DSU Start-Up instructions

Always:

- start with the 10x objective
- properly center the stage around the current objective before changing to another objective
- when done, leave the 10x objective in standby position

To start:

- 1. Remove the dust cover and store neatly folded. Do not place it on the floor.
- 2. Sign the logbook Record the mercury bulb hours
- 3. Start-up the system



4. Turn on the mercury bulb (A)



Mercury bulb hours

5. Turn on the 3 control boxes (B1,B2, B3)



- 6. Turn on the computer and monitor.
- 7. Double click the desired MetaMorph software icon (may take a few moments)
 - a. Glass Only is for imaging through Coverglass using the 60 or 100x Objectives
 - b. Plastic Only only engages the Long Working Distance Objectives (10x, 20x, 40x)
 - c. Expert allows you to move from Plastic Plates to Coverglass Objectives.
 - d. Note: This might allow you to ram an objective into the stage. Be sure you are familiar with the system and focus your coverglass specimens at low magnification!



To begin, first focus your sample in the eyepieces:

- 1. Center the 10x Objective under the hole in the stage.
- 2. Center your sample on the stage and over the objective.
- 3. Select BF to Eyepieces or Fluorescence to Eyepieces
- 4. Rotate the appropriate filter cube using the manual filter turret.a. (Use the number code on the microscope.)







- 6. Open the Shutter manually on the microscope if necessary.
- 7. If transmitted light is being used, Kohler the illumination.
- 8. You should have light to the eyepieces
- 9. You may use the course or fine focus knob to bring your sample into focus.

To change objectives

- Toggle the shutter closed (whenever not viewing your sample, close the shutter to protect your sample from photobleaching)
- 2. Select the desired objective from the pull-down menu.
- 3. Open the shutter.
- 4. Re-focus your sample (as before) through the eyepieces.
- 5. If transmitted light is being used, Kohler the illumination.
- 6. Toggle the shutter closed.

J		
Acquire	Devices	Display
10x BF		4
[None])
10x BF		
10x Dark	field	
10x Pha:	se	
20x BF		
20x Dark	field	
20x Pha:	se	
40x BF (coverglass	8
40x DIC	coverglas	s
40x LW	D BF	
40x Dark	field	
40x Pha	se	
60x BF	coverglass	3
60x DIC	coverglas	s
60x Oil		
4100x BF	coverglas	s
[100x Oil]		





Open Camera Control

Imaging Instructions

- 1. Rotate the manual filter turret to position #1 (DSU)
- 2. Click Open Camera Control



3. Click Experiment Manager





<u>Work</u> 1. 2. 3.	in the Experiment Manager: Select the Options that Apply Work through the left-hand ta acquisition parameters Saving a. Add a description if de b. Select the directory for c. Provide a base name	bs under Main to set the Saving Wavelength Sire the files	Isional Acquisition
	Main Saving Timelapse Wavelengths W1: FITC Camer: W2: TRITC Camer: U2: TRITC Camer: Display	Description: "Images automatically saved with base fill Multi Dimensions Experiment Select Directory C:\Documents and Settings\m_rave Image: Increment base name if file exists Base Name: demo1	e* e* en/My Documents/My Pictures/di s
4.	Wavelength a. Select the number of v Multi Dimensional Action Main Saving Timelapse	vavelengths (transmitted light is or quisition Number of Wavelengths: 2 Allow separate binning for each wavelength	(None) 350ex528em Disk-out 350ex528emDisk-in 402/525 Camera Disk-in 402/525 Camera Disk-out 490/685 Camera Disk-out 8F Camera Disk-out BF Camera Disk-out BF Camera Disk-out BF Eye CFP Disk-out (ex427/em458) CFP Disk-out (ex427/em458) CY7 Camera Disk-in CY7 Camera Disk-out Cy5 Camera Disk-out Cy5 Camera Disk-out

b. Select the wavelengths -

W1: FITC Camer: W2: TRITC Came:

- i. Disk-out is regular epifluorescenceii. Disk-in is DSU confocal (much less light but less out of focus light)

490/685 Camera Disk-out BF Camera Disk-in BF Camera Disk-in BF Eye CFP Disk-in (ex427/em458) CFP Disk-out (ex427/em458) CY7 Camera Disk-in CY7 Camera Disk-in Cy5 Camera Disk-in Cy5 Camera Disk-out	
DAPI Camera Disk-ini DAPI Camera Disk-out	
DAPI Eye	
DIC Camera Disk-in DIC Camera Disk-out	
Disk Stopped Diagnostic	
FITC Camera Disk-in	
HTC Camera Disk-out HTC Eye HcRed Disk-in (ex589/em645) HcRed Disk-out (ex589/em645) TRITC Camera Disk-in TRITC Camera Disk-out TRITC Eye UV Camera Disk-out UV Camera Disk-out YFP Disk-in (ex504/em534) UV Camera Disk-out YFP Disk-out (ex504/em534) unasigned disk CY7 Camera unasigned disk CY7 Camera unasigned disk TRITC Camera unasigned disk TRITC Camera unasigned disk UV Camera unasigned HcRed (ex589/em645) unassigned YFP (ex504/em534) EITC Camera Disk-in	
FII U Lamera Disk-in 🛛 💙	

- c. Adjust the Capture Parameters for each wavelength
 - i. Gain for Fluorescence Usually 0.5x Multi Dimensional Acquisition
 - ii. Digitizer 11 MHz
 - iii. EM Gain 4-1200
 - iv. Exposure >30 ms

In general, you are adjusting the EM Gain And Exposure to capture images in the full 16 bit range

lain	Illumination	FITC Commun Disk in	
Saving	niumination:	FITC Camera Disk-in	
Wavelengths	Gain:	0.5x (High Dynamic Range 😪	
W1: FITC Came	Digitizer:	11 MHz (EM Gain) 🗸	
W2: TRITC Came	EM Gaint		N
Z Series	EM Gain.	4 <u>v</u>	n
Display	Exposure:	30 🗢 ms 🐱	
Summary			Target Intensity:
	Auto Expose:	No Auto Expose 🛛 🐱	3000
	Auto Focus:	No Arito Focus	Configure
	✓ ∠ series with	i the wavelength	
	Alignment Crop	oing 🗶 0 🤤 Y: 0 🤤 Set Alignme	ent
		A Previous	Vevt

in	Illumination	FITC Courses Disk in	
Saving	niuminadori.		
Wavelengths	Gain:	0.5x (High Dynamic Range 💌	
W1: FITC Came	Digitizer:	11 MHz (EM Gain)	
W2: TRITC Came	EN C.S.		
Z Series	EM Gain:	60	
Display	Exposure:	600 😂 ms 💌	
Summary		Targe	t Intensity:
	Auto Expose:	No Auto Expose 🖌	0
	Auto Focus:	No Auto Focus	figure
	Z series with	h the wavelength	
	Aller and Const		
	Alignment Crop	ping X: 0 V T: 0 V Set Alignment	

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- 5. Z-Series
 - a. Set Top (highest objective position)
 - b. Set bottom (lowest objective position
 - c. Set step size
 - d. The number of steps is determined and constrained by Top, Bottom and Step Size

in	- Interactive setting:	5		
Saving	Current Position:	-345 😂	um Increment: 1	
Timelapse				
Wavelengths	Settings for acquis	ition series		
W1: FITC Camer:	Loop order			
W2: TRITC Came	Acquire wave	length set at e	each Z	
Z Series	O Acquire ∠ ser	ies for one wa	velength at a time	
Display	(Mikeep shutter	open betweel	T steps	
Summary	Range:	72.5 🛫	Range Around Current	
	Тор:	503.92 😂	Set Top To Current	
	Bottom:	431.42 😂	Set Bottom To Current	
	Step Size:	0.5 😂	Center Around Current	
	Number of Steps:	146 😂		
	Recommended Step) Size: 2.9 um	Previous 🕨 Next	

- 6. Timelapse
 - The estimated minimum interval is determined by the imaging conditions (e.g. the time required to image the wavelengths (exposure), change filters and image multiple Z planes.
 - b. Time Interval should be greater than the estimated minimum interval.
 - c. Duration is the total experiment time
 - d. Number of time points is constrained by the Duration and Time Interval.

n	Experiment Length				
Saving	Number of time points:	7			
Timelapse			1		
Wavelengths	Duration:	60	sec	×	
W1: FITC Camer:	The Laborat	10	5 <u>1</u> 2002/2		
W2: TRITC Came	i ime intervai:		sec	×	
Z Series	Estimated minimum inte	rval: 9.24 sec			
Display					
Summary					

- 7. Display and Summary
 - a. Set the Display to default or custom
 - b. Check the summary data and confirm all the settings are prepared.
 - c. Click Acquire and the acquisition will begin

n	
Saving	demo
Timelapse	Directory: C:\Documents and Settings\m_raven\My Documents\My
Wavelengths	File Base Name: demo1 incremented to next free name
W1: FITC Camer:	
W2: TRITC Came	Timelapse
Z Series	/ time points every 10 sec, duration = 60 sec
Display	Z Series
Summary	14 z steps of 3 um, range = 41.5
	Wavelengths Wavelength 1 Illumination: FITC Camera Disk-in Collection: Every time point As Z Series: Yes Exposure: 600 ms Gain: 1 EM Gain: 60 Digitizer: 11 MHz (EM Gain)
	Print Previous Next Carter Acquir

8. Select Save State to Save the settings for a future experiment. Save the state file to your My Documents Folder

90 I	Multi Dimension	al Acquisition	
м	ain		
	Saving	Timelapse	Summary
	Wavelength	Multiple Wavelengths	
	Display		Cours Chairs
à	Summary	Stream	Load State
		Run Journals	

Köhler illumination – Inverted Microscopes

In order to get the best image possible from brightfield, phase contrast, differential interference contrast, or polarization optical setups with the light microscope, it is crucial that the light path be set up properly. If the light path is set up properly, you will have the advantages of an evenly illuminated field, a bright image without glare and minimum heating of the specimen.

The following instructions apply to any microscope, upright or inverted which is equipped for transmitted light bright field illumination. Focusing of the field diaphragm as discussed here should be done for phase and differential interference optics as well.

- 1. <u>Light source</u>: Switch on the light source and make sure that light is coming through the field diaphragm. It may help to place a piece of paper over the field stop to see the light.
- 2. **Condenser lens:** Place the condenser lens all the way down toward the stage.
- 3. <u>Objective Lens:</u> Place your specimen on the stage and turn the nosepiece (which holds the objective lenses) to the 10X power objective lens. Make sure the top piece is in place.
- 4. **Other Optics:** Be certain Phase or other optics (fluorescent filter cubes) are out of the light path.
- 5. <u>Focus:</u> Now bring your specimen into focus with the coarse and fine focusing knobs. The best way to do this is to rack the lens as close possible to the specimen watching the objective lens all the time (and NOT looking into the oculars) to make sure that the lens does not run into the slide. Then rack the lens away from the stage (or vice versa) while looking through the oculars to bring the specimen into focus (details are as sharp as they can be).

6. Field Diaphragm:

- a. Close down the field diaphragm to the smallest spot
- b. Use the condenser focusing knob to bring the spot into focus such that you can clearly see the leaves of the diaphragm
- c. Use the centering knobs to bring the field into rough centering.
- d. Open up the diaphragm to almost fill the field. This is the best time to do the final critical centering.
- e. Open the diaphragm to just fill the field.
- 7. <u>Condenser Diaphragm</u>: controls the cone of light. Plays resolution versus contrast. Full open results in flaring, closing it down improves contrast and if closed too much you see the fringe.
 - a. Remove the oculars and look down the viewing tube. Open the condenser diaphragm all the way up. Close it down about 3/4.
 - b. Put the oculars back in.
- 8. Your specimen should be properly illuminated and should give you a great image. If it does not, check to make sure your lenses and other optical components are clean. Then, recheck to see that you have followed each step properly.

Repeat these steps for different objective lenses.

DSU Shut-Down instructions

- A. Wipe the oil off and oil objectives you used and return the 10x objective (use the software) to standby position
- B. Confirm that Nobody is using the system within an hour or less. Leave the system on if they are.
- C. Save what you need to the \\microscopy-nas1 or transfer but other methods via the internet or USB.
- D. Close the software
- E. Shut down
 - a. the computer and monitor (C)
 - b. 3 boxes (B1, B2, B3)
 - c. LASTLY the mercury bulb (A)
- F. Record the final mercury bulb time and finish signing the log book
- G. Cover the A-B with the dust cover

To look at your data use the Review Multi Dimensional Data Set Utility Apps

Review Multi Dimensional Data Window Opens Select Base File Find your folder

👽 Review Multi D	mensional Data		
Select Base File Wavelengths: BF Camer CY7 Came DAPI Cam	C:\D\5-16-13 WELL 7 DSS T	IMESTAIN.ND 8 9 10 11 12 13 14 1	Open Sequential 5 16 17 18 19 20 2
J	<		>
		Timepoints: 1 📚 to	83 📚
Enable Montage	# of Thumbnails: 🛛 👙 x 💈		Apply
Selections [X's] Dis	olay Event marks		
Load Image(s)	Select Best Focus		Clear
Reset Image Display:]	Run Journal Loop	Close



Multi Dimensions Experiment
Time: 1 Time Points
Manather EITC Camera Diak in TRITC Camera Di
Z Steps: 14
Bata Log Not Open
Config beg Open Log
Run Journal View

Other MetaMorph Operations

Removing Planes

To remove out-of-focus planes:

- 1. Open stack and determine which planes to start and end with
- 2. Select Stack
- 3. Select Remove Plane
 MetaMorph Basic
 File Edit Regions Stack Acquire Devices Display
 Projection Images:

 Open a stack file
 Select Process

 File Edit Regions Stack Acquire Devices Display Process Log Measure Journal A
 Mag: 10x Phase
 Note Stack Acquire Devices Display Process Log Measure Journal A
 Note Stack Acquire Devices Display Process Log Measure Journal A
 - 3. Select Stack Arithmetic

Process	Log	Measure	Journal	Apps	Window	Help
Binary Arithm Stack Basic I Detec Morph	Oper netic Arithm Filters, t Edge iology	ations hetic s Filters	(Itrl+B Itrl+Shift+	-А	
Backg	round	ion				
2D De 3D De	convo convo	lution lution				* *
Edit Ko FFT Ratio	ernel Image	, s				

4. Select a method of combination – Average and Maximum are common

To create a scale bar:

Display	Process	Log	Measure	Journa	l Apps	Window	He
Adjus Contr Scale	t Digital Co ast Shorto Image	ontras :uts	t	•			
Graph	nics			•	Calibrat	ion Bar	-
Inten Overl	sity Profile ay Images	 		_	Date/Tii Paint Re	me egion	_
Color Color Color Solit V	Combine Separate. Align /iew				Arrow Grid Text Gray W	edge	
Deint	erlace			-1			
Rotat Show Set Ir Stret	:e /Hide Imag mage Zoon ch and Mirr	ge at F n ror	Full Screen.				

To create a movie:

Select Stack Make Movie

👾 MetaMorph Basic					
File Edit Regions	Stack	Acquire	Devices	Display	
≥	Sele Play Add	ct Plane Preferen Plane	ces		
Open Camera	Rem Set Set Kee	nove Plane Plane Z Di Plane Tim p Planes	e istance e Incremei	nt	
Experiment Ma	Stite Mov Mak	:h Stack 'ie e Movie			
Shutter Toggle	Mon	itage itage Stac	ks		
Evepieces	Aligi Kym	n Stack Iograph			
Fluorescence	3D F Viev Top	Reconstru v Orthogo ographic S	ction nal Planes Surface		

Olympus DSU (Spinning Disk Confocal)

The DSU in this facility is on an inverted microscope base with a manual X, Y stage. The system is configured with phase optics and mercury arc lamp illumination. MetaMorph software controls the camera and microscope allowing for long-term imaging. The microscope is enclosed to allow warming to 37°C and has humidified 5% CO2 available. The stage and samples can be cooled below the ambient temperature in the room (25°C) by special arrangement.

Objectives	Mag/N.A.		
UPlanAFLN	10x/0.30	Ph1	∞/-/FN 26.5
LUCPlanFLN	20x/0.45	Ph1	∞/0-2 FN22
LUCPlanFLN	40x/0.60	Ph2	∞/0-2 FN22
UPlanFl	40x/0.75	DIC	∞/0.17/
PlanApoN	60x/1.42	Oil	∞/0.17/FN26.5
UPlanSApo	100x/1.4	Oil	∞/0.17/FN26.5



Camera

Hamamatsu ImagEM CCD Camera (C9100-13)

The ImagEM camera is a back-thinned electron multiplier CCD camera with a readout rate of 32 frames per second at full spatial resolution and 16 bit digitization.

Imaging Filters

Switching between sets (Sedat, Pinkel, Cy5, and Brightfield) isn't fast because the sets use different dichroic mirrors and moving those is slow but within in sets is fast.

89000 Sedat Quad Filter Set

Excitation		Beam splitte	r	Emission	
AT350/50x	EX	89100bs	BS	ET455/50m	EM
ET402/15x	EX			ET525/36m	EM
ET490/20x	EX			ET605/52m	EM
ET555/25x	EX			ET705/72m	EM
ET645/30x	EX				

Cy5 Cube

Semrock Pinkel Set for CFP/YFP/HcRed					
Excitation	Beamsplitter (triple band	Triple Band Emission Filter			
427/10	diachronic)	Center wavelength (band)			
504/12	444/521/608	464 (31)/542(35)/639(50)			
589/12					

