User's Guide to the

LMD-6000

Laser Micro-dissection

System

Start-up Procedure for Leica LMD-6000.

1. Turn on mercury lamp by pressing the rocker switch;
    a. beige box to left of microscope, on top of laser controller
2. Turn on laser by turning key to "1";
    a. gray box left of microscope
3. Activate the laser by pressing small red button;
    a. red light to the right of this button will turn green after ~10 min.
4. Turn on the microscope controller, pressing rocker switch on its front panel;
    a. located behind the monitor
5. Turn on computer, pressing small dark button above the "Dell" insignia;
    a. located on floor to right of desk
6. [Optional] Turn on monitor by pressing the button located at top center, usually it's left on;
7. Start Laser Microdissection software by double click on its desktop icon.

Note: This computer is running Windows XP. All file transfer must use USB storage devices or optical media. Under no circumstances should it be connected to any network without an approved external firewall.

Shut-down Procedure.

1. Quit software, 'File>Exit';
2. Turn off laser by turning key to "0";
3. Turn off the mercury lamp with the rocker switch;
4. Turn off the microscope controller with its rocker switch;
5. Turn off the computer;
6. Turn off the monitor by pressing its power button.

Do Not Rotate the Camera!!!

The camera should be positioned with the tripod screw hole facing the eyepieces.
Direction of travel of the stage should be the same as it appears on the screen.
Moving the camera to orient your sample may damage the camera mount.

Loading specimen tubes.

1. Click on the rightmost ‘Unload’ button in the software menu bar;
2. Insert or remove tube holder;
3. Choose the correct size tubes for the selected holder,
   a. holders are labeled by size of tube: “0.2” or “0.5” at far right region
4. Load tubes
   a. support tube holder with 1 hand, with openings towards your body
   b. grab tube with other hand and set cap into the inset and pull towards your body until it seats in the indent
   c. fold the tube back under the holder until the tip is gripped by the pair of supports
5. Place tube holder into stage support with caps facing upwards, there is only 1 way it will fit;
6. Click on ‘OK’ in the loading window;
7. Label a tube position by clicking about 4 mm above the desired tube position and entering up to 5 characters;
8. The center position is kept empty for viewing the sample.

Loading specimen slides.

1. Click on the lefthand ‘Unload’ button to lower the stage (to left of tube unload);
2. [Optional] Remove the laser safety shield;
3. Withdraw specimen holder by pulling towards yourself;
4. Slides are oriented with the section and film facing downwards, sections are below the film;
5. Insert the end nearest to you against the wire retainer and press lightly on the far end of the slide until it drops into position;
6. Do not press too hard, or the wire will bend;
7. The wire should be pressing against the near, right corner of the slide;
8. Slide the holder back into position on the stage;
9. Click on “Continue”;
10. Replace the laser safety shield - **Laser will not fire unless the safety shield is correctly in place**.

Laser Control.

1. Under the Laser Menu, select ‘Control...’ to display laser control window;
   a. ‘Power’ - select the fraction of laser output to apply to the cutting beam;
      setting too high may produce an overly wide cutting beam
   b. ‘Speed’ – control how quickly the line is cut, low values increase amount of cutting;
   c. ‘Specimen balance’ - change the current settings to provide more or less cutting;
   d. ‘Factory Settings’ - returns the settings to factory defaults for the selected objective lens;
   e. ‘Offset’ - focus offset for the lens, set at the factory for each lens;
   f. ‘Apply’ - set the current settings as the default for the lens;

Cutting Calibration.

1. Select ‘Draw + Cut’ from the tool window;
2. Open the Control window;
3. Select ‘Calibrate...’ and move to a clear region of the film;
4. Click “Yes” to continue;
5. The system will cut a “+” in the lower right corner of the image;

6. You will position the cursor crosshair centered on this pattern and left-click;
7. Steps 5 and 6 will be repeated for the remaining 3 corners.
8. Ignore that the cut crosses will be opposite as described in the calibration window.

Cutting Line Attributes.
1. Open 'Cut Line Attributes from the 'Options'>'Settings' menu;
2. Line Thickness - sets thickness of the line as it appears on the display, does not affect actual cutting;
3. Line Compression - higher values speed the cut by removing x,y points from the drawn line, but you may get less energy delivered along the trace.

Saving Your Settings.
The settings for your laser microdissection may be saved for future use.
1. Adjust the LMD-6000 to produce to produce satisfactory results for you specimen;
2. Click on 'Save Application Configuration' under the File menu;
3. When needing to replicate these settings, select 'Restore Application Configuration';
4. Default behavior is for the software to open with the most recently used settings;
5. You may revert laser settings to the factory presets for each lens from the Laser Control window;

Selecting Objectives.
1. Under the 'Magnification' menu, choose the desired objective.
2. Obtain brightfield illumination by selecting "No cap" position from the specimen tube controller in the lower left corner of the software window.
3. Select the desired cap position when cutting.

Objective	Working distance	Application
4X/NA 0.1 C Plan		overview
6.3X/NA 0.13 Microdissection	12.8 mm	proteomics, larger areas
10X/NA 0.3 HX PL Fluotar	~11 mm	general cut and viewing
20X/NA 0.4 HCX PL Fluotar*	390 µm	general cut and viewing
40X/NA 0.6 HCX PL Fluotar*	1.9-3.3 mm	narrow line width, 4 µm wide cut
63X/NA 0.7 PL Fluotar*	1.8-2.6 mm	single cell, DIC, 2 µm wide cut

*Coverslip correction collar

Selecting specimens.
1. Select a slide in the 3-position slide holder by a single click on the desired slide position from the icons at bottom of software window;
2. Moving between slide positions requires selecting the desired slide, the manual controls will not move the stage that far.

Obtaining an overview of the specimen.
1. Bring your specimen into focus with a low magnification objective;
2. Select Options>Specimen Overview from the menu bar;
3. From the Specimen Overview window, press 'Create Specimen Overview';
4. Position the upper left corner of the specimen in the eyepieces
5. Click 'Save top/left position';
6. Position the lower right corner of the specimen in the eyepieces
7. Click 'Save bottom/right position';
8. The diagonal buttons will toggle the stage between the 2 corners for adjusting positions;
9. Press 'Scan' at bottom of setup window;
10. If you press 'Save Scan', the montage will be saved;

Viewing the cut specimen in the collector cup.
1. Press the 'Collector' button in the toolbar;
2. The stage will move the the collection cup will come into view.

Selecting regions of interest with DIC.
Differential interference contrast (DIC) is only available for the 60X objective.
The 'Move + Cut' option is not available in DIC because the polarization extinguishes the laser beam.
1. Switch to the 60X objective, Magnification>lens;
2. Select DIC from the Illumination menu, TL-DIC;
3. Adjust DIC optimal contrast:
   a. use the analyzer wheel, located on the front, right corner of the microscope stand, just above the lens turret
4. Select the 'Draw + Cut' option for cutting shapes, right hand panel of the software;
5. Select the type of shape to draw;
6. Draw outlines around the desired structures:
   a. You will be prompted for a cap with each outline;
7. Click on 'Start Cut';
8. The software will switch the microscope to transmitted light, make the cuts, then switch back to DIC, automatically;
9. Remove shapes from the Shape List and proceed with the next field of view.

Directing the Image between Eyepieces and Camera

The eyepieces and the camera are mounted on a trinocular head.
A rod extending from the left side of the trinocular head controls to where the the specimen image is directed.

Position	Image Direction
Fully Out	100% Camera
Middle Position	50% Eyes:50% Camera
Full In	100% Eyes

Selecting regions of interest with epi-fluorescence.

1. Click on the 'Illumination' menu
   a. Select 'Fluorescence'
   b. Alternatively: Toggle with ‘TL-BF’ and ‘Fluo’ buttons in menu bar at top of screen
2. Fluorescence control window commands
   a. Field Diaphragm: a slider controls size of the rectangular field of illumination
      i. This allows reducing photobleaching in adjacent tissue
   b. Light Intensity: a slider controlling output of mercury lamp
   c. Exposure time: choose preset frame rate to select time over which each camera frame is collected (i.e. camera refresh rate)
   d. Preview: Opens live image
   e. Integral: Collects an image for chosen exposure time and freezes frame, but leaves shutter open
   f. Shutter: Open and closes fluorescent shutter
3. Menu Bar Buttons
   a. Live: opens shutter and generates live camera image
   b. Freeze: Maintains current image and closes shutter

Overview of LMD-6000 Epi-fluorescence

This is a very basic system for fluorescent imaging uses a wide spectrum excitation filter to indiscriminately excite blue, green and red fluorophores. Emissions are recorded on a 3-chip analog CCD camera.
Camera: HV-D20 3-chip CCD (Hitachi) analog PAL
Chips: 1/2” diagonal width, 752 pixelsX582 pixels sensing area, 8.44 µm/pixel V, 8.42 µm H

This camera contains a prism that separates the incoming image into blue, green and red portions of visible spectrum. There are 3 separate CCD chips aligned around a prism to accept the respective portions of the spectrum as discrete blue, green and red color images. These images are displayed on the screen as RGB images. This allows full resolution RGB images collected with a single exposure event. The disadvantages of a 3-chip camera for fluorescence are relatively low quantum efficiency and relatively broad spectra recorded in each channel that often results in bleedthrough.

Movies of LMD6000 operations.

http://www.leica-microsystems.com/products/light-microscopes/life-science-research/laser-microdissection/details/product/leica-lmd-software/showcase/

Overview of system.

The LMD 6000 laser microdissection system utilizes a Leica DM6000B upright microscope with a motorized x,y stage (Marzhauser) and automated control for focus, shutters, filters and light paths. It is capable of transmitted light/DIC and epi-fluorescence microscopy, and has a HV-D20 3-chip color CCD camera (Hitachi) for monitoring microdissection or image collection. A 50 mW nitrogen laser producing 355 nm light performs the cutting.