

**Operating Manual for Keck 3DFM Microscope**

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## **Brightfield Imaging**

Turn on stage, microscope, and CCD power supplies.

Turn on microscope.

Select objective. When putting on objective, lower objective turret first. Be careful not to hit linear positioner when lowering turret.

Open the Shutter Control GUI on the desktop (by default, the control GUI will be the "top" application).

If you click "view," and then deselect "always on top," it can be hidden or on top.

Open Zaber Positioner on desktop. When it opens, select "open" and ensure positioner is in middle position in infinity space.

Ensure piezo is turned on (**should not be turned off**). Closed loop light should be on. The display should read 160. If it does not, turn dial to 160. **If dial will not change display, turn unit off and then on again. Press closed loop button again.** Dial should now function.

Select position 1 on the turret selector (100% to ocular).

On shutter control, select "open" for spot option.

You can now see your sample. **If field-of-view is "colored"(red, blue, or green), make sure epifluorescent turret is in proper open position.**

Condenser turret has 5 positions. For brightfield, it should be in position "A." For DIC, it should be in position "DIC M" for 4, 10, 20, and 40x. It should be in position "DIC H" for 60 and 100x. There are also two phase options to choose from.

To capture individual or time-lapse images with Spot camera, turret selector needs to be on position 4. This sends 80% of the light to the CCD and 20% to the ocular. Open SPOT software and snap away.

The Spot software can do individual or time-lapse images. For individual or time-lapse z-stack, open the "Time Lapse Series" GUI on the desktop.

Ensure piezo is turned on (**should not be turned off**). Closed loop light should be on. The display should read 160. If it does not, turn dial to 160. **If dial will not change display, turn unit off and then on again. Press closed loop button again. Dial should now function.**

Select "Time Series" in toolbar and choose options.

For Time-lapse slice using Time-lapse software:

Open Spot software, and choose SPOT TIME-LAPSE in selector at bottom right. Capture live image. When exposure and field of view are what you desire, close software.

The preview window you see in the SPOT software is what will be acquired for the time-lapse data.

In time-lapse series toolbar toolbar, select Spotcam, and then DIC. This option maintains exposure as set.

If you select fluorescence, the exposure will be recomputed before every image capture (if you do not want the exposure to be recomputed (if you are looking for a decrease or increase in signal) choose DIC even if doing fluorescence.).

Choose number of images and time in between.

For brightfield image size, select "Preview Area."

Follow save options as desired. Choose "0" for wait time for a single slice or choose appropriate wait time for time-lapse capture.

Select "Session" in toolbar, and "Start."

For Time-lapse z-stack:

Follow above and choose number of steps and step size.

For DIC:

See above. Move polarizer on top of condenser in. Place condenser ring in the "M" position for 4,10,20, or 40 x DIC. Place in the "H" position for 60 or 100x DIC. Place appropriate Wollanston prism under objective. Slide analyzer in.

## **Single Photon Confocal Microscopy (CLSM)**

Turn on stage, microscope, mercury lamp, Vivascope scanner, and appropriate laser.

Tune laser to desired wavelength and power.

Turn on microscope.

On keyboard, hit "Ctrl", "Ctrl", "B", and then "enter". This will allow you to communicate with Vivascope computer.

Open Imaq Server 0.2.2. **If you get an error at this point, you need to wait and retry. The server needs the scanner to be running for a minute or so before it can communicate with it.**

On keyboard, hit "Ctrl", "Ctrl", "C", and then "enter". This will allow you to communicate with Keck computer again.

Select objective. When putting on objective, lower objective turret first. Be careful not to hit linear positioner when lowering turret.

Open Shutter Control on desktop (by default, the control GUI will be the "top" application.

If you click "view," and then deselect "always on top," it can be hidden or on top.

Open Zaber Positioner on desktop. When it opens, select "open" and ensure positioner is in middle position in infinity space.

**Make sure no Wollaston prism is in place. This will greatly reduce fluorescence intensity.**

Open "Filter Wheel" GUI (FW102) twice. Select "option," "Serial Connection," "Com Port," "Com2" for dichroic filter wheel. Then select "File" and "Open Connection."

Follow this same procedure again but this time select Com3. This will select the filter in the emission wheel in front of the PMT.

The dichroic wheel contains:

Position 1 - 505lp

Position 2 - 568lp

Position 3 - 595lp

The emission wheel contains:

Position 1 - 535/40

Position 2 - 605/55

Position 3 - 630/60

Ensure piezo is turned on (**should not be turned off**). Closed loop light should be on. The display should read 160. If it does not, turn dial to 160. **If dial will not change display, turn unit off and then on again. Press closed loop button again. Dial should now function.**

Select position 1 on the turret selector (100% to ocular).

On shutter control, select "open" for spot option.

You can now see your sample. **If field of view is colored (red, blue, or green), make sure epifluorescent turret is in proper open position.**

Turn off overhead lights.

Turn on 1p power supply in two places.

Select "B" on signal switcher.

Select "Single Photon" in Zaber Positioner GUI.

Open "Confocal Subsystem" on desktop.

Select "grab."

Open confocal shutter in Shutter Control GUI.

If you want one 10 frame averaged image, select "snap" (sometimes the software will take an image of just one frame (you can tell this is happening by the super fast acquisition. If it does happen, close the image, do not save it, and then hit "snap" again. It usually fixes the bug).

To continue capturing any other type of image, including slice, z-stack, or time-lapse, you need to determine your field of view using the confocal subsystem and then close it. Then open the "Time Lapse Series" GUI (**if you do not close the confocal subsystem GUI, and just minimize it, the time lapse software will freeze**).

For slice, time-lapse (slice or z-stack), or just z-stack, open "Time Lapse Series" GUI on desktop.

Select Time Series in toolbar and choose option. **For all confocal imaging, 1P, 2P, or CRM, you must choose 640x480 as your imaging size.**

For slice (time-lapse or individual):

Choose number of images, time in between images (0 if collecting 1), and number of frames to average (it's usually not a good idea to collect less than ten because of the servo speed. If you're image is saturating at 10, reduce laser power and/or gain).

Follow save options as desired.

Select "Session" in toolbar, and "start."

For z-stack (time-lapse or individual):

Follow above and choose number of steps and step size.

Choose "0" for "wait time" for individual or appropriate for time lapse.

## **Two-Photon Confocal Imaging (TPLSM)**

Turn on stage, microscope, mercury lamp, Vivascope scanner, and appropriate laser (choose starting wavelength and power). Nearest spindle (on Tsunami) determines wavelength. Farthest spindle changes the path length in the lasing cavity and allows for cw or mode-locked operation. Complete operating instructions for the Tsunami can be found in the owner's manual.

**For wavelengths over 900nm, purge laser with nitrogen for 45 minutes at 8 L/min and then 2 L/min for the duration of the experiment.**

Turn on microscope.

On keyboard, hit Ctrl, Ctrl, and then B. This will allow you to communicate with Vivascope computer.

Open Imaq Server 0.2.2. **If you get an error at this point, you need to wait and retry. The server needs the scanner to be running for a minute or so before it can communicate with it.**

On keyboard, hit Ctrl, Ctrl, and then C. This will allow you to communicate with Keck computer again.

Select objective. When putting on objective, lower objective turret first. Be careful not to hit linear positioner when lowering turret.

Open Shutter Control on desktop (by default, the control GUI will be the "top" application. If you click "view," and then deselect "always on top," it can be hidden or on top.

Open Zaber Positioner on desktop. When it opens, select "open" and ensure positioner is in middle position in infinity space.

Ensure piezo is turned on (**should not be turned off**). Closed loop light should be on. The display should read 160. If it does not, turn dial to 160. **If dial will not change display, turn unit off and then on again. Press closed loop button again. Dial should now function.**

Select position 1 on the turret selector (100% to ocular).

**Make sure no Wollaston prism is in place and make sure the analyzer is not engaged. This will greatly reduce fluorescence intensity.**

On shutter control, select "open" for spot option.

You can now see your sample. **If field of view is colored (red, blue, or green), make sure epifluorescent turret is in proper open position.**

Eliminate all ambient light (I'll even turn off the monitor for time-lapse).

**Cover OQM port with appropriate cap.**

**Turn on 2p power supply in three places.**

Select "C" on signal switcher.

Select "Two Photon" in Zaber Positioner GUI.

**Place appropriate filter in emission light path either on top of filter turret or in turret.**

Select "2" on microscope turret selector.

Open "Confocal Subsystem" on desktop.

Select "grab."

Open confocal shutter in Shutter Control GUI.

If you want one 10 frame averaged image, select "snap" (sometimes the software will take an image of just one frame (you can tell this is happening by the super fast acquisition. If it does happen, close the image, do not save it, and then hit "snap" again. It usually fixes the bug).

To continue capturing any other type of image, including slice, z-stack, or time-lapse, you need to determine your field of view using the confocal subsystem **and then close it.** Then open the "Time Lapse Series" GUI (**if you do not close the confocal subsystem GUI, and just minimize it, the time lapse software will freeze).**

For slice, time-lapse (slice or z-stack), or just z-stack, open the "Time Lapse Series" GUI on the desktop.

Select Time Series in toolbar and choose option. **For all confocal imaging, 1P, 2P, or CRM, you must choose 640x480 as your imaging size.**

For slice (time-lapse or individual):

Choose number of images, time in between images (0 if collecting 1), and number of frames to average (it's usually not a good idea to collect less than ten because of the servo speed. If you're image is saturating at 10, reduce laser power and/or gain).

For "wait time," choose "0" for individual.

Follow save options as desired.

Select "Session" in toolbar, and "Start."

For z-stack (time-lapse or individual):

Follow above and choose number of steps and step size.

For "wait time," choose "0" for individual.

## **Confocal Reflectance Imaging**

Turn on stage, microscope, mercury lamp, Vivascope scanner, and appropriate laser (choose starting wavelength and power).

**For wavelengths over 900nm, purge laser with nitrogen for 45 minutes at 8 L/min and then 2 L/min for the duration of the experiment.**

Turn on microscope.

On keyboard, hit Ctrl, Ctrl, and then B. This will allow you to communicate with Vivascope computer.

Open Imaq Server 0.2.2. **If you get an error at this point, you need to wait and retry. The server needs the scanner to be running for a minute or so before it can communicate with it.**

On keyboard, hit Ctrl, Ctrl, and then C. This will allow you to communicate with Keck computer again.

Select objective. When putting on objective, lower objective turret first. Be careful not to hit linear positioner when lowering turret.

Open Shutter Control on desktop (by default, the control GUI will be the "top" application. If you click "view," and then deselect "always on top," it can be hidden or on top.

Open Zaber Positioner on desktop. When it opens, select "open" and ensure positioner is in middle position in infinity space.

Ensure piezo is turned on (**should not be turned off**). Closed loop light should be on. The display should read 160. If it does not, turn dial to 160. **If dial will not change display, turn unit off and then on again. Press closed loop button again. Dial should now function.**

Select position 1 on the turret selector (100% to ocular).

On shutter control, select "open" for spot option.

You can now see your sample. **If field of view is colored (red, blue, or green), make sure epifluorescent turret is in proper open position.**

**Make sure no Wollaston prism is in place. This will greatly reduce signal intensity.**

Turn off overhead lights.

Place B1 beamsplitter on pegs in front of polygon mirror.

Place 1/4 waveplate into lens assembly on back of microscope housing.

Select "A" on signal switcher.

Select "Single Photon" (or whatever position places a fully reflective mirror in the infinity space) in Zaber Positioner GUI.

Open "Confocal Subsystem" on desktop.

Select "grab."

Open confocal shutter in Shutter Control GUI.

If you want one 10 frame averaged image, select "snap" (sometimes the software will take an image of just one frame (you can tell this is happening by the super fast acquisition. If it does happen, close the image, do not save it, and then hit "snap" again. It usually fixes the bug).

To continue capturing any other type of image, including slice, z-stack, or time-lapse, you need to determine your field of view using the confocal subsystem and **then close it**. Then open the "Time Lapse Series" GUI (**if you do not close the confocal subsystem GUI, and just minimize it, the time lapse software will freeze**).

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For "wait time," choose "0" for individual.

Follow save options as desired.

Select "Session" in toolbar, and "Start."

For z-stack (time-lapse or individual):

Follow above and choose number of steps and step size.