Laser Capture for "Dummies"

Histological specimen from a membrane-coated slide

Cytospin from a glass slide

Chromosomes from a membrane-coated slide

Living cells from a culture dish

Pipetting

Incubation

Centrifugation

DNA

RNA
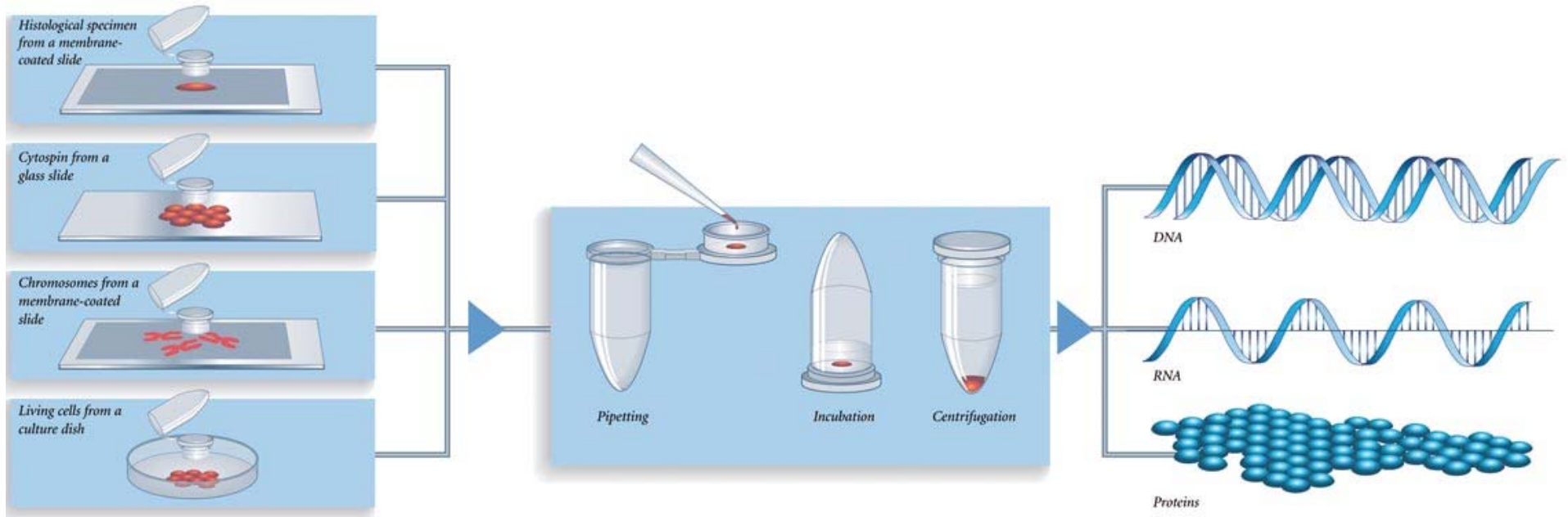
Proteins

Background & Preparation

Zeiss' PALM Microlaser system has many applications, but its power lies in the selective harvesting of multicellular regions of interest (ROI), single cells, or even subcellular structures from histological sections, cytospins etc. The system's UV laser (355 nm) is used to both cut out these ROIs and "catapult" them into the adhesive cap of a microfuge tube with a de-focused laser pulse. The laser has an extremely high energy density at the small focal point only (focus < 1 µm), and there is no heat transfer to the adjacent material hence the term "cold ablation." Also, the UV laser does NOT affect DNA, RNA, or protein in surrounding areas as the 355 nm wavelength of the laser falls short of the absorption spectra for these molecules. To facilitate cutting and catapulting, PALM offers microscope slides covered with a polyethylene napthalate (PEN)-membrane. This membrane acts as a stabilizing scaffold during cutting and allows larger areas to be catapulted in a single de-focused laser shot. It is still possible, although more difficult, to cut and catapult from glass slides.
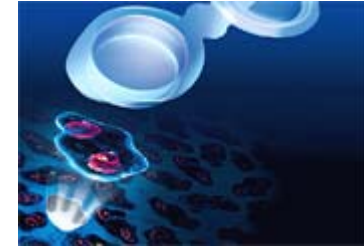
Mouse Tissues	Fold Increase Relative to Brain	RNase Characterization
Pancreas	181,000	**Group A: Very high RNase levels**
Spleen	10,600	Primarily RNase A enzymes*
Lung	5,300	Recommended RNA Isolation Kit: TRI Reagent® or MagMAX™ for Microarrays
Liver	64	**Group B: Lower RNase levels**
Thymus	16	A more balanced mixture of RNase A and other cellular RNase activities*
Kidney	8	Recommended RNA Isolation Kit: MELT™ Total RNA Isolation System
Heart	2	
Brain	1	*Based on inhibition by EDTA and RNase Inhibitor Protein

If your experimental design involves extracting RNA, there are several important factors you should be aware of before beginning a laser microdissection and pressure catapulting (LMPC) experiment. Due to the presence of ribonucleases (RNases), both endogenous to the tissue and from environmental surfaces, you must be very careful in protecting the integrity of your sample's RNA. The figure to the left (courtesy Ambion) shows the relative amounts of endogenous RNase activity in different tissue types. As you might guess, higher RNase levels mean your samples are ever more fragile. As a general rule of thumb, the colder and dryer your slides are, the better off your RNA will be. Therefore, if you are cutting fresh-frozen sections in OCT, it is imperative that you keep the slides cold and begin any staining steps quickly. Before microdissection, OCT *must* be removed. It can easily be washed away with DEPC-treated water or perhaps even 70% EtOH if you don't have much OCT in the sample. Keep staining procedures brief, keep all reagents ICE cold, and *avoid any prolonged aqueous phase steps* as they will allow RNase activity. As long as you fully dehydrate your samples in the final staining steps, your RNA should be stable up to a few hours while you're working on the LCM platform. For example, ending a staining protocol with a quick increasing ethanol series with a longer dehydration in 100% ethanol should keep RNase activity to a minimum; DO NOT, however, use xylenes as they will cause the tissue to become too brittle and cut poorly.

Membrane slides and any glass surface that will come in contact with your sample should be heat-treated at 180 °C for at least 4 hours before use. Alternatively, use RNase ZAP (Ambion) to treat any contact surface in order to inactivate RNases. Do NOT breathe heavily on your samples, and for very sensitive RNA extractions, do NOT use autoclaved pipette tips as water vapor may contain RNases. To overcome the hydrophobic nature of the membrane, UV-treat the slides for 30 minutes. This will also sterilize the slides and destroy potentially contaminating nucleic acids.

Analysis of RNA Integrity. When capturing very small ROIs with this laser system, the small amount of RNA extracted dictates very small RNA concentrations, perhaps only picograms/µl. These concentrations are much too small for either the Nanodrop system to measure meaningful A260/280 or 260/230 values, or even the Agilent Bioanalyzer to measure RNA Integrity Numbers (RIN) based on 28S and 18S ribosomal RNA peaks. To get around this problem, evaluate the RNA integrity of much larger samples both before staining and after you have completed your LMPC procedure. The pre-staining sample will serve as a baseline for RNA integrity and will validate attempts to characterize expression profiles assuming the RNA is intact. The post-catapulting sample can be compared to your baseline RNA integrity. If minimal degradation has occurred between these gross samples, then it is safe to assume that the RNA from your small ROIs is suitable for further experiments!
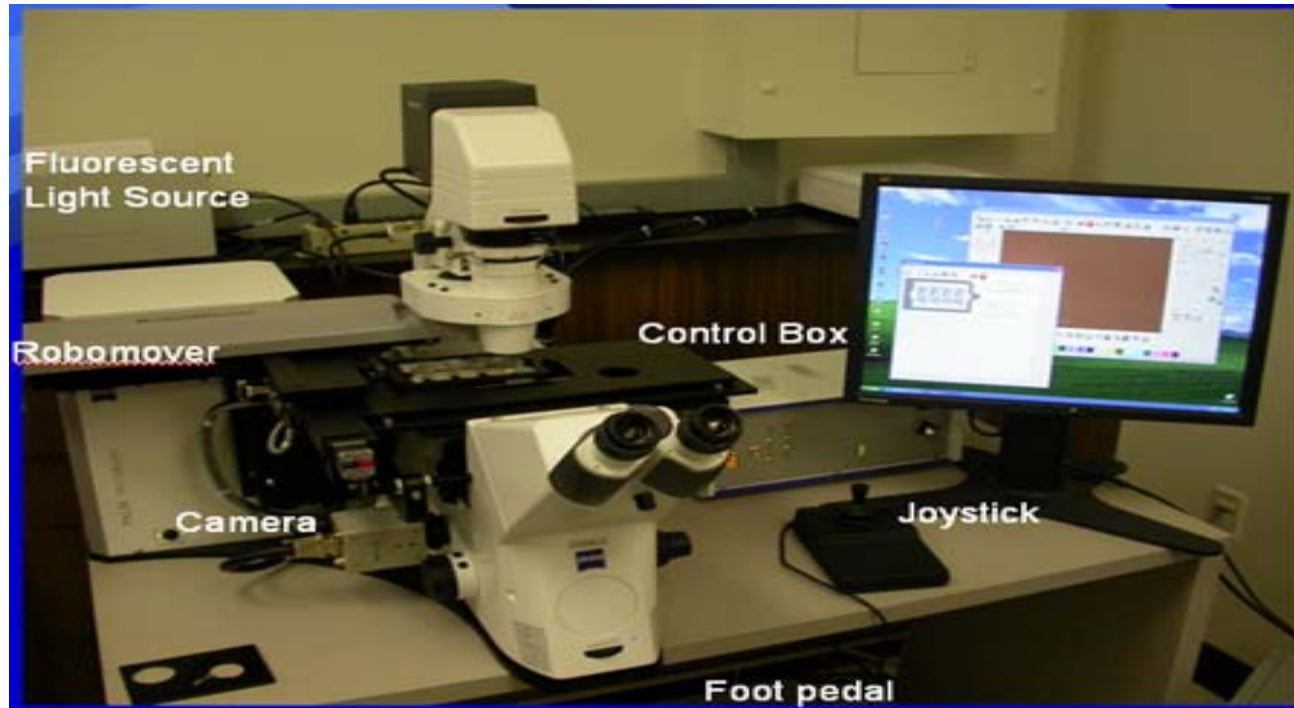
Start Up Procedure

1)   Turn key on control box to the on position.
2)   If using fluorescence imaging, turn on the X-cite flourescence lamp.
3)   Press silver power button on the left of the microscope.
4)   Turn on computer and logon
5)   Double-click Palm@RoboV4

In the main menu of the software, click "Setup" and choose **Load Settings** from the dropdown menu. If no previously established settings have been saved and loaded, default settings will be loaded automatically.
Look at the Laser on/off indicator on the microscope. When both LEDs turn green, click the laser button again. The right LED should turn red indicating the laser has warmed up.

Loading Slides and Adhesive-cap Centrifuge tubes

To begin loading your slides, click on the "Loadposition" icon as shown below. This will move the staging out of the microscope's light path to allow for easier loading/unloading.

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.
To load your capture device (e.g., adhesive-cap centrifuge tubes), select the "Capture Device" icon shown below. Now click "Load Collector," which will move the staging out to allow for easier loading/unloading.

If you are collecting samples for RNA/DNA/protein processing, you can load the adhesive caps directly in the collector.
Note: *If you are catapulting live cell samples for re-culturing, put approximately 30ul of liquid into the cap of the collection tube and place it cap down in the collector. Timing is important when catapulting and collecting live cells. If you have too much water in your cap, it will contact the stage when it moves into position. Due to the small amounts of liquid which can be placed in the cap, it is important to work quickly to ensure your fluid does not evaporate.*

After loading, select "scan for collector types." (Note: If you have already scanned during a previous session, you can simply select "use same collector type"). By clicking on the cap you want to catapult into, the Robomover will place this cap in the microscope's light path (which will actually make the picture clearer and easier to focus). Now, close this window.

Locating Sample

Back in the main work window, you can use the joystick to move the slide around and view different areas of tissue. If the joystick does not respond, click "Setup" and select **PalmRobo** in the dropdown menu. Check the "Activate joystick" box.

Brightfield Microscopy

The sample can be viewed through the monitor, or the eyepieces (larger field of view). If no image is present through the eyepieces, turn the knob located below the eyepieces counterclockwise.
You can change the objective lens using the dropdown menu shown below

Lens: 10x
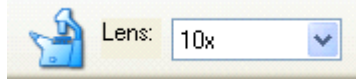
To adjust the microscope's focus, you can either manually turn the coarse and fine adjustment knobs, or change the values in the area under "-Focus-" on the right side of the slide image.

If needed, advanced microscope settings can be accessed by clicking "View" and selecting **Microscope Window** from the dropdown menu.

Adjusting Laser Focus and Intensity

Laser focus and intensity are perhaps the 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. It's important to note that higher magnification objectives automatically give you "finer" cutting. Also, slower cutting speeds allow you to use a *lower* laser energy. LPC focus should always be just a bit lower than the tissue itself, and the LPC energy should always be higher than cut energy. The optimal settings may vary for different tissue types, embedding materials, or even staining procedures. Therefore, it's important to always test your settings before attempting to cut and catapult. Find an area on the slide with extraneous tissue or even no tissue at all (just membrane) to cut in order to determine these settings. Make sure the laser function dropdown menu reads "Cut."

1 x Cut

-Speed-

Cut

µm/sec
200

50
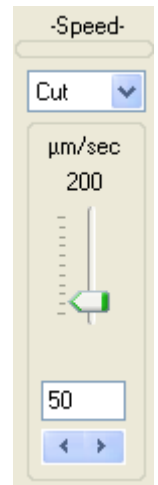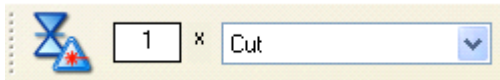
Here, use the "Freehand" drawing tool to draw a long, serpentine line. Also, bring the -Speed- down to about 50 microns/sec to allow you enough time to change parameters as shown to the right. Make sure the Cut Energy is at an intermediate value, i.e. 40-50.

Once this is done, select the "Start Laser" button. While the laser is attempting to cut, adjust the Cut Focus to the lower boundary where cutting just begins and make note of this value. Continue increasing the Cut Focus until cutting nearly disappears again (this is your upper boundary). Now, average these boundary values to find the proper setting for Cut Focus. In the picture below, for example, the Cut Energy was arbitrarily selected at 40 with a 10x objective lens. As the laser started cutting, the Focus was moved up from 84 until, at 90, the laser stopped cutting well. Then, the laser was moved down until 82, where the laser again stopped cutting. Therefore, an average value of 86 was used, which resulted in visibly more precise and defined cutting. To change the *width* of the laser's cut, cut along a line and either increase the Cut Energy for a wider cutting path, or decrease for a thinner cut.
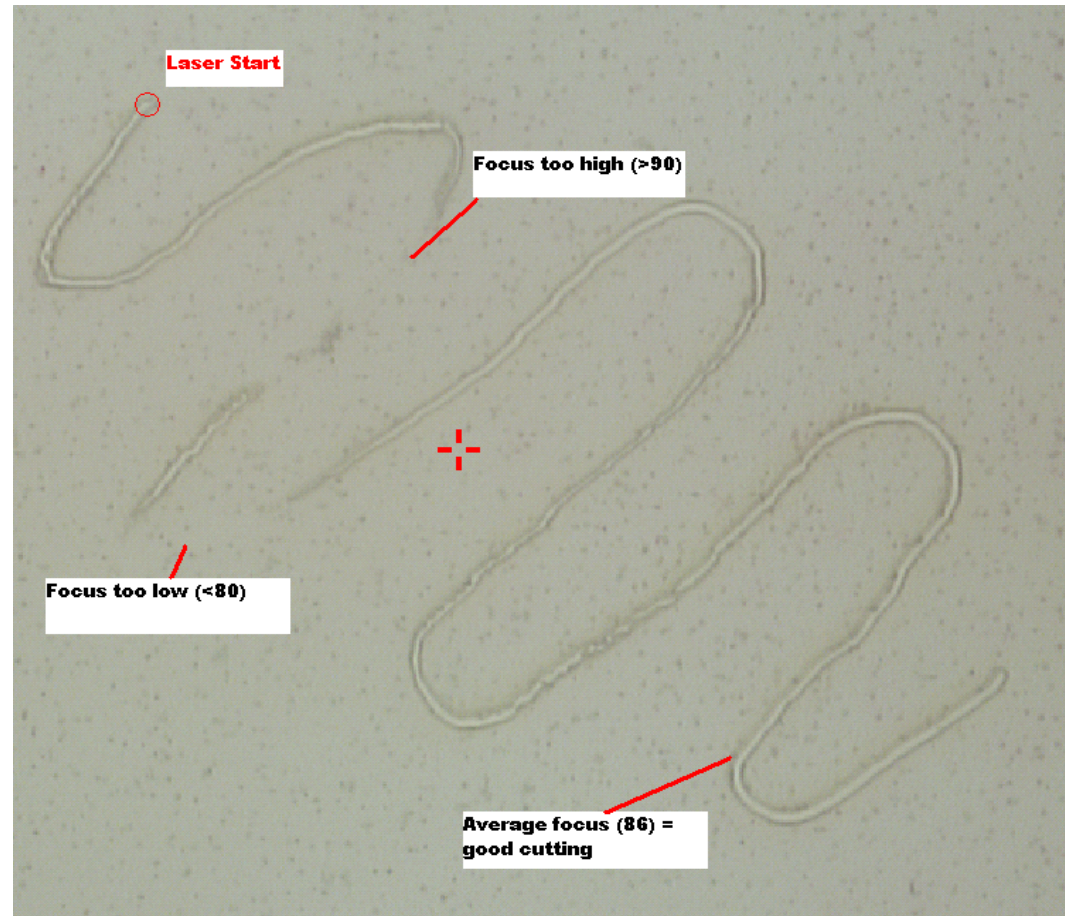
Fluorescence Microscopy

Preparation Note: *A special PET membrane slide is recommended by Zeiss for LCM applications using fluorescence (the normal PEN membrane used for brightfield applications may autofluoresce). POL membrane slides are used for ablation applications.*

To begin fluorescence imaging, make sure that the X-cite power supply is turned on.
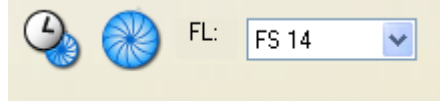
Looking at the toolbar above, the first icon is the "Shutter-Timer" icon where you can set the fluorescence shutter to automatically close after a certain number of seconds to avoid photobleaching. The second icon is the "Fluorescence Shutter Open/Close" icon that, you guessed it, opens and closes the fluorescence shutter. You can select different filters from the fluorescence dropdown menu (FS 10 = FITC and FS 14 = Rhodamine).

To avoid photobleaching, the freeze mode should be used and the fluorescence shutter closed in order to keep the last image captured by the camera on the screen. You can either press the freeze button, shown below, or click "Move" and select **Freeze mode** from the dropdown menu. In freeze mode, you can mark the image for cutting and catapulting, but you will not be able to move with the joystick. After marking this image, you will have to exit freeze mode in order to cut.

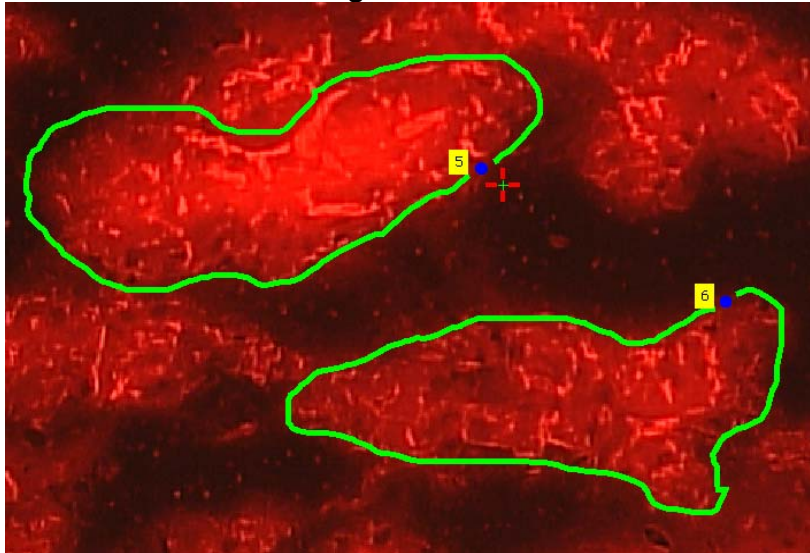
Simultaneous Cutting & Catapulting

Cutting and catapulting at the same time seems to be the most efficient method of capturing tissue features of interest.
Begin by selecting "RoboLPC" in the dropdown menu beside the "Start Laser" button.
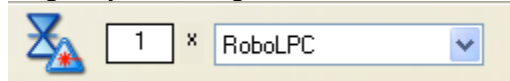

Now, when you select a drawing tool and encircle a feature of interest, the RoboLPC feature will automatically leave a small connecting piece, which ensures the area will not float away after cutting. The system then immediately catapults the entire selected area with a single shot to this "tab." In the example below, elements 5 and 6 were circled in green using the "Freehand" tool. The RoboLPC immediately left a small gap in the figure and targeted it for LPC (marked by the small blue circle). By clicking "Start Laser," the system cut around and catapulted element 5, then automatically proceeded to cut around and catapult element 6.

Note: *In order for the system to recognize the desired functions to be carried out, you must have them highlighted in the "Elements List" before clicking "Start Laser." In the example below, both elements 5 and 6 were highlighted; therefore, the system automatically cut and catapulted both pieces of tissue. Conversely, highlighting only one area in the Elements List would only catapult that specific tissue area.*

Marking areas of interest   **After LPC**
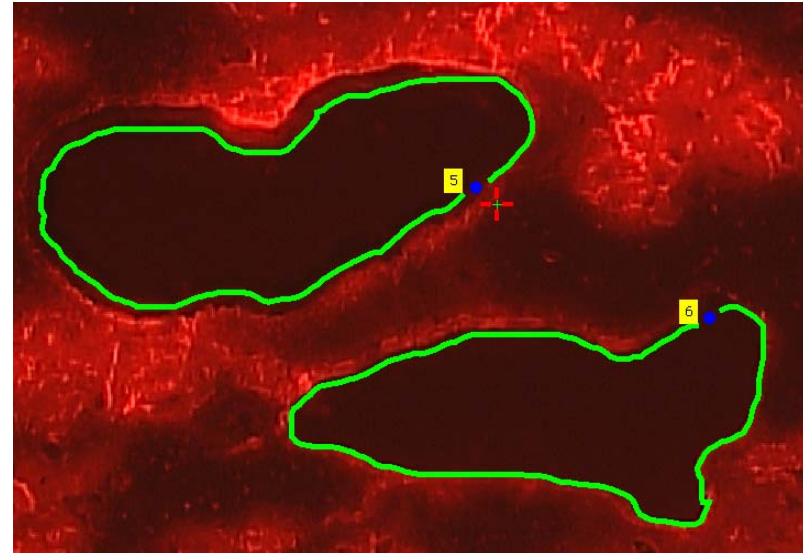
Inspecting Sample

To find the catapulted sample in the adhesive cap, either click the "Check-Point" icon shown below, or select Move and click **Go to check point** from the dropdown menu.


Pan around using the joystick and adjust the focus to locate catapulted items on the cap of the collection tube. Select go to check point again to return the robomover to its previous collection position. You can also click on the load position icon to return the robomover to allow for the loading of new collection tubes.

Saving Images

If at any time you want to save the image on the screen, simply click the "Save Image" button shown below. Alternatively, you can click File and select **Save Image...** from the dropdown menu.

You can also setup auto-imaging that will automatically save pictures from before and/or after cutting and LPC. To do this, click Setup and choose **PALM Robo** from the dropdown menu. First, 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.
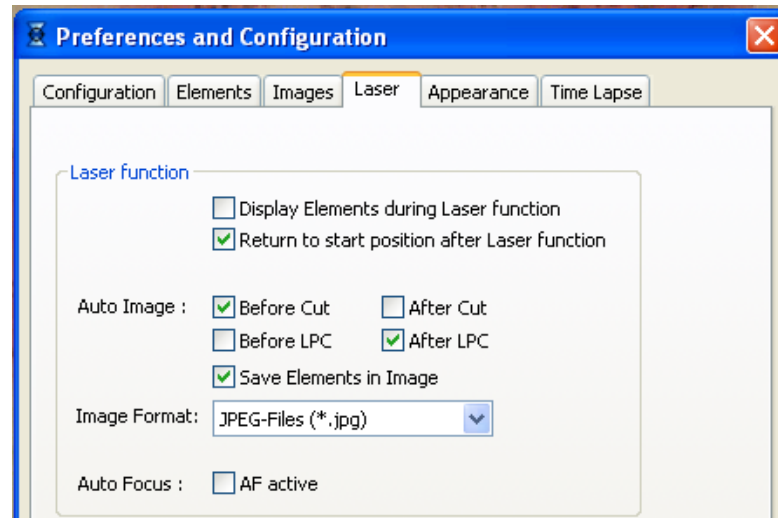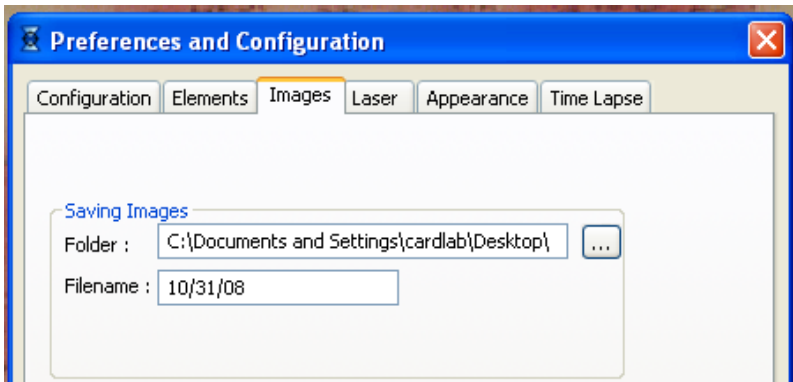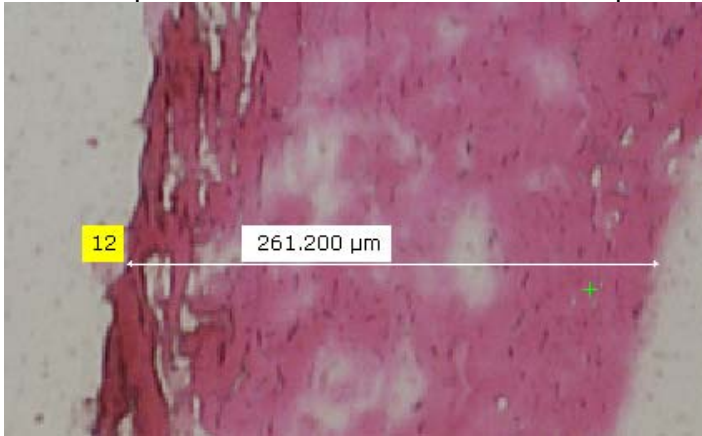
Additional Useful Tools

The **Ruler** tool is very useful for making more accurate measurements of tissue features.

The example shown below illustrates the more precise measurements that can be made rather than "guesstimating" using the scale bar.

The **Flag** tool can be very 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.

Color	Nr	Name ▽	Type	cut,shot	Area (µm²)	Grp	Comment	H x W	Position
	1		Text				Cut Area 1	21.1 x 21.1 (µm)	(101134.4,47833.8)
	2		Text				Cut Area 2	21.1 x 21.1 (µm)	(100590.4,48046.4)

Shutdown Procedure

Exit the PALM Robo software.
Exit Windows and shutdown the computer.
Turn off the X-cite fluorescence light source if in use.
Turn key on control panel to the off position.
Cover laser capture system.

Troubleshooting:

Problem: Can't see through the eyepiece.
Solution: Turn knob under eyepiece to adjust the light path, or under Microscope settings, open the transmitted light shutter.

Problem: Can't acquire a fluorescence image.
Solution: Have not selected the filter set, have the fluorescence shutter closed, or have forgotten to turn on the light source.

Problem: Can't move joystick
Solution: Under the Setup menu, select PalmRobo and check the Activate Joystick box. If the joystick still doesn't work, make sure CAPS LOCK is de-activated.

Problem: Laser Marker Calibration is off, or you cutting performance has generally "fallen apart."
Solution: Load Default settings.

Problem: Fluorescent images are poor due to background "autofluorescence."
Solution: Make sure you are using a PET membrane slide rather than a PEN membrane slide (the latter will autofluoresce). Also, check agreement between the microscopes filters and your fluorophores.

Credits & Contact Info

1st Edition: Ross Gunst

2nd Edition/Updates: Joel L. "Jock" Moore, Jr.
                              Research Specialist
                              Department of Cardiovascular Sciences
                              Brody 3S-16, moorejo@ecu.edu