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# LASER CAPTURE MICRODISSECTION (LCMD)

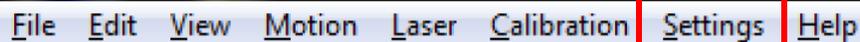
## USER GUIDE

Start-up Procedure .....	2
Shutdown Procedure .....	2
Adjusting Configuration and Settings .....	3
The Touchscreen Monitor .....	4
Loading Slides .....	4
Loading Capture Device .....	4
Moving Stage/Locating Sample .....	5
Camera Selection .....	5
Choosing Objective Lens .....	6
Adjusting Display.....	6
Transmitted Light and Reflected Light .....	7
Cutting under Brightfield Mode .....	7
Cutting under Fluorescence Mode .....	8
Cutting & Collection (for Membrane-mounted specimen) .....	8
Cutting & Collection (for Glass-mounted specimen) .....	9
Inspecting Sample Collected .....	9
Saving Images .....	10
Additional Useful Tools .....	10
Laser Functions/Tools .....	11
Laser focus and intensity/ Energy .....	11
Adjustment of Laser Power and Focus .....	12
Checking Position of Laser Marker .....	12
Elements List .....	12
Pricing .....	13
Online Scheduler .....	13

After-hours Access .....	13
BRC Imaging Contacts .....	13
Sample Preparation .....	14

### Start-Up Procedure:

1. If you plan to work under fluorescence mode, turn on the power of the HXP Illuminator located in front of the PALM Microbeam tower.
2. Flip on the power at the two power strips located underneath the table. This turns on the whole system, including the halogen lamp, microscope, and the lasers.
3. Turn on the computer (if not already on) and log on with your **Cornell net ID and password**.
4. Double click the software – PalmRobo v 4.6.
5. In the main menu of the software, click "Settings" and choose Load Settings from the dropdown menu (if you had previously saved settings). If no previously established settings have been saved and loaded, default settings will be loaded automatically.



The image shows a screenshot of the PALMRobo software's menu bar. The menu items are: File, Edit, View, Motion, Laser, Calibration, Settings, and Help. The 'Settings' menu item is highlighted with a red rectangular box.

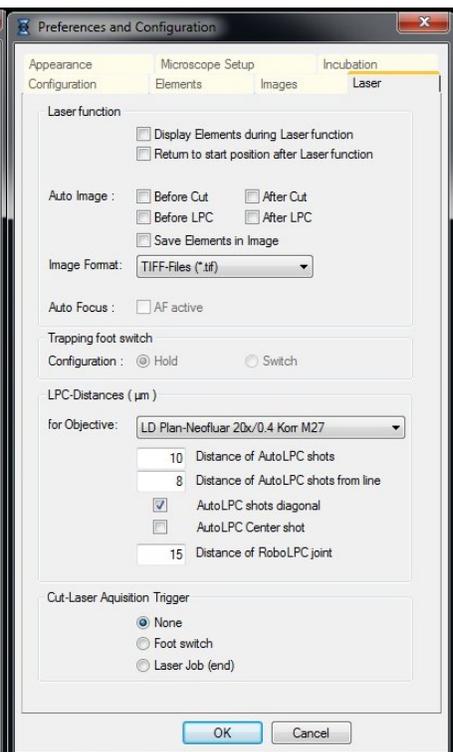
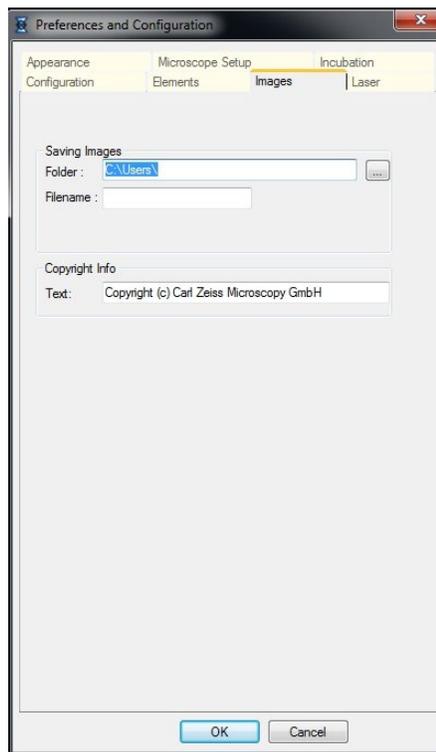
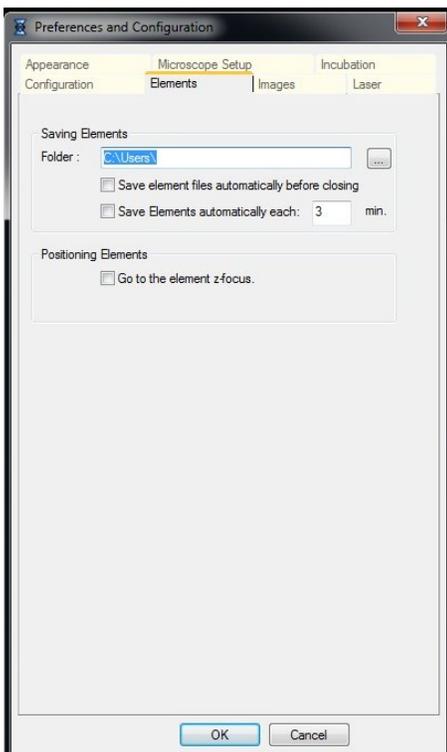
### Shutdown Procedure:

1. Exit the PALMRobo software. When the program is closed, a prompt appears as to whether you want to save your settings or your elements for later use.
2. Copy your files to the Imaging LabShare so that you can access your data from your lab/office.
3. If somebody is signed up in the next hour, please leave the whole system on (just need to log off the computer); Log your usage of the system in the log sheet and proceed to step 7.
4. If no one else is signed up to use the system, exit Windows and shut down the computer.
5. Turn off power strips.
6. Cover microscope.
7. If you got slides/caps from the facility for your imaging session, please indicate the number under the Materials Inventory sheet (hanging just above the table).

## Adjusting Configuration Settings:

Under Settings – Preferences and Configuration, check the following tabs:

1. Elements: If you would like to automatically save your Elements (ROI area, position, laser functions, etc), make sure you choose the folder in which to save the list.
2. Images: Auto saving of images can be activated by choosing the folder in which to save them and providing a base file name.
3. Laser: To auto save images at various time points of the microdissection procedure (before/after cut, before/after LPC), check the indicated boxes.



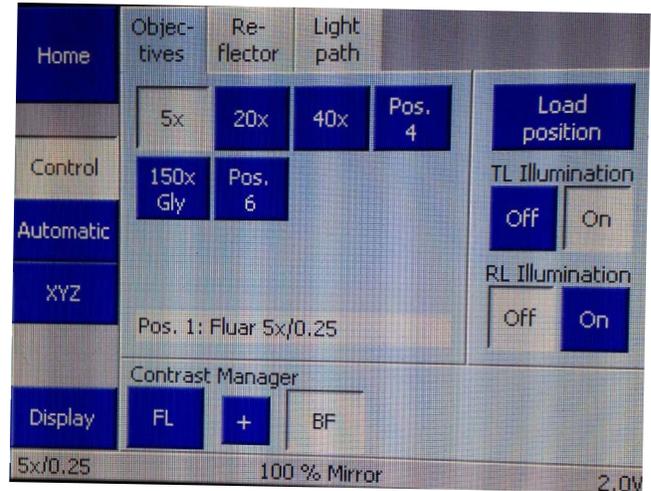
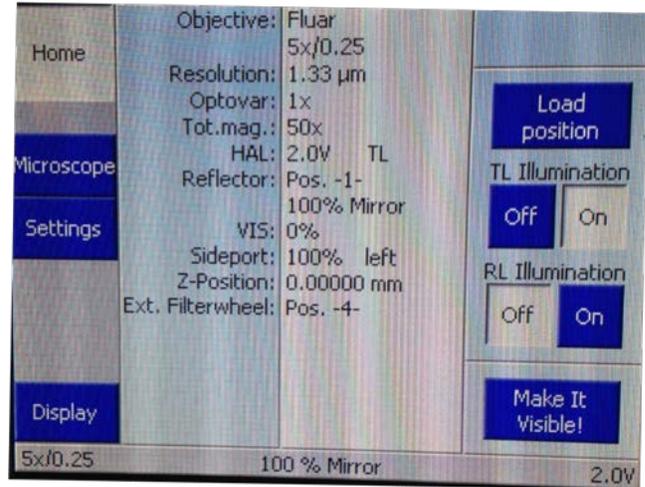
## The Touchscreen Monitor:

The microscope can be controlled via the touchscreen monitor located at the right side of the microscope.

The objectives can be selected from the monitor (Control -> Objectives). Objective specifications such as magnification, NA and resolution are provided under the Home option.

Transmitted light can be turned on and off via the TL Illumination buttons.

The excitation laser for fluorescence can be switched on/off via the RL Illumination buttons. *Take note that RL Illumination will only appear in the monitor if you had previously switched on the HXP Illuminator.*



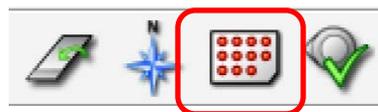
## Loading Slides:

1. To begin loading your slides, click on the "Loadposition" icon. This will move the stage out of the microscope's light path to allow for easier loading/unloading.
2. After your slides have been loaded, select "Return to the working area" in the open window to return the slide to the microscope's light path.



## Loading Capture Device:

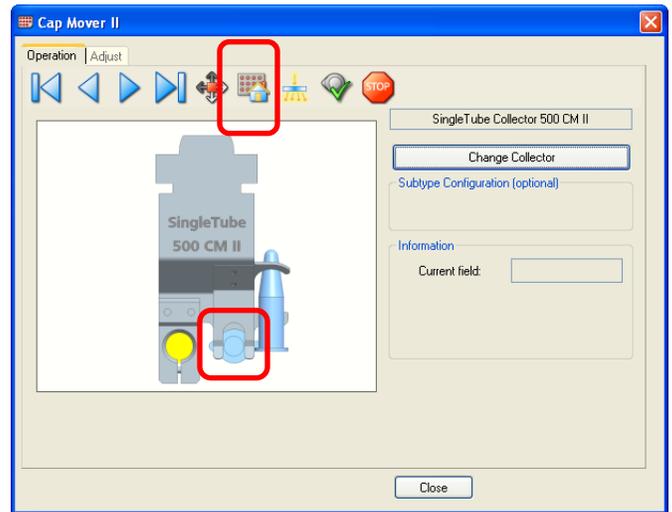
1. To load your capture device (e.g., adhesive-cap centrifuge tubes), select the "Show the Collection Device" dialog.



In the Collection Device dialog (Cap Mover): Under "Operation", you have the option to "Change Collector". Click on this and Select Collector Type if needed.

- By clicking on the cap you want to catapult into, the Robomover will place this cap in the microscope's light path. To return cap to loading position, click on the home icon. Close Cap Mover window by clicking Close or clicking anywhere on your image.

*If you need to adjust the Moving Height and/or Working Height of cap, click on **Adjust** tab, then use the up/down arrows on the left to adjust. Click on Set in corresponding Moving Height or Working Height to adjust new position. Click on GoTo if you would like to check the current moving/working height of cap.*



### Moving Stage/Locating Sample:

There are various ways of moving the stage to locate your sample:

- mouse (Stage Mode and Scroll)
  - In Stage Mode (F7), you control the movement of the stage synchronously using the mouse. Each mouse action is translated into a movement of the stage.
- white scroll arrow bars above, below and to the left/right of the microscope image
- arrow keys on the keyboard
- joystick
- 'Navigator' function 
- 'Centering' tool in the graphic toolbar and clicking a point that is desired to be centered

The sample can be viewed through the monitor, or the eyepieces (larger field of view).

### Camera Selection:

Under Display – Camera, choose the camera you want to use from the dropdown menu.

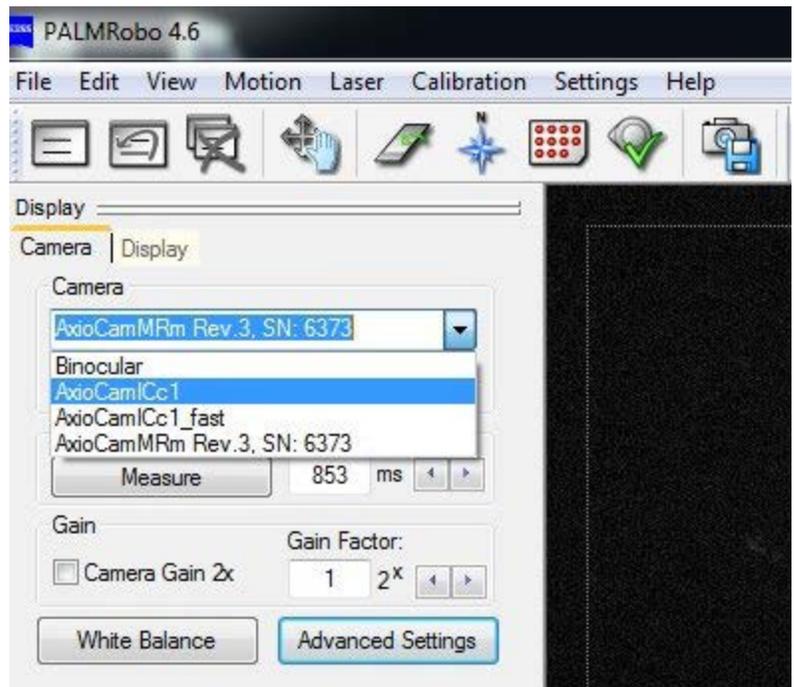
*AxioCamICc1*: recommended for brightfield/phase contrast images; for stained/unstained tissue

- 1.4 megapixel resolution
- Color

*AxioCamICc1\_fast*: faster image acquisition, but lower resolution

*AxioCamMRm Rev 3, SN 6373*: recommended for fluorescent samples

- monochromatic

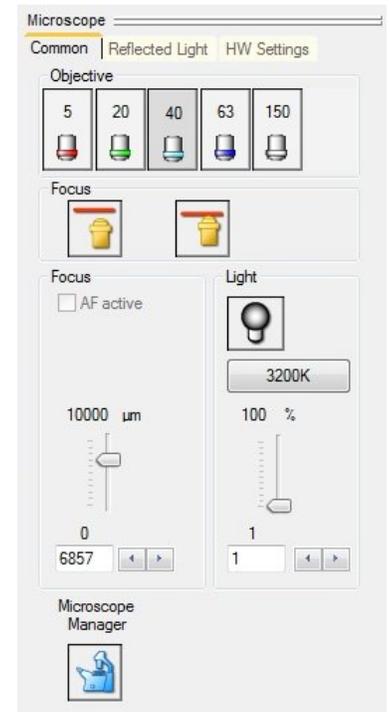


## Choosing Objective Lens:

You can change the **objective lens** by clicking on the icon displayed in the main display window of the software (Objective under Common Tab of Microscope) or using the TouchScreen monitor.

The following objectives are available:

	Magnification	N.A.	Working Distance (mm)	Coverglass (mm)
Fluar	5x (air)	0.25	12.5	0.17
LD Plan-NeoFluar	20x (air)	0.4	7.9 at coverglass 0.75	0-1.5
LD Plan-NeoFluar	40x (air)	0.6	2.9 at coverglass 0.75	0-1.5
Plan-Apochromat (DIC)	150x (gly*)	1.35	0.17 at coverglass 0.16	0.14-0.18



\* You will need to add a drop of glycerin on the 150x objective lens before putting your sample on the stage

\*\* A 63x objective is currently available for demo. You can test out its functionality if you wish.

## Adjusting Display:

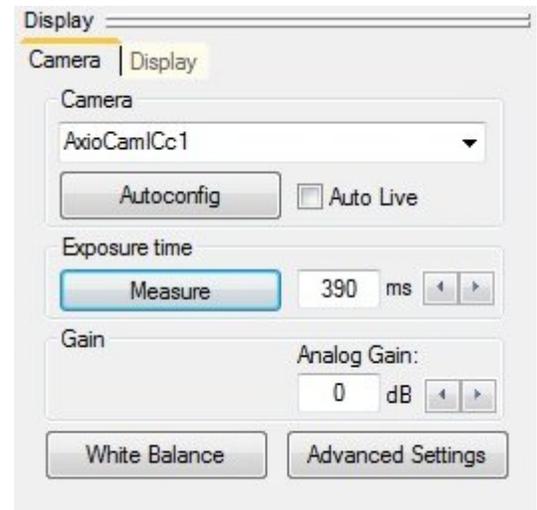
**Autoconfig:** Automatic adjustment of exposure time, white balance, brightness, and contrast.

**Auto Live:** Automatic and continuous adjustment of exposure time of the live image, e.g., if the objective is changed or while a sample is being moved. *If you change the exposure time manually, the Auto Live function switches off automatically.*

**Measure:** Automatic adjustment of the exposure time. If you are not satisfied with the result, you can set other values manually using the arrows.

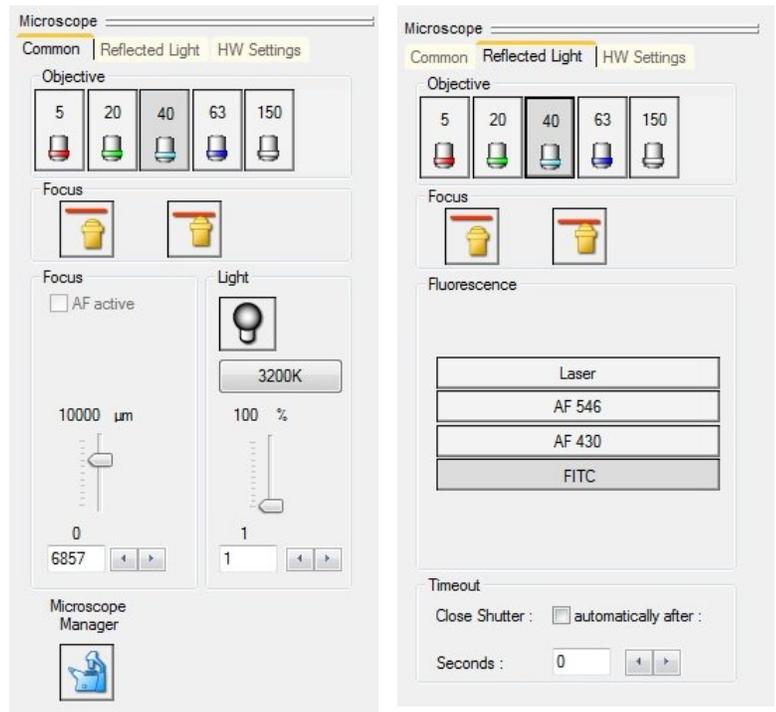
**White Balance:** Automatic white balance. You can adjust the white balance automatically using any microscope image. It is not necessary for the camera to see a completely empty, bright image.

**Gain:** The Gain is usually adjusted only for very dim fluorescence images.



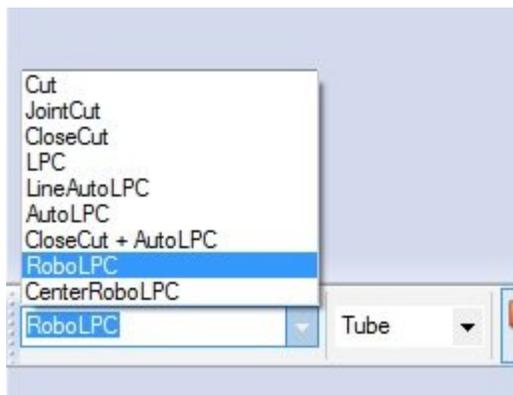
## Transmitted Light & Reflected Light

1. To view brightfield images on screen or on eyepieces, turn on transmitted light (TL on Touchscreen monitor or click on light icon under Common tab of Microscope), if not already on.
2. If you want to look at fluorescence, turn on reflected light illumination (RL on Touchscreen monitor).
3. For fluorescence, make sure you click on the Reflected Light tab under Microscope in the main display. Click on the filter tab that is compatible with your fluorescence label.
4. Use focus adjustment to locate sample.  
*To adjust the microscope's **focus**, you can either manually turn the coarse and fine adjustment knobs, change the values in the area under "Focus" or use the slider located under Focus in the Common tab.*



## Cutting under Brightfield Mode (for stained/unstained tissue):

1. Choose the AxioCamICc1 camera.
2. Make sure you are under the Common tab and lamp (TL) is turned ON
3. Select cutting function from the Laser Tools located at the bottom left of the main display window.



4. Proceed to selecting the regions of your sample that you want to cut. Use the drawing tools located at the bottom of the main display and draw ROI(s).



5. Click on the Activate Laser button to start cutting selected ROI(s).



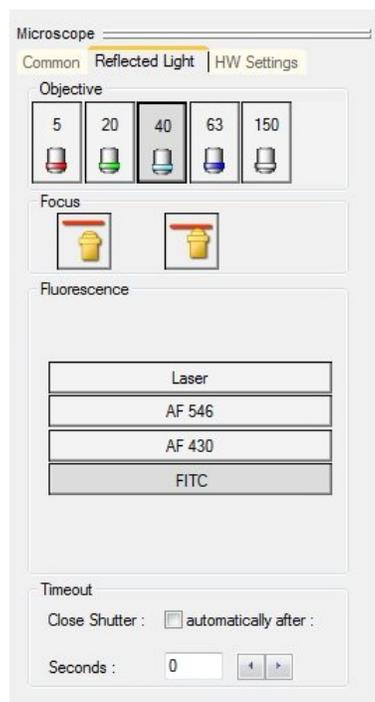
6. Adjust laser energy and/or focus if necessary.

## Cutting under Fluorescence Mode (for fluorescent samples):

1. Choose the AxioCamMRm camera. Note that this camera is monochromatic, so any fluorescence from your sample will be displayed in gray LUT
2. Go to the Reflected Light tab under Microscope in main display window.
3. Turn off TL lamp and turn on the RL Illumination in the touchscreen monitor.
4. Click on the filter tab applicable to your sample. Note that if you have more than 1 fluorophore, you can only view 1 color at a time.

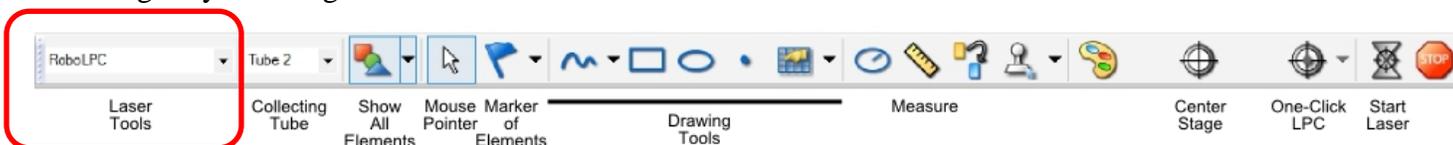
There are currently 4 filter positions:

- a. Laser: no filter (for Brightfield)
  - b. AF546: filter for red fluorescence (Ex 510-560, Em 590-∞)
  - c. AF430: filter for blue fluorescence (Ex 390-420, Em 450-∞)
  - d. FITC: filter for green fluorescence (Ex 455-495, Em 505-555)
5. Adjust focus to check fluorescence of your sample or view fluorescence through the microscope binoculars.
  6. Draw ROIs on fluorescent areas you would like to cut
  7. When ready to cut, switch filter tab from your chosen filter to Laser. This switches light from fluorescence excitation laser to the 355 nm laser.
  8. You can only cut your sample with the 355 nm laser, thus you will be viewing and cutting your ROIs under brightfield mode.



## Cutting & Collection (for Membrane-mounted specimen):

1. Begin by selecting "RoboLPC from Laser Tools or Functions.



2. Select where your sample will be catapulted into (dropdown menu located to the right of laser functions: (Manual- no collection; tube – in collection tube).
3. Select a drawing tool and encircle a feature/ features of interest. The RoboLPC feature will automatically leave a small connecting “bridge” or “LPC joint,” (marked by a small blue circle) which ensures the area will not float away after cutting. The system then immediately catapults the entire selected area(s) with a single LPC shot to this bridge.
4. Check Element List if you have the correct laser function and collector.



*If multiple regions are defined, the regions or elements can be easily managed in the Element list. All regions are organized in the list. Each element can be selected (and highlighted) by clicking on that row. Double-clicking on one element opens a window that laser operations can be further modified for*

*function type, objective, collection tube, etc. Operations on multiple elements (regions) can be executed if those elements are selected while holding down the 'Shift' key on the keyboard.*

- The selected elements can be deleted by clicking the icon Element List → delete selected in Element List, or by simply using 'delete' key on the keyboard; In the icon toolbar, clicking the “delete all” icon  will delete all elements (regions), while clicking on the “delete last element” icon  deletes only the last element (ROI) drawn.
- By clicking "Start Laser," the system cuts around and catapults the designated ROI/ element, then automatically proceeds to cut around and catapult another ROI.

### Cutting & Collection (for Glass-mounted specimen):

- To capture large areas from glass mounted tissue use the AutoLPC function or “CloseCut AutoLPC” under Laser Tools. Choose collecting tube.
- You can predefine an area, outlining it with the graphic tool and let the PALM RoboSoftware automatically perform the catapulting. Once you outline your ROI, the AutoLPC feature will automatically leave small dots marking positions where the laser will shoot like gun fire. As there is no supporting membrane, the cells or the tissue will arrive as flakes within the microfuge cap, which are, however, perfectly suitable for subsequent genetic or proteomic analysis.
- The number of dots and spacing between them and the enclosure can be adjusted by clicking on the Paint palette icon at the bottom of the display.



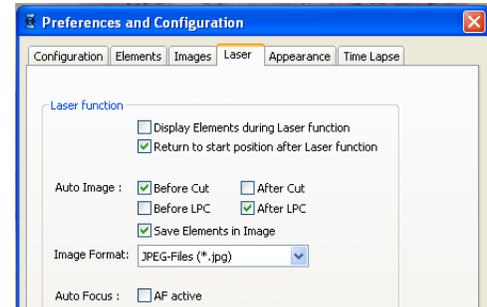
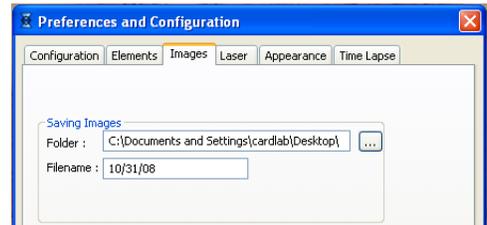
### Inspecting Sample Collected:

- To find the catapulted sample in the adhesive cap, click the "CapCheck" icon (cap icon with a green check mark). 
- Pan around using the joystick and adjust the focus to locate catapulted items on the cap of the collection tube.
- You can save an image of the cap by clicking File > Save Image.
- Select the CapCheck icon (cap icon with a red cross mark) again to return the robomover to its previous collection position.

**Important:** *If you are using the 150x gly objective to cut, make sure you first change to a lower magnification, long working distance objective (5x, 20x) BEFORE you inspect the cap. Lower focus so that the oil on the slide does not touch the objective.*

## Saving Images:

1. Click the "Save Image" icon and choose the desired filepath. Alternatively, you can click File > Save Image.
2. Set up Auto saving: click Settings on the main menu. Then choose "Preferences and Configuration". Select the "Images" tab and choose a folder that you would like your images to save to. Then select the "Laser" tab and check which boxes that apply to the images you wish to automatically save.



## Additional Useful Tools:

The *Ruler* tool (bottom part of screen) is very useful for making more accurate measurements of tissue features.

The *Flag* tool is useful if you want to identify several features and then be able to locate them immediately. You can enter text to identify each area, and by selecting that flag from the elements list and clicking "Go," the Robomover will immediately take you back to that position.



## **Laser Tools/Functions:**

**Cut:** laser cuts your sample along pre-drawn lines so that a clearly split line between required and non-required material is generated.

**Close & Cut:** laser also cuts your sample along defined lines; the start and end dot of a line element are connected here by a straight cut line

**Joint Cut:** similar to Close & Cut, but start and end dot remains a joint. Cut area remains fixed within the tissue and can be catapulted later by a single laser pulse.

**LPC:** only the cells from your sample upon which you have drawn a "Dot" element are catapulted into the collecting cap.

**LineAutoLPC:** A line-shaped structure is catapulted into your collection vessel; line is not catapulted in one piece, but with several laser pulses.

**AutoLPC:** contents of a defined area of glass-mounted samples are catapulted into your cap with several laser pulses.

**RoboLPC:** defined structures are cut out and catapulted intact into the cap. This can only be used when there is an LPC membrane between your sample and the slide.

**Center RoboLPC:** defined structures are cut out and catapulted intact into the cap in a single operation. The laser pulse is placed in the center of the figure for catapulting. It can only be used with membrane-coated samples.

## **Laser focus and Intensity/Energy:**

Laser focus and intensity: most important factors for successfully cutting and catapulting regions of interest. Any changes to these settings will only apply to the current objective lens being used. Therefore, if you will use multiple magnifications for cutting, you must determine and save the optimal settings for the different lenses.

- \* Higher magnification objectives: “finer” cutting
- \* Slower cutting speeds - use lower laser energy
- \* LPC focus: always just a bit lower than the tissue itself
- \* LPC energy: always higher than cut energy
- \* Delta – difference between cutting and lifting energy

## Adjustment of Laser Power and Focus:

It is highly recommended that you test the power AND focus of the laser for cutting and/or catapulting before you start working on your interested regions. In general, the catapulting power is higher than the cutting power and the laser has to be defocused for catapulting.

1. Use graphic tool to draw a 'Z' line in a homogenous area.
2. In 'Speed' tools (left side of display), select “Cutting” and a low speed.
3. To adjust laser power: Start cutting and adjust the laser power by clicking on the arrows at the bottom of the “Energy” column. Select the minimal laser power that generates a clear and fine cut.
4. To adjust laser focus: start cutting and adjust the laser focus by clicking on the arrows at the bottom of the “Focus” column. Use the focus that results in a clear and fine cut.
5. Repeat the above steps if it is necessary.

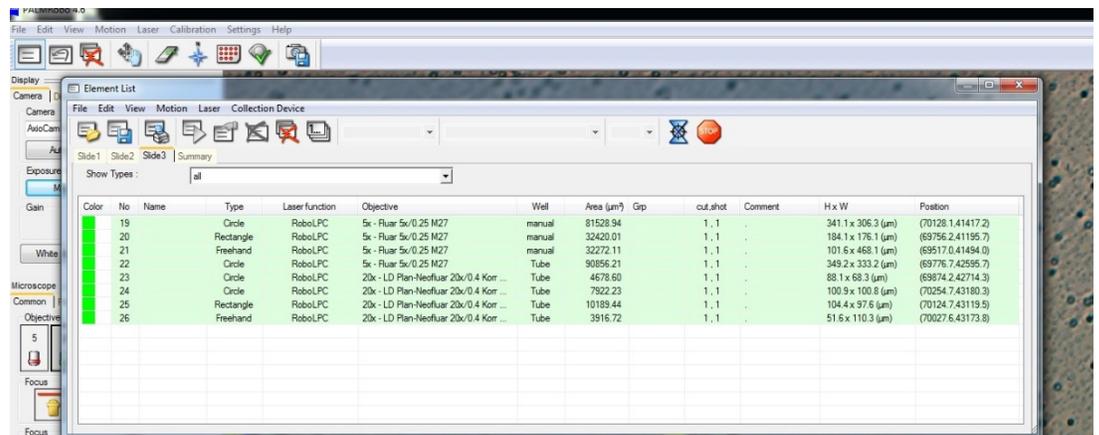
## Checking position of Laser Marker:

Before you start cutting, you have to make sure that the position of the laser marker is aligned with the actual position of the laser focus. Draw an ROI on an empty membrane area; cut. The ROI outline should align with the cut portion. If the positions vary, do the following:

1. Move stage to an area of your sample that you do not require for work (eg. empty membrane area).
2. Switch to Stage Mode (F7).
3. Release laser with a single shot application of pressure to the footswitch.
4. Check result. You should see a small, almost circular area where material has been removed. *Increase laser energy if you don't see anything.*
5. Exit Stage Mode or click on F7 again.
6. To set position of laser marker: In Calibration menu, click on “Position Cut Laser”.
7. Click in the center of the laser focus for the laser beam.
8. Draw ROI and check result.

## Element List:

The element list defines ROI parameters as well as laser/cutting functions used.



Color	No	Name	Type	Laser function	Objective	Well	Area (µm <sup>2</sup> )	Gp	cut_shot	Comment	H x W	Position
	19	Circle	RoboLPC	5x - Fluor 5x/0.25 M27	manual	81528.94			1, 1		341.1 x 306.3 (µm)	(70128.1, 41417.2)
	20	Rectangle	RoboLPC	5x - Fluor 5x/0.25 M27	manual	32420.01			1, 1		184.1 x 176.1 (µm)	(69756.2, 41195.7)
	21	Freehand	RoboLPC	5x - Fluor 5x/0.25 M27	manual	32272.11			1, 1		101.5 x 468.1 (µm)	(69517.0, 41494.0)
	22	Circle	RoboLPC	5x - Fluor 5x/0.25 M27	Tube	90856.21			1, 1		349.2 x 333.2 (µm)	(69776.7, 42595.7)
	23	Circle	RoboLPC	20x - LD Plan-Neofluar 20x/0.4 Kor ...	Tube	4678.60			1, 1		88.1 x 68.3 (µm)	(69674.2, 42714.3)
	24	Circle	RoboLPC	20x - LD Plan-Neofluar 20x/0.4 Kor ...	Tube	7822.23			1, 1		100.9 x 100.8 (µm)	(70254.7, 43180.3)
	25	Rectangle	RoboLPC	20x - LD Plan-Neofluar 20x/0.4 Kor ...	Tube	10189.44			1, 1		104.4 x 97.6 (µm)	(70124.7, 43119.5)
	26	Freehand	RoboLPC	20x - LD Plan-Neofluar 20x/0.4 Kor ...	Tube	3916.72			1, 1		51.6 x 110.3 (µm)	(70027.6, 43173.8)

### **Pricing:**

\$40/hour – peak and off-peak hours

\$90/hour – training fee

#### Membrane Slides and Adhesive Caps:

PEN 1.0 membrane slides - \$6.75/slide

PEN NF 1.0 membrane slides - \$8.00/slide

PEN 0.17 membrane slides - \$9.00/slide

Adhesive cap 500 opaque - \$6.50/cap

\*\*\* Please fill in the Materials Inventory Sheet (on microscope desk) if you take slides and/or caps from our facility

### **Online Scheduler:**

<https://cores.lifesciences.cornell.edu/userdev/>

### **After-Hours Access:**

You must attend the Weill Hall Orientation session to get access to Weill Hall and the basement.

<http://blogs.cornell.edu/whfs/access-to-weill/>

Download the Weill Access Request Form and have me sign under Section 2. You will need access to the BRC Imaging Facility.

For further details on Orientation, please contact [Amy Layton](#) (ael67), 107 Weill Hall

### **BRC Imaging Contacts:**

Johanna Dela Cruz (jmd244) – B31 Weill Hall

Rebecca Williams (rw36) – B35 Weill Hall

Carol Bayles (cjb4) – B46 Weill Hall

## SAMPLE PREPARATION FOR LCMD

- 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.
- Preparing RNA from fixed paraffin sections is difficult due to RNA fragmentation by formalin, which is the common fixative used for preparing paraffin sections.
- Samples for LCMD can also be obtained from archived slides.

*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.*

### Slides

The tissue sections are usually placed on a *UV-absorbing membrane* prior to laser microdissection. 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) is catapulted into the cap of the microcentrifuge tube.

#### I. Sterilizing Membrane Slides

Slides may be sterilized by autoclaving or UV-treatment. However, RNase-free certified slides can also be purchased.

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. Alternatively, regular Membrane Slides can be pretreated with reagents such as RNaseZap or RNase Away.

##### 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 to 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 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.

*Note: Sterilizing methods can be combined and should be done shortly before Membrane Slides are used.*

### **Workflows for Preparing Frozen and Paraffin Sections:**

Frozen sections – fast method preserving DNA, RNA & proteins, but the morphology might be diminished.

Surgery → embed tissue in freezing medium → flash-freeze → section in cryostat → stain → LCMD → isolate DNA, RNA or proteins → downstream analysis

Paraffin sections – time consuming, but morphology is superb; limitations concerning high molecular weight RNA, DNA, and native proteins may occur.

Surgery → tissue fixation → paraffin infiltration and preparation of paraffin block → section with microtome → stain → LCMD → isolate DNA, RNA or proteins → downstream analysis

## **II. 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.

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

1. Dip in 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 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 plastic ware. Alternatively, reagents for RNases decontamination can be used to clean the gel tray, spatulas, slides, and other surfaces.*

### 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.
- Tissue or organs should be flash-frozen (snap-frozen) after surgery followed by cryosectioning.
- Sections should be stained within a very short time, not exceeding in total 30 minutes.
- 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.
- 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.

#### **A. Flash-freezing Methods**

Tissues are embedded in a freezing medium such as 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-methylbutane 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.

## B. Sectioning

### Preparing Cryo Sections

Cryo blocks can be directly sectioned (recommended for RNA preparation) or stored at  $-80^{\circ}\text{C}$ . The temperature of the cryo blocks should be adjusted to  $-20^{\circ}\text{C}$  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  $\mu\text{m}$  sections and immediately place them on the slides for LCMD

*Important: If you are using Frame Slides, the Frame Support 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 PEN- or PET-membranes.*

Typical sectioning temperatures are shown below. 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 LCMD is performed on the same day. Alternatively, they may be stored in sealed slide boxes with a desiccant at  $-80^{\circ}\text{C}$  until needed.

*Important: Dry slides at room temperature for 15 minutes using a drying chamber with desiccant or at  $40^{\circ}\text{C}$  for 10 minutes. This is a critical step for successful LCMD. Sample slides should be stained just prior to microdissection.*

Knife Edge Temperature for Cryo Sectioning:

<i>Knife Temperature</i>	<i>Tissues</i>
-10 to -15 $^{\circ}\text{C}$	adrenals, bone marrow, brain, cartilage, spleen or bloody tissue, testicular
-15 to -17 $^{\circ}\text{C}$	bladder
-15 to -18 $^{\circ}\text{C}$	breast - less fatty, cervix, intestine, liver, thyroid
-15 to -20 $^{\circ}\text{C}$	kidney, lung, pancreas, prostate, ovary, rectal, skin without fat, uterus
-18 to -22 $^{\circ}\text{C}$	heart and vessel
-20 to -25 $^{\circ}\text{C}$	muscular
-25 to -30 $^{\circ}\text{C}$	skin with fat, breast - fatty

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

### A. 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](http://www.dcs-diagnostics.de)).

### B. 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.

1. 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.
2. The tissue block can be stored at room temperature or below. For better cutting results with the microtome, cooling the paraffin block down to  $-20^{\circ}\text{C}$  is recommended.
3. 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  $\mu\text{m}$  sections
  - Place the paraffin ribbons on the surface of the water in a water bath at  $37^{\circ}\text{C}$ – $0^{\circ}\text{C}$ ; 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.
4. 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

#### **IV. Preparation of archived slides for LCMD**

There is a possibility to process regular archived slides to obtain the tissue for 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  $40^{\circ}\text{C}$  up to  $70^{\circ}\text{C}$  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  $37^{\circ}\text{C}$  or for 2 h at  $70^{\circ}\text{C}$ . “Quick Mount” must remain pliable.
  3. Trim excess “Quick Mount” around tissue section.
  4. Place in a distilled water bath at  $50$ – $60^{\circ}\text{C}$  for around 1 h.
  5. Use a fine razor to lift the section and peel off.
- c. Transfer tissue onto the Membrane Slide
  1. Lay “Quick Mount” with tissue onto membrane.
  2. Flatten lightly with finger tip pressure.
  3. Place in oven in a horizontal position at  $56^{\circ}\text{C}$  for 3 h or until “Quick Mount” is completely cured.

- d. Removal of “Quick Mount” from the 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 Membrane Slides for laser microdissection, than working with archived slides. However, direct ablation of tissue without coverslip from glass is also possible, but not comparable regarding the much better results of downstream analysis with laser microdissected samples derived from Membrane Slides.*

## V. Typical Staining Protocols

Before staining, the paraffin should be removed from the tissue section. 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. This is the last step prior to staining. 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.

### A. 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 sub-segment RNA preparation.

You will need:

- Toluidine blue
- DEPC-treated water is used for subsequent RNA preparation, otherwise regular distilled water can be used
- 75% ethanol
- 0.22  $\mu\text{m}$  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

### B. 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

- Eosin (Eosin Y solution, alcoholic with phloxine B)
- 0,1 % NH<sub>4</sub>OH (blueing reagent): 200 µl NH<sub>4</sub>OH 100% + 200 ml H<sub>2</sub>O or Scot's tape water substitute

#### Procedure

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

1. Distilled H<sub>2</sub>O 30 seconds
2. Hematoxylin 1 minute
3. H<sub>2</sub>O 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

### C. *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

#### ***D. 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 H<sub>2</sub>O 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

#### ***E. 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

### **F. 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
- 75%, 95%, 100% ethanol
- 0.22 µm sterile syringe filter
- 0.5% Thionin staining solution
  - Dissolve 1.36 g of sodium acetate (anhydrous) or alternatively 2.26 g sodium acetate trihydrate) in 100 ml H<sub>2</sub>O and fill up to 250 ml
  - Add 0.5 g thionin to 100 ml of the sodium acetate solution.
  - 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

### **G. 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  $-70\text{ }^{\circ}\text{C}$  until needed.
12. Cut the sections on a cryostat (usually  $4\text{--}10\text{ }\mu\text{m}$ ) and thaw the mountant.
13. Wash away the mountant for LMD.

### Average Number of Cells used for LCMD

<i>Analyzed Molecules</i>	<i>Average number of cells</i>
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% or 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. Large tissue areas of 500 $\mu\text{m}^2$ may contain enough cells.

**Average section thickness** used for LCMD: 4–20  $\mu\text{m}$ .

In some cases, where more material is needed and tissue morphology is sufficient, thicker sections of 15–30  $\mu\text{m}$  can be used for LMD. For plant Research, even unfixed fresh tissues above 100  $\mu\text{m}$  can be used.

*Solid state laser 355 nm Advantages:*

1. No harm to DNA, RNA and proteins
2. Long lifetime
3. High precision cutting

**Cutting lines:**

- 10x - ~5  $\mu\text{m}$
- 20x - ~2.5  $\mu\text{m}$
- 40x - ~1.3  $\mu\text{m}$
- 100x - ~700 nm