

SHORT INSTRUCTIONS FOR OPERATING SD2 AT CIAN

Version 6, September 2015

General workflow

1. Turn on microscope, components as needed (see sections 1, 2)
2. Start up computer, log into one of the acquisition software:
 - Volocity (see section 3.2)
 - Metamorph (see section 3.3)
3. Visually inspect objectives; clean as needed (section 2.2)
4. Use test slide to adjust Köhler illumination in bright field (section 2.1)
5. Focus and position experimental sample in transmitted light (section 3.1)
6. Use acquisition software to acquire images (section 3.2, or 3.3)
7. When done, send light to eyepiece, clean microscope workstation as needed
8. Turn off microscope (see section 4)
9. Move data off computer (see section 5), log out of computer

General reminders for SD2

- Save the camera: move light to eyepiece when not acquiring data; avoid overexposure by checking intensity levels
- Optical cables are fragile, don't touch or put things on them
- Avoid bumping into equipment, leaning on air table
- Keep immersion oil bottle tightly closed – except when actively using – to avoid spills
- Only use cover slips of “no. 1.5” thickness (0.17 mm)
- Keep objective variable NA ring all the way open (clockwise)
- Move data off microscope computer – if storage space runs out during an acquisition, all data is lost for that acquisition!
- Limit or omit use of non-confocal (wide field) fluorescence to save sample, signal
- Acquisition software time = pay time; log off when not needed

Useful abbreviations and terms

SD = spinning disk

BF = bright field

TL = transmitted light

IL = incident light, i.e. fluorescent light

AP = aperture diaphragm (also FA = field aperture)

FD = field diaphragm

WFF = wide field fluorescence

DIC = Differential Interference Contrast,
a.k.a. Nomarski Interference Contrast

FRAP = fluorescence recovery after
photobleaching

Volocity = acquisition and analysis software
from Improvion (Perkin-Elmer)

MetaMorph = acquisition and analysis
software from Molecular Devices

Light sources on SD2: halogen lamp (for TL), *Exfo* metal halide lamp (for WFF), lasers (for SD confocal), and the *Mosaic* laser for photo-manipulation (e.g. FRAP applications).

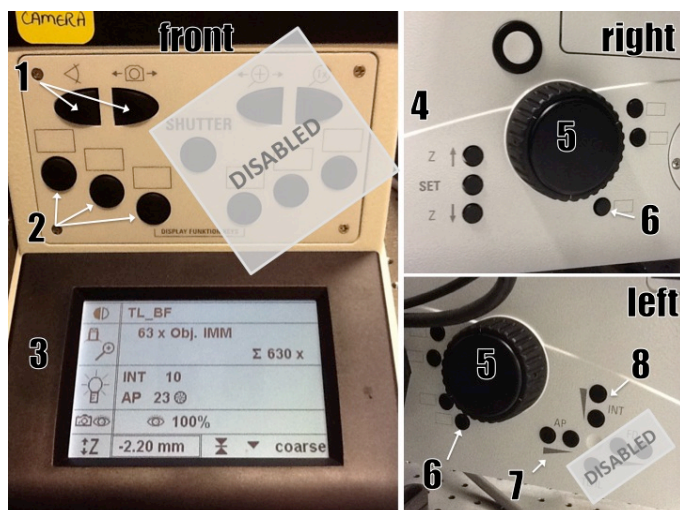
1 - Equipment Setup



Fig 1: Basic components of the SD2 microscope; brand names in italics.

- | | |
|--|--|
| 1. Laser shutter controller | 10. APC UPS (uninterruptable power supply) |
| 2. Laser ignition (4 boxes, “xxx nm” on label) | 11. Computer |
| 3. [<i>Mosaic</i> FRAP unit] | 12. Joystick for XYZ stage control |
| 4. Camera, Faraday cage, “grounded” | 13. Controller for environmental control chamber |
| 5. Emission filter wheel | 14. <i>Leica</i> electronic box, microscope controller |
| 6. Confocal scanning head | 15. [<i>Mosaic</i> laser] |
| 7. Halogen lamp, bright field light source | 16. <i>Exfo</i> metal halide light source for WF |
| 8. <i>Leica</i> DMI 6000B inverted microscope | |
| 9. Air table | |

Fig 2: Microscope front and side panels



- | |
|--|
| 1: 100% eyepiece/camera |
| 2: WFF filter cubes (CFP, GFP, RFP) |
| 3: status display |
| 4: set Z zero/limits (hold set and click up arrow twice to set zero), move objectives in z |
| 5: focus knob |
| 6: XYZ coarse/fine toggle |
| 7: AP control |
| 8: BF light intensity |

2 - Starting up SD2

Note: Diode lasers and *Exfo* lamp require only minimal warm up, but adhere to **15min/15min** rule: leave on at least 15 min before shutting off, leave off at least 15 min before restarting

1. Take the **dust cover** off the microscope, put on hooks by the door, not the floor
2. Remove any **stage inserts**, CO₂ chamber, objective warmer to make sure that the stage does not hit anything when moving on startup
3. Turn on **laser shutter control** box (Fig 1-1)
4. **Turn on** microscope system by pressing the top button on the *APC UPS* (Fig 1-10)
5. Turn on **computer** (Fig 1-11), log in to your account, start acquisition software; in *Volocity*, calibrate stage ('Stage : Calibrate Stage')
6. Optional, as needed: turn on lasers (Fig 1-2), *Exfo* widefield fluorescent light source (Fig 1-16), environmental control (Fig 1-13, Appendix A), CO₂ tank (behind desk)
7. Visually inspect **objectives**, adjust Köhler Illumination (2.1), clean as needed (see section 2.2)

2.1 - Adjusting Köhler Illumination



Fig 3: FD alignment elements

1. Insert your test slide, adjust light intensity for eye comfort, **focus on specimen** in bright field using low magnification objective
2. **Close FD** (Fig 3-1) to see edge of diaphragm, **focus** it by adjusting the condenser height (Fig 3-2), **center** if needed (screws in Fig 3-3, insert in holes Fig 3-4), **open** FD just enough to illuminate field of view (see images in Fig 4)
3. **Adjust AP** (a.k.a. FA) if needed, opening/closing with 'AP' buttons (Fig 2-7); aperture position is shown on microscope status display (Fig 2-3)
4. In case of poor images, re-clean objective (section 2.2), repeat procedure
5. Adjust for every objective to be used; note that 40x and above objectives need FD completely closed

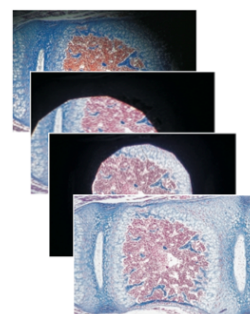


Fig 4: FD alignment

Note: For oil immersion objectives, make sure that the **variable NA ring is completely open**, at highest NA. To open, turn clockwise to stop. To check, look at the diaphragm in the back focal plane of the objective by removing an eyepiece.

2.2 - Cleaning objectives

Clean immersion objectives before and after use.

Use **dry lens paper** to blot off larger amounts of oil or other immersion medium:

- Take a sheet of lens paper, fold it in three into a long rectangle; do not run your fingers over the middle of the paper that will touch the lens
- Wipe the lens by holding either end of the lens paper, dragging it gently across the objective lens three times. Use a fresh area of the tissue each time.
- Repeat as needed with a fresh lens paper to remove excess of immersion oil.

Use **wet lens paper** to remove traces of oil, other dirt:

- Fold as above, put a drop or two of lens cleaner (the blue fluid) on the lens paper.
- Wipe the lens gently by holding either end of the wet lens tissue dragging it gently across the objective lens three times. Use a fresh area of the tissue each time.
- Repeat with water.

NEVER wipe the lens in a circular pattern; NEVER apply any pressure directly to the lens.

Note: If you find something on the lens that you can't remove with this procedure, **please contact CIAN staff as soon as possible, and discontinue use of the objective** until the problem is solved.

3 - Operating SD2 - Report any problems to facility staff (on PPMS, by email, in person)

3.1 - Manual controls (some disabled for safety reasons)

See Fig 2 for layout of buttons on microscope body.

Most functions are also available as software controls in *Volocity* or *Metamorph*.



Fig 5: Leica XYZ joystick

- 1: focus/Z control
- 2: Y control
- 3: X control
- 4: XY fine (top) and coarse (bottom)
- 5: Z fine (top) and coarse (bottom)

(The fine/coarse movement for all three dimensions, XYZ, can be toggled using one of the buttons on the side of the microscope, Fig 2-6.)

3.2 - Using *Volocity* for microscope control and image acquisition

1. Log in to '*Volocity 3DM acquisition*' (or '*AcqVis*') using your license server login and password. Other license types: '*Volocity complete*' (all), '*Restore class*' (deconvolution), '*Visual class*' (3D rendering), '*Presentation*' (free, limited edition, can be run without license).
2. Create a new library, or open existing library.
3. Select **<Show Video Preview>** from the Window menu.

Note: To zoom out in *Volocity*, hold down "control" key while clicking with the zoom tool.

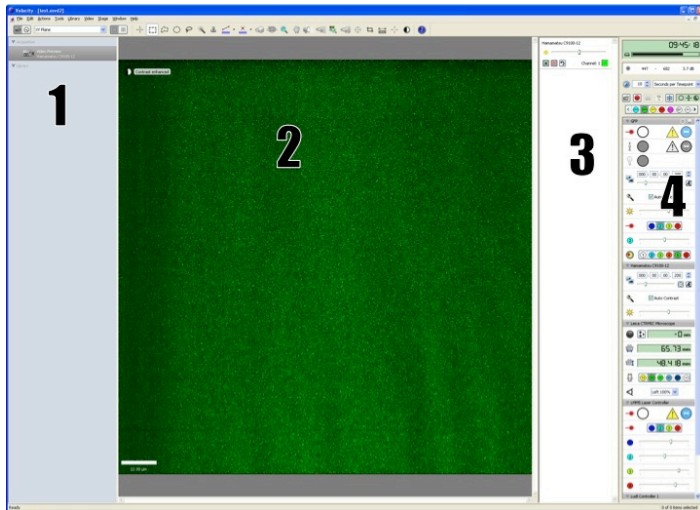
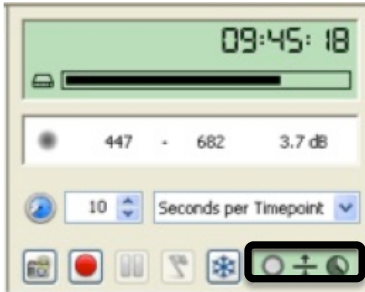


Fig 6: *Volocity* Video Preview window

- 1: Acquisition/library overview area
- 2: Video preview (live camera image)
- 3: Camera display control
- 4: Device control panel; see details in Fig 7

Fig 7: Device controls in Video Preview window; will change dependent on settings



- < Time or elapsed time, toggle through views by clicking on panel
- < hard disk storage space
- < Pixel intensity min. and max. – can toggle to different views of signal intensity, e.g. signal/time graph for bleaching evaluation
- < Acquisition rate/frequency – pull down to select for manual control
- < **Acquisition setup** – double-click to see menu (see section 3.2.2)

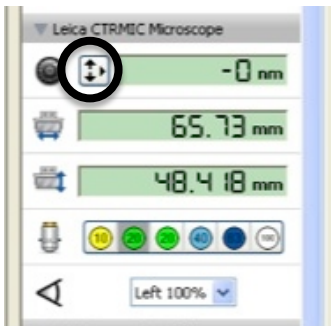
Shoot – click to shoot a single image, **Acquire** – start recording with current settings (turns into stop button, Pause and Mark Event will become available), **Freeze** preview and close shutter



- < Button to save light path changes
- Confocal, WFF, TL shutters
- < Exposure time
- < Auto exposure (use with caution)
- < Auto contrast (use recommended)
- < Camera sensitivity
- < Laser line indicator (do not use to switch laser lines)
- < Laser intensity control (neutral density filters) – linear between 20 and 80%
- < Confocal emission filter indicator – should match light path

Light path manager
 many light paths are preconfigured, save changes by clicking button in 'O', specific configurations can be made upon request

- CFP, GFP, YFP, RFP, FRFP confocal
- BF, bright field
- DIC
- POL, polarization
- WFF, wide field fluorescence; not false colored



- Focus controls, Leica focus drive**
- < Leica controller will be used for Z stack setup; use button in circle to pull up the focus control window (see section 3.2.1)
 - < XY stage position indicator
 - < switch light between camera ("left") and eyepiece (use this OR buttons on microscope body)

3.2.1. Z-stack setup

1. **focus** on the sample in Video Preview
2. click on the **Leica focus control** button (highlighted in Fig 7) to open control window (Fig 8); slider arrow on the right can be used to control Z position
3. [optional: choose **<Set Zero>** to set current position as 0 μ m]
4. for **stack definition**:
 - a. find the top/bottom of the sample, click **<Set Top>/<Set Bottom>**
 - b. alternatively, enter relative distances from current position in the up/down arrow fields, e.g. '5' and '-5' for a 10 μ m stack around zero, or enter stack size when position is zeroed for even stack around current position

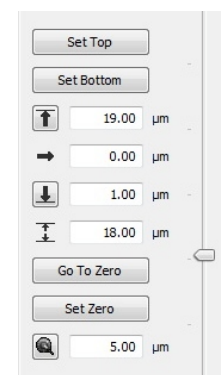


Fig 8

3.2.2. Acquisition protocol setup

Remember to balance laser power vs. bleaching, exposure time vs. dynamics; set gain accordingly, avoiding constant use of 100% gain.

1. Open **Acquisition Setup window** by double-clicking green rectangle in device control panel (Fig 7, boxed)
2. Select the **channel(s)** for acquisition
3. For **Z-stack** acquisition, select Leica stage, then choose one of:
 - Capture using Z spacing
 - Capture this many slices
4. Specify the **order** for channels and Z stacks
5. **Shutter management** – “balanced” usually works well
6. **Time**: Setup the duration and frequency of capture
7. **Points**: For multiple XY positions, select ‘Leica XY stage’, set up as in 3.2.3
8. **Other tabs**: ‘Stitch’ for tiling images across an area larger than the field of view (avoid stitching the images during acquisition); ‘Reference’ allows to exclude one channel from Z stack, e.g. BF or DIC, capturing only one slice; refer to user manual for more information on the other options

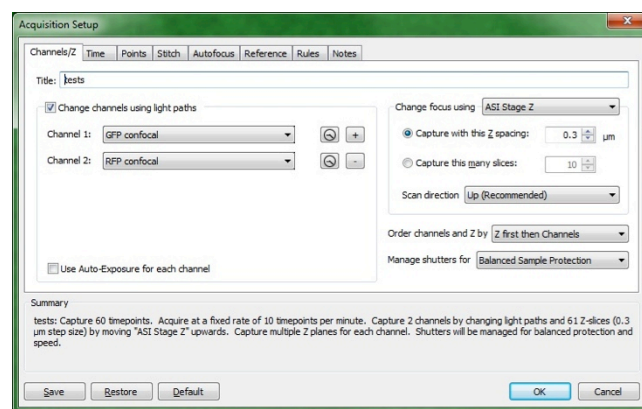


Fig 9: Acquisition Setup window

Save acquisition settings using a descriptive name (‘save as – export’, e.g. to Desktop) for future use with “Restore”, or click OK to use immediately.

3.2.3 Multi-point acquisition

Change to stage view by right clicking into preview, checking ‘XY stage’

1. Make sure that **stage calibration** was done **before** any stage inserts were put in place, ‘Stage : Calibrate’
2. **Clear all points** if needed (program remembers last set of points)
3. Find an XY(Z) position of interest using Video Preview, save position with ‘Stage : **Add A Point**’
[XY points can’t be modified, but have to be removed and re-added; to remove a point, select a region of interest around point, then do ‘Stage : Clear Selected Point’]
4. **Keep track** of point placement by sketching a map on a piece of paper
5. When all points are set, **recheck focus** by one of two methods:
 - All points sequentially: ‘Stage : Review Points’; for each point, adjust focus, click ‘next’
 - Points individually: ‘Stage : Go to next point’, adjust focus, click ‘update point’, repeat
6. Before starting the acquisition, quit and relaunch Volocity to **save points**, or save under a descriptive name using ‘Stage : Save Points’

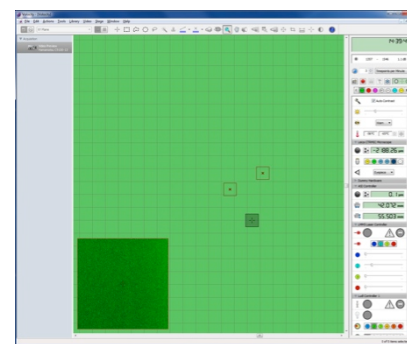


Fig 10: XY Stage View, with video preview inset

3.3 - Using *Metamorph* for microscope control and image acquisition

3.3.1 Starting *Metamorph*, basic controls

- Launch the software by clicking on the desktop icon “**Quorum WaveFX**”.
- Enter your *Metamorph* login and password.

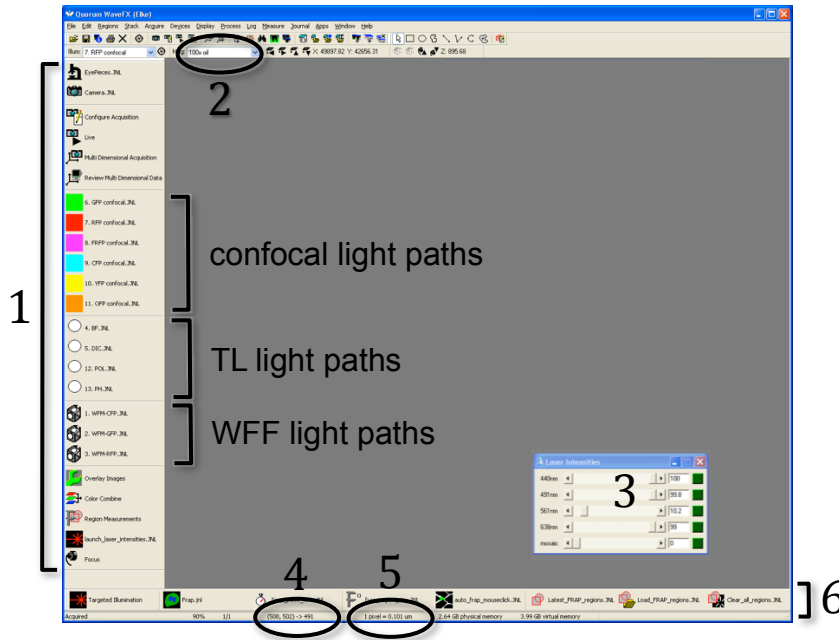


Fig.11: *Metamorph* window

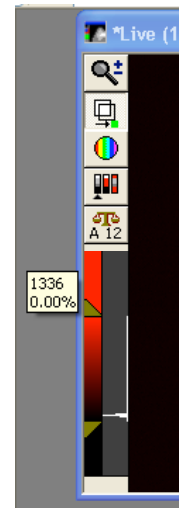
- 1: Buttons/journals for common tasks
- 2: Objective selection
- 3: Laser shutters and intensity control sliders
- 4: X, Y coordinates, pixel intensity
- 5: Objective calibration value
- 6: Buttons/journals for FRAP functions

- Make sure the **objective** in use is selected, and that the right calibration is in place.
- Switch between **eyepieces and camera** using buttons in software OR buttons on microscope stand.
- Click on one of the transmitted light, wide-field fluorescence or confocal **light path buttons** to set up the illumination, open the shutter, and start a “Live” preview.
- The “**Live**” preview is stopped/started by clicking on the icon in the left side buttons/journals, or buttons in the acquisition windows (see below).
- The **shutter** for the active light path can be opened and closed by clicking on the icon to the left of the objective selection drop-down, or in the icon bar above.



3.3.2 Display controls

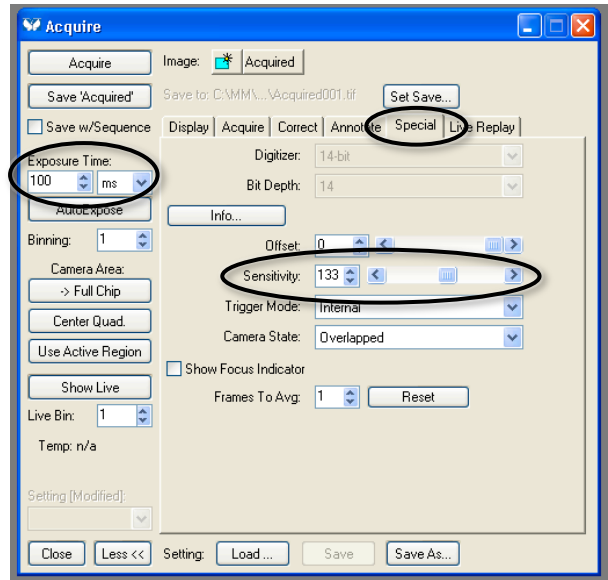
- Image **display window** (live and acquired image) can be **zoomed and resized** with the mouse wheel for the active window.
- To **zoom** within the window, use the **magnifying glass** icon from the side bar of the window.
- To select the **display color** (lookup table, LUT), use **rainbow button**.
- The **scale button** allows to select the display range for the histogram (best fit range, or bit scale), checkbox for auto-scaling display.
- Vertical **histogram bar** displays number of pixels at each intensity level, with triangles indicating top and bottom of display scale. Hover mouse over triangle to read the value; if in auto-scaling, the value reflects measured maximum or minimum intensity.



3.3.3 Acquisition with the “Configure Acquisition” functions

Click “Configure Acquisition” in left-hand side column of buttons/journals to open “Acquire” window.

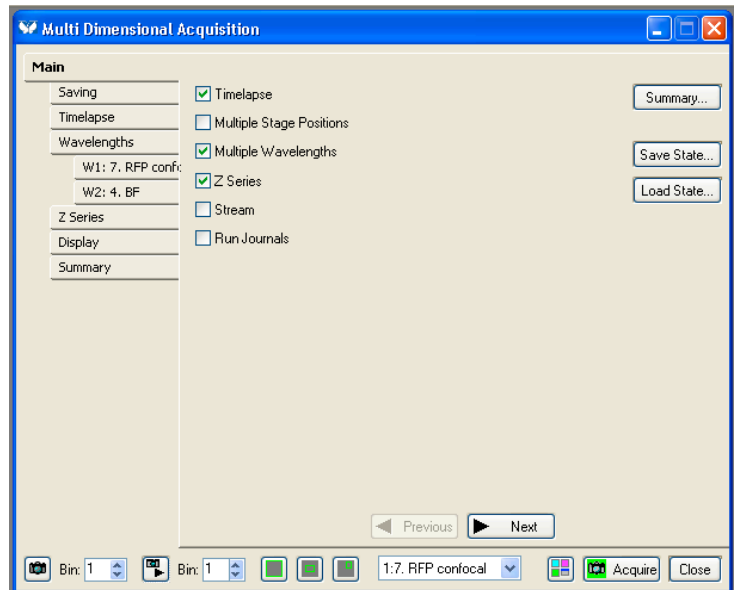
- Set up **exposure time**, **camera gain/sensitivity** (in “Special” tab); adjust **laser intensity** in separate window.
- Click **“Acquire”** button in top left corner to acquire an image.
- Picture will be unsaved (asterisk before name in window header), so **save** by using “Save ‘name’” in Acquire window, or save file later. As a file format, use “MetaSeries Single/Multi-Plane TIFF”.
- Further controls:
 - Camera area: Select full chip, center quadrant, or active region
 - Show live/stop live
 - Save/load settings
 - Display tab: auto-scale
- To set up z-stacks, time course, use further controls from Acquire menu in top menu bar, or control acquisition with MDA (see below).



3.3.4 Acquisition with “Multi-Dimensional Acquisition” (MDA) window

Click “Multi-Dimensional Acquisition” in left-hand side column of buttons/journals to open MDA window.

- **Select dimensions** from **main tab**, then set up acquisition parameters in sub-tabs:
 - Timelapse
 - Multiple Stage Positions
 - Multiple Wavelengths
 - Z Series; around current z, or absolute positions
 - [Stream – n/a on SD2]
 - Run Journals
- To **save settings** for reuse, use “save state”/“load state” buttons on main tab
- **Buttons** on the bottom:
 - Snapshot at current position, wavelength
 - Go live/stop live
 - Camera area: full chip, center quadrant, active region
 - Display/select current wavelength
 - Preview
 - Acquire runs entire acquisition protocol, automatically saves images in MetaSeries Single/Multi-Plane TIFF format



4 - Shutting down SD2

1. While in the acquisition software, close the **confocal fluorescence shutter** (Fig 7, second panel)
2. Switch **light path** to eyepiece (Fig 2-1, or in software)
3. Log out of **Volocity/Metamorph** by closing window
4. Turn off **lasers** (Fig 1-2), **controller** (Fig 1-1), *Exfo* light source (Fig 1-16) if used
5. **Log out** of the PC, [optional: turn off the computer using Windows if finished using]
6. Lower **objectives**, turn turret to 10x objective or empty position
7. **Clean** objectives by wiping off excess oil (see section 2.2)
8. Remove **stage insert**, shut down **CO₂** tank, empty and dry **humidifier bottle** if used
9. Turn off **SD2** - long button on *APC* UPS (Fig 1-10), push and hold until you hear second beep, then release
10. Carefully replace the **dust cover** on the microscope

5 - Saving data on the Server

1. Make sure to **exit Volocity or Metamorph** before moving any files
2. **Connect** to the server (start menu, run: \\10.1.0.3), open window for your folder on the server
3. Locate your library on the PC and drag it into your **server folder**
4. **Delete** the library from its original location on the hard disk as soon as possible; very large projects will have to be removed immediately after backing up. **No long-term storage** will be allowed on the computer.
5. To access your data from outside CIAN, connect to the server “Perola” via its public IP address: 132.206.213.90
You must be on the McGill network, either via a wired connection, or by VPN, to connect to Perola. For access from Windows computers you may need a tool like WinSCP.]

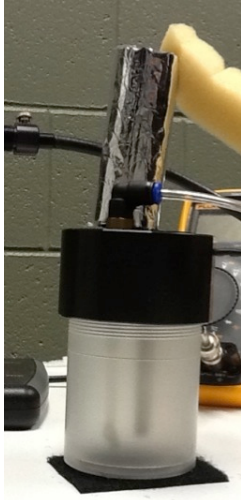
Note:

NEVER ACQUIRE DATA DIRECTLY ONTO THE SERVER (i.e. over the network) but acquire on the PC and then move or copy files.

NEVER OPEN A VOLOCITY LIBRARY OVER THE NETWORK, but move or copy it off the server to the computer you work on, and then open. The library could otherwise be irretrievably damaged.

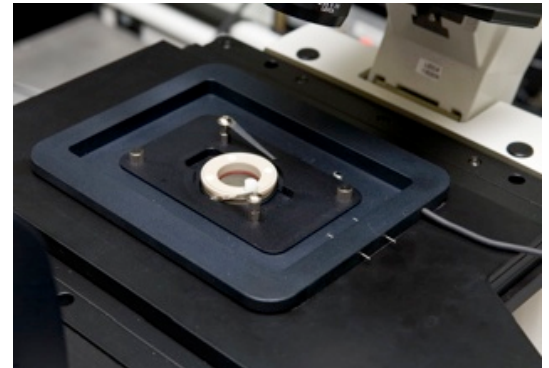
Appendix A: Chamlide TC environmental chamber

- Ask CIAN staff for the location of all the accessories.
- For long-term imaging, the system needs to be warmed up for at least an hour.
- Perfusion option: First get your own tubing if using the perfusion chamber.



At the beginning of the session:

1. Put water in the **humidifier**, a little more than half (there are bottles of water in the room for that purpose)
2. Identify the **5% CO₂** tank for this microscope and open the main valve all the way
3. Adjust size of the **objective heater** and slide it onto the desired objective, avoiding the variable NA ring; BE CAREFUL NOT TO SCRATCH THE SURFACE OF THE OBJECTIVE IN THE PROCESS; attention with turning objective on the turret after this point
4. Carefully and correctly insert the **main body of the chamber** on the stage, then put on the **glass top frame**
5. **Turn on** the chamber to start heating the assembly
6. Ensure proper **bubbling of the CO₂** in the humidifier (40cc/min)



At the end of each session:

1. Turn **CO₂** tank all the way off
2. Remove **chamber** from stage, clean and put away
3. Remove **oil** from the objective
4. CAREFULLY remove **objective heater**
5. **Turn off** control unit
6. EMPTY THE WATER AND DRY UP THE **HUMIDIFIER**



Appendix B: Technical data for SD2

Microscope: Quorum WaveFX spinning disk confocal system, on a Leica DMI6000B inverted microscope, fully motorized, with a Hamamatsu EM-CCD camera; Live Cell Instruments ChamSlide TC environmental control system; Mosaic Targeted Illumination system (Andor)

Objectives:

Position	Objective	Phase/DIC	µm/pixel	FD	FA
1	10x/0.3 dry	PH1	0.9940		9
2	20x/0.7 oil-glyc-water*	DIC	0.4987		12
3	20x/0.5 dry	PH2	0.4987		12
4	40x/1.25-0.75** oil	DIC	0.2532	Completely closed	12
5	63x/1.40-0.6 oil	DIC	0.1592	Completely closed	12
6	100x/1.40-0.7 oil	DIC	0.1010	Completely closed	12

* can be adjusted for oil, glycerol, or water immersion

** make sure to adjust for highest NA

Confocal mode: Lasers, fluorescence emission filters:

Lasers (all diode)	Emission filters	Typical fluorophore
440nm, 15mW	483/32	CFP
491nm, 25mW	520/35	GFP
	543/40	YFP
561nm, 50mW	624/40	RFP/mCherry
638nm, 30mW	692/40	FarRedFP
	593/40	OFP/Cy3
	645/75*	L-Cy5

*Available on demand

Wide-field fluorescence:

Light source: X-Cite120 metal-halide fluorescence lamp, 120W

Fluorescence filter cubes:

Filter set	Excitation	Dichroic	Emission	Typical fluorophore
CGFP	BP 436/20	455	BP 480/40	CFP
GFP	BP 470/40	500	BP 525/50	GFP
TX2	BP 560/40	595	BP 645/75	Texas Red

Appendix C: Nyquist suggested settings for Z-spacing**10x/0.3**

Fluorescent molecule	Expected Z resolution ($1.4\lambda/NA^2$)	Z-step (+2 or 3)
CFP/483nm	7.51 μm	2.50 to 3.76 μm
GFP/520nm	8.09 μm	2.70 to 4.04 μm
YFP/543nm	8.45 μm	2.82 to 4.22 μm
RFP/624nm	9.71 μm	3.24 to 4.85 μm
FRFP/692nm	10.76 μm	3.59 to 5.38 μm

20x/0.5

Fluorescent molecule	Expected Z resolution ($1.4\lambda/NA^2$)	Z-step (+2 or 3)
CFP/483nm	2.70 μm	0.9 to 1.35 μm
GFP/520nm	2.91 μm	0.97 to 1.46 μm
YFP/543nm	3.04 μm	1.01 to 1.52 μm
RFP/624nm	3.49 μm	1.16 to 1.75 μm
FRFP/692nm	3.88 μm	1.29 to 1.94 μm

20x/0.7

Fluorescent molecule	Expected Z resolution ($1.4\lambda/NA^2$)	Z-step (+2 or 3)
CFP/483nm	1.38 μm	0.46 to 0.69 μm
GFP/520nm	1.49 μm	0.50 to 0.74 μm
YFP/543nm	1.55 μm	0.52 to 0.78 μm
RFP/624nm	1.78 μm	0.59 to 0.89 μm
FRFP/692nm	1.98 μm	0.66 to 0.99 μm

40x/1.25 (CHECK NA IS 1.25 AND NOTHING ELSE!!)

Fluorescent molecule	Expected Z resolution ($1.4\lambda/NA^2$)	Z-step (+2 or 3)
CFP/483nm	0.43 μm	0.14 to 0.22 μm
GFP/520nm	0.47 μm	0.16 to 0.23 μm
YFP/543nm	0.49 μm	0.16 to 0.24 μm
RFP/624nm	0.56 μm	0.19 to 0.28 μm
FRFP/692nm	0.62 μm	0.21 to 0.31 μm

63x/1.4 and 100x/1.4 (CHECK NA IS 1.4 AND NOTHING ELSE!!)

Fluorescent molecule	Expected Z resolution ($1.4\lambda/NA^2$)	Z-step (+2 or 3)
CFP/483nm	0.35 μm	0.12 to 0.17 μm
GFP/520nm	0.37 μm	0.12 to 0.19 μm
YFP/543nm	0.39 μm	0.13 to 0.19 μm
RFP/624nm	0.45 μm	0.15 to 0.22 μm
FRFP/692nm	0.49 μm	0.16 to 0.25 μm