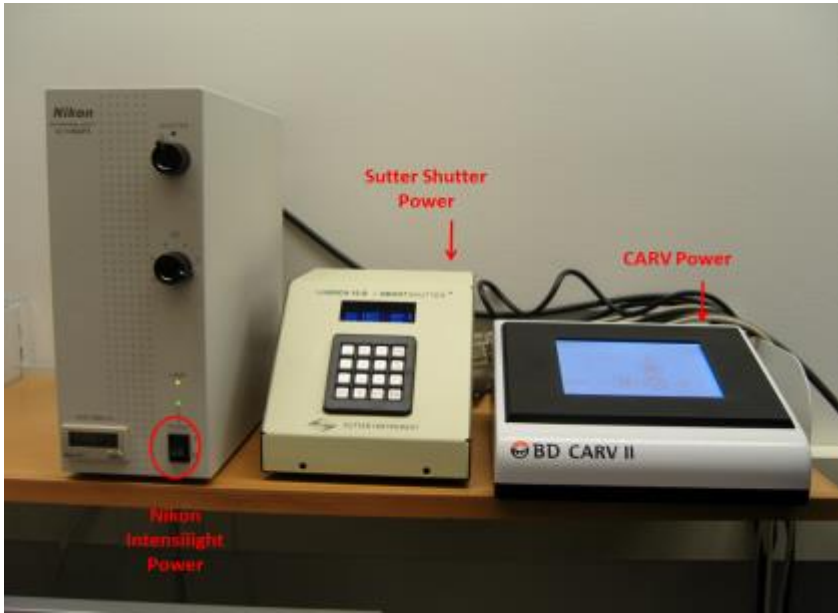
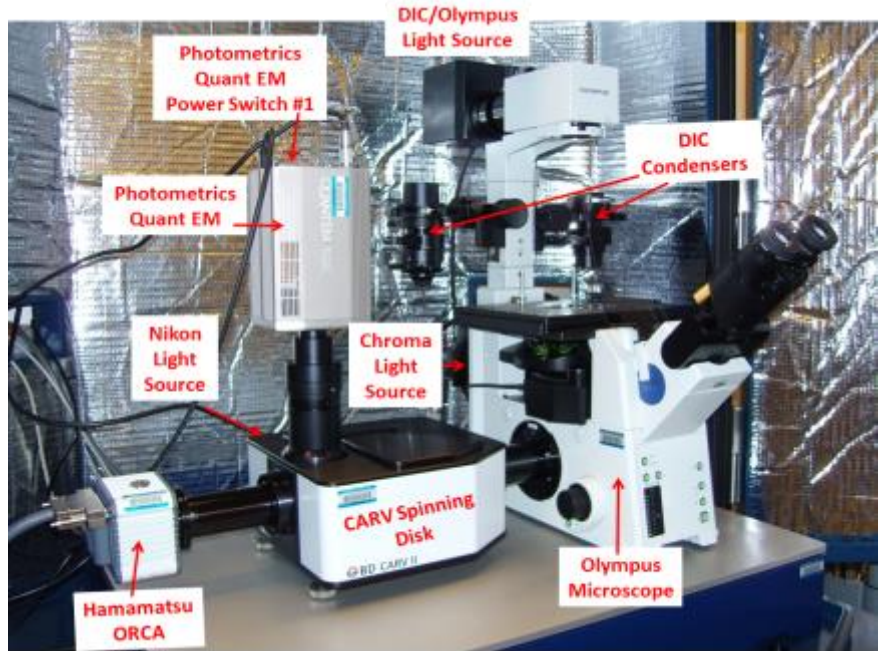


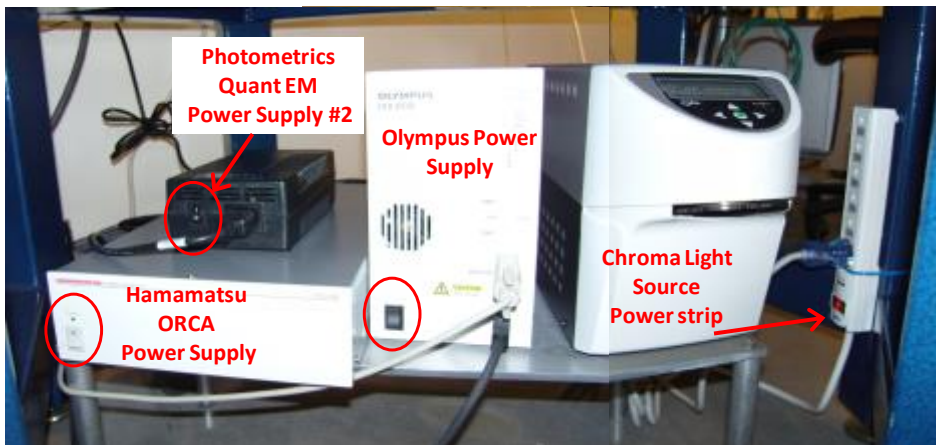
## CHANL Confocal Checklist

Familiarize yourself with the microscope and all of its components. You may not need to use all of the components for your experiment, but an understanding of the whole system will make it easier to use. This guide will walk you through which components are needed for the type of experiment that you want to do.



### Light Sources

- 1) DIC/Olympus light source: This is the light source that illuminates the sample from above, when you want to detect light that is transmitted through your sample. (Brightfield, DIC or phase imaging)
- 2) Olympus light source (Chroma): This light source comes in through the lower back side of the Olympus microscope. You will use it when doing WF/fluorescence by eye, and you can also send this signal to the cameras.
- 3) CARV light source (Nikon): This light source comes in through the back side of the CARV spinning disk system. You will use it when doing confocal imaging, and you can also do WF/fluorescence to one of the cameras with it.



## Filters

Filter Set	Excitation	Emission
DAPI	381-393 nm	417-477 nm
GFP	464-500 nm	516-556 nm
TxRED	542-582 nm	604-644 nm

## Detectors

Detector	Resolution	Sensitivity	Maximum Frame Rate
Hamamatsu ORCA	High (1344 X 1024)	Medium	~5 full frames/sec
Photometrics Quant EM (QEM)	Medium (512 X 512)	High	~30 full frames/sec
Binoculars	N/A	N/A	N/A

Either camera works well for widefield, but the ORCA has ~4x the pixels which gives better spatial resolution. The QEM, however, has more sensitivity and much more speed. For faint or dynamic samples, the QEM is recommended (confocal or fast streaming movies). For static bright samples, the ORCA is recommended.

## Operating Procedure

- 1) Determine what type of experiment you want to do.
  - a. Brightfield: Transmitted light experiment that gives no contrast enhancement. This is good for viewing thick sections.
  - b. DIC: Can view unstained thin samples. The image has the appearance of a three dimensional object under very oblique illumination, causing strong light and dark shadows on the corresponding faces. The direction of apparent illumination is defined by the orientation of the Wollaston prisms.
  - c. Phase: Can view unstained samples. Small phase shifts in the light passing through a transparent specimen are converted into amplitude or contrast changes in the image.
  - d. Widefield (WF)/Fluorescence: Samples must be stained (or otherwise contain a fluorescent component) to employ this technique. Light of a wavelength which will excite your fluorophore is incident on your sample, and the fluorescent light from your sample is collected. **Widefield fluorescence does not omit out-of-plane light, so good sample preparation is needed.**
  - e. Confocal: Confocal is a fluorescent microscopy technique that uses a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. This technique allows you to collect images from different heights from within your sample. **As a result, much less light makes it to the detector, so unless your sample is very bright, you need the Photometrics EM CCD camera (QEM).** You will also need longer exposure times, and may need to increase the EM gain levels (~200).

**Once you know the experiment, light source, filter set, and detector you want to use, you can select the appropriate illumination pathway through the Metamorph software.**

- 2) Turn on all of the microscope components you will need as shown in the images on Page 1. (Nikon and Chroma light sources, CARV, Sutter Shutter, Hamamatsu ORCA and Photometrics QEM cameras – the Photometrics QEM has two switches to turn on)
- 3) Open the Metamorph software by first opening the Meta Imaging Series 7.5 folder on the desktop and double clicking on the Metamorph icon.

- 4) Next decide what magnification you will use. All of these objectives below can be used for fluorescence work, but only certain ones can do DIC or phase imaging.
  - a) 10x, 0.3 NA, air, phase 1: Low resolution; large field of view. Use this objective to locate a region of interest on your sample, or for use with thick samples. Can do phase imaging.
  - b) 20x, 0.45 NA, air, phase 1: Low-medium resolution; large field of view; can do phase imaging.
  - c) 40x, 0.6 NA, air, phase 2: Medium resolution; can do phase imaging.
  - d) 60x, 1.2NA, Water: Medium-High resolution; can be used with DIC. Must put a drop of water between your sample and the objective lens.
  - e) 100x, 1.3NA, Oil: High resolution; small field of view; can be used with DIC. Must put a drop of oil between your sample and the objective lens.
  - f) 100x, 1.4NA, Oil: High resolution; small field of view; can be used with DIC. Must put a drop of oil between your sample and the objective lens.
- 5) Select the objective you want to use from the Mag pull down menu at the bottom of the Metamorph software. If necessary place a drop of oil (100x objectives) or water (60x objective) on the bottom side of your sample.
- 6) Place your sample on the sample stage, over the objective. Use the clips to hold your sample in place if necessary.
- 7) In Metamorph, select Devices from the top pull down menu and then select Configure Illumination. On the right side of the page, there are different illumination pathways that describe: i) the type of experiment you are doing, ii) the origin of the light source, iii) The filter set used, and iv) the detector as described in the table below. **DO NOT change the settings on the left side of this page. If you do need to do so (to access a setting that's not in the given list, for example), please do NOT save the changes.**

Imaging Mode	Light Source	Filter set	Detector	Illumination Pathway
Confocal	CARV	DAPI	ORCA	Confocal DAPI ORCA
Confocal	CARV	DAPI	QEM	Confocal DAPI QEM
Confocal	CARV	GFP	ORCA	Confocal GFP ORCA
Confocal	CARV	GFP	QEM	Confocal GFP QEM
Confocal	CARV	TxRED	ORCA	Confocal TxRED ORCA
Confocal	CARV	TxRED	QEM	Confocal TxRED QEM
DIC/Brightfield/Phase	Olympus - DIC	None	Binocular	DIC Binoc
DIC/Brightfield/Phase	Olympus – DIC	None	ORCA	DIC ORCA
DIC/Brightfield/Phase	Olympus – DIC	None	QEM	DIC QEM
Fluorescence	CARV	DAPI	ORCA	WF CARV DAPI ORCA
Fluorescence	CARV	DAPI	QEM	WF CARV DAPI QEM
Fluorescence	CARV	GFP	ORCA	WF CARV GFP ORCA
Fluorescence	CARV	GFP	QEM	WF CARV GFP QEM
Fluorescence	CARV	TxRED	ORCA	WF CARV TxRED ORCA
Fluorescence	CARV	TxRED	QEM	WF CARV TxRED QEM
Fluorescence	Olympus	DAPI	ORCA	WF Olym DAPI ORCA
Fluorescence	Olympus	DAPI	Binocular	WF Olym DAPI Binoc
Fluorescence	Olympus	GFP	ORCA	WF Olym GFP ORCA
Fluorescence	Olympus	GFP	Binocular	WF Olym GFP Binoc
Fluorescence	Olympus	TxRED	ORCA	WF Olym TXRed ORCA
Fluorescence	Olympus	TxRED	Binocular	WF OlymTxRED Binoc

The illustrations and descriptions in Appendix A further describe the possible light paths available.

- 8) Select one of the binocular settings to first find your sample and focus on it by eye.
- 9) Once you've found your sample, you can select one of the camera settings depending on the type of experiment and the type of sample you have. (For low light samples, use the QEM, for high resolution images, use the ORCA.)
- 10) Select Acquire from the top pull down menu and click on Select Camera/Board. Ensure that the detector you want to use is selected. If you decide to change detectors, you will need to not only select the correct illumination pathway, but you will also need to change it here.
- 11) Click on the Acquire Config button on the right side of the Metamorph software. In this window you can set the dwell time and some of the detector settings to acquire an appropriate image.
- 12) Click on the Live button on the right side of the Metamorph software to view a live image of your sample as viewed at the camera.
- 13) You may also need to adjust the light intensity from the source you are using.
  - a. For transmitted light experiments (brightfield, DIC, and phase) you can adjust this on the front of the microscope.
  - b. For experiments using the Chroma light source through the Olympus, you can use the black dial on the front of the power supply to adjust the light intensity.
  - c. For experiments using the Nikon Intensilight, you can either change the CARV Intensity Iris through the software, or adjust the dial on the front of the power supply to adjust the light intensity.
- 14) To acquire an image, press the Acquire button in the Acquire Config window. You may save this image by going to File->Save As at the top of the page. Save your data in C:/MM\_7.5/images/advisor's name/your name/
- 15) If you would like to do a **z-stack**, click on the MDA Acquire (multidimensional acquisition) button on the right of the Metamorph software. You can choose to do a z-stack in either confocal or widefield mode. In any confocal experiment, out-of-plane light is omitted, thus allowing you to image one plane of focus at a time. As a result, much less light makes it to the detector, so unless your sample is very bright, you will need the Photometrics EMCCD camera (QEM). You will also need longer exposure times, and may need to increase the EM gain level to ~200 (this is found under the wavelength tab in the MDA window when the QEM detector is selected).
  - a. In the Main tab on the left make sure that Z-Series is selected.
  - b. In the Main tab, select Multiple Wavelengths if you would like to collect data at more than one wavelength.
  - c. Under Saving, make sure to select the correct directory to save the data and also give your file a Base Name.
  - d. Under Wavelengths select the number of wavelengths you want to collect.
  - e. Under W1, W2, etc., select the illumination pathway(s) that you want to use and the exposure time (and EM gain if using the QEM) for each acquisition.
  - f. Under Z-series, select the appropriate z heights for the top and bottom positions which will define the z range over which you will collect data. Enter the step size you want to use. You can also select to take all of the wavelengths at each height or to acquire the z series for one wavelength at a time.
- 16) If you would like to take a **timelapse series or a streaming movie**, click on the MDA Acquire (multidimensional acquisition) button on the right of the Metamorph software.

- a. In the Main tab on the left make sure that Timelapse is selected. If you want to take a streaming movie, also select the Stream box.
- b. In the Main tab, select Multiple Wavelengths if you would also like to collect data at more than one wavelength.
- c. Under Saving, make sure to select the correct directory to save the data and also give your file a Base Name.
- d. Under Timelapse enter the Number of Time Points (number of images) and the Time Interval between images. This will determine the Duration of the scan. When streaming has been selected, the Time Interval between images is zero.
- e. Under Wavelengths select the illumination pathway(s) that you want to use and the exposure time for each acquisition.

17) To **overlay images** there are two options:

- a. Go to Display and select Color Combine. At the top of the window, click the components box, and then select the images you want to display in the blue, green, and red channels. Note: your images must already be open in Metamorph to combine them in this way.  
-OR-
- b. Go to Acquire and select Multiple Wavelengths. Select the number of wavelengths you want to combine and select the appropriate illumination pathways. Input exposure times or select the auto expose checkbox. Click on the Acquire button at the top right corner. Go to Display and select color combine. At the top of the window select stack and then press Color Combine at the bottom left corner of the window. Your combined image should appear.

18) To **create a three dimensional image**, go to File and select Open in 4D Viewer. Select the nd file you wish to open. You can rotate this image by clicking on it and dragging. [Files with the nd extension can only be read with the Metamorph software. These files tell Metamorph which individual tif files to load into a stack.](#)

19) To **calibrate an image** so that you can measure distances:

- a. Open the image to be calibrated.
- b. Go to Measure and select Calibrate Distances.
- c. Press the Load From File button and make sure the most recent calibration file is selected from C./MM\_7.5/images/Distance Calibrations.
- d. Select the row that gives the appropriate magnification and camera that were used to take the image. While imaging, pay attention to the 1.6x magnification slider on the right side of the microscope to ensure accurate measurements. Hit the Apply button to apply these calibration settings to the image. (DO NOT press the Calibrate By Region button – each objective and camera combination have already been calibrated and you do not need to do this yourself.)
- e. Use the line tool to draw a line on your calibrated image, and it will display a length in  $\mu\text{m}$  as the line is being drawn. After the line is drawn, you can go to Measure and select Region Measurements. This will bring up a table that will display the distances and other stats for all lines drawn.

20) To **display a calibration bar** on your image:

- a. First calibrate the image as described above.
- b. Go to Display and select Graphics and then Calibration Bar.
- c. In the window that pops up, select the size of the calibration bar you wish to display, its thickness, X,Y location, and orientation.
- d. Make sure that the Erase Image Behind Label checkbox is unchecked. (This option is only displayed after the More button is pressed if it is not already displayed.)

- e. Press Stamp, and the calibration bar will appear on the image.

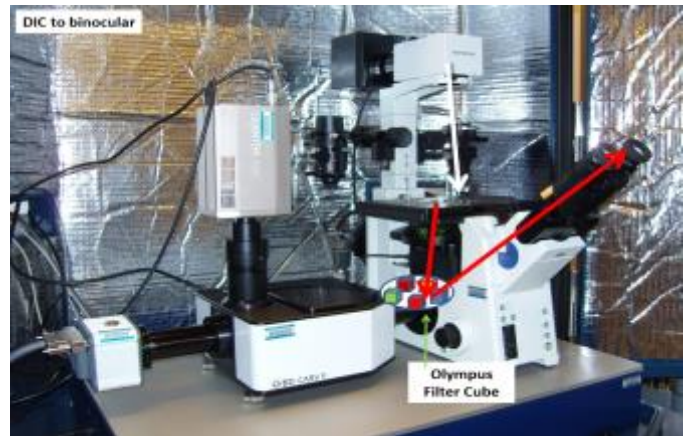
#### 21) Shutdown

- a. Remove your sample from the microscope. Gently wick away any oil or water left on the objectives. **CAUTION: Use the edge of some lens paper to *wick* the liquid away. You should not put *any* pressure on the surface of the objective with your finger, as you will likely scratch it.**
- b. Close the Metamorph software.
- c. Make sure to turn off all of the microscope components, especially the light sources and the cameras. The Olympus power supply, the CARV controller, and the Sutter shutter controller can be left on. Please also make sure that the Olympus lamp is off by pressing the button on the front of the microscope with the light bulb symbol.

## Appendix A

### a) DIC Binoc

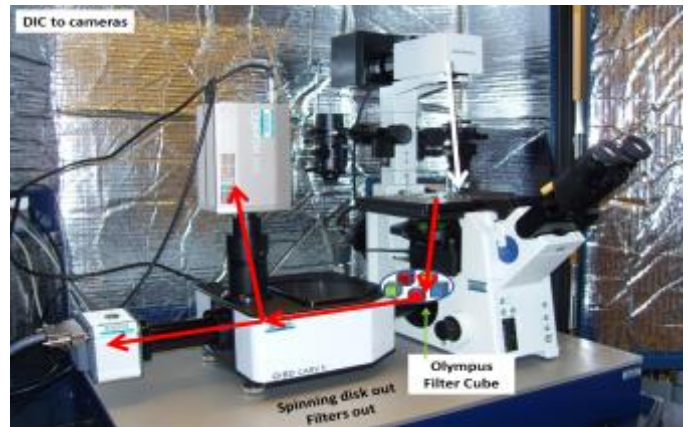
Illumination from the Olympus source at the top of the Olympus, through one of the DIC condensers and then through the sample. This light goes to the Olympus filter cube, and the signal is sent to the binoculars.



### b) DIC ORCA

DIC QEM

Illumination from the Olympus source at the top of the Olympus, through one of the DIC condensers and then through the sample. This light goes to the Olympus filter cube, and the signal is sent to the one of the cameras.

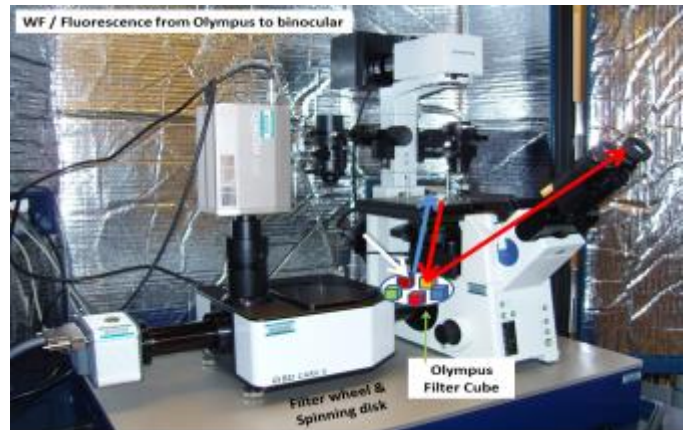


### c) WF Olym DAPI Binoc

WF Olym GFP Binoc

WF Olym TxRED Binoc

Illumination from the Chroma source at the back of the Olympus, through the Olympus filter cube and up to the sample. Fluorescence is collected back through the Olympus filter cube, and the signal is sent to the binoculars.

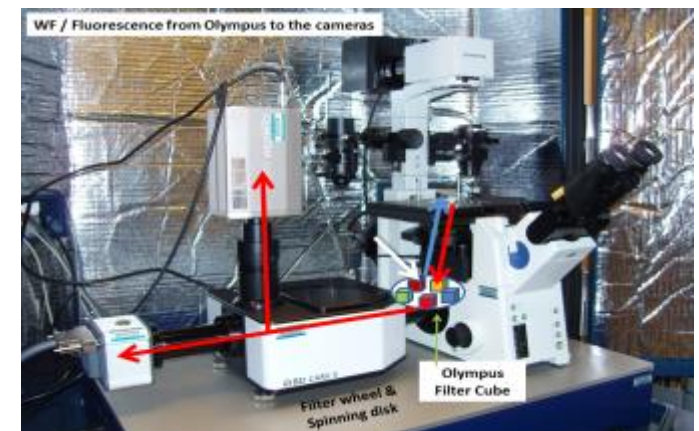


### d) WF Olym DAPI ORCA

WF Olym GFP ORCA

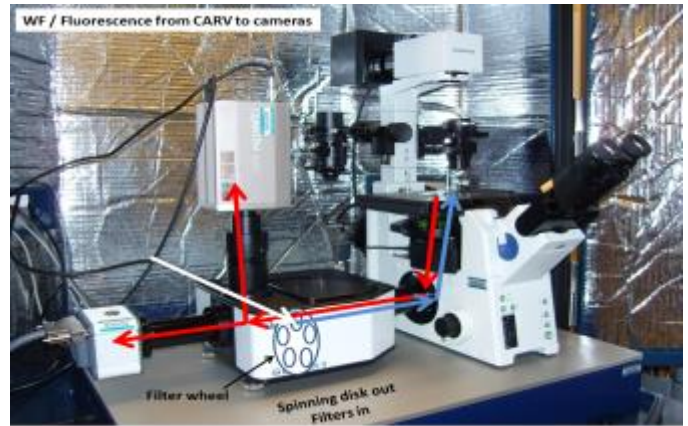
WF Olym TxRED ORCA

Same as #c above, but the signal is sent to the ORCA camera. (Signal can be sent to QEM if needed.)



- e) WF CARV DAPI ORCA
- WF CARV DAPI QEM
- WF CARV GFP ORCA
- WF CARV GFP QEM
- WF CARV TxRED ORCA
- WF CARV TxRED QEM

Illumination from the Nikon source at the back of the CARV, through the CARV filter set and up to the sample. Fluorescence is directed back to the CARV, and the signal is sent to one of the cameras.



- f) Confocal DAPI ORCA
- Confocal DAPI QEM
- Confocal GFP ORCA
- Confocal GFP QEM
- Confocal TxRED ORCA
- Confocal TxRED QEM

Illumination from the Nikon source at the back of the CARV, through the CARV filter set and the spinning disk and up to the sample. Fluorescence is directed back to the CARV, through the CARV filter set and the spinning disk again, and the signal is sent to one of the cameras.

