 µl of HRP-conjugated streptavidin and incubate at room temperature for 10 minutes. The dilution of the streptavidin has to be determined experimentally and might range from 1:300–1:1000 (see manufacturer's instructions). For example, the 1:300 dilution is prepared by adding 1.7 µl streptavidin to 500 µl 1x PBS.
10. Wash briefly once with PBS.
11. Apply 500 µl of DAB staining solution onto the slide for 10 min at room temperature. The DAB staining solution is prepared as follows: A drop of DAB plus solution is added per ml buffered substrate solution (both from the DAKO DAB plus solution kit).
12. Wash briefly once with PBS.
13. Wash briefly with RNase-free water.
14. Air-dry the slides in the dark.

General LMD Guidelines using Leica Microsystems Microdissection Systems

Section 5. Placement of LMD Systems

A laser microdissection system should be located away from doors and windows that can be opened and air conditioning. Air draft should be avoided, especially during cutting.

Scientists who work with RNA should ensure that the LMD is placed in an RNase-free zone in the lab.

Important: All working steps and technical data on LMD are included in the Instruction Manual. Please read the Instruction Manual entirely.

Section 6. Benchmark Data on Cell Number and Section Thickness for LMD

Standard numbers for laser microdissection are given in Table 7.

Table 7. Average Number of Cells used for LMD.

Analyzed Molecules	Average Number of Cells
DNA	Number of cells is not critical because DNA is easily amplified by PCR. Usually up to 5000 cells can be dissected.
RNA	30% of all researchers dissect between 100 –1000 cells 30% of all researchers dissect over 1000 cells However, gene expression profiling by dissecting a single cell or as few as 10-30 cells has been reported.
Proteins	Thousands to tens of thousands of cells are required. The collection of cells can be facilitated by Leica Auto Vision Control (AVC) software (see page 35), which enables automatic cell recognition and automatic microdissection from a tissue section(s). The region of interest can also be cut from several consecutive slides and pooled. In this case, regular, thinner sections with the best morphology are used. Alternatively, large tissue areas of 500 μm^2 may contain enough cells.

The average section thickness used for LMD is between 4–20 μm . In some cases, where more material is needed and tissue morphology is sufficient, thicker sections of 15–30 μm can be used for LMD. For plant Research, even unfixed fresh tissues above 100 μm can be used.

Section 7. Application Software

Live Cell Cutting (LCC)

Microdissecting cells from a cell culture is termed live cell cutting (LCC). Since the laser beam does not come into contact with the live cells, it does not injure them. Hence, this technique is also safe, and it is possible to recultivate the microdissected cells.

Leica Microsystems offers an optional LCC module including additional software and hardware, which allows special Petri dishes to be used for LMD. This optional module lowers the stage every time the objective is changed in order to avoid collision with the Petri dish, and then brings the specimen back into focus. This software option is included within the basic software version.

Either membrane bottom Petri dish or 18-well μ -Slides can be used for cell cultivation (see pages 8–11). The protocol below describes LCC when using the Petri dish.

Procedure:

1. The membrane of the Petri dish can be coated with poly-L-lysine or collagen prior to inoculation. This may improve cell attachment and growth.
2. The cells are inoculated in the Petri dish.
3. The seeded membrane Petri dish can be inserted into a larger Petri dish and incubated under usual conditions for 24–48 hours. The cells should not grow to confluence, in order to distinguish single cells for microdissection. Cells can be inspected with the LMD system using brightfield (BF), phase contrast (PH), differential interference contrast (DIC) or fluorescence.
4. Just prior to microdissection, the PBS, base medium (without serum), and complete culture medium (with serum and antibiotics) should be warmed up to 37°C in a waterbath.
5. The stage, substage, holder, and UV shield should be cleaned with alcohol. 300 μ l of complete culture medium are added to each well of the 8-well strip (see Instruction Manual).
6. Next, the cells are carefully washed three times with 2 ml PBS. Then 150 μ l – 1 ml of PBS or base medium is added to the membrane Petri dish to just cover the cells that have to stay alive.

Important: The amount of liquid in the dish is critical because if there is too much medium (more than just covering the cells), the laser causes “cavitation” (bubbles), which reduces the efficiency of cutting!

7. The Petri dish without cover and the collector with the 8-well strip are mounted onto the LMD stage.
8. Cells of interest are selected, followed by microdissection

Tips:

- Be careful to leave wide margins around the cells.
 - Typically, nearly rounded cells are easier to dissect.
 - The selected area should not be too large.
 - Single cells or groups of two to three cells fall down the best.
 - The laser settings are critical to release the dissected pieces during the first cut. The best settings for the investigated cells have to be defined empirically.
9. The microdissected cells can be inspected in the wells, although the presence of phenol red makes this difficult. Another hindrance: When the specimen drops to the bottom of the well, focusing is not possible.
10. To re-culture the cells, the 8-well strip is removed from the sub-stage after laser microdissection. 50 μ l more of the medium is added on top of each well and spun down for a short time (up to 1 minute). The microdissected cells are inoculated in the regular cell culture vessels and incubated as usual.

LCC Applications using membrane μ -Slides and Petri dishes

Single-slide LCC

A solution for live cell cutting. Single membrane μ -Slide is mounted on the regular slide holder. μ -Slide can be used with the cover that is UV translucent up to 20x objective. This increases sterility of the experiment. Cells are collected in 8-well strips (see Fig. 5).

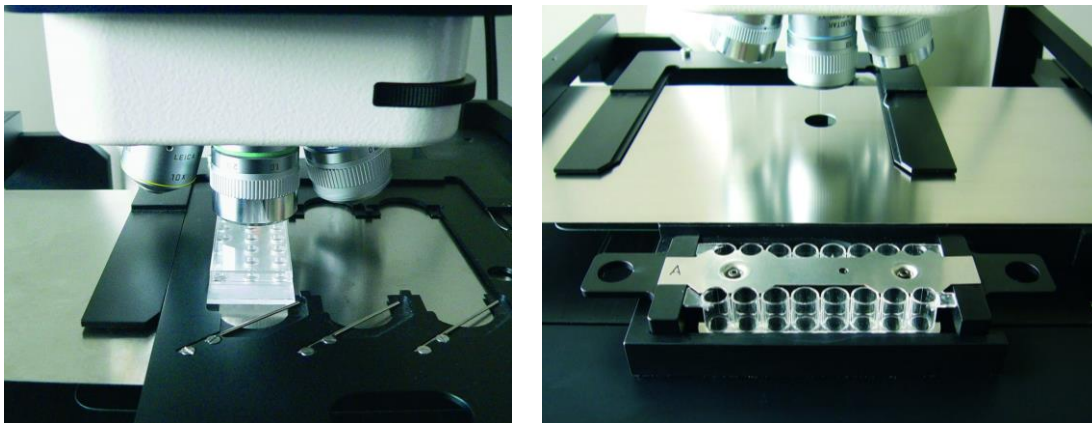


Fig 5. Single-slide LCC with the scanning stage and 8-well stripe for LCC collection

Stack-slide LCC

Completely sterile solution for live cell cutting that is available for scanning stage. Membrane μ -Slide with grown cell culture and regular plastic bottom μ -Slide (e.g. Plasma coated, Leica Microsystems order no. 11532520) are fixed in the slide-stack holder under the clean bench. Then the holder is mounted on the regular three-slide holder. The stack can be used with up to 20x objectives (see Fig. 6 and 7).



1. Cover
2. Slide with membrane bottom for LMD
3. Slide with regular plastic bottom
4. Slide-stack holder

Fig 6. Scheme of the stack-slide

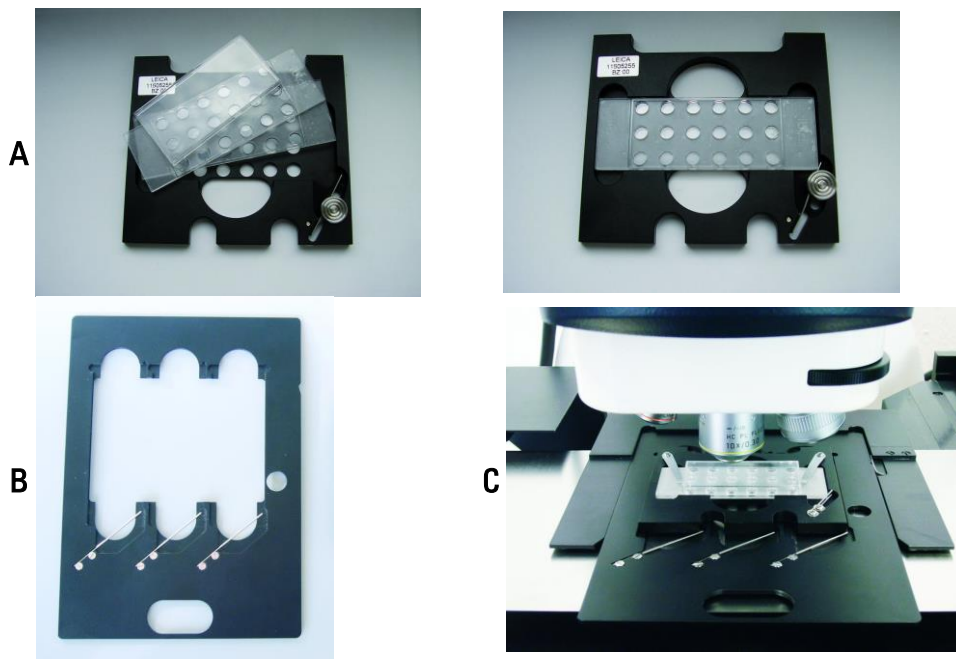


Fig 7. **A** View on the mounted stack-slide in the holder. **B** Stack-slide holder **C** View on the stackslide on the scanning stage.

Classical LCC with Petri dishes

This is the semi-sterile solution for live cell cutting, because cutting occurs without dish cover and the cells drop into 8-well strips or opened Petri dish (membrane dish or any regular culture Petri dish with 5 cm diameter and 7 mm height). Petri dish LCC with open cover can be used with up to 40x objective (see Fig. 8).

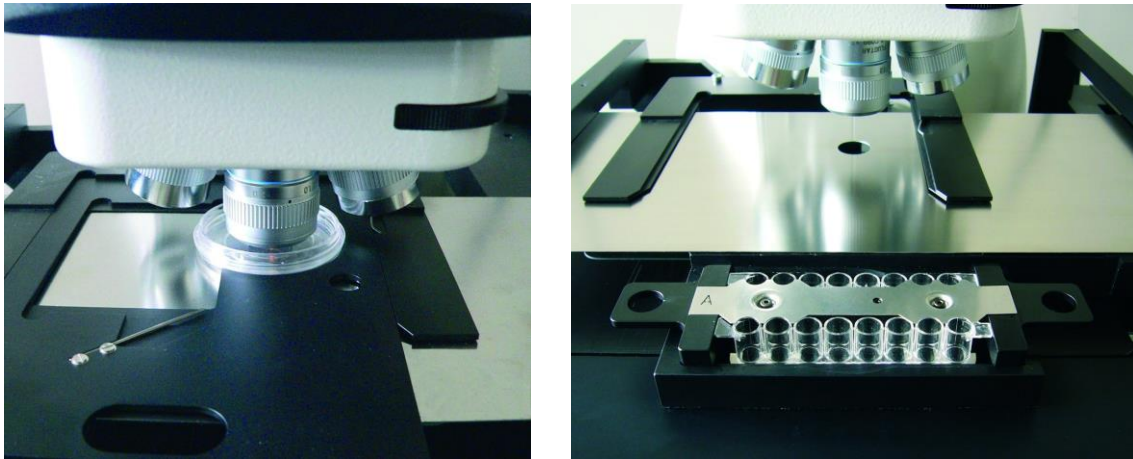


Fig 8. LCC using Petri dish with the scanning stage, 8-well stripe for LCC collection

Order numbers of holders and consumables are given on pages 8–11.

General conditions for successful cutting

- Use as little growth media as possible.
- The best dropping results are obtained with shapes with a diameter < 200 μm .

Auto Vision Control (AVC)

Auto Vision Control (AVC) is a software option for automatic recognition and selection of cells for laser cutting. It is not included within the basic software version and must be ordered separately.

This software module is designed for investigators requiring higher throughput, where many single cells will be microdissected and preparation cannot be contaminated by other tissue types. AVC is useful when analyzing low abundance transcripts of specific cells where the adjacent cell may mask the result of a gene expression profile.

The AVC module is also a viable alternative for collecting single cells for protein research. Once cells are defined and automatically selected, the laser dissects them quickly and drops them automatically into the microcentrifuge cap. The ability to automatically isolate hundreds or thousands of cells offers a tremendous time-saving.

Please refer to the Instruction Manual for operating application software.

Nucleic Acids and Protein Preparation for Downstream Analysis

Section 8. Preparation of Nucleic Acids

Laser microdissected tissue specimens present a particular challenge for molecular analysis, as nucleic acids must be purified from very small samples. It is a particular issue for RNA, which cannot be directly amplified in PCR like DNA. In addition, fixation and staining steps may compromise the integrity of nucleic acids. If the yields and quality of the nucleic acids are not satisfactory, tissue section preparation has to be optimized (see Section 2), and the method of RNA or DNA preparation must be verified.

There are many “home-brewed” techniques that may work for larger amount of starting material. However the less material to start with, the more risk of losing the precious sample. Leica Microsystems recommends the high-quality QIAGEN kits for preparation of nucleic acids. These kits have been tested with the Leica Microsystems LMD systems.

These “Micro Kits” have been designed for fast, reproducible isolation of high-quality genomic DNA (QIAamp® DNA Micro Kit) or total RNA (RNeasy® Micro Kit) from laser microdissected tissues. The preparations are free of proteins, nucleases, and other impurities. They can be immediately used in downstream applications such as PCR, sequencing, quantitative, real-time PCR, or can be stored at –20°C until needed. Please refer to www.qiagen.com for details.

Section 9. Total Protein Preparation

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

- The entire area of interest can be microdissected using low magnification objectives such as the 5x, 6.3x or 10x.
- Cut regions or cells of interest from several consecutive tissue sections.
- Hundreds of single cells can be cut out from the tissue using the Leica AVC module (page 35)

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

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

Other Information

Section 10. Ordering Information

Specimen Preparation Instrumentation

Please have a look on: www.leica-microsystems.com

Please note that these are typical products from a broad range of Leica Microsystems specimen preparation portfolio. Please contact your local Leica Microsystems representative to select the best solution for your needs.

QIAGEN Kits for Nucleic Acids Preparation

Order No.	Product Description	Remarks
56304	QIAamp® DNA Micro Kit for 50 DNA preparations	Kit for genomic DNA preparation from microdissected specimens
74004	RNeasy® Micro Kit for 50 RNA preparations	Kit for total RNA preparation from microdissected specimens
74034	RNeasy® Plus Micro Kit for 50 RNA micropreparations	Kit for total RNA preparation from cells and tissues
56404	QIAamp® DNA FFPE Tissue Kit for 50 DNA preparations	Kit for genomic DNA preparation from paraffin-embedded tissues
73504	RNeasy® FFPE Kit for 50 RNA preparations	Kit for total RNA preparation from formalin-fixed, paraffin-embedded tissue sections
217504	miRNeasy® FFPE Kit for 50 miRNA/RNA preparations	Kit for microRNA and total RNA preparation from formalin-fixed, paraffin-embedded tissue sections
37623	Qproteome™ FFPE Tissue Kit for 20 protein preparations	Kit for protein preparation from formalin-fixed, paraffin-embedded tissue samples

Please visit www.qiagen.com for more information on additional kits.

Section 11. General References

1. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G. and Smith, J.A. (eds.) (1995) Current Protocols in Molecular Biology, Wiley
2. RNeasy® Micro Handbook, available at www.qiagen.com
3. Sambrook, J., Russel D.W. (1991) Molecular Cloning, A Laboratory Manual, 3rd ed., Cold Spring Harbor (N.Y.) Laboratory Press
4. QIAamp® DNA Micro Handbook, available at www.qiagen.com
5. Sambrook, J., Fritsch, E.F. and Maniatis, T., Cold Spring Harbor (N.Y.) Laboratory Press

Section 12. Useful Websites

Leica Microsystems LMD system:

www.leica-microsystems.com/LMD

Leica Microsystems Science Lab:

www.leica-microsystems.com/science-lab

QIAGEN

www.qiagen.com

Section 13. Protocol Form

Please fill in the form and fax to +49 6441 29 2255. You can also request the form as a hard copy or electronic copy from your local Leica support.

Please briefly describe your current workflow for downstream applications:

1. What type of specimen(s) do you work with?

a. Tissue _____ Cell type _____ Species _____

b. Cultured cells (please specify) _____ c. Stem cells: _____

2. What is the starting material for laser microdissection? (indicate all that apply)

- | | |
|-----------------------------------------------------|------------------------------------------------------|
| <input type="checkbox"/> Frozen sections | <input type="checkbox"/> Smears |
| <input type="checkbox"/> Paraffin-embedded sections | <input type="checkbox"/> Cytospins |
| <input type="checkbox"/> Cultured cells | <input type="checkbox"/> Other, please specify _____ |

3. Do you use a tissue fixative? If yes, which one (indicate all that apply)

- | | |
|-----------------------------------|------------------------------------------------------|
| <input type="checkbox"/> Formalin | <input type="checkbox"/> Methacarn |
| <input type="checkbox"/> HOPE | <input type="checkbox"/> Other, please specify _____ |

4. How thick are the tissue sections?

From _____ μm up to _____ μm

5. Do you store paraffin-embedded or frozen tissue blocks before sectioning and LMD?

- | | |
|------------------------------|-----------------------------|
| <input type="checkbox"/> Yes | <input type="checkbox"/> No |
|------------------------------|-----------------------------|

a. If yes, how long and at what temperature? _____

b. Do you observe decreased quality of the preparation (nucleic acids and proteins) over time?

- | | |
|------------------------------|-----------------------------|
| <input type="checkbox"/> Yes | <input type="checkbox"/> No |
|------------------------------|-----------------------------|

c. Do you know, how to overcome this? _____

6. Do you store sections before LMD?

a. If yes, please describe how long and at what temperature: _____

b. Do you observe decrease of the quality of the preparation (nucleic acids and proteins) over time?

- | | |
|------------------------------|-----------------------------|
| <input type="checkbox"/> Yes | <input type="checkbox"/> No |
|------------------------------|-----------------------------|

c. Do you know, how to overcome this? _____

7. What is your staining protocol? Please indicate reagents and the incubation time as well as precautions necessary for successful RNA, DNA, and protein isolation after LMD:

8. How many cells do you dissect for downstream experiments? (indicate all that apply)
- | | |
|----------------------------------------|-------------------------------------------|
| <input type="checkbox"/> Single cell | <input type="checkbox"/> 101 – 1000 cells |
| <input type="checkbox"/> 2 - 50 cells | <input type="checkbox"/> Over 1000 cells |
| <input type="checkbox"/> 51 –100 cells | |
9. What do you analyze after LMD?
- DNA RNA Proteins Live cells
- Other (please specify) _____
10. If you isolate nucleic acids (A) or proteins (B), what analyses do you perform? (indicate all that apply)
- | | |
|-----------------------------------------------------------|-------------------------------------------------------|
| a. <input type="checkbox"/> PCR | b. <input type="checkbox"/> Western Blotting |
| <input type="checkbox"/> RT-PCR | <input type="checkbox"/> 2DGE |
| <input type="checkbox"/> Real-time RT-PCR | <input type="checkbox"/> HPLC |
| <input type="checkbox"/> Microarrays | <input type="checkbox"/> MALDI |
| <input type="checkbox"/> in vitro transcription | <input type="checkbox"/> SELDI |
| <input type="checkbox"/> Whole genome amplification (WGA) | <input type="checkbox"/> Other (please specify) _____ |
| <input type="checkbox"/> Other (please specify) _____ | |
11. Do you use a commercial kit, or “home-brewed” procedures? Please indicate the kit(s) or briefly describe your protocol(s):
- _____
- _____
- _____
12. Do you have any comments about Leica Microsystems’s LMD systems or service?
- _____
- _____
- _____

We would like to acknowledge your feedback with a gift. **Please tell us about you ...**

Title: _____

Name: _____

University/Company: _____

Institute/Department: _____

Address: _____

City: _____

ZipCode: _____

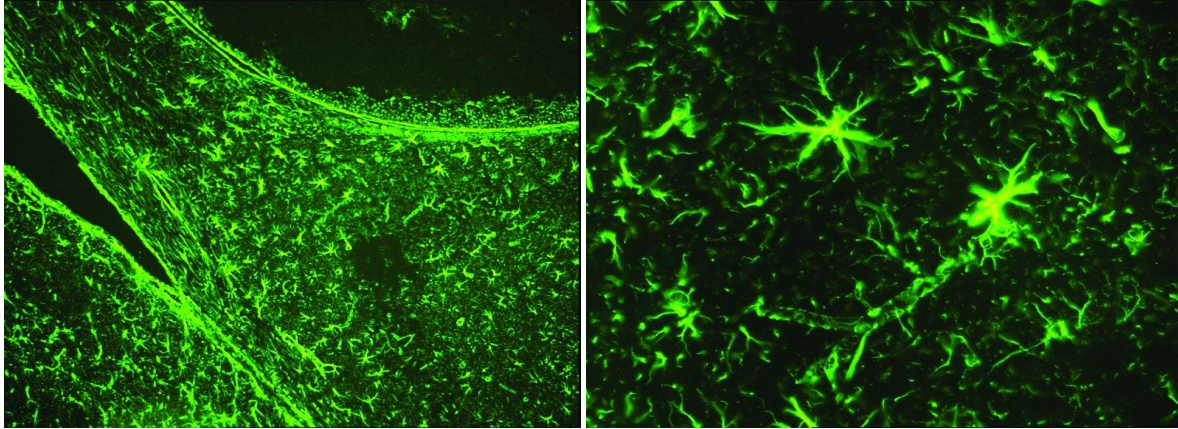
Country: _____

Phone: _____

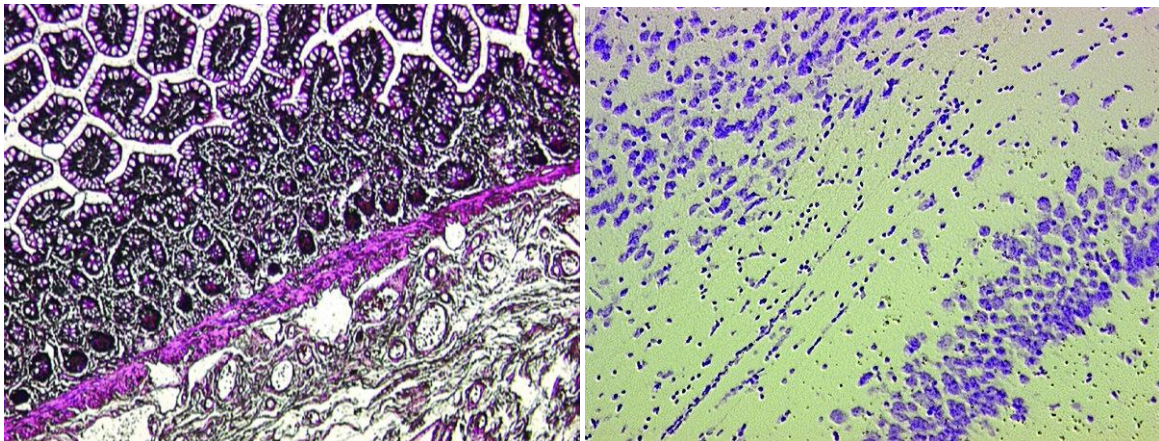
Fax: _____

Email: _____

Sample images of tissue sections

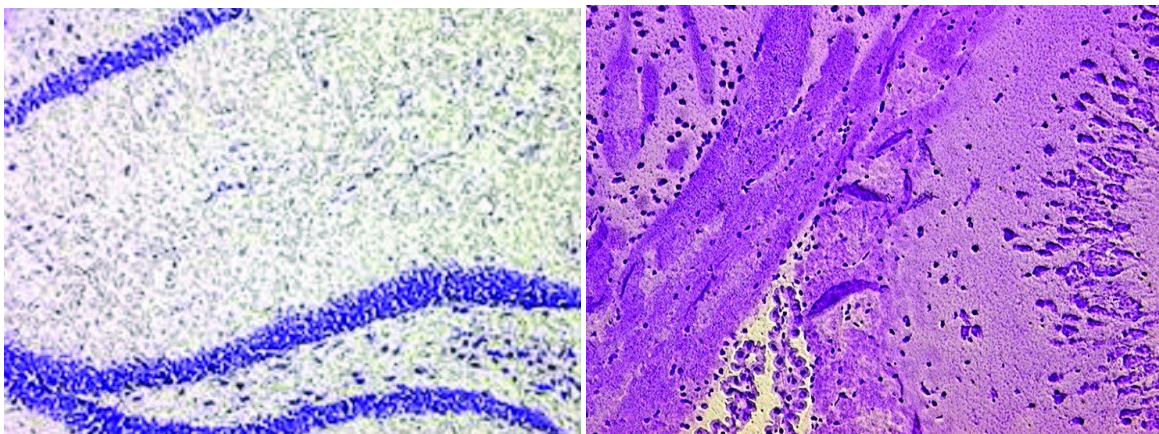


Cryo section 6 μm , Magn.: 40x, 400x, Mouse brain, corpus collosum, Astrocytes, IHC with GFAP



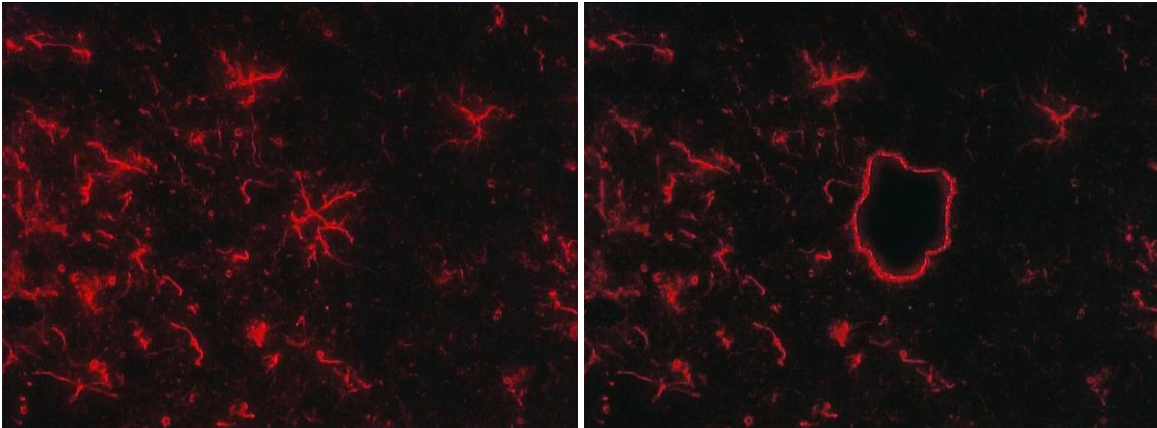
Left: Paraffin section 15 μm , Magn.: 100x, Rat colon, H&E staining

Right: Cryo section 6 μm , Magn.: 400x, Mouse brain, Thionin staining

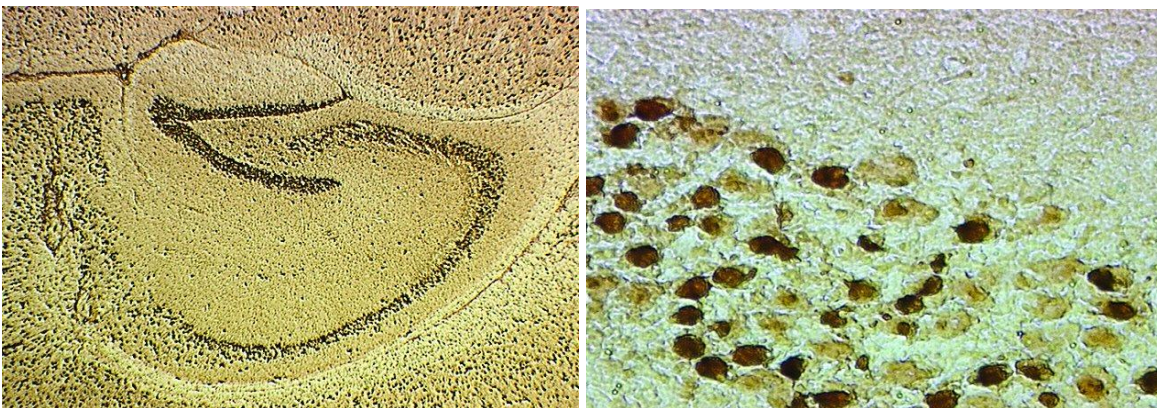


Left: Cryo section 6 μm , Magn.: 400x, Mouse brain, hippocampus, Toluidine blue staining

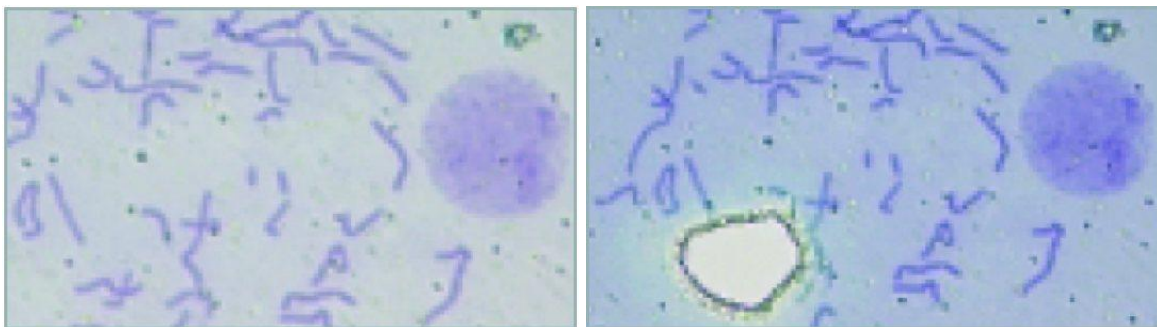
Right: Cryo section 6 μm , Magn.: 100x, Mouse brain, Cresyl violet staining



Cryo section, Mag.: 630x, Mouse brain, IHC with GFAP



Cryo section 6 μm , Magn.: 40x, 400x, Mouse brain, hippocampus, IHC (DAB as a substrate)



Methaphase chromosomes cut with 150x HCX Fluotar dry objective