

Nikon C1si
Spectral Laser Scanning Confocal Microscope

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Contents

1 Overview	2
2 Start-Up	2
3 Sample Alignment by Eye	5
4 Confocal Imaging	7
5 Shut-Down	11
6 Troubleshooting	12
7 Pinhole Alignment	13

1 Overview

The Nikon C1si is a confocal laser-scanning microscope with four lasers for multi-color imaging. The laser lines include: 404 nm, 488 nm, 561 nm, and 639 nm. The microscope has 2 types of detectors: one with 4 virtual band pass detectors and one with a spectral detector that can image 32 different channels at the same time (adjustable widths of 2.5 nm, 5 nm, and 10 nm). The instrument is built around an upright Nikon FN1 microscope with a large electrophysiology stage. The maximum penetration depth into tissue is approximately 100-200 μm depending on the opacity of the sample.

2 Start-Up

1. Turn on **arc lamp** (Nikon Intensilight on shelf above table: Figure 1). The lamp should be left on for at least 30 minutes at a time and turned off for at least 30 minutes prior to turning on again. This prevents the arc from flickering and wearing out prematurely. Please check the online scheduler to see if a user is signed up directly after you.
2. Turn on the **lasers** that you will use (Figure 2).
 - (a) 2x Diodes on the laser bench: turn key from 12 o'clock to 3 o'clock.
 - (b) 1x Diode on top of the control box: turn key and then push 'laser on' (green button).
 - (c) Obis 488 laser: turn the switch on the power supply and wait a full minute, then turn the key from 'standby' to 'on'.
3. Turn on the **remote focus accessory**: switch is on left hand side of box (Figure 3a).
4. Turn on the **Nikon D-Eclipse C1 control box** located on the floor under the table (Figure 3b).
5. Turn on the **computer**.
6. Choose and **carefully** load one or two **objectives**. Center the lever so that both lens sockets are in the up position. Raise the nose piece to its maximum. Using both hands, unscrew any loaded objectives, and thread the objectives you want to use. Only finger tighten. Overtightening will lead to objectives getting stuck.

Available Objectives

Number	Objective	Theoretical Resolution (nm)	Depth of field (μm)	Suggested step size (μm)	Transmitted Light	Working Distance	Imaging Conditions
1	20x/0.75 NA air CFI Plan Apo	407	2.31	0.924	DIC N2/20x	0.75 mm	Air
2	40x NIR/0.8 NA W	381	2.03	0.812	DIC N2/40x III	3.5 mm	Water Dipping
3	40x/1.30 NA oil Plan Fluor	235	0.77	0.308	DIC N2/40x	0.20 mm	Oil Immersion
4	60x/1.2 NA W NIR Apo	250	0.7	0.4	DIC N2/60x I	0.27 mm	Water Immersion
5	60x/1.4 NA oil Plan Apo VC	218	0.66	0.264	DIC N2/60x I	0.21	Oil Immersion

Dipping objectives are designed to form an image without a coverslip, whereas immersion lenses require a coverslip. For ideal imaging, always use #1.5 coverslips.

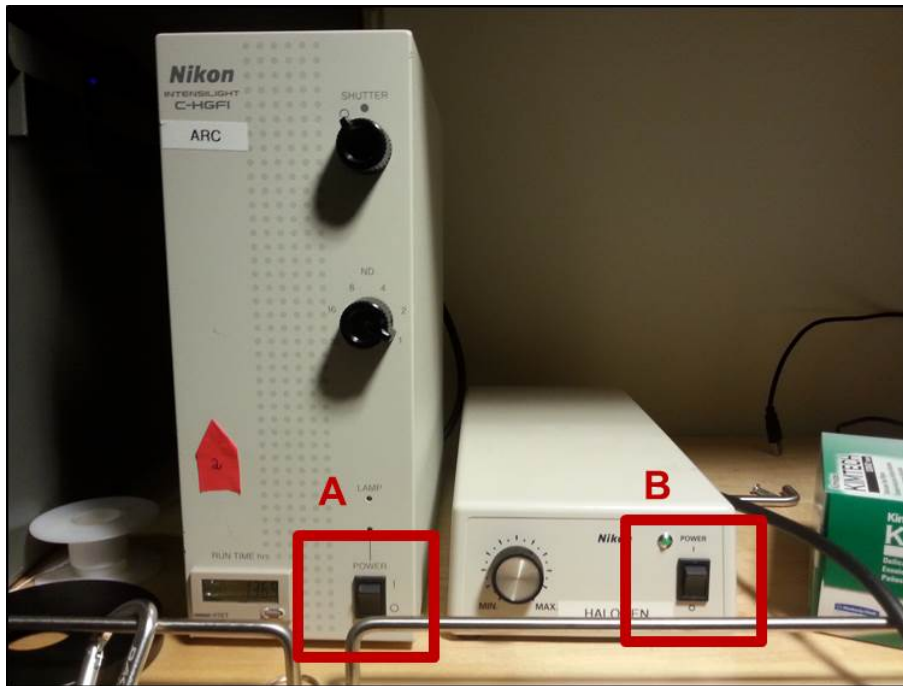


Figure 1: Arc Lamp (left) and Halogen Lamp (right) .

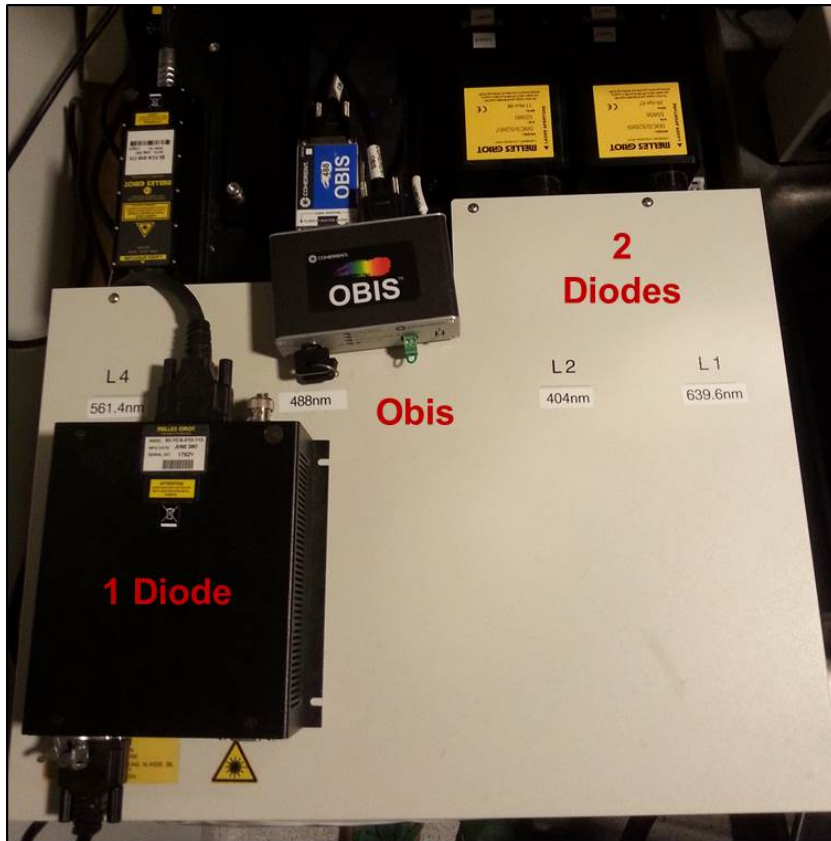


Figure 2: Available Lasers.



(a) Z-stepper Controller



(b) Control Box

Figure 3: Accessories.

3 Sample Alignment by Eye

1. Disengage z-stepper motor by pushing switch down (Figure 4).
2. Raise nose piece to its maximum.
3. Select smaller magnification objective (typically the 20x).
 - (a) Make sure the lever is centered (Figure 5A).
 - (b) Press the button on the upper right of the carriage and push or pull to move objective (Figure 5B).
4. Add any immersion or dipping fluid to your sample if needed.
5. Roughly place sample under objective by eye.
6. Use the objective lever bar to carefully lower the objective.
7. Using the coarse adjustment knob, lower the objective until it is just above your sample surface.
8. On the right side of the microscope push the slider to 'bino' (Figure 6).
9. Choose **dichroic #1** (Figure 7A).

Note: This is a triple band pass filter that is useful for aligning most typical fluorophores.
10. Open the **shutter** at the epi source on front of microscope (Figure 7B) (move to 'O' from 'C').
11. While viewing through the eyepiece slowly raise the objectives into the focal plane.

Note: rotate the adjustment knob so your thumbs point up to ensure the objective does not crash into your sample

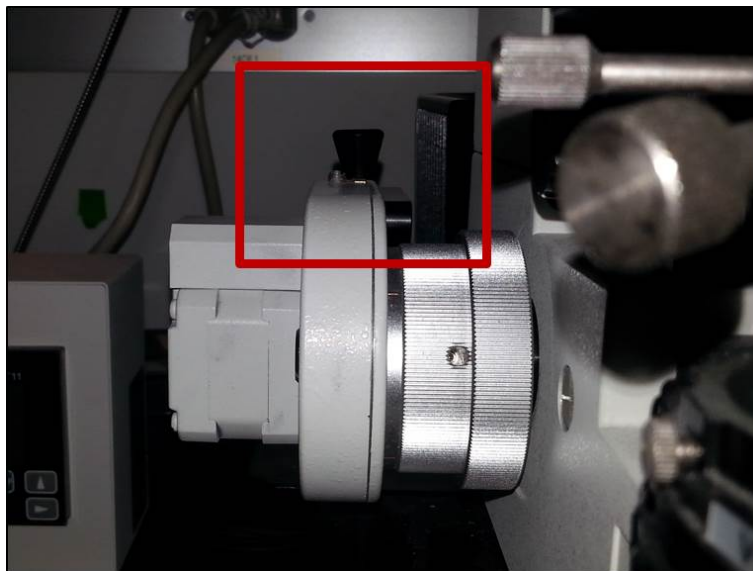


Figure 4: Push the knob down to disengage, up to engage the z-stepper.

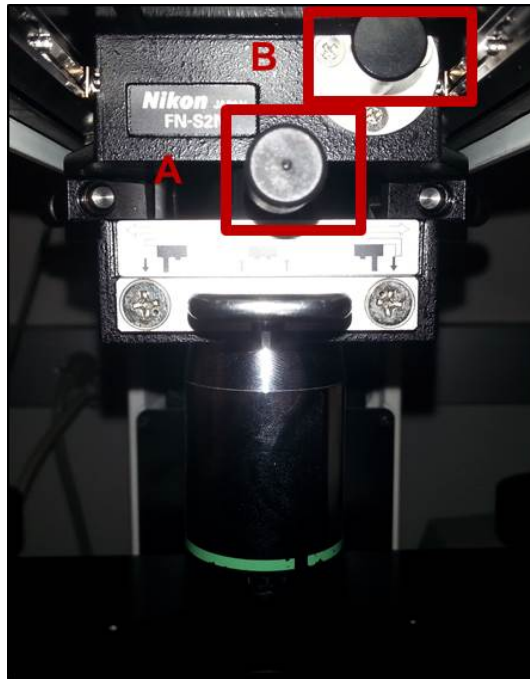


Figure 5: Objective Lever (A) Push Button (B).



Figure 6: Light Pathway Slider.

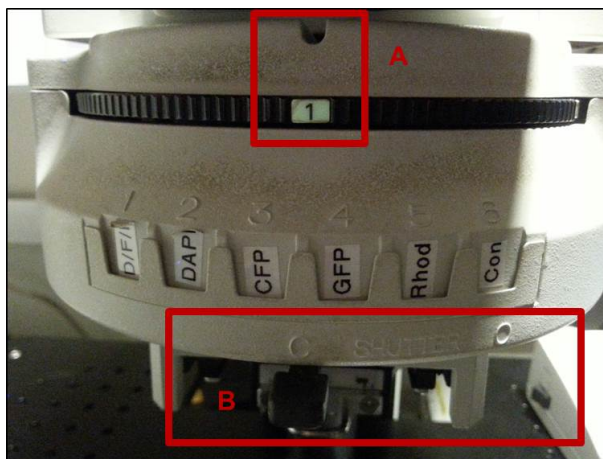


Figure 7: Filter Wheel (A) Shutter (B).

4 Confocal Imaging

1. Choose position '6' on the filter wheel.
2. Close the shutter on the front of the scope (from 'O' to 'C').
3. Pull out the slider to the 'conf' position.
4. Launch the software EX-C1 3.80.
 - (a) Open the appropriate parameter list. The '408_488_561_637' parameter file is a good starting point.
 - (b) A visual basic window will pop up during loading. Close it without changes/saving.
 - (c) A second window will appear listing errors. Close it.
5. Choose the objective you are using to image in the top right box (Figure 8 top). This is important to get the correct scaling.
6. Turn on and off the detectors and lasers that you want to use (Figure 8 bottom). Remember that they are 'on' when the background is colored, and the are 'off' when the background is black.
7. Set your pinhole (Figure 8 bottom). Typically the 30 μm (S) pinhole is acceptable. A larger pinhole allows more light at the sacrifice of resolution and vice versa.
8. Select all the imaging parameters wanted (Figure 8 middle). A selected box changes from dark green to bright green.
9. Click 'Single' to acquire an acquisition series.

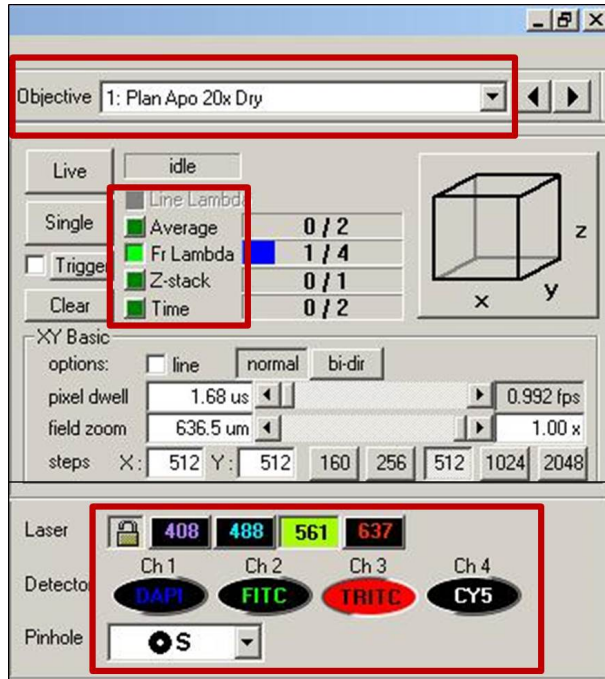


Figure 8: Objective selection, imaging parameters, laser and detector options.

Typical Imaging Parameters

Frame Lambda

- Set laser power and gain for each laser being used (Figure 9).
 - Under the 'Frame Lambda' tab highlight a particular channel and check the 'preview pass' box. When going 'Live' only one color is imaged.
 - While in 'Live' mode adjust the laser power and detector gain.

Note: A typical starting gain level is around '6.5' and laser power is between 20-50% depending on sample labeling.

Note: The Cy5 channel is white in color while saturation is black. If you see an 'outline' of your structures, trying turning down the gain/laser power.
- Uncheck the 'preview pass' box to image all colors sequentially.

Protip: Save a copy of the original running order in your user folder to easily re-load if needed.

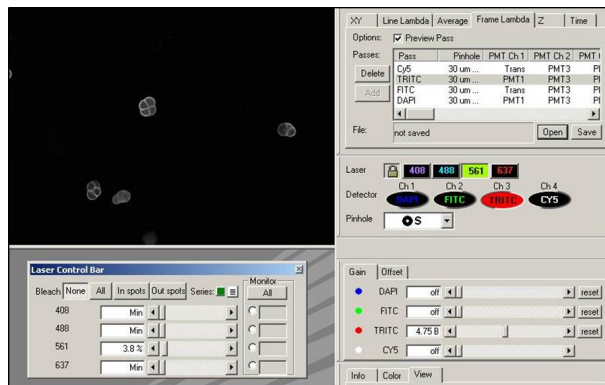


Figure 9: Frame Lambda tab, gain settings, and laser control bar.

Average

1. Increase the amount of frames averaged to increase the S/N without increasing laser power.
2. Press the green box near the 'Live' button to engage averaging.
3. Each channel can have a separate averaging condition.
 - (a) 'Stop at count' is the number of frames averaged.
 - (b) Check the 'Stop when Quality decreases' box, if your sample is at risk of photobleaching at a rate that negates the advantage of averaging.
4. Engaging the 'Average' box near the 'Single' button will open a new window with the averaged images.

Z-stack

1. Be sure the stepper motor is engaged by pulling up on the lever located on the back-left side of the microscope.
2. Clear everything by pressing the red bars.
3. Set a reference point by selecting 'Ref' and moving to a z-position (Figure 10). This can be any point between the top and bottom of your sample.
4. Set the 'top' by either moving the stepper motor via the control to the limit of your sample, or enter a number.
5. Set the 'bottom' using the same procedure.

Note: If you notice the imaging is not changing as the objective is being moved by the stepper motor, the piezo is probably at its limit. To re-center the piezo, raise the objectives, and press the button near the z-stepper display. Re-align your sample following the procedure above.
6. Set the step size. The 'N' button sets the step size to the Nyquist sampling frequency based on your sampling parameters.
7. Engage the 'Z-stack' setting by the 'Single' button.
8. Hit 'Single' to record a z-stack. A new 'Volume' window will appear.
9. When the stack is finished recording, use the 'View' tab and select '3D orthogonal' to slide through the z-position.
10. Colors can be displayed or removed by using the 'channel enable' buttons on the top.

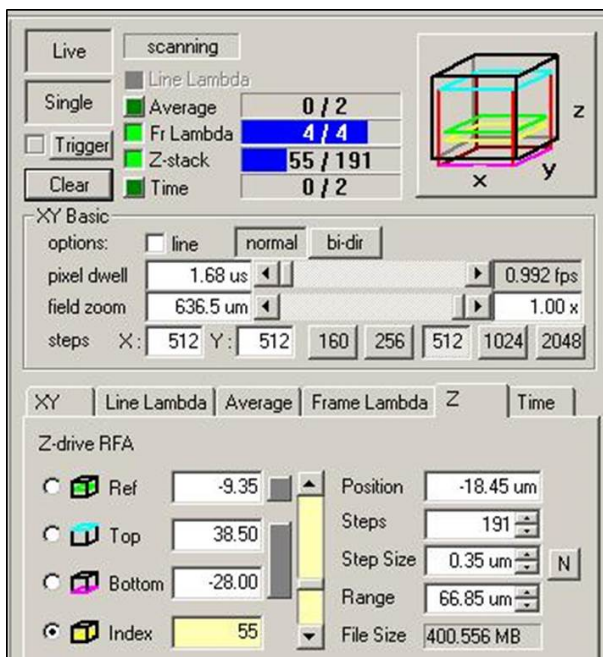


Figure 10: Z-stack settings.

Zoom

1. Click on the top right box with the cube in it (Figure 11 top).
2. Use the corners of the box to zoom in.
3. Grab the middle to move it around.
4. To zoom out, drag the field zoom bar in the XY window all the way to the right (Figure 11 bottom).
5. Check the XY tab to check the pixel size and whether you have reached the Nyquist sampling frequency. The NON OPTIMAL PIXEL SIZE warning disappears when you have reached this.

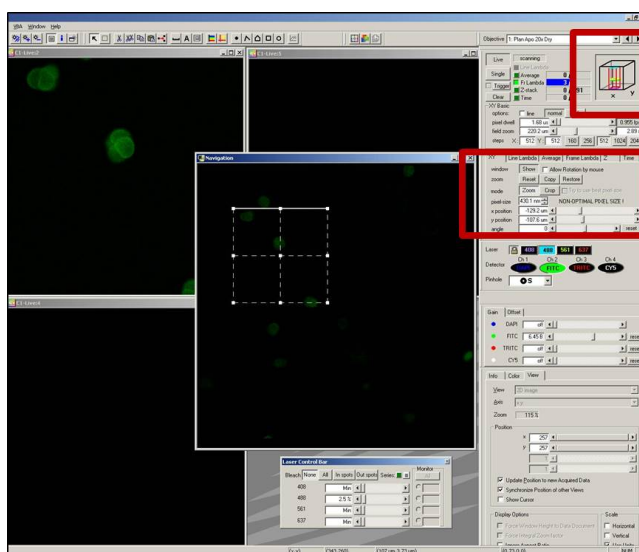


Figure 11: Adjust software zoom.

XY basic

1. Pixel dwell time: this is how long the laser stays on one pixel. The minimum type is typically sufficient.
Note: Changing the zoom or pixel number can change the dwell time. If imaging seems slow after changing those settings, check the dwell time.
2. Field zoom: The zoom that was performed using the 'cube'.
3. Pixels: 512x512 is a typical viewing area, but can be changed with experimental imaging speed and detail requirements.

Time

1. Count: The number of acquisitions (Figure 12).
2. Speed: When the next acquisition is started. Common selections are 'As fast as possible' and 'With fixed delay'.



Figure 12: Time lapse settings.

5 Shut-Down

To keep the computer's hard drive available for recording data, a BIDC member will clean up all files that are older than 2 weeks. It is a good idea to start transferring your collected data to an external hard drive before you start your clean up and shut down procedure.

1. If no one is signed up for two hours after your session is over:
 - (a) Raise objectives with lever and move the focus to the top.
 - (b) Clean off dipping and immersion objectives with lens paper.
Note: If you have not cleaned objectives before, please have a BIDC member show you a proper method.
 - (c) Close software **WITHOUT** overwriting software parameters.
 - i. Save changes to C1? **No.**
 - ii. Save profile? Select 'No' or 'Save as' your own profile if you plan on using it again.
 - (d) Reverse startup of items 4 → 1.
2. If someone is signed up after you:
 - (a) Turn off EZ-C1 software following above directions.
 - (b) Leave everything else on.

6 Troubleshooting

The z-stepper motor is not working.

1. Make sure the knob is pulled up on the rear left of the microscope.
Note: Be careful not to pull the knob out too far. This will completely disconnect the device and requires additional work to re-engage the device.

You cannot see anything through the eyepiece.

1. Check the arclamp (Intensilight) is turned on.
2. Check the shutter above the nosepiece is open.
3. Check the slider is set to 'bino'.
4. Check the filter wheel is set to '1'.
5. Check the focus of your sample.
6. Make sure the aperture on the arclamp is set to '1' (max transmission).

You cannot see anything on the computer.

1. Check the filter wheel is set to '6'.
2. Check the slider is set to 'conf'.
3. Check both the detector and laser are turned on.
4. The detector gain is set to '6.5' or higher.
5. The lasers are turned on by moving the slider to the right.
6. Check you are focused on the sample.
Note: As with most microscopes there is a slight difference in the focus between the eyepiece and the detectors. This becomes more apparent with sparsely labeled and/or thin samples.

Your sample is photobleaching too quickly.

1. Try using less laser power and more detector gain.
2. Focus on your sample using single frames instead of live.

Needing higher spatial resolution.

1. Use a higher NA objective.
2. Zoom in by clicking on the cube in the top right corner of the screen. When you reach the Nyquist sampling frequency, the NON OPTIMAL PIXEL SIZE warning disappears. The Nyquist sampling frequency: $(0.61 * \text{emission wavelength}) / \text{NA} = 2.7 \text{ pixels/diffraction limit}$.

Needing higher temporal resolution.

1. Use the 'bi-dir' option in the XY basic. This option collects photons on both scan directions of the galvo.
2. If the image appears 'double' go into the Tools window, select 'Bi-Directional Scan', check 'Bi-Directional' and Adjust the scan lines. Negative 1 is a good starting point.
3. The scan lines will need to be adjusted for each XY basic setting.

You accidentally close a 'Live' channel window.

1. Hit the 'New Window' button at the top. This will open a new window and assign it to whichever channel was closed.

7 Pinhole Alignment

Depending on your particular application you may want to choose different scanhead dichroics:

	Scanhead Dichroic	Left Filter Cube	Right Filter Cube
Beam splitter (spectral detector)	BS 20/80	N/A	N/A
Blue/Green/Red/FarRed	408/488/561/640	595/50 525/50	DAPI/CY5
CFP/YFP	457/514	550/50 485/30	

Note: While CFP/YFP imaging is possible, there are no longer the appropriate laser lines for excitation. Imaging conditions will be sub-optimal.

After changing the scanhead dichroic, the pinhole will need to be re-aligned (Figure 13A).

1. Place a fluorescent slide under your objective and focus on it using the arc lamp and eyepiece. The slides can be very bright, so neutral density filters can be put in the light path. These are located on the back right of the microscope.
2. Switch to confocal: pull out slider to 'conf', close shutter, and move to filter cube '6'.
3. Choose an appropriate laser line and detector and check 'preview pass'.
4. Go live and turn up the gain until you get signal.
5. Use a 2.5 mm hex key to align the pinhole using the two screws on the bottom right of the scan head (Figure 13B). This is done by 'walking' the pinhole: Move one screw until the image gets as bright as possible. Then move the other screw to the brightest point. Return to the first screw and repeat the process, continually switching screws until the image is maximally bright at the center of the field of view. If the image becomes saturated, turn down the gain.
6. Remove alignment slide and switch to your sample.

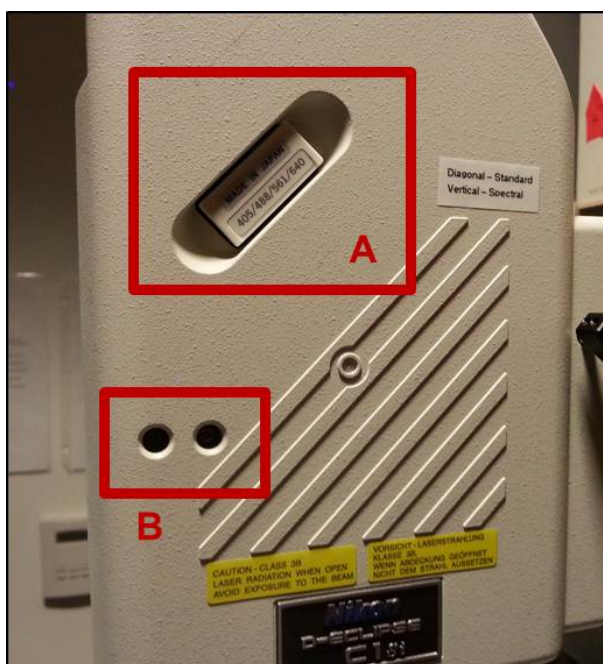


Figure 13: Scanhead Dichroics (A) and Alignment Screws (B).

Contact the BIDC

The BIDC office is located in Medical Sciences Building Room S1109.

The BIDC office phone number is 415-476-4550.

If you need immediate assistance and no one is available in the office, or it is after business hours, please call the **BIDC Hotline** at 415-745-2432.