LaVision Ultramicroscope – Jul 2020

Start Up

1. **Start your session on the iLab Kiosk**
2. Turn on the power strip
3. Turn on all laser lines
4. Make sure the computer is on.
5. Open "ImspectorPro" software program

Mounting the sample

1. Raise the zoom body all the way up, using the focus knob.
2. Rotate the objective out of the working position.
3. Loosen the hex screw on the right side of the gray stage plate.
4. **PUT ON GLOVES.**
5. **<u>Carefully</u>** lift off the gray stage plate.
6. Remove the reservoir's black lid and place it inside the hood on a paper towel.
7. **<u>Carefully</u>** replace the gray stage plate.
8. Mount sample in the correctly sized sample holder. Try to mount your sample as level and as near the top of the holder as possible.
9. Insert the holder into the sample cradle such that the screw of the holder is at a 45° angle to the supports of the cradle.
    - *You need the light sheet to be able to pass unobstructed through your sample.*
10. Place the cradle into position such that the sample is oriented perpendicular to the light path.
    - *Note: if you will be imaging using a single sheet only it should be the right side. Therefore, make sure to place your sample such that the most interesting part is facing the right side so that your area of interest is accessible to the sheet: e.g., if interested in spinal cord, place it to the right side so it will not be shadowed in the image.*
11. **REMOVE YOUR GLOVES!!**
12. Tighten the hex screw on the right side of the gray stage plate.
13. Rotate the objective into the working position.
14. Set the correction collar to appropriate position.
15. Check that the second light from the right on the joystick is off. If it is on, press this light (it is also a button) to restore control of the stage to the joystick.

Finding the sample

1. Set joystick to fast mode by pressing and holding the rightmost button until it beeps.
2. Select the lowest zoom setting (0.63X) on the microscope and the software.
3. In the software UltraII window:
    a. Select the 561nm ("OBIS") laser
    b. Set laser power to 10%
    c. Set NA to the highest possible setting (optics section)
    d. Adjust the light sheet width to 20% (optics section)
    e. Select the right light sheet (lightsheet section)
4. Click on the video button to begin rapid scanning
5. Look into the reservoir: you should see the light sheet hitting the sample holder and your sample. If not, maneuver the sample in XYZ until you can see the light sheet going through your sample. Once the light-sheet is hitting your sample, lower the Z stage until the light-sheet is going through the top of your sample. **The light-sheet must be in the sample to avoid damage to the system.**
6. Disengage joystick fast mode by pressing the rightmost button on the joystick once.
7. Select the proper laser for your fluorophore.
8. Hit the min/max display icon (this is a sun and moon with stars picture on the right side of the imaging window).
9. Lower the objective until it is close to touching the DBE. Once it is close, lower it more slowly, constantly monitoring the image and adjusting the display to avoid hitting your sample. As you continue to lower the objective your sample should begin to come into focus.
10. Adjust the width of the light sheet to 100% to illuminate the entire field of view evenly.
11. Center the sample in the field of view using XY controls. Focus on a structure within the sample using the focus knob on the microscope.
12. Go to the top of the sample by rotating the Z knob counter-clockwise. Set as zero in the XYZ-Table Z window.
    Never go deeper than 5.2mm into the sample with the objective.
13. Set the top of the sample as the end of the Z stack in the XYZ-Table Z window.
14. Adjust your zoom as desired. Adjust your focus and readjust light-sheet width (if needed) after adjusting the zoom. Adjust the zoom setting accordingly in the software.

Imaging setup – the basics

Focusing evenly across the X dimension
If the sample does not seem to be fully in focus across the entire field in the X dimension, you can try the following:

1. Use the light-sheet from the other side.
2. Adjust the horizontal focus position, by clicking on the button with 2 red horizontal triangles to bring up the indicator on the imaging window (measurement tab, UltraII window). Click and drag the indicator to adjust the position of the horizontal focus until the focus of your structures of interest improves
3. Lower the sheet NA. This makes the light-sheet dimmer, and thicker in Z in the middle, but more even as a function of X position.

If none of these adjustments leads to an image that looks fully even and in focus across the field of view, try imaging with 2 light-sheets or with multiple horizontal foci.

Exposure and laser power

1. Exposure will be the same across multiple channels
2. There are two ways of adjusting laser power:
    - Adjust the power of desired laser using the laser slider in the Ultra-II window (move the slider and then hit apply). NOTE: scale is logarithmic. Having different settings for multiple channels can lead to slower imaging with more than one channel if channels are acquired at each Z plane before moving the sample in Z.
    - Adjust the power of desired laser using the OBIS laser module (recommended for more than 1 channel). Ask MSL staff about this.

When looking at the sample, it is better to use long exposure (hundreds of ms) and low laser power (10-20%); this avoids bleaching. When imaging the sample, it is better to use very short exposures (10ms) and high laser power; this speeds up the acquisition. To get equivalent intensity between one mode and the other use the power curve posted on the wall.

Imaging with both light sheets (OPTIONAL):

1. Activate both light sheets (button with 2 horizontal triangles in the light sheet section of the UltraII window).
2. Set horizontal focus optimally for each of the sheets.

Imaging with multiple horizontal X sheet foci (OPTIONAL):

You can set the start and stop position for the horizontal focus and choose how many images you would like to capture as the focus moves across the field.

1. **Check that the zoom factor in the software is the same as on the microscope knob**
    - If this is not set properly the dynamic focus will give very poor results.
2. Set dynamic focus to "ON" (horizontal focus section, measurement tab, UltraII window).
3. Click on the icon with multiple horizontal triangles and set the area in the image over which you want to move the focus.
4. Input the recommended number of focus positions.
5. Enter the desired exposure time, ensuring that it is higher than 100ms (the software will adjust it slightly automatically).

Cropping

If you only care about a subregion in the field of view, cropping will significantly reduce your file sizes and save you transfer and processing times later on.
To crop, draw an ROI on the image and use the box inside a box button. To ensure optimal quality, your ROI should be centered in the field of view.
To uncrop, click on the Full field button in the UltraII window (Measurement tab, sample section)

Autosave settings

Make sure you are writing to the correct directory, with a proper basename. Note that the date and time will be embedded in the name automatically. We highly recommend embedding the zoom you used as part of the basename.

Experiment designs

All experiments are set up using the devices menu. Depending on what devices are selected, the system can do multiple Z positions, multiple channels, multiple positions, and any combination thereof in whatever order desired.

Typical setups are described in what follows.

Acquiring Z stack – 1 channel:

1. **Check that the zoom factor in the software is the same as on the microscope knob**
    - If this is not updated properly before you begin imaging, your metadata will be incorrect later.
2. While imaging in video mode, move to the top of your sample (move the joystick counterclockwise).

In the Table Z dialogue box:

3. Click the "Set as Zero" button.
4. Click the End target button.
5. Move to the bottom of your sample (move the joystick clockwise).
   DO NOT EXCEED 5.2mm deep in the sample with the 4X objective
6. Click the Start target button.
7. Set z step size. We recommend using 1/2 or 1/3 of the light-sheet thickness for optimal sampling. Do not use less than 2um Z-step size.
8. Stop Video Mode
9. Add "xyz-Table Z" to the device list in the measurement wizard window.
10. Make sure that "autosave" is on. In "autosave settings" make sure to write to the D (normal) or F (hotswap) drive, NOT the network, and that every step is saved.
11. Press START

Acquiring Z stack – more than 1 channel:

In addition to steps 1-9 above:

1. Check the boxes for the filter sets you would like to use.
2. Check to see if chromatic correction is needed:
    - Go to your shortest wavelength channel:
        i. If your shortest wavelength is 488, focus on your sample.
        ii. If your shortest wavelength is NOT 488, set the chromatic correction lens to the zero position for your shortest wavelength.
    - Go to your other channels and check focus in each one. Do not move the focus knob. If the focus is off, use the chromatic correction slider to adjust focus. Once it is properly positioned, click the Set button to save the position.
    - Note that higher magnifications require more movement of the chromatic correction lens. Also note that some combinations of fluorophores and magnifications cannot be corrected (example 647 and 488 at 6.3X zoom).
3. Adjust the laser power for each channel.
    - You can adjust the power of each laser using the laser slider in the Ultra-II window. Choose a channel by highlighting it (not checking it) and make the proper adjustment, then click "Apply" for each. Note that if you use different laser powers for different channels, imaging will be slower, particularly if channels are acquired sequentially at each Z plane.
    - You can also adjust the power of each laser using the OBIS laser module (recommended for faster acquisition, see MSL staff)
4. Adjust the exposure for the camera. Note that there is only one exposure setting allowed for all the channels:
5. Add "Ultra Filter" to the device list in the measurement wizard window.
    - Note that the order in the device list is the order in which things will be done (for example: UltraII Filter 1st, Table Z 2nd will take images in each channel, and then move to the next Z position; the opposite order would take an entire Z stack in one channel, and then in the other).
6. Make sure that "autosave" is on for all devices
7. Make sure that the Ultra Filter setting has the "split" engaged.
8. Press START

Generating a Mosaic Image:

Notes:

- For best results you will likely need to crop each image (see MSL staff to determine how much to crop). This will increase you acquisition time but dramatically improve your quality.
- Be careful with the objective bumping into the edge of the travel range when tiling at high magnifications

In addition to steps 1-9 (Z stacks), and 1-7 (multiple channels) above:

1. In xyz Table Visual XY, select the "Mosaic" tab.
2. In set parameters, under advanced options, set the overlap between 10%-20%.
3. Verify that the lock button is closed.
4. While imaging in video mode, adjust the position of the stage to one corner of where you want mosaic.
    - This can be done using the joystick or by dragging the red box in the XY Table window.
5. Double click red box then click and drag it to create area for your mosaic.
    - The size of the mosaic area can be adjusted by dragging the sides of the box delineating the mosaic area.
    - You can also increase the size of the mosaic by dragging the red box to an area outside of its borders and double clicking.
6. To generate an overview of the mosaic area:
    1) Remove all devices that will not be included in the overview image from the device list (Z certainly, possibly UltraFilter).
    2) Add xyz-Table X and xyz-Table Y, **in this order**, to the device list in the measurement wizard window.
    3) Deactivate autosave for each device in the list by clicking the small disk in the "AS" column next to it. The disk should now be greyed out.
    4) Activate the split view for xyz-Table X and xyz-Table Y by checking the box next to it (at the top of this column "split" is written).
    5) Hit the "Start" button to begin imaging.
    6) After imaging, adjust the display in one tiles and hit CTRL-Q to equalize those settings to all tiles. If the mosaic looks good, proceed with complete imaging.
7. Decide on the imaging order. A typical example would be to do Z first, then channels, then mosaic positions. The order of the devices in the device list should reflect this order. Note that for the tiling, you will need to add xyz-Table X and xyz-Table Y, **in this order**.
8. When ready for final acquisition, check that all channels have autosave and that—if used—Ultra Filter has the "split" option engaged.
9. Press START

In the name of the image, the first parenthesis is the Y value and the second is the x value [YY x XX], even though that is the reverse order of that used when adding it to the device list.

Viewing data in Imspector

1. Open “Series Viewer”. This is the icon along the top that has 3 squares, 2 arrows and a line on it.
2. In the window that opens, select the folder containing the data set you wish to view.
3. Select the data set in the “image series found” part of the window.
4. Click the “down” arrow to add that file to the bottom box of the window.
5. Click on “load”.
6. Use the “Series Viewer” window to move through your images, not the bars along the image.
7. Close the image window when you are done.

Changing samples

1. Raise the zoom body all the way up, using the focus knob.
2. Rotate the objective out of the working position.
3. **PUT ON GLOVES.**
4. Remove sample cradle and move it to the hood. Use paper towels to avoid DBE spills.
5. Swap sample
6. Insert the holder into the sample cradle such that the screw of the holder is at a 45° angle to the supports of the cradle.
7. Place the cradle into position such that the sample is oriented perpendicular to the light path.
8. **REMOVE YOUR GLOVES!!**
9. Rotate the objective into the working position.
10. Set the correction collar to appropriate position.

Shut Down

1. Raise the zoom body all the way up, using the focus knob.
2. Loosen the hex screw on the right side of the gray stage plate.
3. Rotate the objective out of the working position.
4. **PUT ON GLOVES.**
5. **Wipe off all DBE from the objective using lens paper.**
6. Remove the cradle that holds the sample with a paper towel and put it in the hood.
7. Carefully lift off the gray stage plate. Put the reservoir's black lid back on.
8. Moisten a paper towel with ethanol and clean off any DBE from the stage or gray plate. Discard the towel contaminated with DBE in the bag in the fume hood.
9. Put the gray stage plate back on.
10. Put away your sample and clean the sample holder, cradle and tray in the fume hood with ethanol.
11. **Discard your waste bag, set up a clean one for the next user.**
12. **REMOVE YOUR GLOVES!!**
13. Exit the software.
14. Log off computer.
15. Turn off all lasers.
16. Turn off the power strip.
17. **Finish your session on the iLab Kiosk**