**Intelligent Imaging Innovations (3i) Spinning Disk Confocal System**

**Caution!!**

 **\*The confocal lasers are very intense. Use caution when working with these lasers.**

 **\*Never look down the barrel of the objective where the laser exits the objective lens.**

# **Instrumentation**

**Yokogawa CSU-W1 T1 Spinning Disk with 50um pinhole disk**

 17mm x 16mm FOV

**Prime 95B Back Illuminated sCMOS camera**

 1200x1200 pixels, 11x11um square pixels, 18.7mm FOV

 95% Quantum Efficiency

Speed 0 = 12 bit/82 fps or **12msec/image** - Gain only works in 12-bit mode

Speed 1 = 16-bit/41 fps or **24msec/image**

Note: Binning on a CMOS camera will make the image brighter but does not significantly increase imaging speed.

CMOS – 4-pixel binning – 4 readouts

 CCD – 4-pixel binning – 1 readout

 To Crop – use Marquee tool to mark area and then select camera to update number of pixels

*To center an object within the field of view of the camera, double click left on the object within the image.*

**Lasers:** 445nm – 100mW

 488nm – 150mW

 514nm – 150mW

 561nm – 150mW

 637nm – 140mW

**Multi-Pass Excitation Dichroic/Single Emitter Laser Position Emission Filter Wheel**

**c445** 440/514/561 2 – 445nm 4 - 482/35 = 464.5-499.5nm BP

**c488** 405/488/561/640 3 – 488nm 5 - 525/30 = 510.0-540.0nm BP

**c515** 440/514/561 4 – 514nm 6 - 542/27 = 528.5-555.5nm BP

**c561** 405/488/561/640 5 – 561nm 7 - 617/73 = 580.5-653.5nm BP

**c640** 405/488/561/640 6 – 637nm 8 - 692/40 = 672.0-712.0nm BP

**Quad-Pass Ex Dichroic/Quad BP Emitter Laser Position Emission Filter Wheel**

**q488** 405/488/561/640 3 – 488nm 1 – 440/521/607/700

**q561** 405/488/561/640 5 – 561nm 1 – 440/521/607/700

**q640** 405/488/561/640 6 – 637nm 1 – 440/521/607/700

**Triple-Pass Ex Dichroic/Tri BP Emitter Laser Position Emission Filter Wheel**

**t445** 440/514/561 2 – 445nm 2 – 475/543/702

**t515** 440/514/561 4 – 514nm 2 – 475/543/702

**t640** 440/514/561 5 – 561nm 2 – 475/543/702

# **Instrument Set-up**

1. Turn all components of the 3I Spinning Disk hardware “ON”.
2. Open the **3I Slidebook 2022** software.
3. Click on the **Focus** icon. The **Camera** window and **Focus** window will appear.
4. Click on the **Capture** icon. The **Capture** window will appear.
5. If the Focus and Capture icons are not available, be sure that a SlideBook is open. To create a new SlideBook, select File/New SlideBook.







# **Microscope Control**

1. The Zeiss automated microscope can be controlled through the **Focus** widow, the microscope itself, and the microscope handset controller.
2. The Zeiss **Objectives** that are available include:

10x Air NA 0.3 Working Distance = 5.2mm

20x Air NA 0.8 Working Distance = 550um

40x Oil NA 1.3 Working Distance = 201um

63x Oil NA 1.4 Working Distance = 190um

40x Water NA 1.2 Working Distance = 280um

63x Water NA 1.2 Working Distance = 280um

1. **Focus Window / Emission Selection**

**100% Left** = Spinning Disk/Camera

**100% Right** = not an option

**100% Eyes** = Ocular/Eye Port View

**Lamp** = Halogen Lamp, **%** = lamp intensity

**Condenser Aperture** = Contrast setting for Brightfield or DIC images

**Condenser Position** = BF (Brightfield)

 DIC II, for 10x and 20x objectives

 DIC III, for 40x oil, 63x oil, 40x water, and 63x water objectives

 Phase – no phase objectives are available

Note: For DIC imaging, you must also insert:

 Polarizer (located above the condenser)

 Analyzer (position 5 on the filter wheel)

 Matching DIC prism inserted below the objective

1. **Focus Window / Filter Set**

**Eyes** – Ocular view of conventional fluorescence (blue, green, or red) or transmitted

light (BF or DIC).

**Wide Field** – Camera recording of conventional fluorescence (blue, green, or red) or

transmitted light (BF or DIC).

**CSU-Single Band** – Spinning Disk, Single Laser Excitation/Single Band Pass Emission Filter

**MultiBand Em** – Spinning Disk, Single Laser Excitation/Multi Band Pass Emission Filter

**Open Fluor** – Mercury Arc Lamp Shutter

**Open Bright** – Halogen Lamp Shutter

**Open Alt** – Laser Shutter

1. **Focus Window / XY Stage**

Automated control of XY Stage, in 10um, 1um, 0.1um or FOV (Field of View) increments.

1. **Focus Window / Z Stage**

Automated control of Piezo Z-focus, in 10um, 1um, 01.um or user-defined increments.

Please note that the Piezo Z-focus has a 300um total focus limit. It is recommended that the microscope coarse/fine focus knob be used to set the initial focus of the sample.

1. **Focus Window / Neutral Density**

Not functional on our system.

1. **Focus Window / Laser Power**

Controls intensity of the laser power from 1-100% of total power.

# **Camera Control**

1. **Focus Window / Camera**

**PMUSBCAM00** = Prime 95B Back Illuminated sCMOS camera

**Exposure** – Increase exposure time to increase the brightness of your image.

 Exposure time should be greater than 10msec to avoid lines within the image.

**Bin** – 1x1 and 2x2 are available. 4x4 and 8x8 binning are not available.

 2x2 binning will increase the brightness of your image. However, the increased

pixel size will result in a decrease in the image resolution.

 **Update** – Use the Marquee tool to draw a region of interest (ROI) and then select

**Update** to crop the image. The image will have the same pixel size, but fewer

pixels. Select **Full Chip** to return to the full field of view.

 **Guide** – Useful for centering your field of view.

 *To center an object within the field of view, double click left on the object.*

 **Pseudocolor** – Useful for distinguishing intensity within your image.

 Blues/greens represent low intensity. Yellows/reds represent high intensity.

 **Live** – turns the camera on

 **Stop** – turns the camera off

 **Snap** – takes a snapshot of the current image

1. **Focus Window / Camera tab**

**Scale Image** = Pixel Histogram = Look Up Table = LUT

 **Manual** – Manually set your Low value and High value for your LUT.

 The Manual option is recommended.

 **Auto** – Computer automatically and continuously rescales your image for

the current dynamic range.

**0-99%** - Leave this option selected.

**Range** – Number of pixels about center for auto scale

**Fan** - Leave this option selected.

**Speed** **– 0** – 12-bit display, 0-4095 intensity values

 **1** – 16-bit display, 0-65,535 intensity values

**Gain** – 1x, 2x, 3x – *only available in Speed 0, 12-bit display mode.*

1. **Capture window / Extent, Offset, Binning (pixels)**

**Bin Factor** – 1x1 and 2x2 are available. 4x4 and 8x8 binning are not available.

 **Width/Height/X Offset/Y Offset** – Use to crop the current field of view.

 Options include 256x256, 512x512, 1024x1024, 1200x1200

*To center an object within the field of view, double click left on the object.*

Alternatively, use the Marquee tool to draw a region of interest (ROI) and then select

**Update** to crop the image. The image will have the same pixel size, but fewer

pixels. Select **Full Chip** to return to the full field of view.

# **Setting 3D Focus Boundaries**

1. Find the approximate center focal plane of your sample using the manual microscope coarse/fine focus knob.
2. Use the Piezo Z-drive up/down arrows (**Focus Window / Z Stage**) to find the lower boundary and the upper boundary of your sample. It is recommended that the **Scale Image** be set to **Manual** before locating your sample 3D boundaries. Once the lower boundary is located, click the **Bottom** button within the **Focus/Z** tab. Once the upper boundary is located, click the **Upper** button within the **Focus/Z** tab.

Enter the **Step Size** and the software will automatically calculate the **Number of Planes** (optical sections) and the **Total Travel** (total thickness).

Click on **Optimal** to set the **Step Size** to Nyquist.

1. To capture an XY-Z series, from the **Capture window / Capture Type**, select **3D**.
2. Within the **3D Capture** window, select:

**Use Current Position** – The first image in the series will be the current position. The series will then focus up (go deeper into the sample) using the specified Range, # Planes, and Step Size.

**Use Reference Position** – The first image in the series will be at the designated **Reference Position**, which is set in the **Focus Window / Z** tab. The series will then focus up (go deeper into the sample) using the specified Range, # Planes, and Step Size.

**Use Top and Bottom Position** – The XY-Z series will use the top and bottom boundaries which were set in the **Focus Window / Z** tab, using the specified # Planes, and Step Size.

**Range Around Current** – The first image in the series will focus down by 1/2 the specified Range. The series will then focus up (go deeper into the sample) through the specified Range using the specified # Planes and Step Size.

# **Setting Multiple XY Locations or Montage Boundaries**

1. Use the **Focus Window / XY** tab to set multiple XY Locations and Montage boundaries.
2. Once multiple XY locations have been identified, the **Multipoint List** option will be available in the **Capture / Multiple XY Location Capture** window.
3. For a montage, once the montage boundaries have been identified, the **Montage** option will be available in the **Capture / Multiple XY Location Capture** window.

# **6D Imaging**

1. Within the **Focus Window** tab, optimize the **Exposure** and **Laser Power** for each dye.
2. Go to the **Capture** taband set the image parameters for the first phase of the experiment.

Remember to set:

 -the **Filter Set**

 -the **Exposure**

 -the **Laser Power** (“Current” is not recommended)

 -**Full Chip** or ROI (**Width** and **Height**)

Draw ROI box on image and select **Update** to update the camera dimensions.

 -**Timelapse**: enter the **# of Time Points** and **Interval** (set to 0 for no delay)

 -**3D / 3D Capture**: Set Z focus boundaries as described in Part V.

1. To save these image settings for the first phase of the experiment, go to **Capture / Capture Settings**, select **Save As** and enter a **Description** that will be the filenamefor the first phase of the experiment.
2. Repeat steps 2/3 for each phase of the experiment.
3. From the **Focus Window / XY** tab, select **Set Point** to identify the X, Y, Z position for the first phase of the experiment. *Remember to use the computer-controlled piezo Z Stage to determine the focal position. Do not use the microscope coarse/fine focus.*
4. Repeat step 5 for each phase of the experiment. You should have an XYZ location that can be assigned to each experiment phase.

Note: If you have 3 phases to your experiment, you must have 3 XYZ locations, even if it is exactly the same XYZ location. To copy a location, double click on the position to be copied (not single click) and then select **Set Point**.

1. From the **Focus Window / 6D** tab, all of the designated locations (Set Points) from steps 5/6 should be listed. Again, there should be a location for each experiment phase.
2. Next, each location will be assigned to the image parameters/experiment phase that was saved in steps 2/3. To assign the saved image parameters for each experiment phase to the saved XYZ locations, click on the XYZ location and then from **Capture script** select the name of the associated experiment phase (determined in steps 2/3). For a delay between phases, enter the desired delay time in the **Pause After Script**.
3. Repeat step 8 for each phase of the experiment.
4. To begin the experiment, click on the **6D icon**. Enter a desired filename under **Name** and select **Start**.

# **Saving Data**

1. Raw Data: Select **File / Save** or **Save As** – Saves raw data in SlideBook in .sld format.

To copy a single image within a SlideBook to a second SlideBook, click on the image filename and select control/copy. Then, click on the new SlideBook and select control/paste. You must use the keyboard control (Cntrl/V) for the paste option. The software selection (right click/Paste) does not work.

1. Quantitative Data Export: Select **Export** icon (yellow arrow) **16-bit TIFF (OME)** or **MATLAB**



1. Qualitative Data Export: Select **Export** icon (yellow arrow)

**RGB TIFF (24-bit)** – saves current view exactly as displayed as 24-bit color TIF

**TIFF Series** – exports entire series, each image in the series as its own color TIF

**All Default Views as TIFF** – exports all files within the SlideBook, each image in each file/series as its own color TIF

**Export All Channels as 8-bit TIFFS** – exports each channel of current view as its own grey-scale TIFF

1. To re-use previous settings, Right Click on the image filename within the SlideBook and select **Recapture Experiment.**

# **Image Speed Comparisons**

For comparison, a two-color image was collected under different camera conditions.

Total exposure time for the two-color image was 120msec (green 20msec, red 100msec).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Pixel Density** | **Emission Dichroic** | **Binning** | **Speed** **(Bit Depth)** | **Gain** | **Time per image** | **Pixel Size** |
| 1200x1200 | Single | 1x1 | 0 | 1 | 654 msec | 0.529 um |
| 1200x1200 | Single | 1x1 | 0 | 2 | 656 msec | 0.529 um |
| 1200x1200 | Single | 1x1 | 0 | 3 | 655 msec | 0.529 um |
| 1200x1200 | Single | 1x1 | 1 | 1 | 703 msec | 0.529 um |
| 1200x1200 | Single | 2x2 | 0 | 1 | 644 msec | 1.058 um |
| 1024x1024 | Single | 1x1 | 0 | 1 | 646 msec | 0.529 um |
| 512x512 | Single | 1x1 | 0 | 1 | 610 msec | 0.529 um |
| 256x256 | Single | 1x1 | 0 | 1 | 593 msec | 0.529 um |
| 256x256 | Single | 2x2 | 0 | 1 | 593 msec | 1.058 um |
| Crop 92x92 | Single | 1x1 | 0 | 1 | 593 msec | 0.529 um |
| 1200x1200 | Quad | 1x1 | 0 | 1 | 322 msec | 0.529 um |
| 512x512 | Quad | 1x1 | 0 | 1 | 278 msec | 0.529 um |
| 512x512 | Quad | 2x2 | 0 | 1 | 275 msec | 1.058 um |
| Crop 92x92 | Quad | 1x1 | 0 | 1 | 256 msec | 0.529 um |