

Leica DM6B User Guide

Last Modified 08-18-2023

You must read the MIC Facility Manual before training. It covers lab and laser safety, training policy, scheduling, and biosafety requirements.

USERS MUST RECEIVE TRAINING FROM MIC STAFF. Getting trained by another user is neither sufficient nor permissible for independent operation of the microscope. In other words, without the official training, you can be in the room and watch, but you are not allowed to touch the microscope.

Acknowledgment policies

The use of the facility must be properly acknowledged in any publication (including web pages). You can use the following statement:

“The use of the Microscopy and Imaging Center facility at Texas A&M University is acknowledged. The Leica DM6B microscope acquisition was supported by the Office of the Vice President for Research at Texas A&M University.”

Users are also required to file a copy of any relevant publication containing the acknowledgment with the MCF administrative office.

Table of Contents:

Microscope slides and other types of sample carriers:	2
Software for viewing and analyzing captured images	2
Startup	3
Shutdown	4
Operating the Microscope	5
Focusing:.....	5
Finding the samples, using the microscope for brightfield and fluorescence imaging:.....	5
Setting up Köhler Illumination.....	6
LAS X Software User Interface	7
File/Project Settings Tab.....	8
Acquisition Tab	9
Z-stack acquisition:.....	10
2. Light Path panel	10
3. Image Display Panel.....	11
Color Balance (for Color Camera imaging):.....	11
Show Saturated Pixels:.....	11
Filter Cubes for Fluorescence Imaging.....	12
Microscope Objectives, Calibrated Pixel Size for the Cameras	13
Switching Fluorescent Filter Cube Configuration.....	14
DFC9000GT MONOCHROME CAMERA PERFORMANCE SHEET.....	17

Microscope slides and other types of sample carriers:

Microscope objectives are designed to view the sample through a thin glass window, 0.17 mm thick (this is the #1.5 coverglass).

If you use thinner or thicker coverslips (or no coverslips), you will not be able to get the best quality images especially with the 20x dry objective. When using the 40x objective, the correction collar on the objective must be set correctly for the coverglass thickness).

Software for viewing and analyzing captured images

Always save the original leica Image files (.lif). They contain both the images and the metadata (acquisition parameters).

1. LASX Office

Free software to review existing files. Allows annotation and export, but not analysis or 3D visualization or image acquisition.

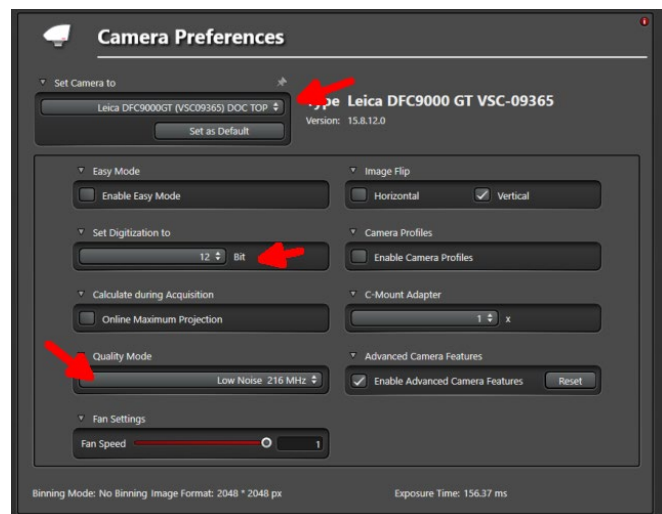
<https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/downloads/>

2. ImageJ/FIJI <http://fiji.sc>

Freeware image processing and analysis software with many functions. It can open the Leica .lif files directly.

Startup

1. Look in the microscope room if the instrument looks ready for use
2. Start your iLab kiosk session
3. Fill out the log book
4. Turn on the Microscope control box (Labeled as #1)
5. If doing fluorescence imaging, turn on the LED fluorescence illuminator (#2 and #3). If not doing fluorescence, leave the illuminator off.
6. Turn on the monochrome camera (a small switch on top of the camera). It has to be on before you start the software, otherwise it may not be properly recognized.
7. Turn on the computer
8. Log into Windows as DM6B user.
9. Start the LASX software (NOT the “LASX Hardware configurator”)
10. Go to Camera preferences and select the camera you want to use (Monochrome DFC9000 or colorDMC4500).
11. Select the desired bit depth. For the monochrome camera, digitization to 12-bit is usually a good choice. The Quality Mode (read-out speed) for the monochrome camera should be set to “Low Noise” unless you need to take high speed video images.
12. Make sure the Easy Mode is NOT selected.
13. Load a slide, focus using a 10x objective and set up Köhler illumination



Shutdown

1. Remove the slide, clean the area around the microscope
2. If oil immersion was used, blot off oil from the objective with lens paper.
3. Switch to a 10x objective
4. Save the Leica project file (.lif) to your folder on the Data (E:) drive. If you need to export some images into a different format, do it now.
5. Exit the software, copy your image files (.lif and also the .lifex if present) to your portable storage
6. Shut down the PC (labeled #4), the fluorescence illuminator (#3, #2) and the microscope controller (#1)
7. **TURN OFF THE MONOCHROME CAMERA (if it was on)**
8. Replace the dust cover
9. Sign out in the log book
10. Go to the kiosk computer, finish your iLab session.

Operating the Microscope

Focusing:

Use the focus knob on the XYZ joystick, or the focus knob on the microscope itself. Turning the focus knob on the microscope away from you (clockwise when looking at the right hand side of the microscope) will move the microscope stage UP.

Finding the samples, using the microscope for brightfield and fluorescence imaging:

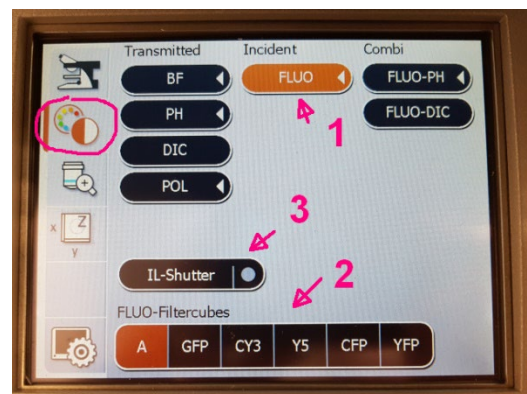
Most functions are controlled through the front panel. You can turn on the Brightfield (BF) or Fluorescence (FLUO) mode, open and close the illumination shutter, move the objective turret to a pre-set focus (zero) position, remember and recall XY coordinates on the motorized stage, change the intensity of illumination,

1. **Start with a 10x dry objective.** The microscope starts with the stage in lowered position, several mm below focus. Bring the objective close to focus using the Z control on the microscope front panel: push and hold the “focus” (1) button. Caution: if you have an unusually thick sample, you will hit and possibly damage the objective.



2. **Set up Köhler illumination to get a good brightfield image (see next section).**

3. **If desired, switch to Fluorescence mode (#1), select a suitable filter set (#2) and open the IL shutter (#3) to view the sample.**



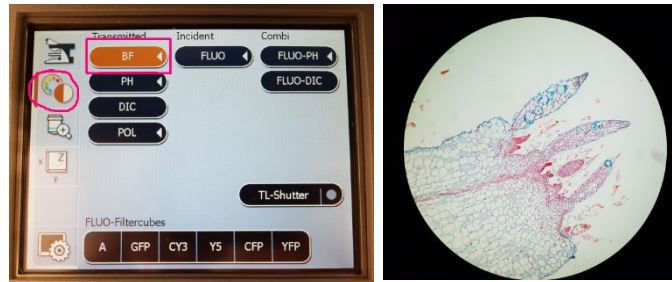
4. **Once you found the area of interest, close the illumination shutter and set up the imaging parameters in the software**

Setting up Köhler Illumination

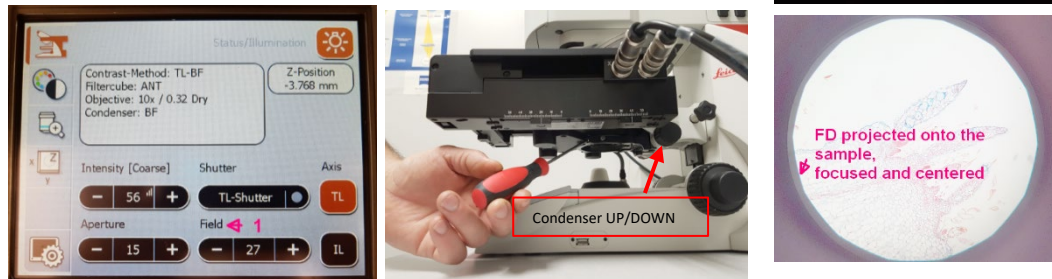
Köhler illumination produces an even field of illumination in TRANSMITTED LIGHT and does not show dust and dirt that accumulated on optical surfaces in the illumination path.

Adjust the illumination using the 10x dry objective. When you switch objectives, you do not need to repeat the process, except to check that the condenser aperture is set properly to get a good balance between contrast and resolution.

1. Place specimen on the stage, turn on the Brightfield mode. (BF button on the microscope front panel) and focus on the specimen.

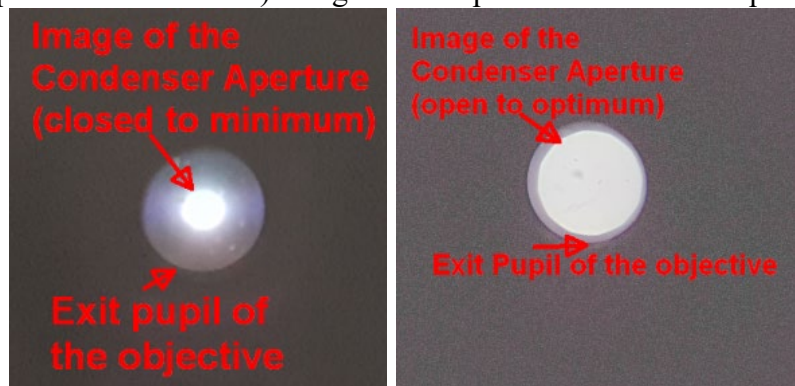


2. Close the Field Diaphragm, FD (“Field” on the control panel) to see its edge in the specimen image and focus the FD by turning **the condenser** knob. If necessary, center the FD using two Allen wrenches (stored in the desk drawer) to turn the adjustment screws on the underside of the stage. Open FD just enough to illuminate the entire field of view. Stow the wrenches when finished.



Adjust Condenser Diaphragm (“Aperture” on the LCD) using the front panel of the microscope.

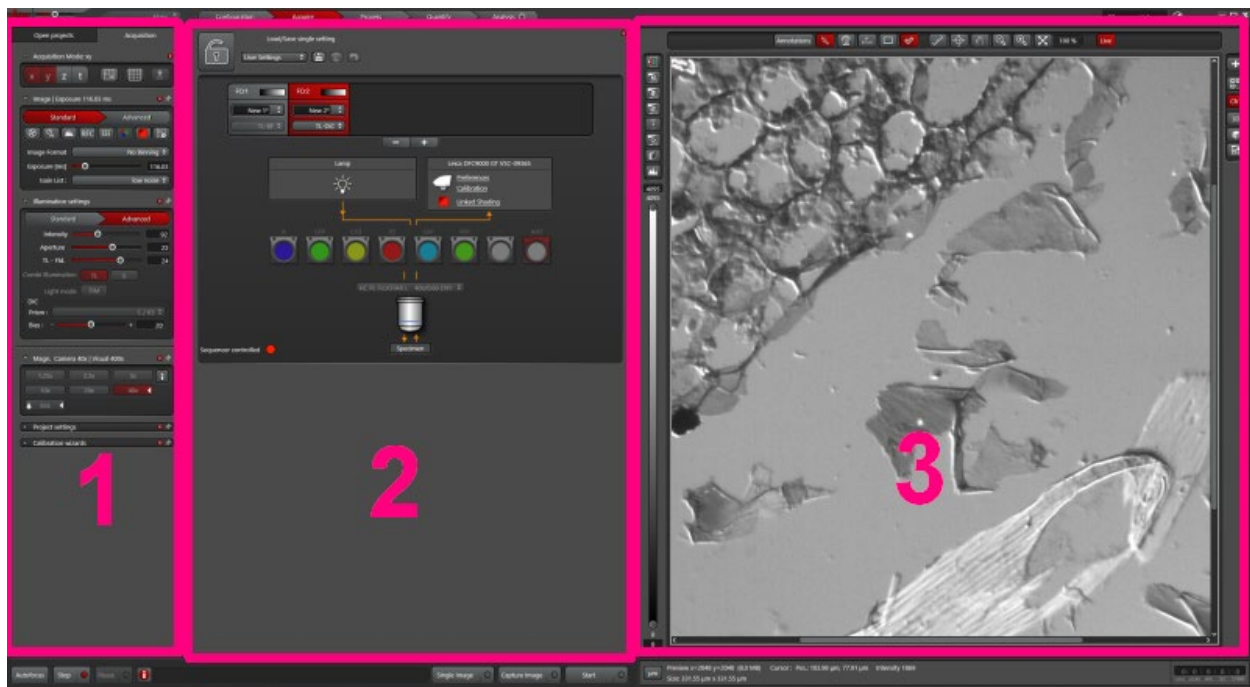
Decrease the aperture size to minimum, then start increasing it. View the aperture by removing an eyepiece. When the Aperture size almost reaches the size of the objective exit pupil, stop. Closing the Condenser Aperture will provide more contrast but less resolution.



LAS X Software User Interface

The LASX user interface for Image Acquisition has three sections:

- 1: Acquisition parameters and File/Project settings
- 2: Light Path section: Imaging mode selection, multi-channel imaging setup, Camera selection settings
- 3: Image display



File/Project Settings Tab

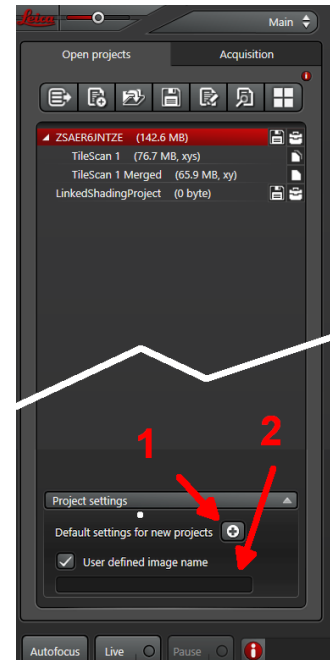
The Leica “Project” file (.lif and .lifex) is a container (collection) that can contain multiple images, z-stacks, time-lapse series or tile scans.

You can set the default name for a new project and also choose to automatically save the image after each capture (#1). Also set a desired name for captured images (#2) so that you do not have to re-name them manually.

Loading image settings from a previously saved image:

This function loads most of the image parameters from an existing image. Certain settings, like the condenser aperture, do not always load correctly (please check).

1. Open a previously acquired Leica project file (.lif)
2. Right-click on the image file of choice, and select “Apply Image Settings”.



Acquisition Tab

Select imaging mode (XY, or XYZ, or T, i.e. time lapse)

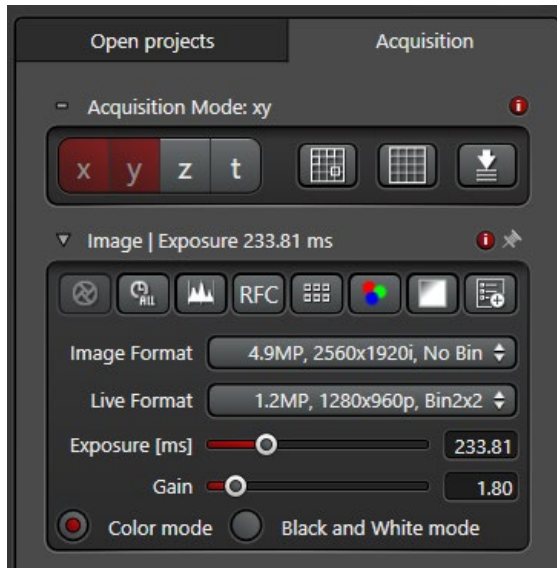
BINNING: combine 2 x 2 (or more) pixels on the camera chip to acts as one big pixel. Reduces the amount of data, increases sensitivity, but reduces spatial resolution.

If using the **MONOCHROME CAMERA**, choose the desired “Gain list” mode: The “**Low noise**” mode has higher sensitivity and is best for regular and weak fluorescence signals. “**High Well Capacity**” for imaging objects with very high contrast – This mode has a lower gain, so much longer exposure or higher intensity illumination will be needed.

Other controls include light intensity, field and condenser aperture settings, and if you chose the Z or T options, also z-stack and time-lapse options.

You can perform Stage Experiments (button labeled “1”) where you can set up multiple locations for imaging, and also the Navigator (button labeled “2”) for image tiling and stitching. Ask MIC staff for additional training in using these features.

When Using the **COLOR CAMERA**, you can choose binning for the live view and for the acquired image independently.

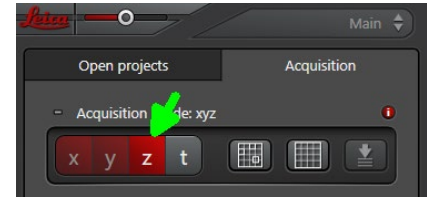


The higher the gain, the brighter the image, but also more noise will be introduced.

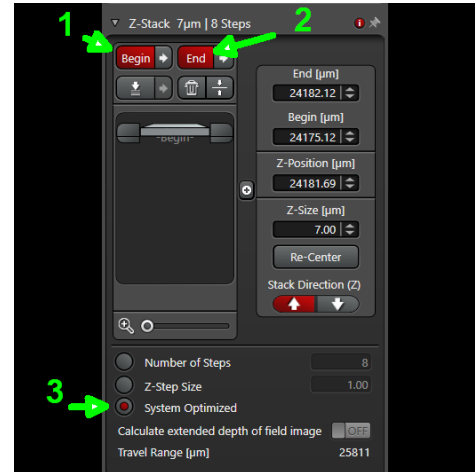
If you are considering the black and white mode with the color camera, you should rather switch to the monochrome camera. It is much more sensitive.

Z-stack acquisition:

Activate the “z” button in acquisition mode, so that the XYZ mode is set. Z-stack setting will appear in the control panel.



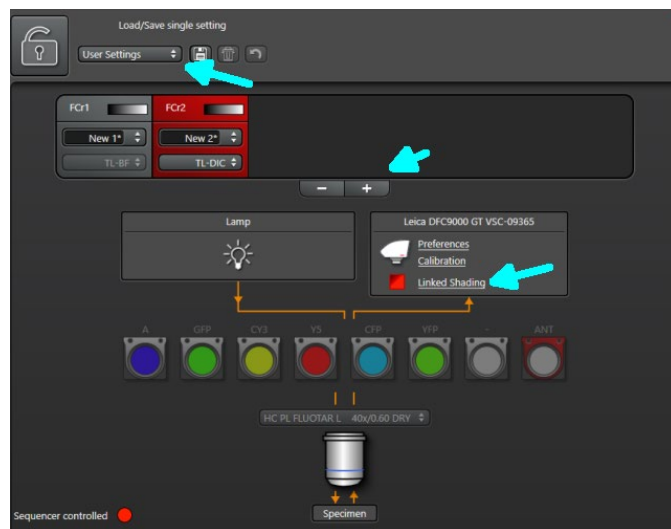
- 1) In live view, focus the microscope to where you want to start the stack, and click the “Begin” button in the z-stack setting
- 2) Change the focus to where you want to end the z-stack, and click the “End” button
- 3) Check that z-step size is set to “System Optimized”. The software sets the z-step to ensure proper Nyquist sampling for the current objective and wavelength of the fluorescence signal.



2. Light Path panel

Useful functions to remember:

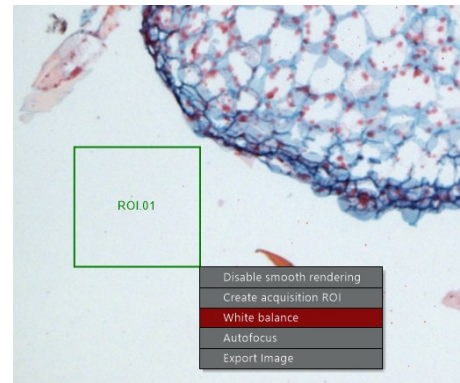
- User defined or Leica-standard presets (for instance, 3-color fluorescence imaging). Click on the “Save” icon to save your current imaging settings as a user-defined pre-set that you can apply next time. **After applying a setting, make sure to check in “Configuration-Hardware” that your imaging bit-depth (“Bit Resolution” is correct (e.g., 12-bits for monochrome camera imaging)**
- Adding and removing imaging channels (the “+” and “-” buttons)
- Linked Shading, a.k.a. Flat Field Correction: will subtract the illumination background for better image stitching (without the obvious edges of the individual tiles).
- Camera Preferences – switch between the color to monochrome cameras.



3. Image Display Panel

Color Balance (for Color Camera imaging):

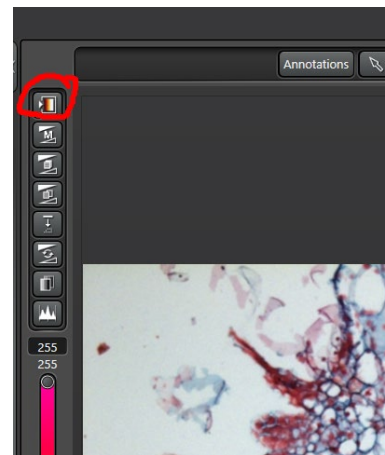
Select the Rectangle ROI tool on top, draw a box in a blank area of the sample. When you release the button, select the “White Balance” button.



Show Saturated Pixels:

Top left button, toggles between the color image, monochrome image and a pseudocolor image (Over/Under) where saturated pixels are shown in red (or blue), and zero-intensity pixels are shown in Blue (or green, in some modes).

Lower the Illumination intensity or exposure time to avoid saturation.



Filter Cubes for Fluorescence Imaging

Filter specifications – DM = Dichromatic Mirror; LP = long-pass filter; bandpass filters are described by central wavelength and the full bandwidth (525/50 = 50 nm bandpass centered at 525 nm, i.e. transmission is in 500-550 nm range)

- A : (DAPI, Hoechst; Calcofluor White); LED365; Ex 360/40; DM 400; Em LP425
- GFP: (GFP, FITC, Alexa Fluor 488) LED470; Ex. 470/40; DM 495; Em 525/50
- Y3: (Cy3, Alexa 546, Alexa 565) Ex 546/26; DM 565; Em 605/70
- Y5: (Cy 5, Alexa 633, Alexa 647) LED620; Ex 620/60; DM 660; Em 700/76
- CFP: Ex. 436/20 DM 455; Em. 480/40
- TR: Ex 560/40; DM 585; Em 630/75
- YFP: LED: 505; Ex. 500/20; Dm 515; Em 535/30

Note: the CFP filter is typically not present, instead the TXR is in place. Instructions how to change the configuration are given in the next section of this User Guide.

Microscope Objectives, Calibrated Pixel Size for the Cameras

The most important property of the objective is not magnification, but the numerical aperture (NA), which determines resolution¹². The 40x/0.6 objective has resolution that is not much higher than the 20x/0.55 objective, so the 20x objective is usually a better choice, even if the monochrome camera does not quite capture the image at full resolution. The 40x objective is better suited for imaging through thick windows, or without a coverglass, it has a correction collar that can be adjusted between 0 (no coverglass) to 2 (looking through 2 mm of glass). Make sure the settings are correct for your sample, otherwise good resolution and image contrast will not be achieved.

In order to record images at full resolution (Nyquist sampling), the effective pixel size in the image should be ~2.3x smaller than the lateral resolution.

Table 1: Theoretical resolution of microscope objectives, effective pixel size in images captured with the monochrome and the color camera. Green: Nyquist sampling is achieved; Gray: very close to Nyquist; Yellow: Nyquist sampling NOT achieved. Resolution for the oil immersion objective is calculated for the aperture fully open (NA = 1.4).

Please note that smaller number means higher resolution.

Objective Order Number	Obj. Magnif/NA	Lateral Resolution [μm] *	Pixel size [μm] (no binning)		Axial (z) Resolution for 500 nm light [μm]
			MONOCHR. CAMERA DFC9000GT	COLOR CAMERA DMC4500	
11506215	1.25x/0.04	7.63	5.188	3.889	625
11506523	2.5x/0.07	4.36	2.613	1.964	204
11506224	5x/0.15	2.03	1.301	0.976	44
11506522	10x/0.32	0.95	0.652	0.489	9.8
11506518	20x/0.55	0.55	0.324	0.243	3.3
11506203	40x/0.6	0.51	0.162	0.121	2.8
11506349	63x/1.4-0.6 Oil	0.22	0.102	0.077	0.77

¹ Lateral resolution (Rayleigh formula) $d=0.61 * \lambda/\text{NA}$ where λ is the wavelength of light and NA is the numerical aperture of the objective

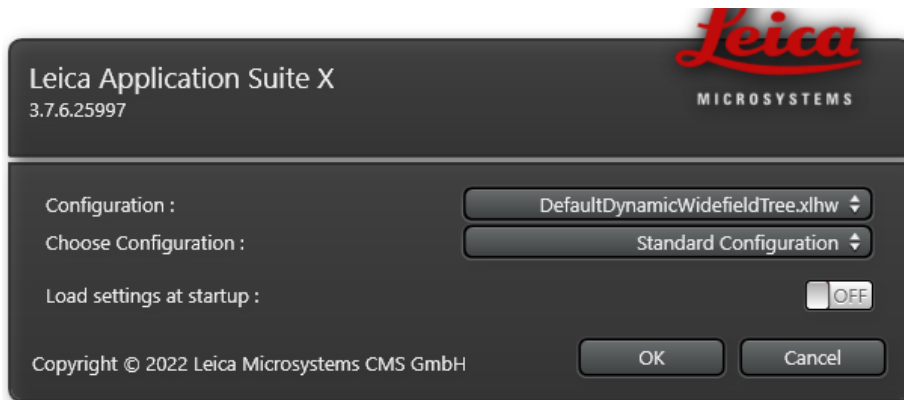
² Z-resolution improves with the square of NA; $d_z= 2 \lambda\eta/\text{NA}^2$ where λ is the wavelength of light, η is the refractive index of the objective immersion medium (air, water, glycerol, oil, ..) NA is the numerical aperture of the objective

Switching Fluorescent Filter Cube Configuration

8-30-2023 -updated procedure - see also video on the control pc desktop

If you need to swap the Texas Red cube for a CFP filter set (or the other way around), follow the steps below. In the new procedure, filter cube in position 5 is designated as a custom C/T cube (either CFP or Texas Red). Thus, do not change the hardware cube definition in the filter turret settings in LASX or in Hardware Configurator. Instead, you just have to choose the appropriate cube description in the LASX software in the light Path panel.

1. Start LASX in Standard Configuration



2. Remove the filter turret cover. If the filter that you need to remove is not pointing to the front, **push and hold the locking pin** and click a filter filter cube in the software that is 4 positions away from the cube that you want. For position #5 which holds the CFP or Texas Red cube, you will need to click the "A" filter cube in the software.



3. Take the old cube out, replace with the new cube (have MIC staff show you how to do this first time). Take care to not touch the filters. Store the extra filter cube in its storage box. Replace the filter turret cover.

- In the following example, you have removed the TXR cube and installed the CFP filter cube. In the Channel settings, select FLUO as a Contrast Method. Then, in the Fluorescence Channel (FCr1) in the pull-down menu select CFP. This will assign the CFP name to this channel, load the pre-defined lookup table (Cyan color) and switch to the filter cube #5 (C/T). When you capture an image, the Image Properties will indicate that the CFP filter set was used in the C/T cube position.

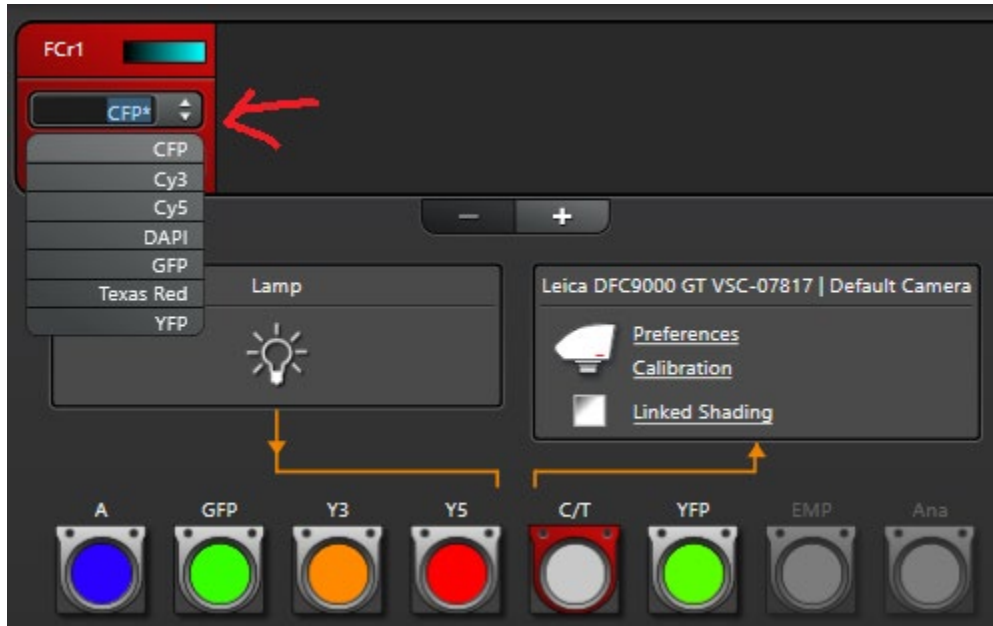


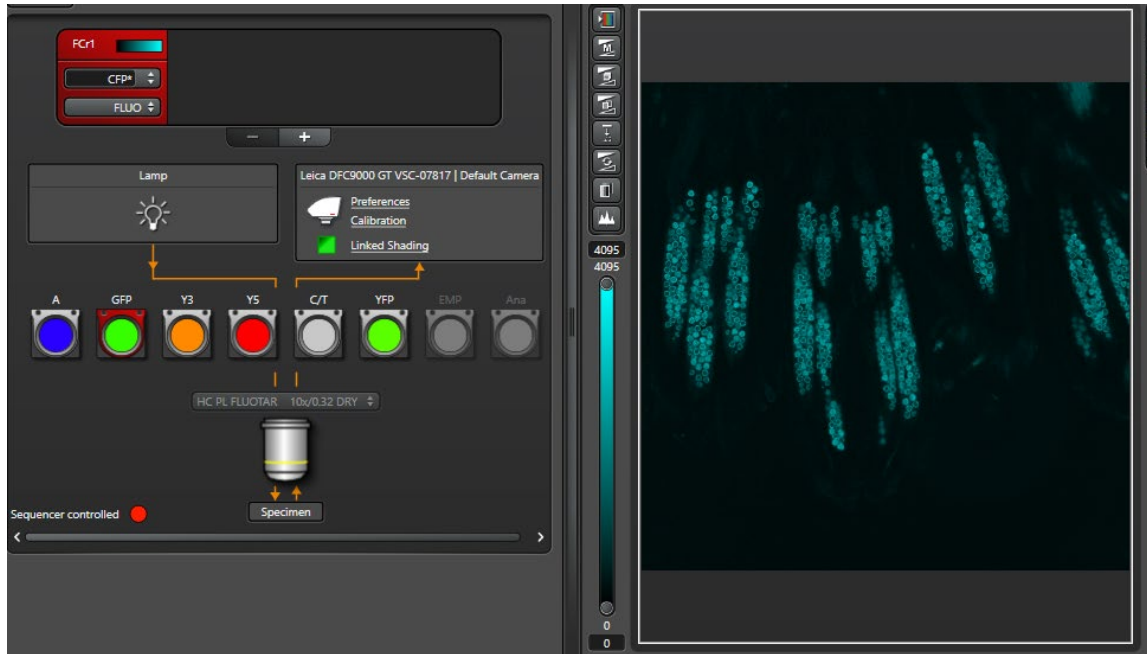
Image Properties:

Channel Name	Name	Cube	Contrast Method	Emission Wavelength	Peak Emission	Peak Excitation
Channel 1	CFP*	C/T	FLUO	550 nm	0 nm	nm

Channel Name	Lookup table name	Exposure Time	Gain	Differential interference contrast	Condenser	Resolution XY	Resolution Z
Channel 1	Cyan	37.1 ms	low noise	-		1.048 μ m	5.371 μ m

- Important: If you just click the filter cube icon, the microscope will switch to that cube, but WILL NOT change the name of the filter set or the lookup table.** So if you do this, the name of the dye will not be correctly recorded in Image Properties. Therefore, it is best to switch between cubes using the pull-down menu in the Fluorescent Channel, as shown above.

6. To reiterate, **here is the wrong way to do it:** We have the CFP filetr in the C/T cube position, but now clicked on the GFP cube and captured an image.



In the Image Properties, the cube is correctly recorded as GFP, but channel name is (incorrectly) CFP:

Channel Name	Name	Cube	Contrast Method	Emission Wavelength	Peak Emission	Peak Excitation
Channel 1	CFP	GFP	FLUO	525 nm	0 nm	nm

Channel Name	Lookup table name	Exposure Time	Gain	Differential interference contrast	Condenser	Resolution XY	Resolution Z
Channel 1	Cyan	34.917 ms	low noise	-		1.048 μ m	5.371 μ m

DFC9000GT MONOCHROME CAMERA PERFORMANCE SHEET

This camera was installed June 3rd, 2021; please contact MIC if you need the performance sheet for the previous camera, s/n VSC-09365)

.eica

VSC-07817
PERFORMANCE SHEET

SYSTEM OVERVIEW

COMPONENTS

Description	Model	Serial Number
Leica	DFC9000 GT	VSC-07817

WINDOW VARIANT

Window Variant	VIS-NIR Enhanced Parallel

SENSITIVITY & READ NOISE

System Readout Rate	Gain settings	Rolling Shutter	
		CMOS Sensitivity* ¹ (e ⁻ per A/D count)	Median Read Noise* ² (e ⁻ RMS)
540 Mhz	High well capacity	8.31	6.78
	Low noise	0.3	1.24
	Low noise & high well capacity	0.53	1.28
200 Mhz	High well capacity	6.76	5.73
	Low noise	0.29	0.97
	Low noise & high well capacity	0.54	1.01

SATURATION LEVEL

Pixel Well Depth* ³	33707	electrons

SENSOR DARK CURRENT

Median Dark Current Achievable	0.089	electrons / pixel / sec

SYSTEM PASSED FOR SHIPPING

Test Technician	Date
Michael Stewart	4th May 2021

NOTES

- *1 Sensitivity is measured in photoelectrons per A/D count from a plot of Variance [Noise squared] against Signal
- *2 Median noise distribution of the sensor
- *3 Pixel well depth measured using high well capacity gain setting