General Information for Cryo-Confocal.

Use Safety Googles and Cryogenic Protective Gloves When Filling Cryogenic Reservoirs-Make Sure All Participants In Experiment Are Using Safety Equipment!!

Wavelengths Available on Cryo-Confocal

Excitation lasers: **405**, **491**, **561**, 640 nm (AOTF in the control panel, MPD should be set to CSV)

Emission Filters, Wheel A: **460/50**, 525/50, **545/30**, **590/30**, 600/40, 620/60, 700/75.

Emission Filters, Wheel B (slave camera): 660/20 & 690/50

The confocal is based on a spinning disk confocal microscope Yokogawa CSU-X1. The system has an integrated dichroic mirror which prevents excitation wavelengths to passing through to the camera and an iris with which one can adjust the incoming illumination on the sample.

Set Up Procedure

1. Turn on power supply at the bottom of electronic rack
2. Turn on power supplied from top to the bottom for all devices including computer (Yokogawa power supply switch is on the back)
3. Turn on spinning disk with the key and turn on the light supply from Thorlabs.
4. Turn on power with voltage: 24V on the A power supply, set B to 18V to keep the window between microscope and the objective lens dry
5. Turn on power to PI z step controller.
6. On the PC: Start up Labview code for the stages.
7. Start uc480 viewer code, this is low magnification camera (LMC) for alignment. Start the camera.
8. Start Andor software, if one camera (wheel A) is used get “Cryolight master time lapse code” if both cameras needed (wheel A+ B) open Andor software again and start “Cryolight slave 2 code”.
9. Check that the wheel A reads out camera du897_bv5157 and wheel B reads out du897_bv2035.
10. Make sure that all cameras are working (make dark current acquisition). Setup wheel positions to open. Set up trigger control to free run. System is now initialized.

Warm Test (prior to cool down)

1. Take an empty glass capillary, fill it with rainbow beads and put it in the sample holder by hand.
2. Open sample loading port and push the sample manipulator to load position.

3. Pre-align capillary with the manipulator by adjusting X and Z to the black marks on the sample port.
4. Put capillary down into imaging chamber.
5. Setup LMC for bright field acquisition: set Filter wheel A to 5, close iris, laser off. Light intensity can be adjusted with Thorlabs manual controller.
6. Find capillary in X and Z again, put the tip of the capillary in the middle of the camera field of view. Mark position on the screen. Record X,Y,Z positions of the stage.
7. Move 1100um towards high magnification camera (HMC).
8. Setup imaging for high magnification: Filter wheel A to 1, iris open. If necessary setup the trigger and set Andor control to “low”, i.e. high magnification.
9. The capillary should be in the middle of the FOV and in focus.
10. Setup a new protocol on the camera.
11. Warm up required lasers, setup filters and exposure time, z stack spacing, etc following Andor manual.

Cooling Down Cryolight Microscope

1. Connect gaseous Helium and liquid nitrogen pipes. Open He tank. Open the In and Out valves to the zeolite canister to start flow.
2. Fill Dewar with liquid nitrogen to cool the zeolite canister.
3. Turn up DC power supply to 24V to keep the window between microscope and the objective lens ice free.
4. Flow dry nitrogen through cryo-LM by opening the front valve to flow control.
5. Fill Dewar with liquid nitrogen to 3-4 inches deep (10cm).
6. Wait until the temperature falls to -162 degrees. Let it settle down for a bit.
7. Bring sample transfer device from the beamline. Connect it to dry Helium gas supply.
8. Add propane to the propane vessel.

Loading The Sample

1. Make sure to check Liquid Nitrogen level and refill propane before loading.
2. Start flow of He in the transfer machine, add liquid nitrogen.
3. Meanwhile adjust flow of dry nitrogen in the microscope (left one -bottom flow- should go to zero).
4. First grab capillary with the grabber (without He flow as it gets very steamy and one can not see anything). Detach it from the crocodile clip and start the He flow.
5. Put transfer device on the kinematic mount on the cryo-light microscope, make sure it is positioned correctly!

6. Open the iris on top, load the capillary such that the top 1cm is still accessible, close iris and dismount transfer machine.
7. Grab top of capillary with the specimen holder, open iris and put specimen into imaging chamber.

Image Acquisition.

Confocal z-stack acquisition

1. Close iris, put filter wheel to 1, open shutter for lasers (AOTF control panel), open shutter in Yokogawa.
2. Go to LVPZT control panel (that is scanning motor for z stack), move it to 25. Its range is from 0 to 53.
3. Insert the channel of interest in the control panel. Start to edit it, set the emission filter position appropriate to the fluorescence wavelength of choice. Start live acquisition. Adjust EM gain to a minimum of 10, exposure time of 200ms.
4. Adjust Z position in Labview panel until you are in the middle of capillary.
5. Now you are ready to setup your Z scan. Make sure to close the shutter on Yokogawa while you are not imaging.
6. Start with any protocol. From top to bottom it should be setup as follows: Image, Camera binning- Use Current, Wait allocation-ON, Camera Selection – Use current, Events, Repeat T- 1 times, Repeat Z (um range in X planes), move channel of choice, trigger out – Out3 High, snap, end Z, move scan to 25, endT.
7. Move Z scan to 25um this assures that you always align with the complete range of motion available.
8. Any camera and microscope setting can be adjusted within the chosen protocol. For sensitive samples it is recommended to put Yokogawa shutter in open-closed mode.

Confocal Two Camera Z-stack Acquisition

1. Follow protocol above to set up camera A with wheel A.
2. Change the position of wheel B for the fluorescence of interest, typically red.
3. Setup protocol for camera B. This one should be: Image-Protocol1, wait allocation-off, camera selection- Use current, Events, Repeat T- number of planes in camera A settings, Move channel – no settings required, snap.
4. Make sure of camera settings and go to ext trigger.
5. The actual readout between camera A and B is almost 2.5x longer. Thus it is crucial to adjust the intensity in camera A, either electronic gain, binning or decrease the laser for camera A (if different lasers are used). Similar image quality should be obtained on both cameras with similar exposure time.
6. Check the actual frame rate by running both cameras simultaneously.

7. If the frame rate is different. In camera A control pane add after snap – wait time. Put as many seconds as required for the difference in frame rate.
8. Setup the save directory for camera B, make it auto. Set up saving directory for camera A make it auto.
9. First run protocol for camera B, it will wait for trigger signal, so start camera A as well. If you set up them correctly, you will have the same number of images in folders you specified.

Shut Down Procedure

1. Move the capillary to low magnification, then remove it.
2. Remove propane with venturi pump, 2 minutes later disconnect liquid nitrogen line and close the valve on the Dewar.
3. Close both valves to zeolite canister.
4. Turn voltage on heaters to 12V
5. Close all the camera programs and Labview
6. Shut down the computer
7. Switch off all the power supplies in the electronic rack and spinning disk confocal.