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Start Up:

Upon entering the laser room turn on the wall mounted Laser Power Button by pulling it away from the wall.

Turn on Shutter controllers (far left box on shelf and also 785nm shutter controller box).

Turn on Lasers and allow to warm up for 15 min – 1hr [1hr is best]. (lasers are not connected to the computer in any way):

633 nm (red): turn key to on position

532 nm (green): Flip switch for power on back of box, wait 5-10 min, then turn key to turn on

488 nm (blue): Flip switch for power on front of box, wait 5-10 min, then turn key to turn on

593 nm (orange): (Rarely used) Flip switch for power, wait 5-10 min then turn key to turn on

785 nm (near IR): only turn on when needed (autofocus) {it has a loud fan}

Turn on other equipment:

LAMDA 10-3 Filter Wheel Controller: needs to be turned on before Labview program on computer is launched

Stage controller box (MCL box 1) - Flip switch for power on front of box

QPD Control (MCL box 2) – Quadrant Photo Detector - Flip switch for power on front of box

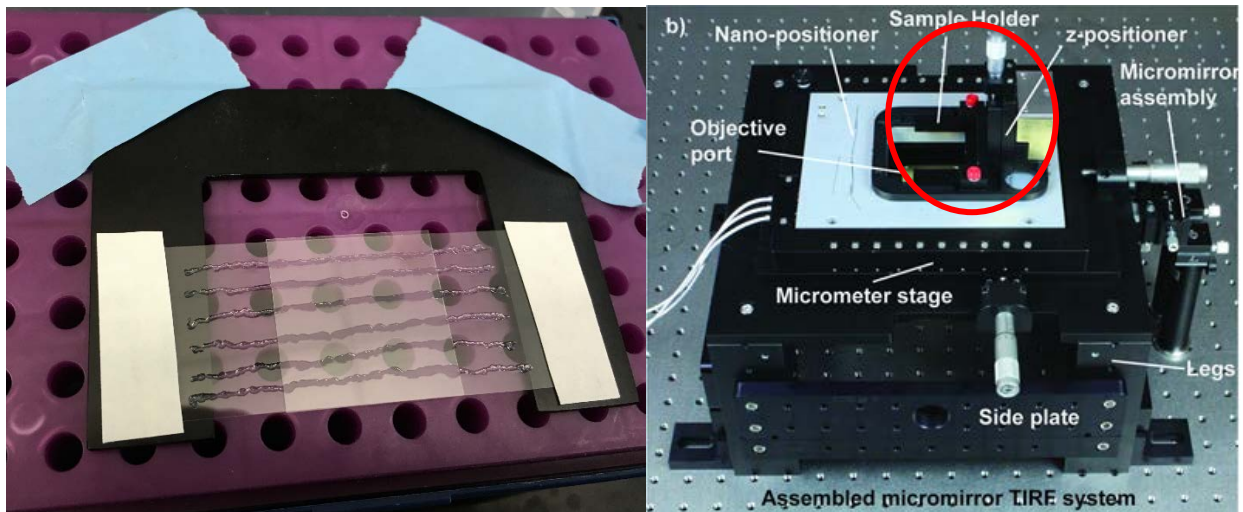
Load Slide onto Sample Holder:

Place a drop of immersion oil on the objective lens. [Immersol type 518F Immersion Oil]

Position the Sample holder with taped-on slide assembly onto the z-positioner stage.

Tighten two red thumb screws to fasten the assembly in place.

Use the z-positioner thumbwheel to lower the stage such that the slide just makes contact with the oil droplet.

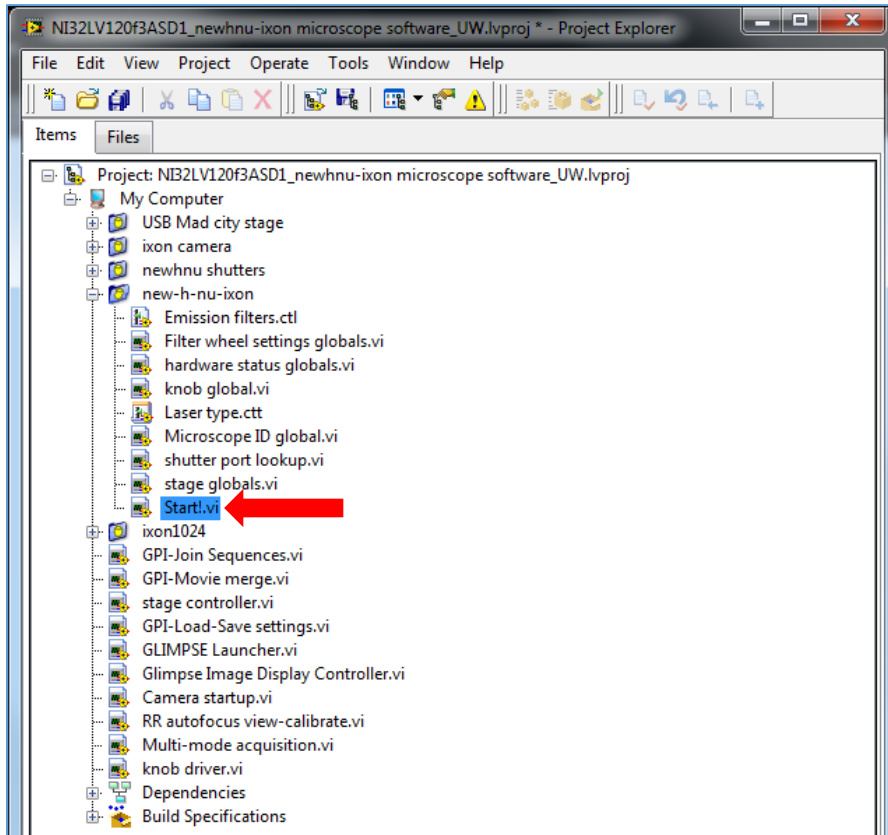


Turn on Computer/ Start Labview/Glimpse:

Open LabView icon (NI32LV120f3ASD1_hnu-ixon microscopesoftware UW.lvproj) on Desktop.

[If this file is not yet saved on your desktop there is a copy saved in C:\users\public\publicDocuments\

Make a copy and save it to your desktop for future use.]



This will open NI32LV120f3ASD1_hnu-ixon microscopesoftware UW.lvproj window


Double click on "Start!.vi"

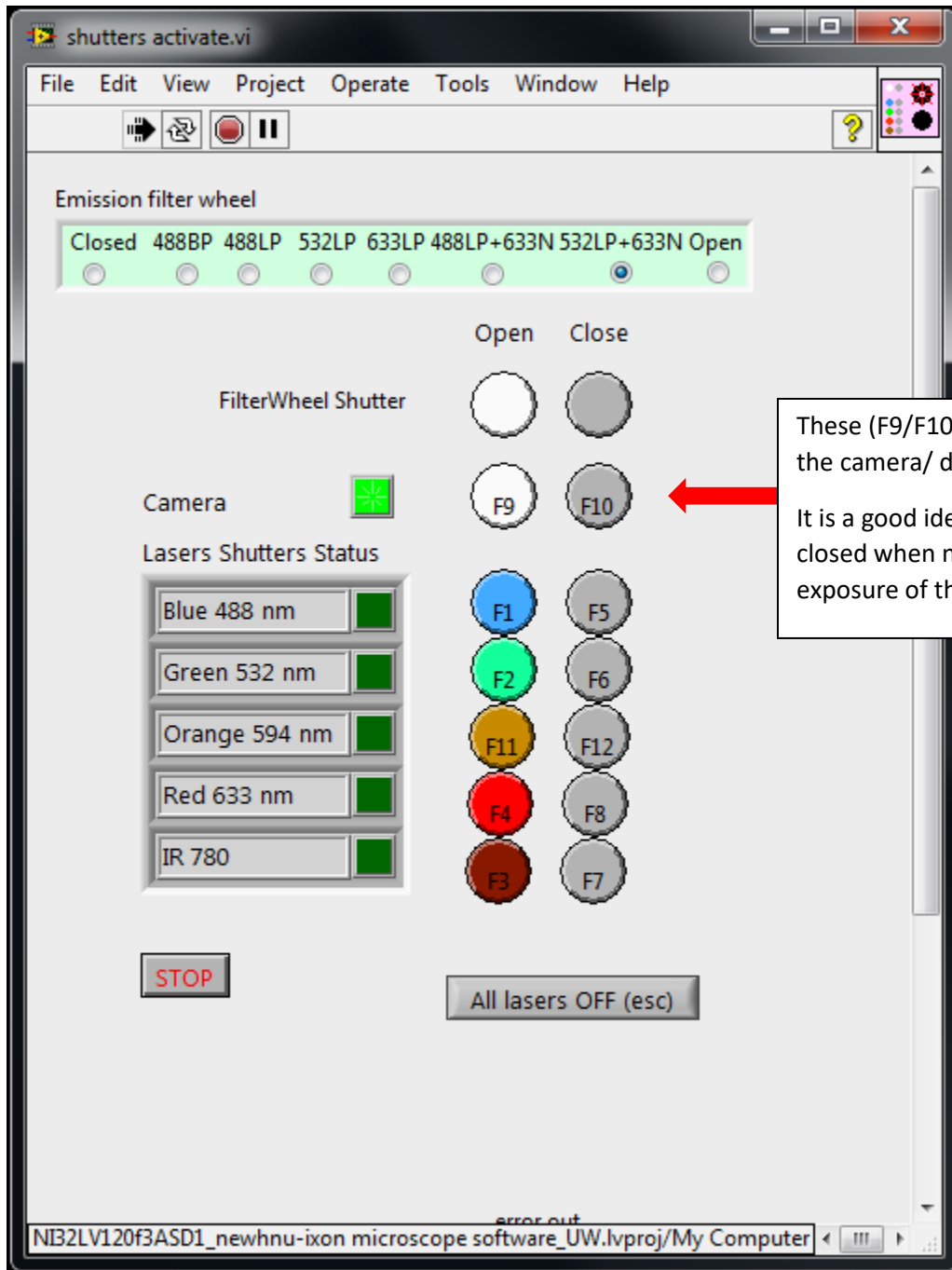
This opens main Labview/Glimpse window

Click on  to "run", this starts all windows you should need:


Shutters Activate.vi window:

Test out Emission Filter Wheel control (you should be able to hear carousel turning after making selection and pressing the “run” arrow button)

Turn on Red Laser (F4) and select 633LP shutter, press the “run” arrow button  to check alignment - you should be able to visually observe red laser exiting from sample stage (if not, see following pages for stage controller movement.)



Camera startup.vi window:

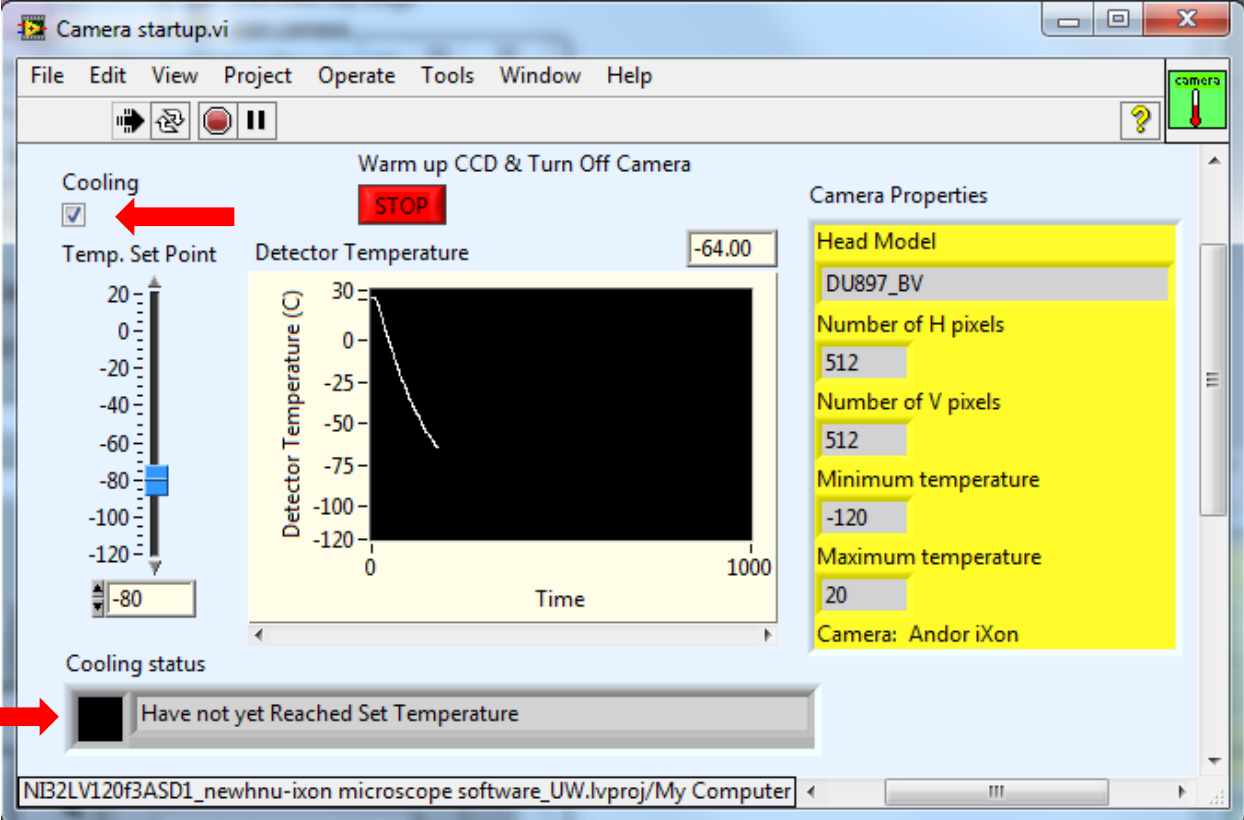
Press the “run” arrow button 

Select the box below “Cooling”

This will start cooling the camera and display the CCD Detector Temperature graph

The graph tracing the Detector Temperature should start decreasing towards -80C

The “Cooling status” at the bottom of the window will change once it is ready and the box will turn green. Do not start a Camera Acquisition until the Cooling status indicates it is ready.



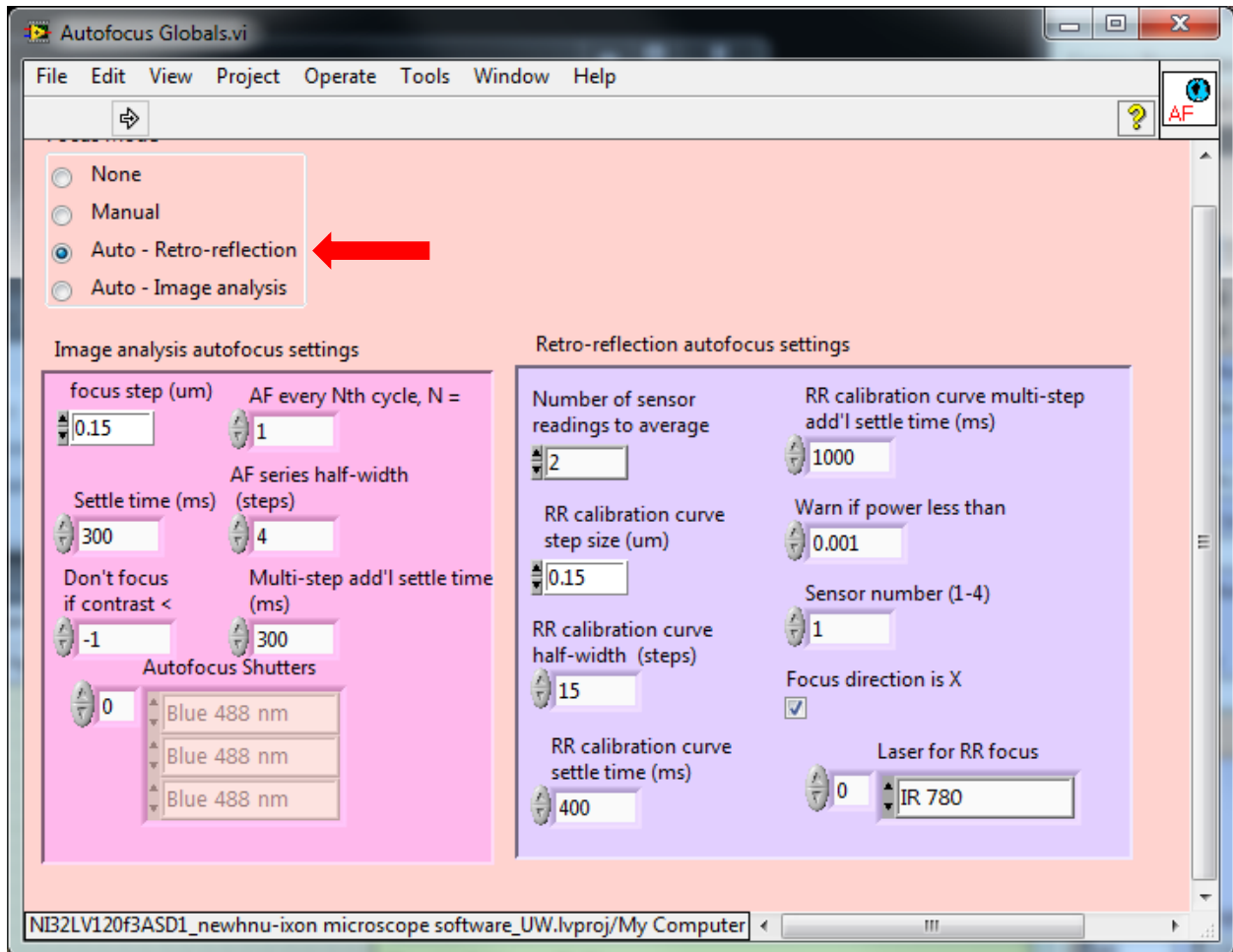
For Shut Down:

When done with your experiments the detector cooler needs to be shut down by unchecking the Cooling box. The temperature should be back up to +25 before shutting down the software.

Autofocus Globals.vi

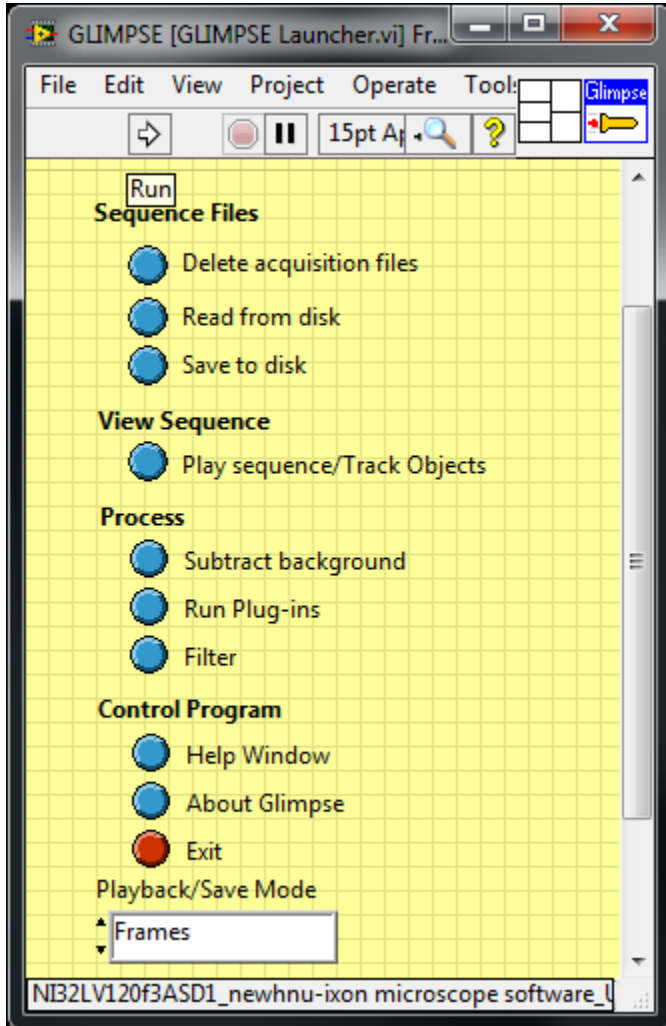
In normal operation you should not change anything on here except the four options in the white outlined box at the top:

- None
- Manual
- Auto – Retro-reflection (uses the IR laser)
- Auto – Image Analysis (we do not use this one)




Glimpse Launcher.vi window

Press the “run” arrow  to launch a Glimpse window.

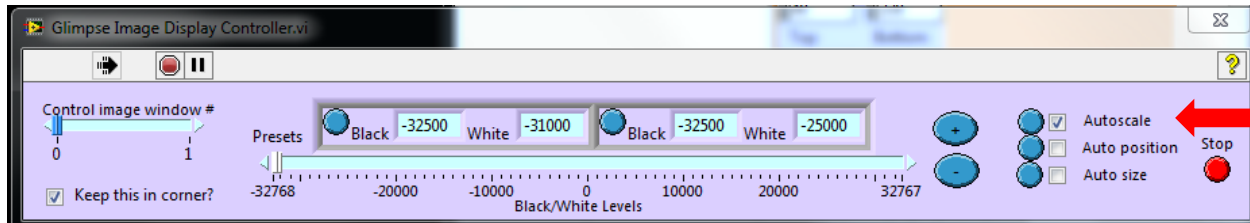


Glimpse Image Display Controller.vi window:

In Multimode Acquisition.vi window select the “run” arrow  and select the yellow circle “Preview” in Camera Setup Acquisition.vi window – full window image on next page.



The Glimpse Image Display Controller.vi window will open, make sure the box next to “Autoscale” is checked.



Turn on the Red (633 nm) and Green (532 nm) lasers, and select “532LP + 633N” filter

Slowly adjust the z axis with the z-positioner thumb screw on the stage, turning clockwise, lowering the slide, to get lasers aligned (you should see the beam exiting out of the emission port, you also should see two beams on the underside of the shelf directly above your sample after passing up through the objective – these two beams should just about converge as they get into focus).

Now is a good time to adjust and record the power output of the lasers [using power meter on laser table]

1. Press the Power button on the Power Meter to turn it on
2. Press the “ λ up” or “ λ down” buttons to change to wavelength to match the laser you are checking
3. Press the “save λ ” button
4. Angle the power meter’s sensor into the path of the laser (just before the sample stage)
 - a. The head of the sensor pivots by 90 degrees into the laser path
5. With all shutters closed (lasers blocked) press the “zero” button to reset the reading
6. Open the shutter for the laser you are interested in
7. Make note of the power reading on the meter
8. To adjust power of the laser reaching your sample adjust the thumbscrew on the quarter-wave plate/ “vertical polarizer” (marked with a little white tape flag) in the laser path
 - a. The vertical polarizers are continuously variable and will rotate back to the original settings if the thumbscrew is rotated enough, they can be rotated indefinitely in either direction and will just cycle through settings
9. Repeat steps 2-8 for all lasers you are using
10. Please remember to turn the power off by pressing the power button until the display goes blank

Camera Setup Acquisition.vi window:

Load 40 uL of streptavidin labeled component onto slide

Wait 5 Minutes

Wash channel with 200 ul of mock buffer

In **Camera Setup Acquisition.vi** window press the Yellow Preview Button to start live acquisition

Add 20 ul of ~200pmol/L RNA solution to channel

Look around towards the corners of the acquisition area to check for good coverage, add more RNA if needed. Open and close shutter depending on if you need to view image or are prepping solutions, so as not to overly photobleach your sample.

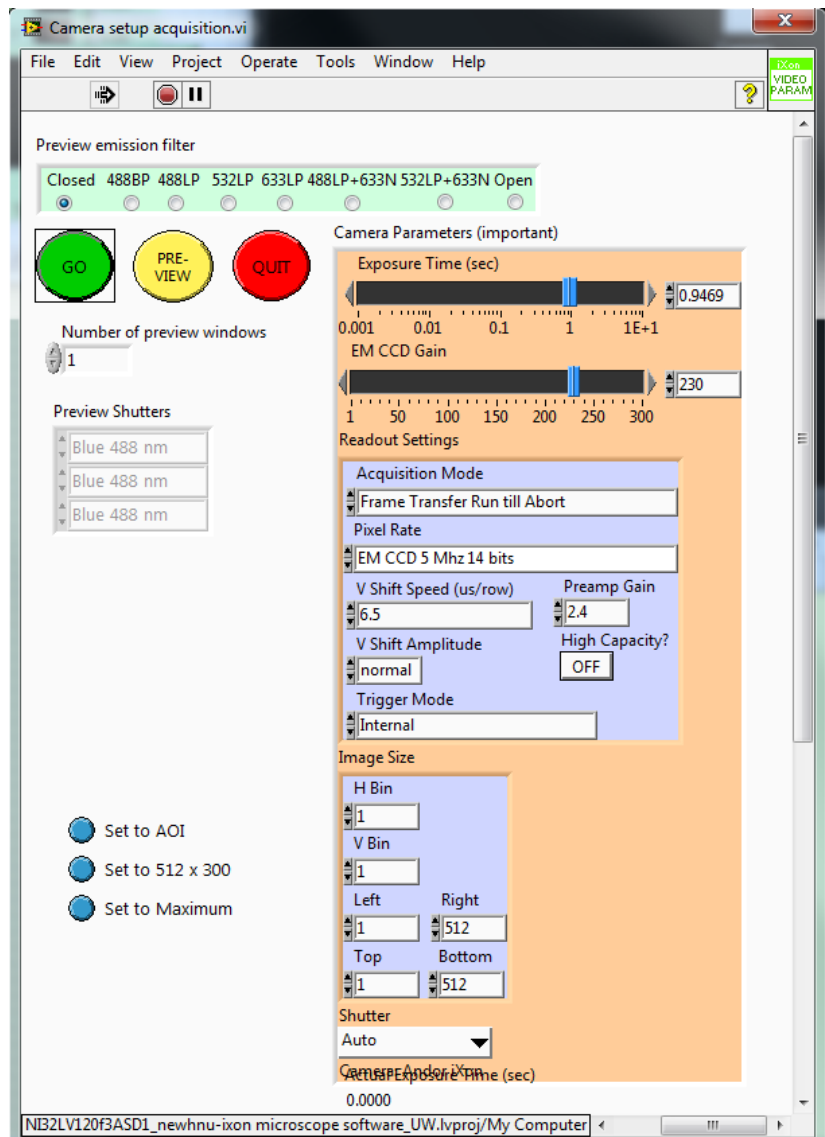
After loading RNA check a few locations on slide with live (Preview) acquisition and moving the slide with stage controller.vi window (next page)

Can add Max RNA if needed.

Wash channel with 200 uL of mock buffer + OSS

Now is a good time to check focus on all lasers

Once you are happy with Laser focus prepare your extract / final sample mixture.

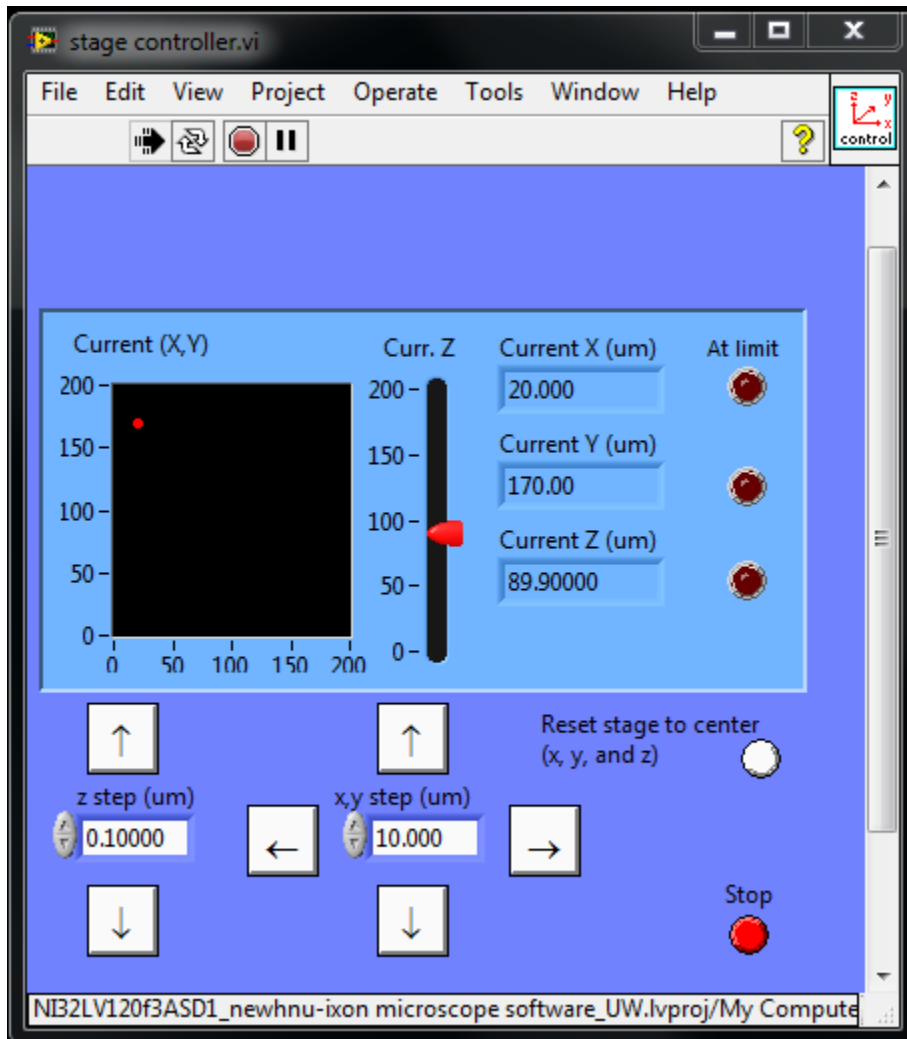


Stage Controller.vi window:

Allows you to control the movement of the stage in the x, y & z axis from the computer.

You can manually fine tune focus by using the up and down arrows for the “z step” (um.)

Further focusing can be done with the Retro-Reflect Autofocus window (next page).



RR autofocus view calibrate.vi window:

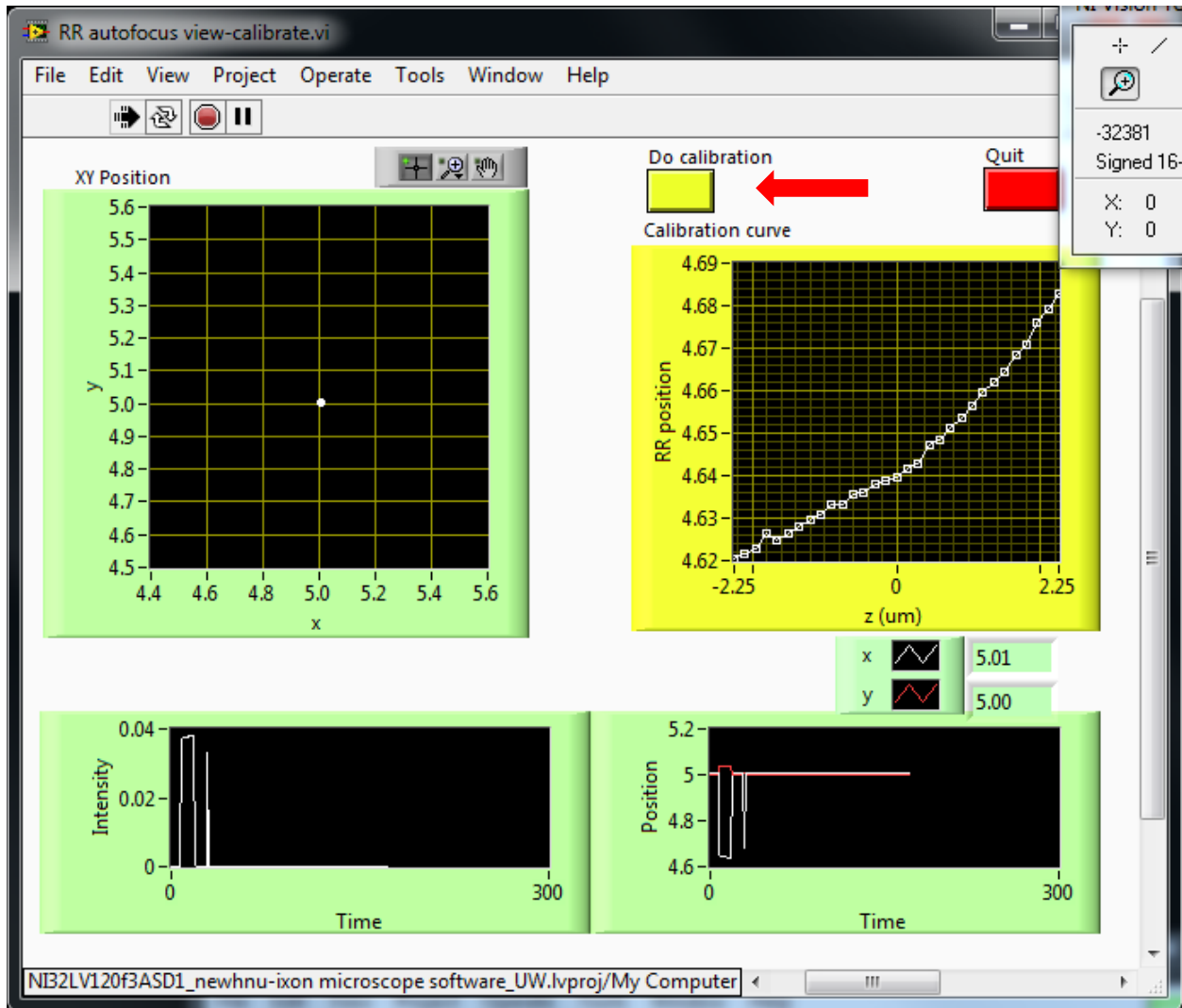
Make sure the Near IR (785 nm) laser is turned on.

Click on arrow button to “run”,



Click on the yellow “Do calibration” box to start calibration curve.

A “good” calibration curve is one where increasing “z(um)” correlates, roughly linearly, with increasing “RR position” (you may see either a positive or negative correlation, as long as it looks linear it is OK)



Now is a good time to recheck all acquisition parameters (# frames, # repeats, etc.)

Add 40 ul of Extract to your sample channel on slide

Focus with z-step on stage controller.vi window

Start collecting acquisition by clicking the go arrow button on the Multimode Acquisition.vi window

Multimode Acquisition.vi window: Set most acquisition parameters, experiment specific:

Exposure time: 1 sec (typical, but dependent on your experimental design)

Em CCD Gain: ~100 (increases signal and noise)

Upper lilac box (Acquisition mode, etc.): do not change these settings

Lower lilac box: Sets image size by selecting which pixels on sensor to record (full 1-512:1-512)

(e.g. Left:40 , Right: 330, Top: 1, Bottom:512)

Acquisition Protocol (yellow Box): Set up acquisition protocol/steps – runs leftmost box first, then right sided box – select which lasers are on and filter setting.

-usually keep “Number of frames to acquire” at 1

-“Wait after frames” can insert a waiting period in seconds after this box is run

-“# times to repeat” enter number of frames you want to collect under these settings

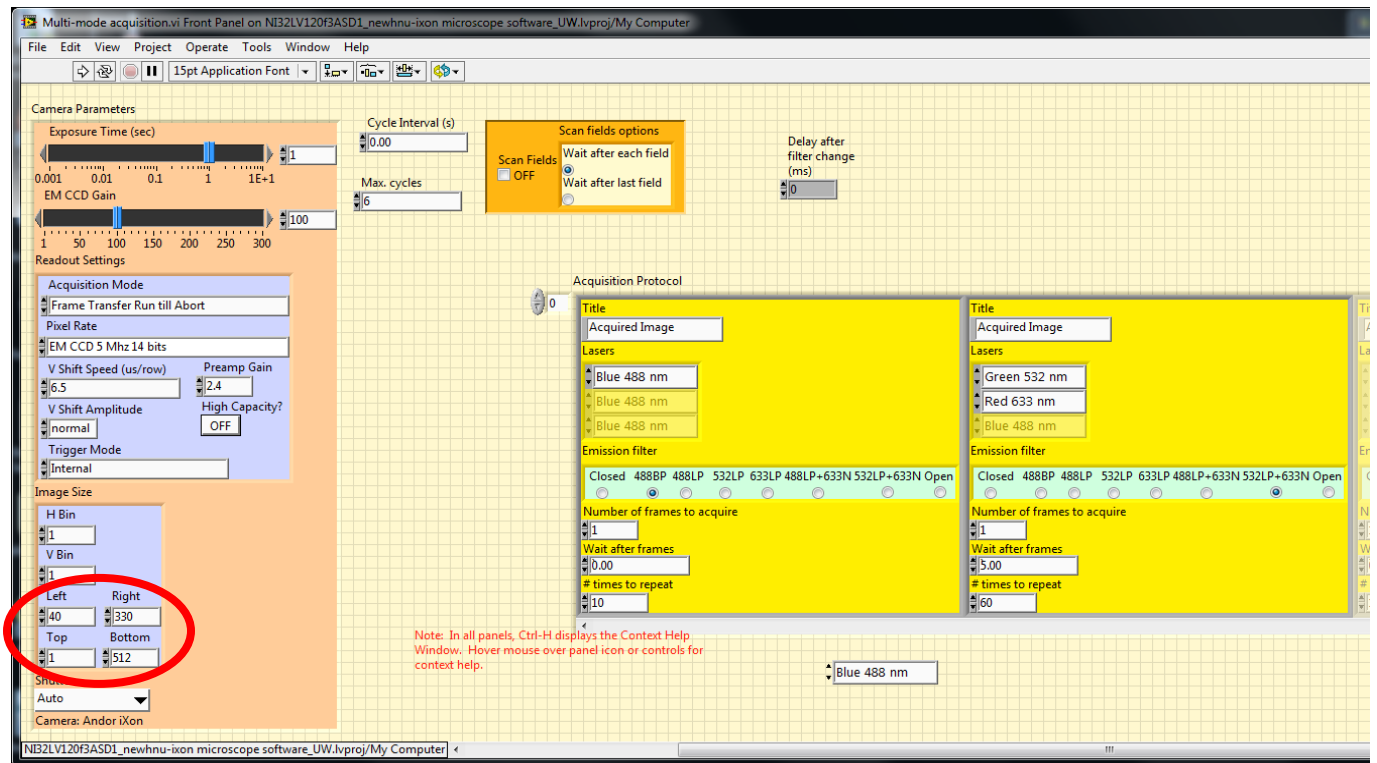
Max cycles: (e.g. 6) the number of times the program will repeat the conditions in the Acquisition Protocol box to complete the experiment

Cycle Interval(s): how long the computer pauses between each cycle of the experiment (e.g. 0)

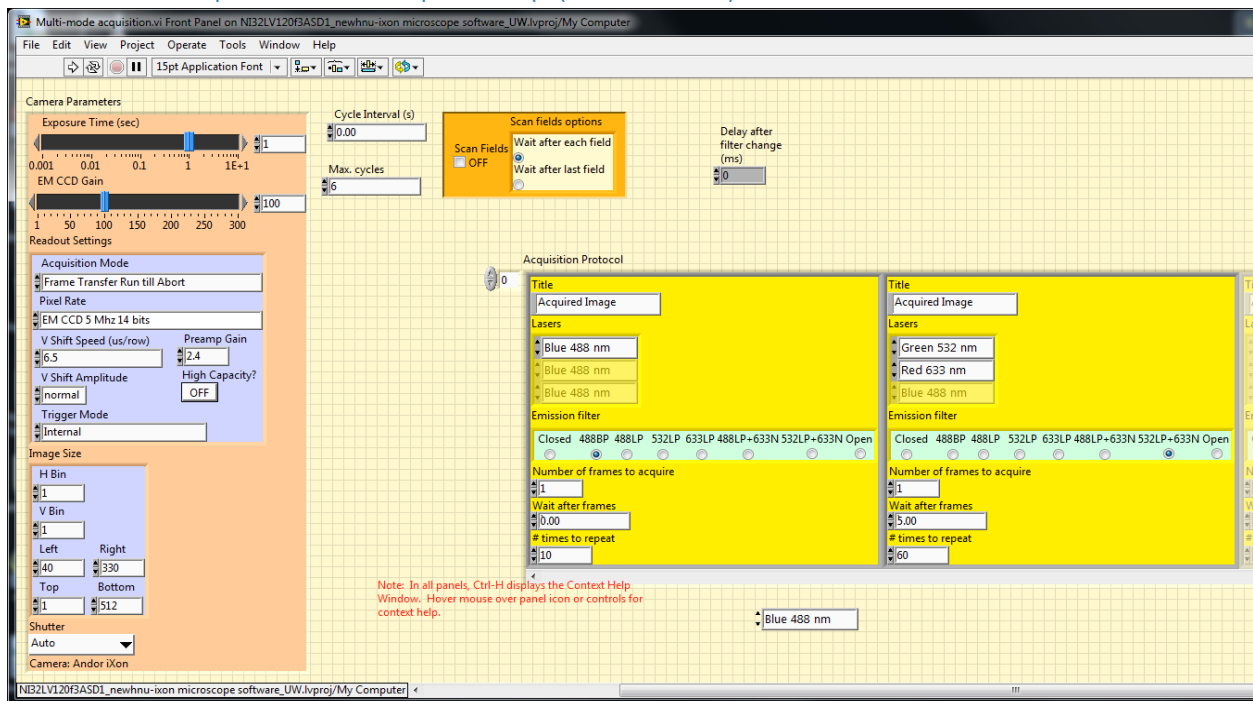
When you are ready to start your acquisition select the “run” arrow



Press the Green Go button and remember to select the box next to Autoscale



Multimode Acquisition example setup (Josh Larson)

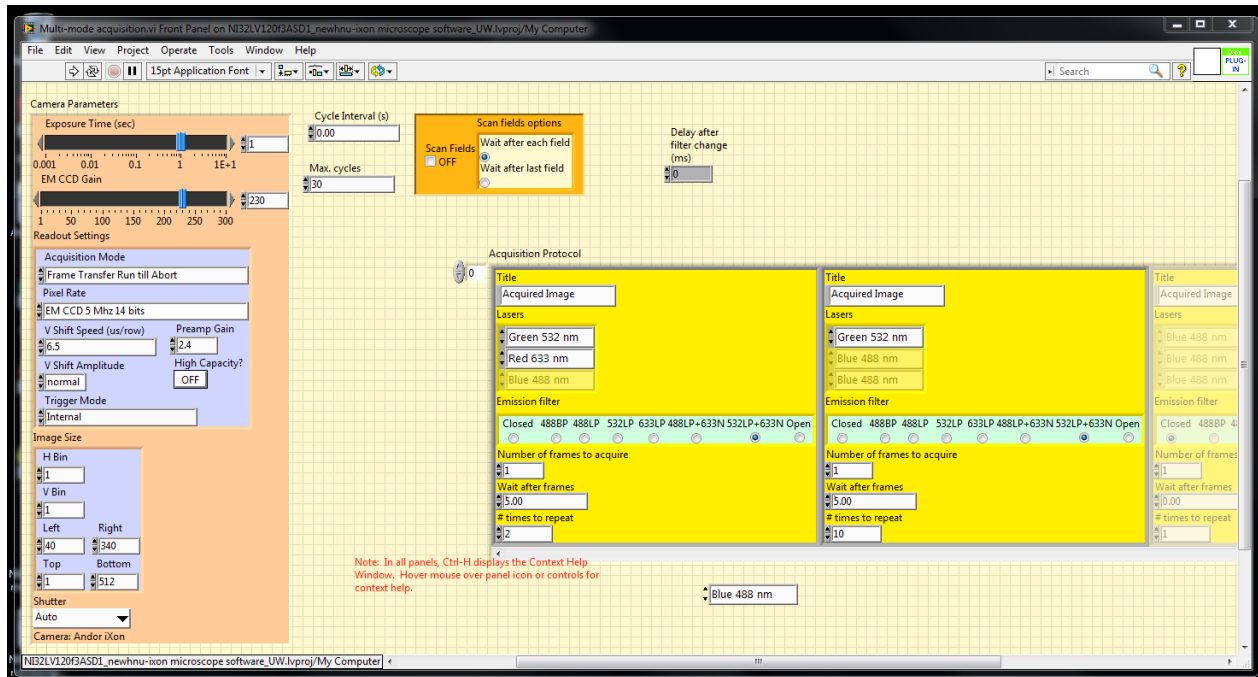


The length of time for each frame is defined as one second.

The area the detector being captured for each frame is defined by positions 40 Left, 130 Right and 1 Top and 512 Bottom.

1. The Blue Laser shutter is opened. The emission filter wheel rotates to the 488P position. Data is collected for 10 frames with no additional pause time (total approximate time 10 seconds).
2. The Blue Laser shutter is closed. The Red and Green Laser Shutters are opened and the emission filter wheel rotates to the 522LP + 633N position. Data is collected for 60 frames, followed by a 5 second pause (total approximate time 65 seconds).
3. The Red and Green Laser Shutters are closed and the IR Laser shutter is opened. The sample is focused using the IR laser Retro Reflect protocol. The IR Laser shutter is closed.
4. Repeat steps 1-3 for an additional 5 times (6 times total)

Multimode Acquisition example setup (Sarah Hansen)



The length of time for each frame is defined as one second.

The area the detector being captured for each frame is defined by positions 40 Left, 130 Right and 1 Top and 512 Bottom.

1. The Red and Green Laser Shutters are opened and the emission filter wheel rotates to the 522LP + 633N position. Data is collected for 2 frames, followed by a 5 second pause (total approximate time 7 seconds).
2. The Red Laser shutter is closed. The emission filter wheel stays at the 522LP + 633N position. Data is collected for 10 frames, followed by a 5 second pause (total approximate time 15 seconds).
3. The Green Laser Shutter is closed and the IR Laser shutter is opened. The sample is focused using the IR laser Retro Reflect protocol. The IR Laser shutter is closed.
4. Repeat steps 1-3 for an additional 29 times (30 times total)

Filter Wheel Position	0	1	2	3	4	5	6	7
Filters	None/ Closed	488BP	488LP	532LP	633LP	488LP + 633N	532LP + 633N	594LP/ Open
Useful for	Blocking Light to the camera	Observing Alexa488 in the presence of Cy3/Alexa594 (green laser shuttered)	Observing Alexa488 by itself or in the presence of Cy5	Observing Cy3 while red laser is shuttered	Observing Cy5 only	Observing Alexa488 and Cy5	Observing Cy3 and Cy5	Observing Alexa 594
Type of Experiment	Blocking light to the camera	Colocalization of Alexa488 signals with other dyes	Colocalization of Alexa488 with Cy5 or Alexa488 FRET	Colocalization of Cy3 when green laser only is open, Cy3/Cy5 FRET	Colocalization of Cy5 when red laser only is open	Colocalization of Alexa488 and Cy5 when both blue and red lasers open	Colocalization of Cy3 and Cy5 when both green and red lasers open	Observing Alexa594 (shows up in both dual view channels)

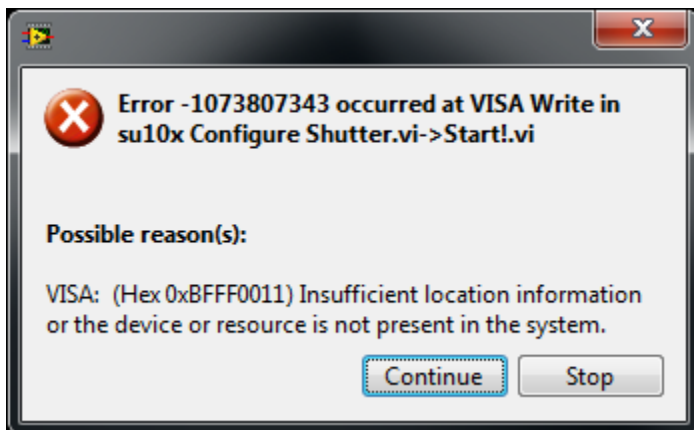
Merging/Joining multiple videos into one video

Can be performed from Glimpse with microscope “on” or “off”

If starting up Glimpse while microscope (lasers, shutters, etc.) is off, follow procedure as written on page 2. You will probably get some error messages when Glimpse tries to communicate with the shutter controller (“Filter wheel controller”) and does not receive a reply. Select “Skip” for this:



and cancel / acknowledge / continue through any other error messages that pop up:



You can close most of the windows to clear up the screen, do not close **Glimpse Launcher.vi**, you will need it.

From here on instructions should be the same whether the microscope is on or off.

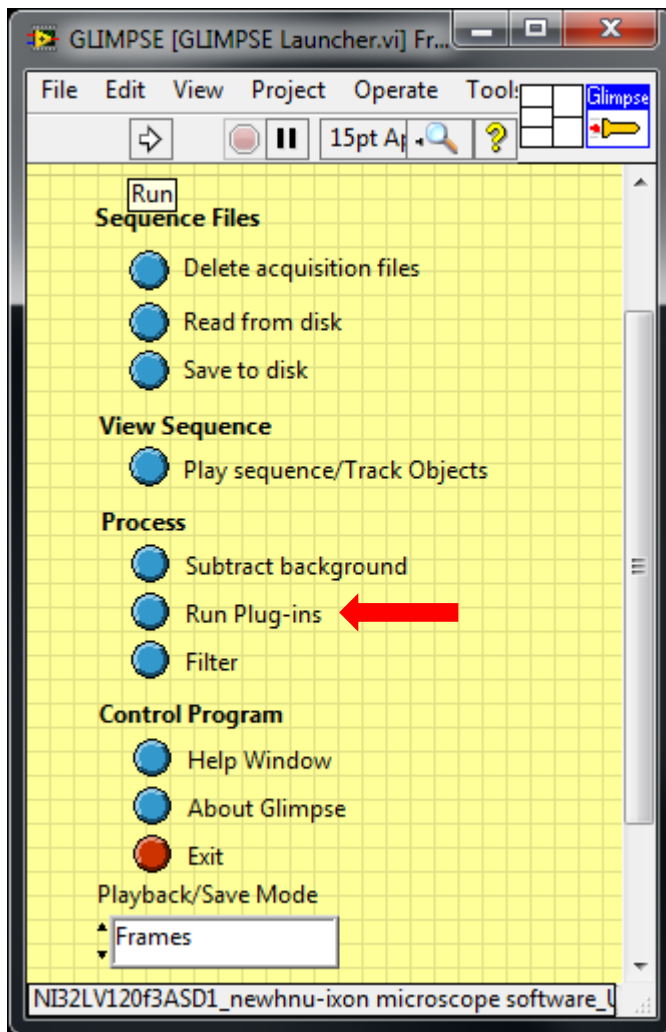
Glimpse Launcher.vi:

Select the “run” arrow



Select the blue circle next to “Run Plug-ins”

This will open the **Glimpse Plugin Handler.vi** window

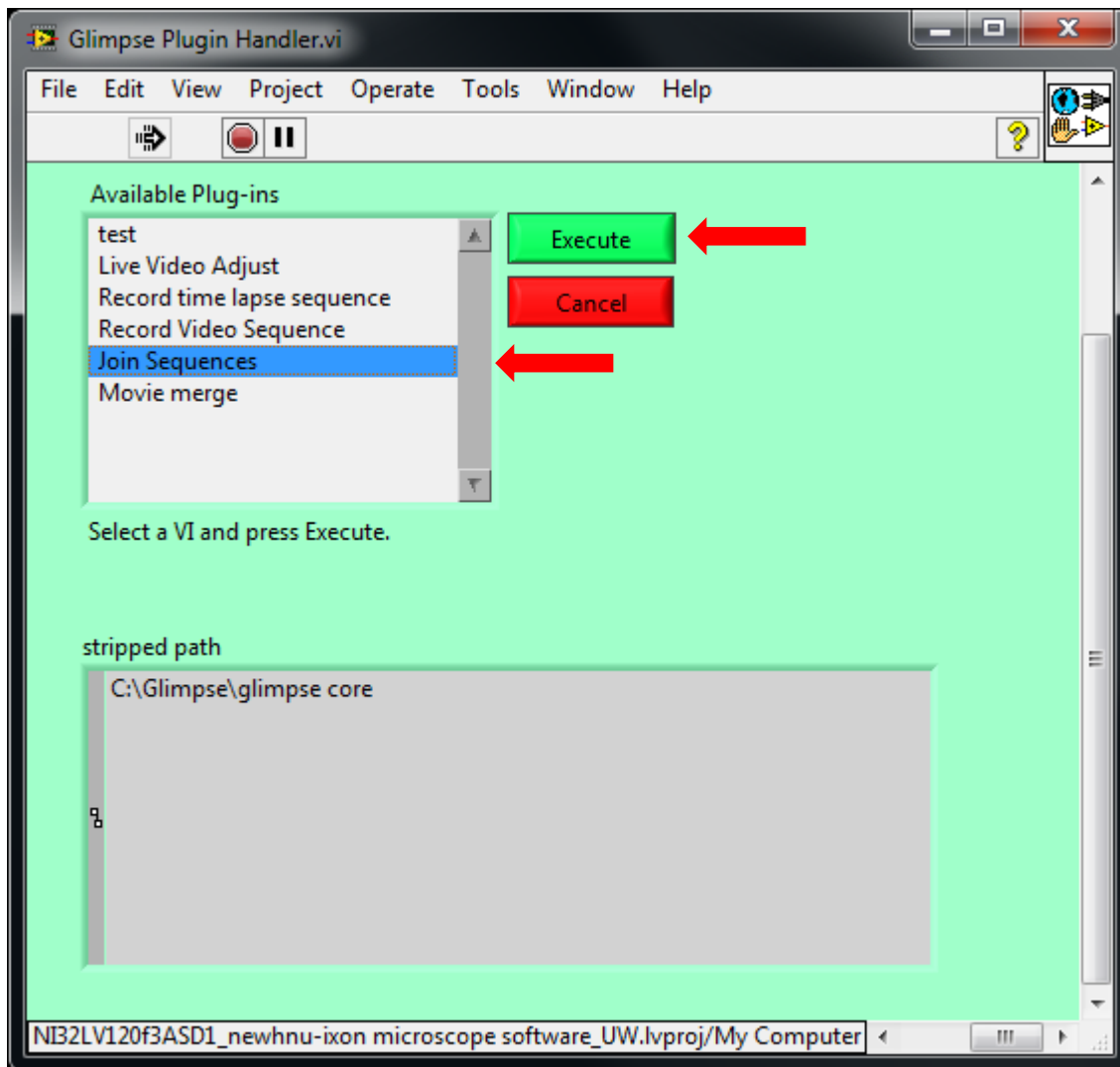


Glimpse Plugin Handler.vi

Select "Join Sequences" from the "Available Plug-ins" menu

Press the "Execute" button

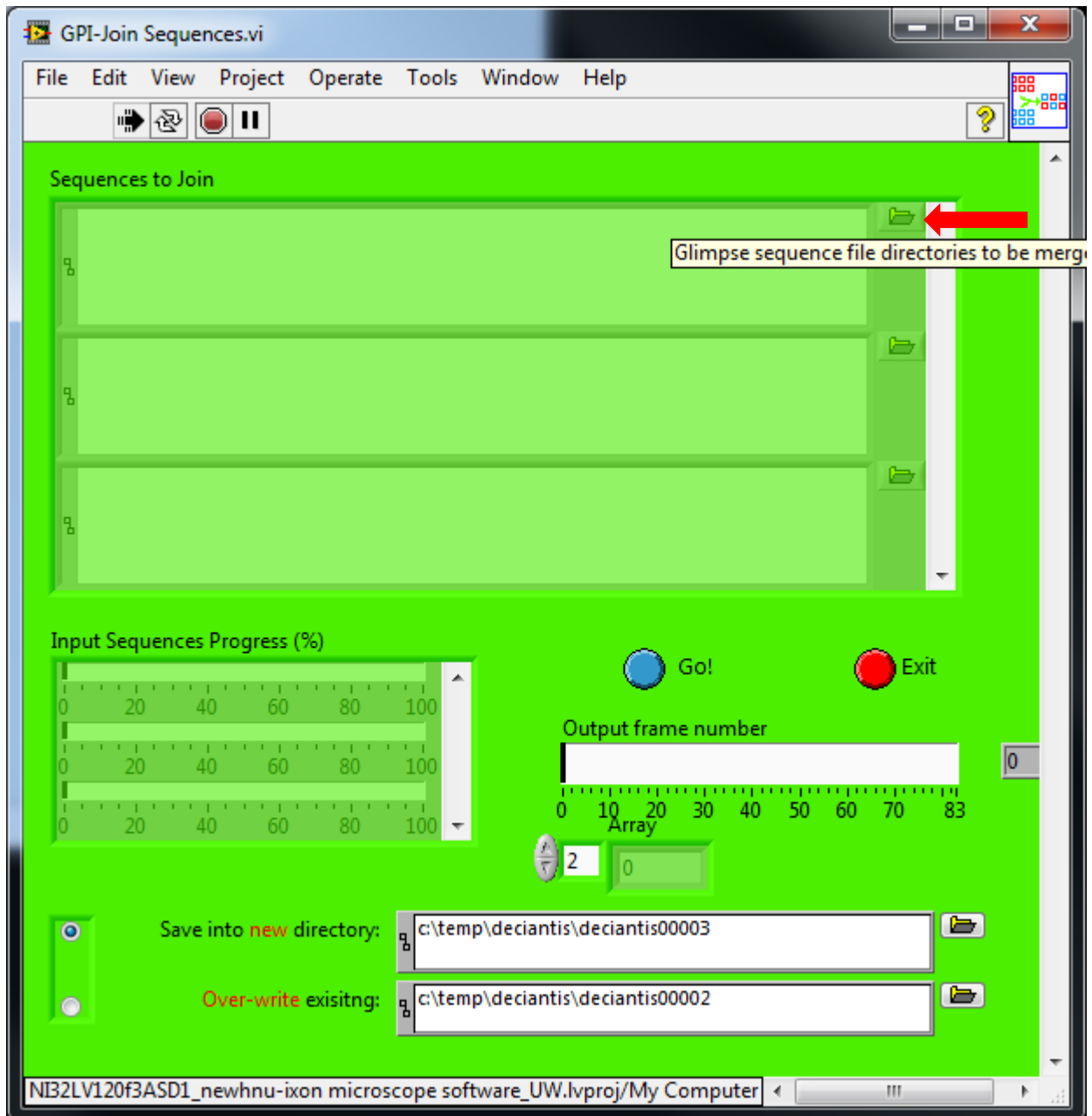
This will open the **GPI-Join Sequences.vi**



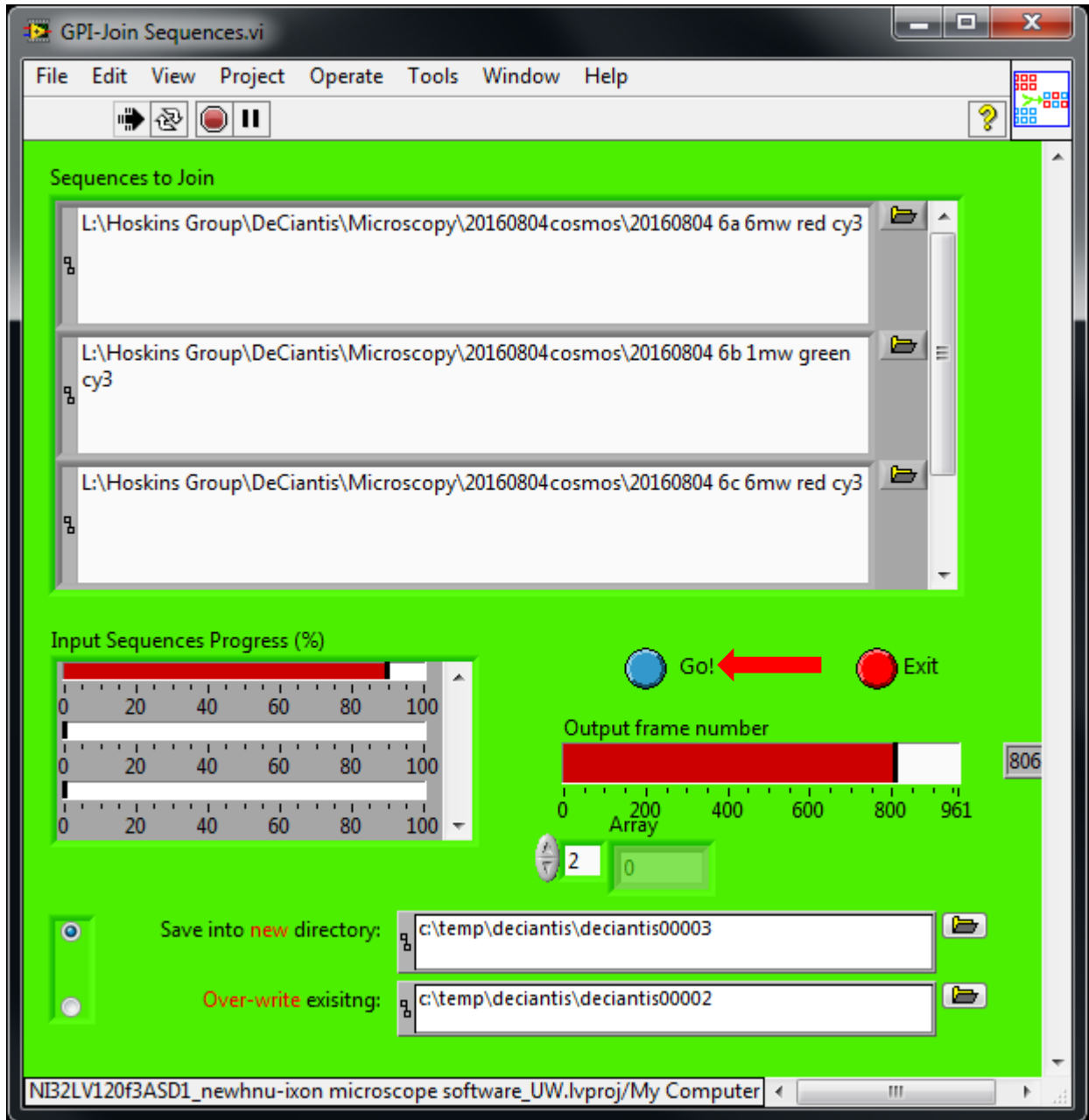
GPI-Join Sequences.vi

Select the uppermost file icon to browse for and choose the file you want to be at the beginning of the merged movie.

Subsequently, select the movie segments you want second, third, ect. in the merged movie by clicking on the following folder icons.



Once all segments have been selected, check the output directory that new movie will to save to listed at the bottom of the window. Press the “Go!” button and the program will start joining the sequences into a new glimpse movie in the designated directory.



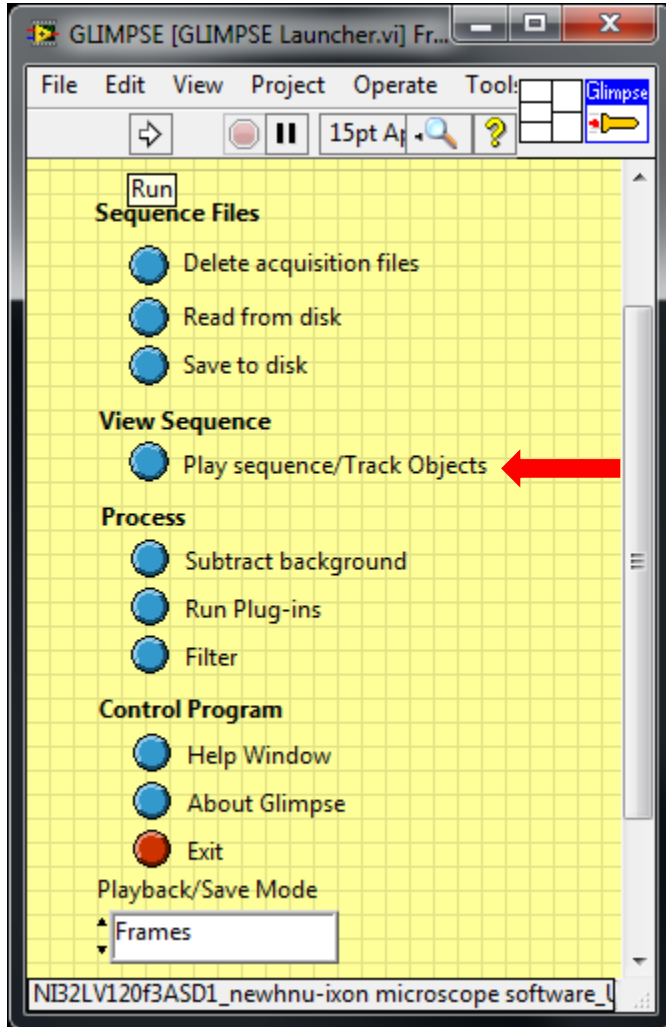
When done select the red exit button.

Playback of merged video:

Select the “run” arrow



Select “Play sequence/Track Objects” in the **Glimpse Launcher.vi** window



Select the movie you want to view and hit OK

