Use the “Ocean view” program to check what wavelength the laser is tuned to. It should be at 780 or 800 and as we are using a 390/20 emission filter for SHG.

The scope uses a 20X objective lens.

If for whatever reason WiscScan ever crashes, after you quit out of it you need to turn off the Qcamera itself (power switch right there on it) and turn it back on and then re-launch WiscScan.
1. Turning things on (Figure 2):
   a. Laser is always on you don’t need to change anything on that.
   b. shutter control (switch in back, the channels are always closed),
   c. turn the ASI box on,
   d. The pockel cell (please don’t use the knob on front panel, you can adjust the pockel cell value using WiscScan),
   e. The galvos (which is just that black box there, switch in back).
   f. Turn on the camera itself (1 = on; 0 = off).

Important note: please turn off everything you turned on at the end of your imaging. But DO NOT turn the laser off.
2. Launch WiscScan from the desktop (don’t use any other version).

Sign in using the following credentials:

Username: service

Password: loci
3. Put your slide on the stage. For this it is easier to navigate the stage down toward yourself a bit. But make sure not to move it too much because it will damage the condenser. When mounting your slide, invert it and have the white tab of the slide on the right side (Figure 3).

![Figure 3](image)

4. Under the z-motor tab, click on “Qcam” and click Start Scan so that you bring up the camera display. The default thing that happens if you click Start Scan is to take an SHG image (in this case the box for “channel 1” is checked) however if you have “QCam” on, then it takes a digital camera pic. So to take an SHG image, you need to uncheck “QCam”. All this is true for when you are just previewing things and getting the focal planes etc. (Figure 4)

   a. When you want to actually collect an array of images, then you have to worry about which imaging channel boxes are selected.
      
      i. H & E: Channel 0 box checked ; Channel 1 box unchecked.

      ii. SHG: Channel 0 box unchecked ; Channel 1 box checked.
b. The SHG and Brightfield images are not parfocal. They are not aligned with each other in x-y either. When you have something in focus in brightfield, when switching to SHG you will need to rotate the focus wheel to be close to that focal plane. (Figure 5)
5. For now, we are no longer using the auto-focusing program called CRISP, but if you decide to use it, here are the instructions. Using the auto-focus (CRISP): check “Crisp” checkbox in “Z Motor” tab. Get the image in focus, then press “ZERO” on the ASI controller (this will also zero out the xy and z position), then press the @ button to get it out of the “D” state, then press it again to get it in “RL” state, then press and hold the @ button for 10 seconds and then the number that is second from the left on the readout on the ASI box itself should go to zero (KL= finding the focus, RL= Ready Lock, FL = found the focus), the number there is the “deviation/error from the home focal plane” value that will change as you migrate around the slide (a kinked slide will have diff z-planes that are in focus)).

a. You can test to see if you set this focal plane correctly by getting out of focus and then pressing the @ button and see if it goes back to your home focal plane. Always a good idea to do this.

b. ALL of this will have to be repeated for an SHG image, which will have a slightly different in-focus focal plane.

c. Also, when setting up for SHG imaging, you can adjust the detector gain right there in Wiscscan to the point where you have just a tiny bit or no red pixels (i.e. large dynamic range). Keep in mind that in the end your image will actually be comprised of a collapsed stack of images so you don’t need to make things too obnoxiously bright at the outset since it will get a little brighter upon collapsing.

6. Acquiring a tiled image. By the camera view, the sequence of tiled images is acquired starting at the upper left to lower right.

a. Setting up your xy image collection: (this is done sequentially)

   i. First, you need to set the zero for your z. Keep in mind that since it’s not parfocal you will need to do this separately for both the H&E image, and the SHG image. Find a nice location with a decent amount of collagen and get it in focus. Then click set zero under the z tab. (don’t touch the focus knob from here on out) this is critical, when you click this zero button you are ONLY zeroing the z and not x and y.

Here we are proposing two method but we suggest to use second method unless for some reason you have to use the first method.

   ii. First method: For the first method you need to either know how much is the area on your sample that you want to image, or you know the number of images in your XY-grid:
a) To know how much you need to move on your sample navigate to the far top-right corner of your imaging area and click “set 0” under “XY stage” tab. Then navigate to the bottom-left corner of your imaging area and click “manual update” under “XY stage” tab. Right down these values, then click “Generate” under “XY stage” tab. set the “Compute Overlap” slider to 10%. Enter the coordinates of bottom-left corner of the imaging area that you just wrote in “Total size” box. Then click of “Calc # Pos from Total Size” and then click “Ok” to generate the grid. (Figure 6)

![Image of XY stage settings](image)

Figure 6

b) If you know the number of images in your grid’, click “Generate” under “XY stage” tab. Enter the number of images in X and Y direction in “#X” and “Y” boxes, respectively. set the “Compute Overlap” slider to 10%. Click “Calc Total Size from # pos” and then click “Ok” to generate the grid.
iii. Second method: Since usually we don’t know the number of grids or the total size that we are going to image we use this method. Go to the upper right corner of the region you want to image. Click on “Set current pos as START corner”. Set the overlap using slider. Navigate to the lower left corner of the region you want to image and click on “Set current pos as END corner”. Now by clicking Ok it will generate your grid.

iii. Click on “Generate” under the xy stage, set the slider to a 10% overlap. Now that you are at your start position, you can click that button.

iv. Now you navigate to your far right and bottom parts of the tissue that you will be imaging. This time around when you click update these are the “real” dimensions of the array you are about to image since you have already set the upper left (xy) at 0,0. Again, you write down the numbers as you find them, then enter them into go to target and then you will have navigated to the bottom right ending corner.

v. Go back to the Generate window (you should not have closed it) and then click on the button to set this location as your end point. Upon doing this then the number of images to be acquired and the total dimension should be calculated for you. Write these numbers down. Click OK and it will generate the xy info you need. (Figure 7)
7. Turn the live scan off, i.e. the live capture of the dig cam. Now set up your channels to collect or not. (Figure 8)

![Figure 8](image1.png)

8. If you are taking a SHG tiled image, under the z-motor tab, set your z top and bottom respectively and your current focal plane should be in focus. For this you can use 4, -4 and 4 for “Z Top”, “Z Bottom” and “Z Step” respectively. Or you can go to any z position using ASI controller knob and set that as your top, and bottom and define you z step. Make sure you click “calculate” (next to number of images) so that these numbers you have entered are now input. (Figure 9)

i. If you are doing a digital camera acquisition, you should set these numbers at 0, 0, and 0. You still need to click calculate so that it locks in a value of 1 for number of z planes.

![Figure 9](image2.png)
9. For SHG image first set the PMT gain slider (in “DCC-100 Controller” box) to a number around 0.4. Then set the Pockel’s cell slider to a value from 50 to 100. Now you can change these two slider values to see your desired image which has few or no red pixels. Don’t use values higher than 120 for Pockel cell, this will burn your sample. (Figure 10)

10. For SHG image, to get large field of view and a flat image at the same time set the zoom slider to 3 in the main panel of WiscScan. (Figure 11)

11. For the resolution you have two options: 512X512 and 1024x1024. You can use either one, but 512x512 usually is enough and faster. (Figure 11)

12. Click “Use XY” and click start sequence (not the “regular start button”) and give it a file name. When it’s done it gets saved automatically. If you are taking a digital camera tiled image make sure you have “Qcam” on, and if SHG off. (Figure 12)

13. After the acquisition is finished, WiscScan will take some time to populate the metadata.
14. After taking a xyz series, Crisp goes back to the idle state, so if you want to do another xyz series you will have to press @ button to get back to RL.

**Important note:** please turn off everything you turned on at the end of your imaging. But DO NOT turn the laser off.

**Stitching:**

If you are working with an SHG image, you need to stitch images **FIRST** and then you can collapse the 3 z planes into one.

a. In plugins → Stitching → Grid/Collection stitching you select “positions from file” as type and the order is “defined by the metadata”.

b. Browse to and select the first file in your xy image series. Use linear blend as your form of stitching. Set “increase overlap” to zero (default). Uncheck the subpixel accuracy box. For SHG images (only) don’t even bother to save this stitched image yet, we need to collapse those three z planes.

   i. Have the “compute overlap” box Unchecked. This helps if your dataset contains several images that are blank, which can confuse the stitching and you can get some weird results and is faster.

c. To collapse the 3 z planes of the SHG data into one, perform a maximum intensity projection (Image → Stacks → z project).