Confocal Microscopy Core Facility

Brigham and Women's Hospital

Confocal Startup Procedure

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New steps are in red.

1. Turn on Mercury lamp power supply. It is a large white box labeled with a red "1."

2. Turn on the shutter driver. It is a large black box labeled "2." It turns on in the back. The shutter starts in the closed position. To fix this, move the silver switch on the front to "N.C." then "N.O."

3a. Turn on the computer.

3b. Turn on the AOM controller (a large white box below the lasers labeled "3a." It turns on in the back. This will not turn on if the computer is not on.

3c. Turn on the C1 controller (a large, white, computer-like box next to the AOM controller).

4. Turn on only the lasers necessary for your samples.

   For green fluorescence (FITC, GFP, YFP, Cy2, Alexa 488, etc.) turn on the 488 laser in two steps:
   488a. Switch the large toggle switch on the large black box below the lasers.
   488b. Turn the key of the smaller black box on top of the larger box.

   For red fluorescence (RFP, Cy3, Alexa 546, PI, etc.) turn the small black key on the medium black box below the lasers.

   For far-red fluorescence (Cy5, Draq 5, Alexa 635, etc) turn the small silver key on the back of the box connected to the actual laser. Be careful!

5. If it is not already on, turn on the digital "Remote Focus Accessory," a small white box with a dial to the left of the microscope.
   To start, disengage this accessory by flipping the lever opposite the focus knobs on the microscope away from you, towards what is labeled "confocal."

6. Turn on the nitrogen tank to float the table.

7. Lower the objectives by moving the large focus knob clockwise.

8. Choose an oil objective for confocal microscopy. **Do not use the 100x oil objective.** The 60x oil objective will yield the highest resolution of any objective our facility owns.

9. Place a very small dot of oil on the lens of the objective (or directly to your coverslip). A small dot is often enough for a few samples. **Too much oil will ruin our microscope by seeping into the lower components.**

10. Slowly raise the objectives by rotating the large focus knob counter-clockwise until you see the oil make contact with the coverglass and lens.

11. Open the shutter by switching the shutter drive to "N.O." and possibly moving a second shutter below the filter wheel to the back position. Rotate the filter wheel until the correct excitation filter is seen.

12. Adjust the microscope to position 1 by turning the knob located next to the focus knobs to "1."

13. Engage the remote focus accessory by flipping the lever opposite the focus knobs towards you.

14. Make fine adjustments to focus using the remote focus accessory, and move the field until a specimen is found and focused.

15. Once a specimen is found and focused, move the filter wheel until no light is passing through the objective (UV is hard to see, make sure there is no purple UV light seen as well). There should be two blank spots on the wheel between the far red (orange) and UV (purple) filter sets.

16. Open the EZ C1 software (not the "Viewer" or "Thumbnailer" software).

17a. Adjust the laser power for all the lasers to be used. Be careful not to make the power too high, as it will bleach your fluorophores. Generally, 488= 5-15%, 543= 15-50%, 640=5-30%. These powers are general guides, and will vary based on the photostability and brightness of your fluorescence, the pinhole used, the number of z stacks, etc.*

17b. Click the "Frame Lambda" tab on the right side.
Click "Pass 1"
Select the laser used in pass 1. Deselected lasers will be black. Selected lasers will be colored.
Select the detector used in pass 1 (usually, right below the corresponding laser). Deselected detectors will be black. Selected detectors will be the color of the fluorescence.

18. Adjust the gain for the selected detector by moving the "gain" bar for that detector. Generally, gain will be between 100 and 140. Much higher than 140 may show noise from the PMT.*

19. Choose a pinhole for the pass. Larger pinholes yield brighter fluorescence, but will look at a thicker optical slice.*

20. Add other passes for a second or third color, or delete unnecessary passes.

* A balance between pinhole size, gain, and laser power must be reached based on what is most important to you. A small pinhole size may make a sample too dim. A high laser power may bleach. A high gain will make a noisy image. For specific help with making these decisions, seek guidance from literature or Tyler.