

Basic Instructions for Zeiss LSM900 Confocal Microscope

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NOTE: these instructions only describe the most common features. For questions about other functions, please contact the MSL staff at msl@med.unc.edu or visit MSL's YouTube site with [detailed instructional videos](#).

1. Getting started with a sample

1.1) Follow the instructions on the startup sheet.

Problems during startup

These are rare, but if you run into problems, contact the MSL staff. If staff is not available, most problems can be solved by:

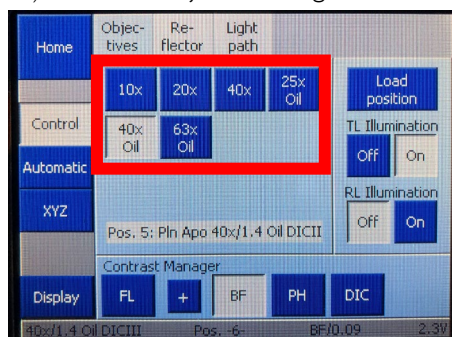
1. Exiting Zen.
2. Turning off the components and system switches (#3 and #2)
3. Waiting 30s.
4. Following the startup checklist from the appropriate step.

Look for more help in these instructions:

[Dealing with problems](#)

[Deeper explanations](#)

1.2) Select an objective using the touchscreen. If using an oil objective, place a small amount of oil on the lens.



How to select an objective

For cellular work, the 20X/0.8 is usually the best choice. The 10X/0.45 can help navigate more quickly in large pieces of tissue, or to search for rare events. For navigation in even larger samples, you can try using the 5X/0.16, though keep in mind that the brightness will be quite low and rejection of out of focus light will be limited.

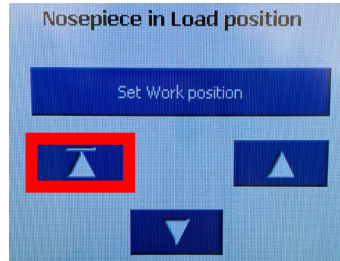
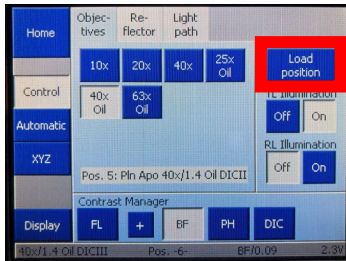
For subcellular work, the PlanApo 63X/1.4 oil and 40X/1.4 oil are both good options. They have the same resolution, but the 40X/1.4 oil gathers more light (images are brighter) and has a shorter working distance (130 vs 190 μm). If you are looking at cells plated on a coverslip, the 40X/1.4 oil is usually the best choice. If you are looking at cells plated on a slide, with a coverslip placed on top after staining, the 63X/1.4 oil is a safer choice, as the working distance will allow you to go through the mounting media and reach the cells you want to image.

There is another, specialized objective for imaging thick samples like organoids (25X/0.8 multi).

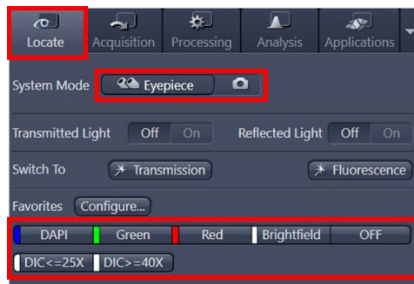
1.3) Verify that sample holder is flat. It should be flush with the stage, not wobble and not go down further if you push on it.

1.4) Put on sample:

- If using an air objective, put on the sample, coverslip side towards the objective.
- If using an oil objective, use the “Load Position” button on the touchscreen to lower the objective before you place sample in the holder. Then raise the objective with the triangle and line button. If the oil does not touch the objective, raise it with the focus knob until it does.



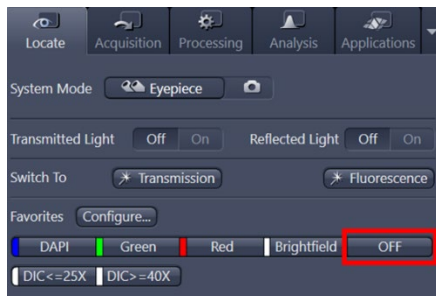
1.5) Using the locate tab in the software, select the fluorescence, brightfield or DIC channel you wish to visualize. Make sure the system mode is Eyepiece.



1.6) Look through the eyepieces to find objects of interest. Focus on them using the focus knob and center them in the field of view using the joystick. The joystick can be toggled between slow and fast mode with a button on top of the joystick itself.

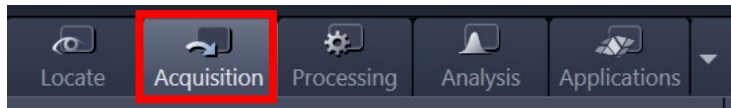
1.7) If applicable, look at other channels by changing settings in the locate tab.

1.8) Once you are done, close the shutter for the illuminating light by using the OFF button in the software, the RL button on the right side of the microscope stand (if doing fluorescence) or the TL button on the right side of the microscope (if doing brightfield or DIC).



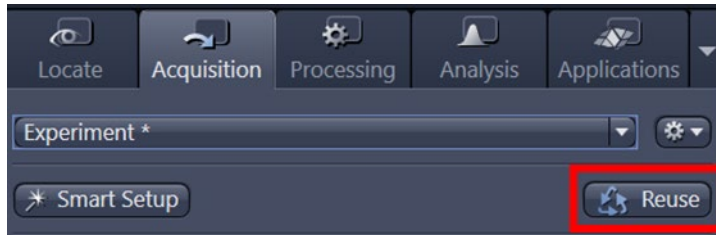
2. Loading Acquisition Parameters

2.1) Switch to acquisition mode

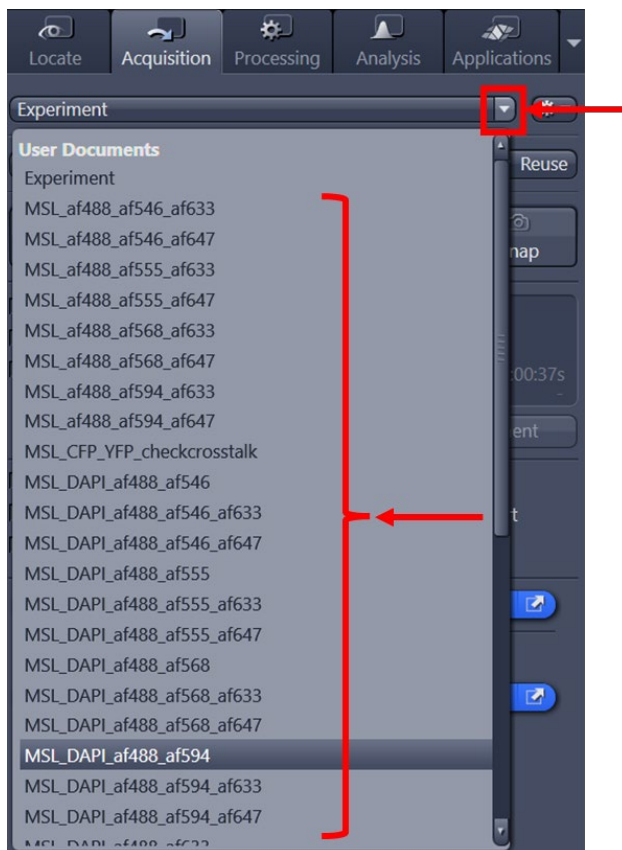


2.2) Load acquisition parameters:

- a. If you already acquired an image with the settings you want, open that image and reuse the settings, by clicking on the recycling symbol. All the settings **except** the objective will be loaded.



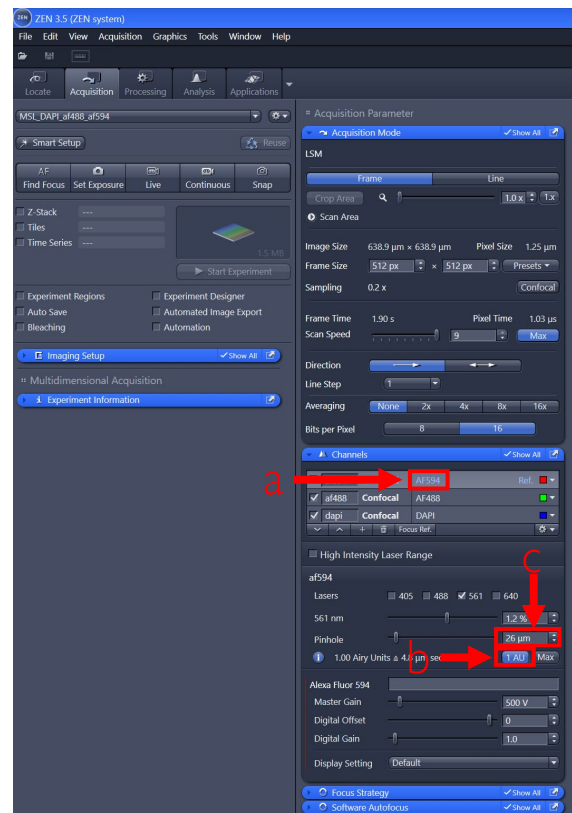
- b. If you are using a new combination of fluorophores, load a setting from the list of all acquisition parameters. Use the following guidelines:
 - i. Only use settings with names that start with MSL.
 - ii. Find a setting with the fluorophores you need. Example: MSL_DAPI_af488_af594.
 - iii. Ask the MSL staff for help if you do not find a setting with the combination of fluorophores you want.



3. Adjust Pinhole Size

3.1) In the Channels panel, go to the channel for longest wavelength fluorophore by clicking on its name [a], and set the pinhole size to 1 Airy Unit, by clicking the 1 AU button [b].

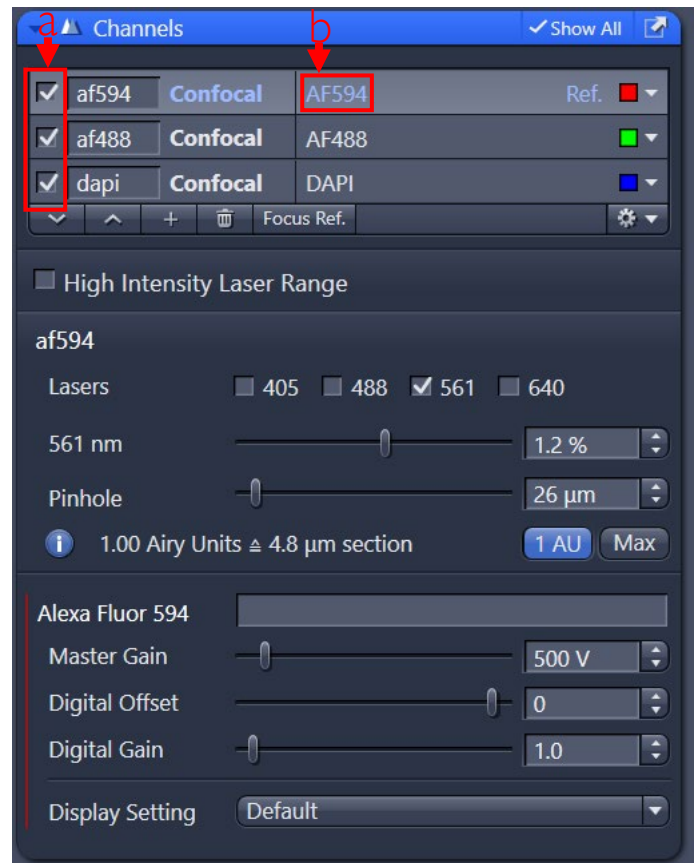
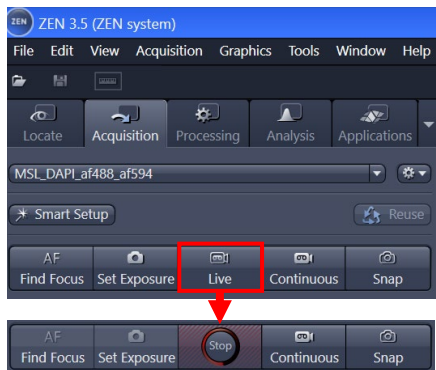
3.2) Verify that the pinhole size is the same in other channels. If not, type in the correct number [c], copying whatever the number is for the longest wavelength fluorophore. Note that the Airy units can be different between channels. That is fine, what you need is for all channels to have the same pinhole size [c].



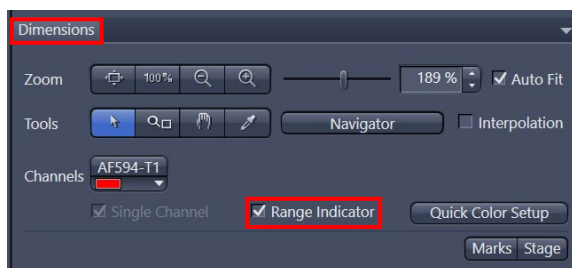
4. Begin live imaging with the confocal

4.1) Adjust initial settings for channels one at a time, using Live mode. Make sure all channels have checkmarks [a] and select the channel you want to adjust first by clicking on it in the channel list so that it is highlighted in gray [b]. Only the highlighted channel will be scanned by the confocal in Live mode. The channel highlighted in gray is also the one whose parameters you will be able to adjust in the bottom of the Channels panel.

4.2) Click on Live. The microscope will begin illuminating the sample with laser light. The Live button will turn into a Stop button that you can press to end live imaging.



4.3) Engage Range Indicator, in the dimensions tab under the image. This will indicate pixels that have a zero intensity as blue, pixels that are saturated as red, and everything in between on a grayscale. It is much easier to see the sample and adjust the settings using this display mode.



4.4) The display tab will show a histogram of pixel intensities on a log scale (in Y).



4.5) You can adjust how your image looks, without changing the underlying data, by making changes in the Display tab. Particularly useful buttons are:

- Min/Max and Best Fit [a]: clicking one of these will usually make things visible in the image, even if intensities are low and contrast is poor.
- Top right handle on the line [b]: if you move this to the left it will make the image look brighter.
- Reset [c] goes back to the initial display conditions.



What if you cannot see anything?

- Make sure that the microscope arm above the sample is not tilted back. If it is, bring it down.
- Stop the live mode, go back to the Locate tab and look at the sample by eye [1.6]. You should have something bright and in focus in the middle of the field of view.
- Verify that you are using the range indicator display [4.3]. Note that whenever you stop live imaging and start it back you will need to turn on the range indicator again.
- Click on Best Fit [4.5]
- If you are sure that something is in focus by eye but cannot see anything in the software, move the fine focus knob very gently while imaging live until something comes into focus. If you still cannot see anything, try focusing by eye again, before you try the next step.
- While imaging in Live mode, Increase the "Gain (Master)" setting using the slider, until something appears.

5. Initial contrast adjustments: Digital Offset, Master Gain and Laser Power

What are you trying to do?

Your goal is an image with good contrast, appropriate intensity levels and no saturated pixels. You will balance the Master Gain and Laser Power settings while keeping track of image quality and pixel intensities using the Display tab.

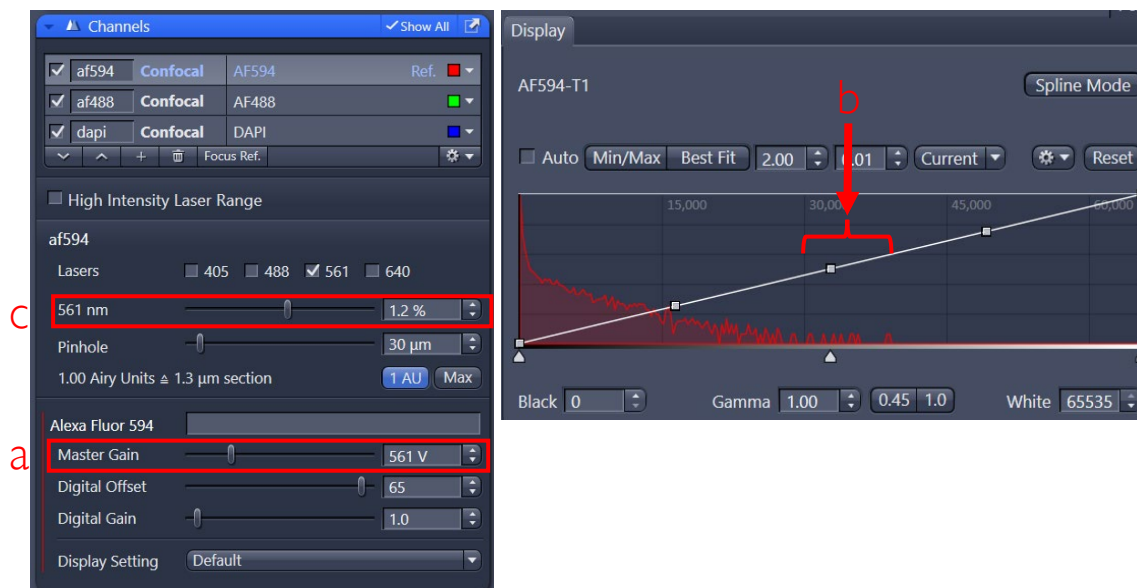
The following adjustments should be performed one channel at a time [4.1], in Live mode.

5.1) Find an area of your sample that is bright, but within the range of fluorophore levels you are interested in. Do not optimize your settings in a dim part of the sample as your pixel intensities will likely be saturated in brighter regions.

5.2) While imaging in Live mode [4.2], find the brightest Z plane, by gently moving the focus knob and monitoring the resulting image. Make sure you are in Range Indicator display mode [4.3] and adjust the display settings such that you can comfortably evaluate image quality [4.5].

5.3) If you have a lot of blue pixels in the image (this is not typical), in the Channels panel, increase the Digital Offset by holding the up arrow until all the blue pixels are gone. Do not use the slider to adjust this setting. Typical values at which all blue pixels turn black are usually less than a few hundred units.

5.4) Adjust the Master Gain [a] until the brightest pixel intensities in the histogram in the Display tab (the right edge of the histogram) are near the middle, around 30,000-40,000 [b].



5.5) Adjust the Master Gain [a] and Laser Power [c] to get the desired image quality while keeping pixel intensities below 40,000. If you want higher image quality, lower Master Gain and increase Laser Power. If you want lower bleaching, lower Laser Power and increase Master Gain. Bleaching will show up as a progressive shift of the histogram's right tail to the left. You will check bleaching more carefully later (see below [7.6]).

5.8) Once you are satisfied with the settings for one channel, repeat these steps for the other channels.

Tips when adjusting laser power

A good way to adjust the laser power is by factors of 2. Lower your gain until your brightest pixels are around 20,000. Then, double your laser power. Now, check the image quality and bleaching to decide whether the change was worth it. If so, try the same trick again. If you double the laser power and the brightest pixel intensities do not double, that means your laser power is too high, even if you do not see bleaching.

Shorter wavelength lasers are much more damaging than longer wavelength lasers and may bleach other fluorophores, aside from the one being imaged with that laser. Be particularly careful when increasing the intensity of the 405nm laser.

Note that on the LSM900, the system operates by default in a mode where laser powers are capped at 3.5% (405nm), 4.5% (488nm), 5% (561 and 640nm). If you need higher power (this is not typical), turn on the High Intensity Laser Range option in the Channels panel.

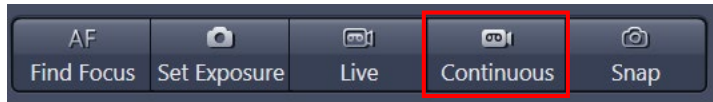
If you previously used the LSM700 at MSL, powers on the LSM900 are quite different. The power settings on the LSM900 that are equivalent to 2% on the LSM700 (this was our default) are:

- 405nm: 0.7%
- 488nm: 0.7%
- 561nm: 1.2%
- 640nm: 1.2%

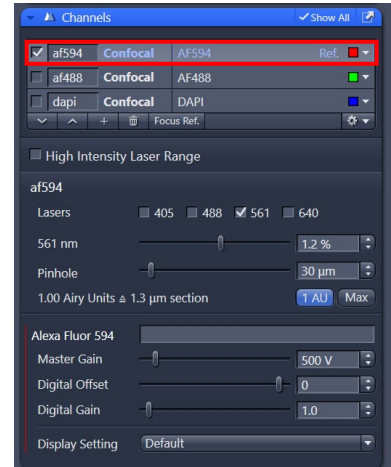
These values are our new defaults on the LSM900 for MSL-created settings.

6. Further contrast adjustments: Averaging, Scan Speed, Direction

The following adjustments should be performed one channel at a time, in Continuous mode.

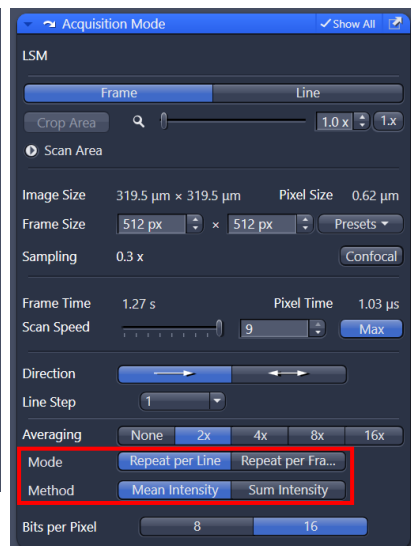
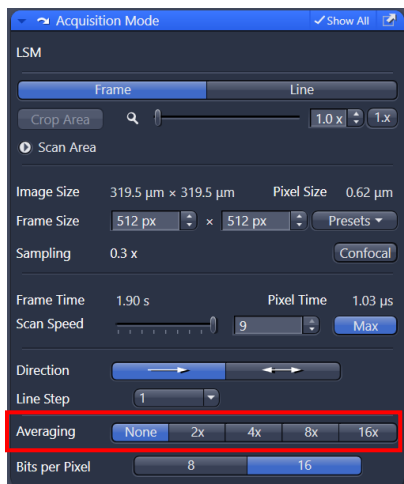


We use Continuous because Live is a fast mode that will not properly reflect many of the adjustments possible in the Acquisition Mode panel. In Continuous mode, the microscope will scan all channels that are checked in the Channels panel. You will need to have only one channel checked at a time. You will need to highlight that channel to access the controls for it below.



6.1) In the Acquisition Mode panel, adjust Averaging until you obtain an image of acceptable quality, time to acquire and bleaching. Make sure Repeat per Line and Mean Intensity are selected. Keep in mind the following:

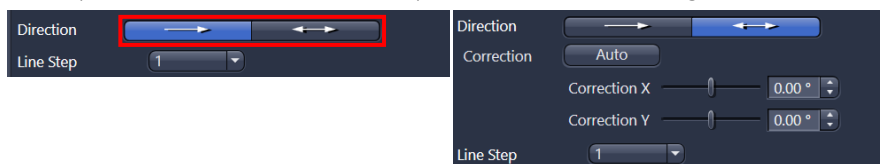
- Increasing averaging number improves image quality but there are diminishing returns: the more you average, the less noticeable the improvements.
- Very noisy features in images improve more noticeably than features that already have high contrast.
- Averaging is applied to all channels. Consider reducing laser power and increasing gain if another channel looked good without averaging.



6.2) Scan Speed should almost always be set at Max, for reasons explained in the [Averaging vs Speed](#) box below.



6.3) Compare Direction set to --> or <--> in Continuous Mode. If the images look the same, use the <--> setting. This will reduce the Frame Time. Leave Corr X and Corr Y settings on 0.00 and Line Step on 1. If the images look different (for example, edges of certain structures seem fuzzy with <-->), set Direction to the --> setting OR, reduce speed and see if that fixes the problem while remaining faster than -->.



Averaging vs Laser Power

Averaging will improve image quality, while also increasing bleaching and the time to acquire an image. Increasing the laser power and lowering the gain will also improve image quality and increase bleaching, but without making the imaging slower. Why then, should one average at all, if there is an alternative that does not slow down the imaging?

One answer is that for the same improvement in quality, with the same total amount of light delivered to the sample, providing those photons all at once by increasing the laser power is more damaging than spreading them over multiple sweeps with averaging. So, for the same increase in quality, averaging does not increase the bleaching *as much as* increasing the laser power.

A second reason for using averaging is that, in some cases, it may not make sense to increase the laser power further. At high laser powers all the fluorophores in the focal plane in the sample can be excited and increasing laser power beyond that does not lead to higher intensities or better image quality. Under those conditions (which should be avoided), averaging is a way of increasing image quality.

Averaging vs Scan Speed

For the same investment of time, increasing averaging and speed will lead to similar improvements in image quality. However, lowering imaging speed is more damaging to the sample than increasing averaging. So, for a given increase in quality, lowering speed pays a higher cost in bleaching. As a result, speed should usually be adjusted so it is at the maximum possible. That said, there are a few scenarios where lowering the speed may be convenient:

- If there is a desire for a slight increase in quality with a small increase in imaging time. Averaging can only be increased by factors of two, whereas speed changes can be more subtle. Thus, changing speed can be useful for cases where a slight quality increase would be beneficial.
- If bidirectional scanning is used but imperfections show up. In these cases, lowering the speed can remove those imperfections while still being faster than unidirectional scanning.
- In conditions with extremely weak signals where laser power and averaging have been maxed out and speed is the only option left. This is rare, but under these circumstances lowering the speed can be useful.

Direction

This parameter controls whether the laser scans the sample in a typewriter pattern—turning off as it swings back—or in a snake pattern—constantly on. Having the laser constantly on means imaging is around twice as fast, which means you should always try to use the bidirectional scan pattern. However, under certain conditions, that are hard to predict in advance, the bidirectional (<-->) or “snake” pattern can lead to problems in the image. If that is the case, it is better to lower the speed slightly (see above) or use the default (-->) unidirectional or “typewriter” scanning pattern.

7. Adjusting Frame Size and Zoom to optimize resolution

What are you trying to do?

Your goal is to obtain an image with high enough resolution for your analysis, which covers a large enough area in a reasonable amount of time. To do so, you will balance the Frame Size and Zoom settings while keeping track of Image Size, Pixel Size, and Frame Time. You may not need to use the highest possible resolution settings in every case; for example, counting nuclei in a tissue section does not need the same resolution as studying mitochondrial morphology.

Cellular vs Subcellular imaging

If you only need to distinguish cells from each other, you will typically use the PlanApo 20X/0.8 air objective. If you need subcellular information, you will typically use a high NA oil objective: PlanApo 63X/1.4 or 40X/1.4.

Zoom, Image Size, Frame Size and Pixel Size

- Zoom is *under user control* and reflects what area of the sample is scanned by the laser. Bigger numbers mean the laser scans a smaller area.
- Image Size is the physical size (in X μm by Y μm) of the sample region that is being imaged by the microscope. Adjusting the Zoom changes the Image Size.
- Frame Size is *under user control* and is the number of pixels in the image in the X and Y dimension.
- Pixel Size is the physical dimensions in X and Y in the sample represented by one pixel. It is calculated by dividing the Image Size by the Frame Size. Smaller pixels result in higher resolution images, to an extent (see below).

To increase resolution you can increase Zoom or Frame Size. The former will make the field of view smaller, the latter will take longer. Both will increase the bleaching. If you need the highest possible resolution for a given objective, you can click on the Confocal button in Acquisition Mode. This will adjust Frame Size to get the smallest recommended pixel size for your current imaging conditions.

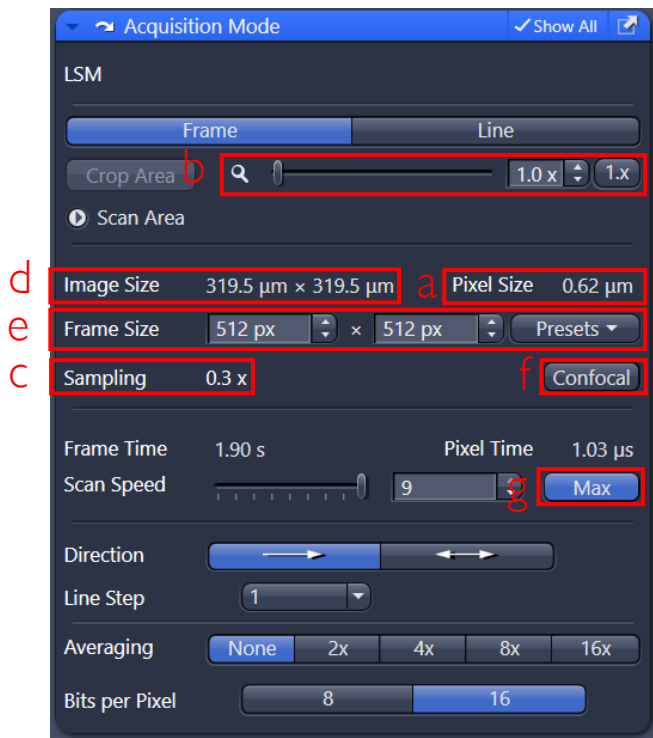
How small should the Pixel Size be?

Beyond a certain point, decreasing the Pixel Size will not increase the resolution of your images, as you will be beyond the physical resolution limits of the microscope. If Sampling is at 1.0X or lower you will not have this problem. Setting the Pixel Size unnecessarily small can make imaging slower, reduce your field of view, and/or bleach your sample more. The following table shows recommended pixel sizes for the highest possible resolution, assuming you are using a PlanApo 63X/1.4 or 40X/1.4 objective, for different fluorophores. These values are similar to what you will get if you press the Confocal button when using those objectives. You may not need this level of detail, but if you do these tables give you a sense of the limits. If you use smaller pixel sizes you will see no benefit.

Recommended Pixel Sizes (in μm) for different fluorophores

(if more than one fluorophore, use the one with the shortest emission wavelength to set the pixel size)

	DAPI	GFP/AF488	AF546/AF555/Cy3/Rhod	AF568/AF594/mCherry/TxRed	AF633	Cy5/AF647
Highest resolution	0.066	0.073	0.081	0.087	0.093	0.096



7.1) Decide what Pixel Size [a] you need, based on the level of detail required for your analysis. You can explore the level of detail at different pixel sizes by adjusting Zoom [b]. You should not use pixel sizes that result in Sampling > 1.0X [c] (which correspond to pixel sizes smaller than those in the [Recommended Pixel Sizes](#) table above).

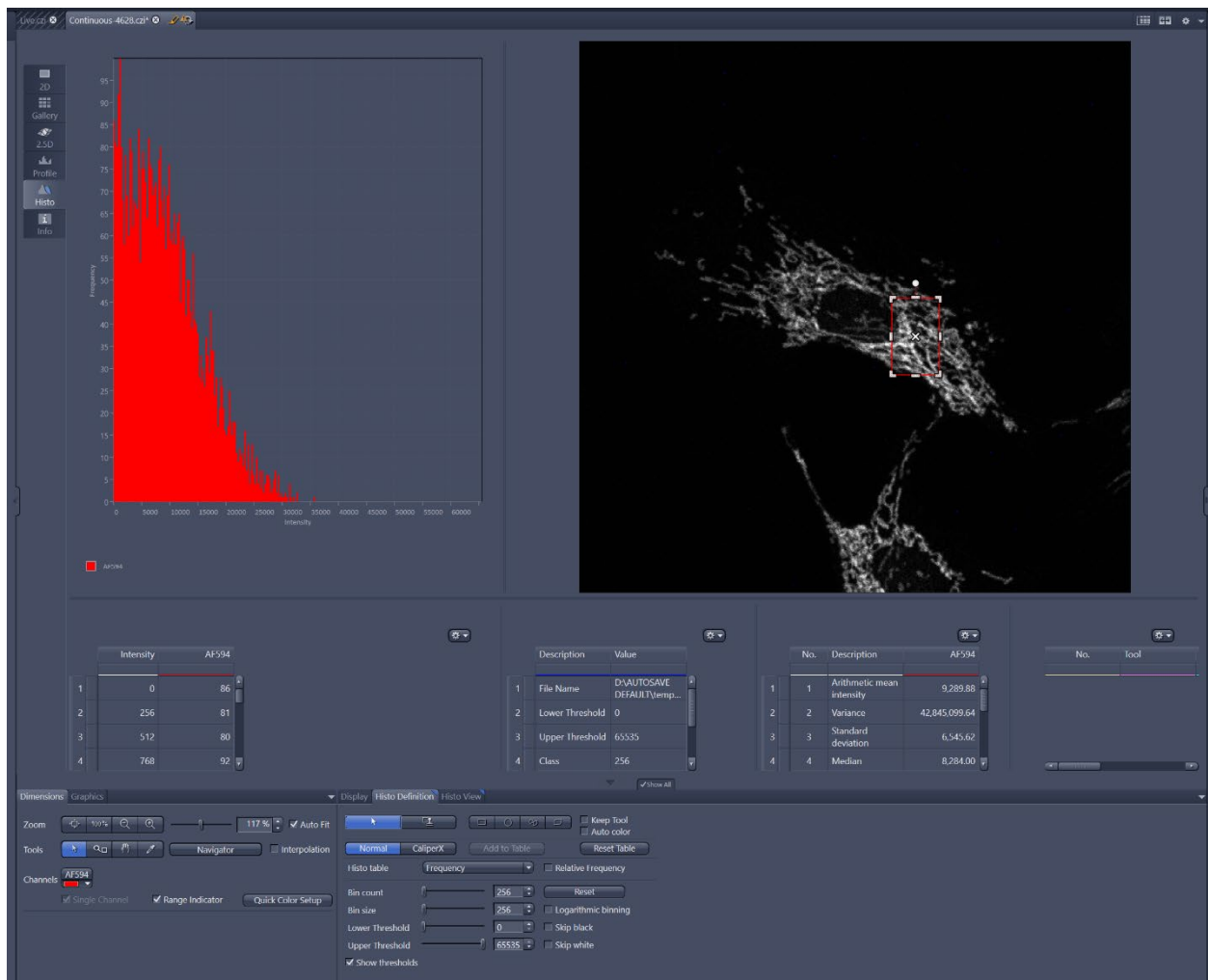
7.2) Decide what Image Size [d] you need, based on the size of structures you need to include in a single image (one cell, one group of cells, one part of cell, etc.). You can explore different image sizes by adjusting Zoom [b].

7.3) Adjust Zoom [b] to get desired Image Size [c].

7.4) Adjust Frame Size [e] to get desired Pixel Size [a]. You can:

- Select from one of the Frame Size Presets Frame Size options (512 x 512, 1024 x 1024, etc.)
- Type numbers in the X and Y boxes to do this more precisely.
- Press the Confocal button [f] for the highest resolution, lowest recommended pixel size. Clicking on the Confocal button will result in Sampling = 1.0X.

7.5) Confirm Speed is on Max [g], to minimize bleaching.



7.6) Verify that bleaching and image quality are acceptable, making sure you are using the Continuous mode. Continuous imaging uses the proper frame size, speed, and averaging. Live imaging mode will use approximate speed and frame size settings, and thus not give you an accurate sense of how much bleaching will occur during imaging. Image quality can be assessed visually and adjusted by making changes to Laser Power, Master Gain, Averaging, Scan Speed and Direction (see above). To check bleaching:

- Switch to the Histo display mode. In this mode the top left will be a histogram of pixel intensities, the top right will be the image, and the bottom will be summary statistics.
- In the Histo Definition tab at the bottom, select the square tool and draw a square on the brightest object in the field of view. The histogram will now be limited to the pixel intensities inside the square in the image. The first item on the third column in the bottom panel of the Histo Display will show the Arithmetic Mean Intensity of the pixels inside the square in the image.
- Confirm that the brightest pixels are below 40,000. If not, adjust Laser Power and Master Gain, as needed.
- Track the Arithmetic Mean Intensity as you scan the sample in Continuous mode. If you have bleaching, the value of this parameter will trend down. Recommendations on what to do if there is significant bleaching below.

What if it takes too long to take an image?

- Try bidirectional imaging.
- If your analysis does not need as much detail, try reducing the frame size.
- If you are imaging a large area unnecessarily because you are including a lot of empty space, try zooming in.
- If your analysis will still work with an image with lower contrast, try reducing the amount of averaging.

What if there is a lot of bleaching?

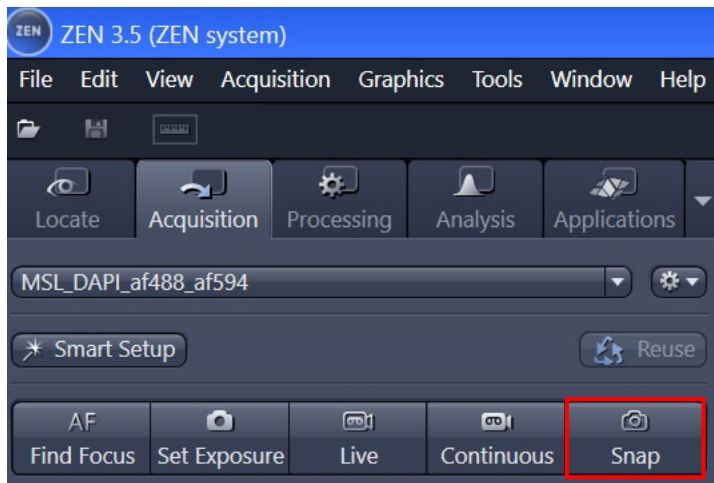
Determine what you are willing to trade off to reduce bleaching:

- If you do not mind spending more time, use averaging instead of laser power and gain.
- If you do not mind an image with lower contrast, reduce averaging and/or laser power.
- If you do not mind an image with lower resolution, reduce frame size.

Imaging is all about tradeoffs.

You will always have to balance parameters. Figure out what is necessary for your analysis and do not spend time or bleaching on things that are not.

8. Acquiring and Saving an Image



8.1) Once you are satisfied with your settings, acquire an image with the Snap button.

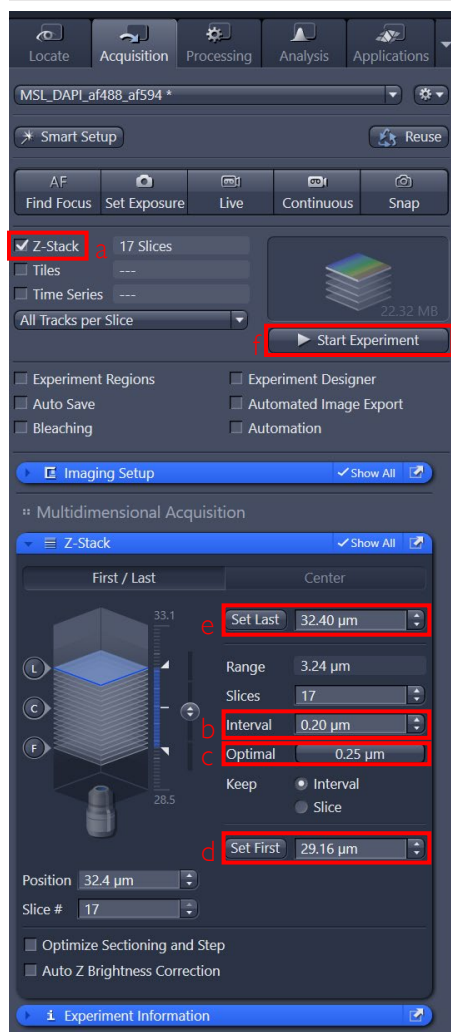
8.2) Images can be saved with File/Save. Make sure you save in .czi format so you retain image metadata. This will allow you to properly report your acquisition settings and reload them for future imaging.

9. Z stacks

Recommended spacing of slices in a Z stack

The spacing between slices in a Z stack is a critical parameter that will affect your axial resolution, the quality of 3D reconstructions, and your ability to accurately capture all elements of interest in a sample. Guidelines:

- If you are imaging with the highest possible resolution (see table in section 7), use an interval of 0.2 μ m. If you don't need the highest possible resolution, consider using the Optimal recommendation.
- Pick an interval that adequately samples your structures of interest. To determine this, you can move the Z position up and down with the focus drive, estimate the size in Z of the objects you are interested in and sample at half or a third of that size. For example, if you don't want to miss any nuclei, and the smallest ones in your sample are 3 μ m in diameter use an interval of 1-1.5 μ m
- Do not use interval values below 0.2 μ m; this will only bleach your sample, slow down your imaging and not provide any significant additional details in the Z dimension.



10.1) Click on the Z-stack checkbox in the top left corner of the Zen interface [a], to activate the Z-stack window

10.2) Set the interval to the desired value [b]. If using highest resolution settings input 0.20 μ m. Optimal [c] also provides high resolution, though lower than 0.20 μ m. For other options see [Recommended spacing of slices in a Z stack](#).

10.3) If possible, to set the limits of the Z stack, pick a channel with easily visible features that extend over the entire Z range of interest.

10.4) In Live acquisition mode [4.2] move down in Z, by moving the focus knob towards you, until you are just below your region of interest and click on Set First [d]. This will be where the Z-stack begins.

10.4) Still in Live acquisition mode move up in Z, by moving the focus knob away from you, until you are just above your region of interest, and click Set last [e]. This will be where the Z-stack ends.

10.5) Stop Live Acquisition.

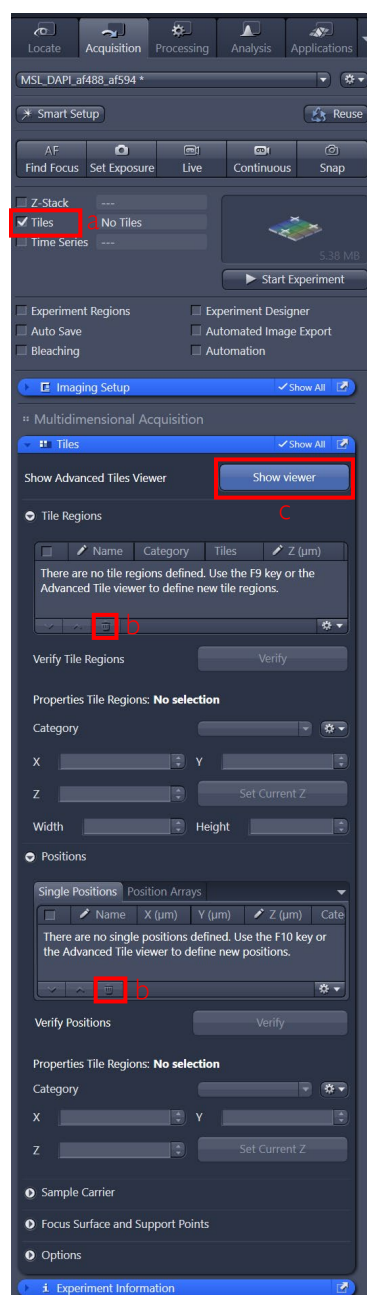
10.6) Press the Start Experiment button [f] to acquire the Z-stack.

10. Tiles mode

Tiles mode is a flexible, comprehensive module in the software that allows you to:

- a) acquire multiple adjacent images (tiles) in a rectangular grid that can subsequently be stitched.
- b) draw irregularly-shaped, large regions in a sample and automatically cover those areas with a non-rectangular, efficient grid for tiling.
- c) create preview images that can be used for navigation or to set further imaging areas.
- d) create multiple independent points that can be imaged separately, each with their own verified focal position.
- e) create random sampling points in a defined area.
- f) take tiles of tilted samples or samples that have an irregular height in Z.
- g) do all of the above in the same experiment.

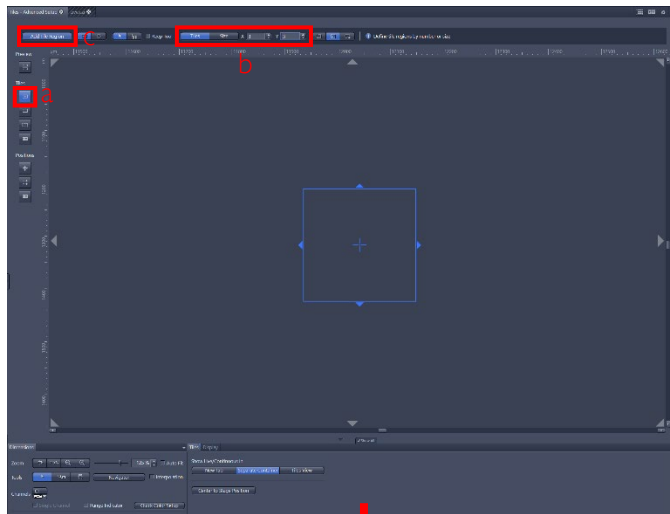
The following instructions only explain how to do a). Consult MSL staff or training videos for other workflows.



10.1) Click on the Tiles checkbox in the top left corner of the Zen interface [a], to activate the Tiles panel

10.2) Remove any preexisting Tile Regions, Single Positions or Position Arrays by using the corresponding trash can icons [b]

10.3) Click on Show Viewer [c]. This will open the Tiles – Advanced Setup panel to the right.

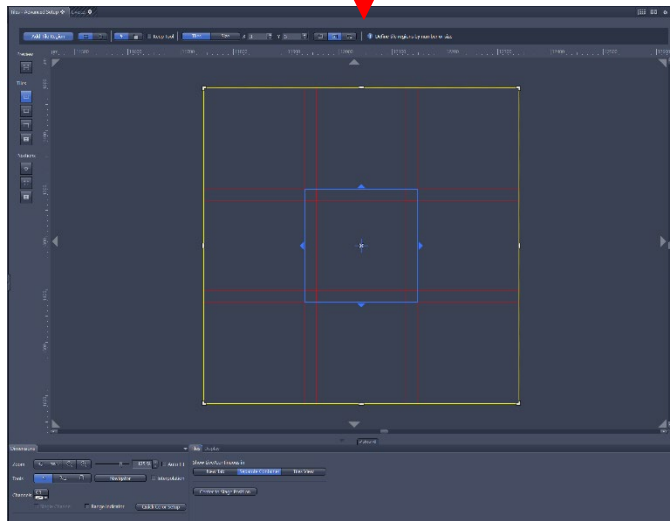


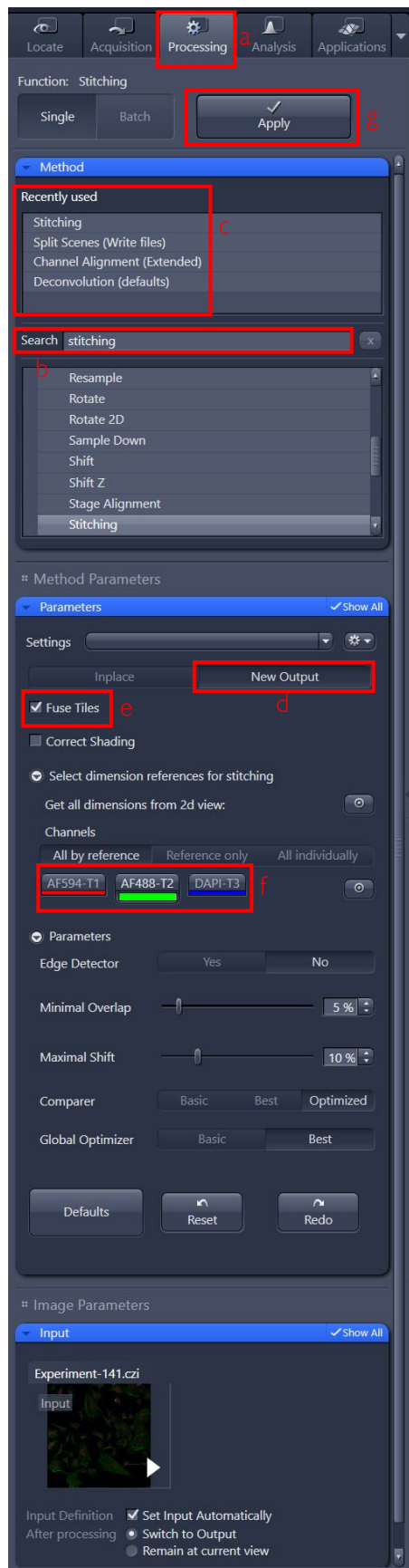
10.4) Click on the first option in the Tiles section [a].

10.5) Click on the Tiles mode on the top ribbon and set the dimensions of the grid in number of positions in X and Y [b].

10.6) Press Add Tile Region [c]. A diagram of the desired tiling will appear.

10.7) Press Start Experiment to acquire the Tile Region. Note that the resulting image will not be stitched, and there may be imperfections along the “seams” between positions.





10.8) To stitch the image you acquired:

- Switch to the Processing tab [a]
- Find the Stitching method either by searching for “stitching” [b] or clicking on it if it was recently used [c]
- Click on New Output [d] to avoid overwriting your original image
- Click on Fuse Tiles [e] to merge your images together.
- Select a channel to use as a reference for stitching [f]. Pick a channel that has good contrast and widespread distribution across the field. Channels that are noisy and/or only show sparse objects will lead to poor results.
- Click on Apply [g]. This will create a stitched image.