Instructions for Zeiss LSM700 and LSM710 microscopes

Microscopy Services Laboratory

University of North Carolina at Chapel Hill

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NOTE: these instructions only describe the most commonly used features in Zen software. For questions about other functions, please contact the MSL staff at msl@med.unc.edu

1. Getting started with a sample

1.1) Follow the instructions on the startup sheet.

1.2) Once Zen is open, press start system.

Look for more help in these instructions:

Dealing with problems

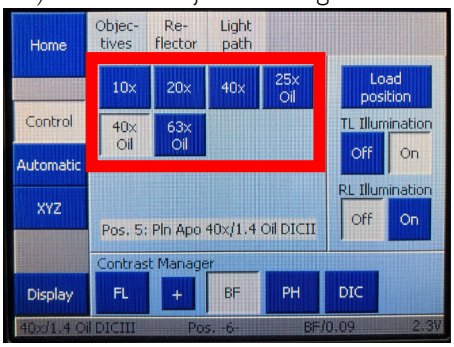
Deeper explanations

Problems during startup

These are rare, but typically involve a white box with error messages that shows up in the lower left-hand corner of the screen. If that happens, take a screenshot and contact the MSL staff. If the progress bar is stuck without any error messages, contact the MSL staff. If staff is not available, most problems can be solved by:

1. Exiting Zen.
2. * On the LSM710: Turning off the components switch, turning off the systems/PC switch.
   * On the LSM700: Turning off both power strips.
3. Waiting 30s.
4. Following the startup checklist from the appropriate step.

1.3) 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. Once something appropriate is in the field of view, you can switch to the 20X/0.8.

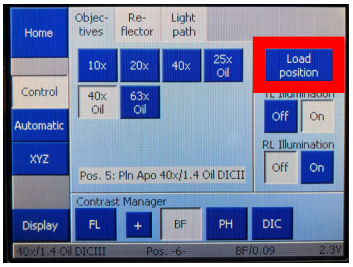
For subcellular work, the PlanApo 63X/1.4 oil objective is the best choice on the LSM700. On the LSM710 the PlanApo 63X/1.4 oil and 40X/1.4 oil are both good options. They have the same resolution, but the 40X/1.4 oil gathers more light (images are brighter) and a shorter working distance (130 vs 190 um). 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.

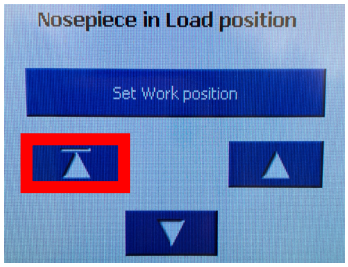
There are other, specialized objectives for imaging using multi-well plastic plates (40X/0.6 on the LSM700) or thick samples (25X/0.8 multi LSM710). Consult the MSL staff if you need more information about these options.

1.4) 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.

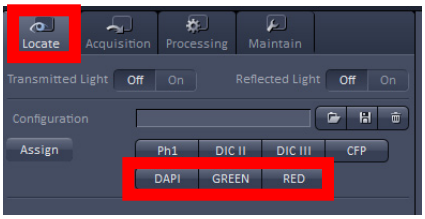
1.5) Put on sample:

a. If using an air objective, put on the sample, coverslip side towards the objective.
b. 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.





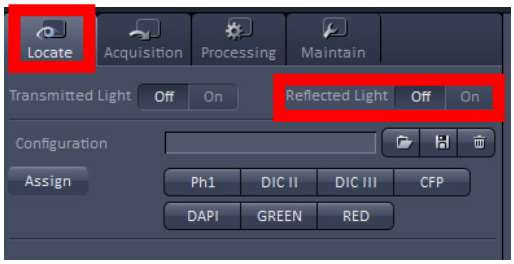
1.6) Using the locate tab in the software, select the fluorescence channel you wish to visualize.



1.7) 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 the side (LSM710) or on top of the joystick itself (LSM700).

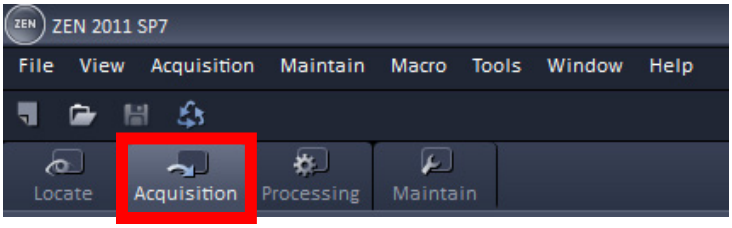
1.8) If applicable, look at other fluorescence channels by changing settings in the locate tab.

1.9) Once you are done, close the shutter for the illuminating light by using the Reflected Light button in the software or the RL button on the right side of the microscope stand.



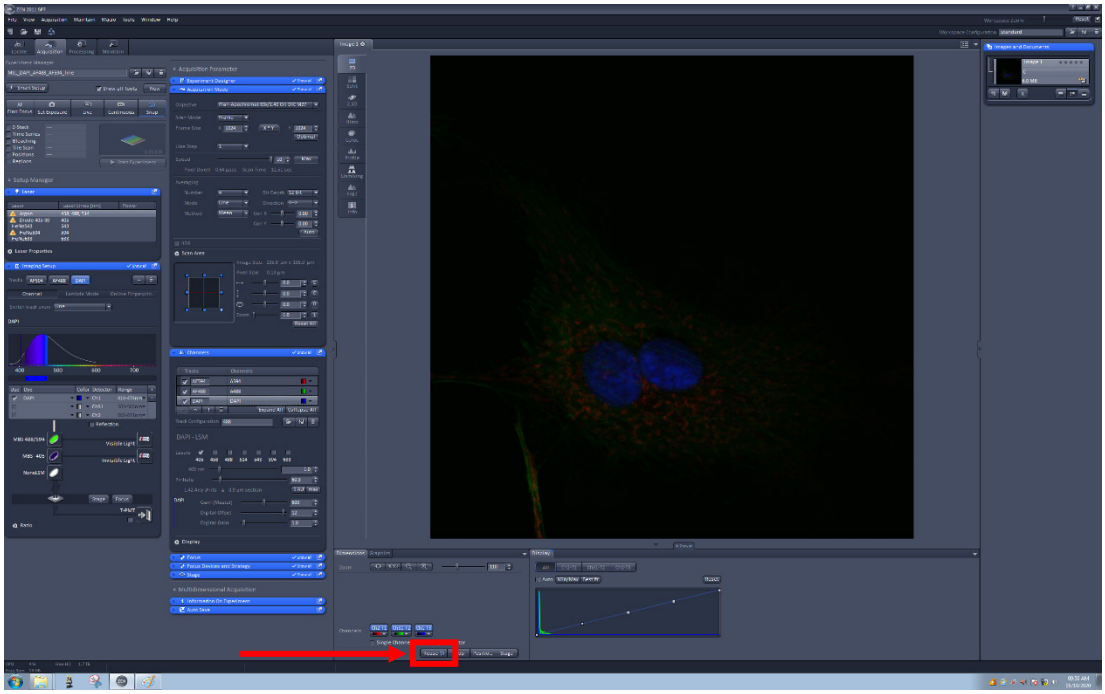
2. Loading Acquisition Parameters

2.1) Switch to acquisition mode

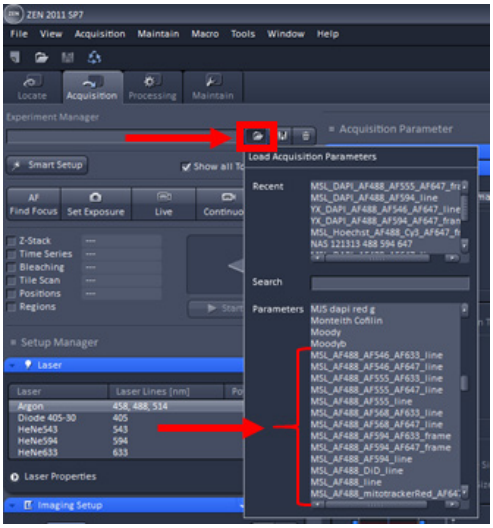


2.2) Load acquisition parameters:

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.

iii. *On the LSM700:* When using 3 or 4-channel settings, load the one that includes "multi" in the name.

iv. *On the LSM710*: When using a 4-channel setting, use the one that includes "frame" in the name.

v. Ask the MSL staff for help if you do not find a setting with the combination of fluorophores you want.

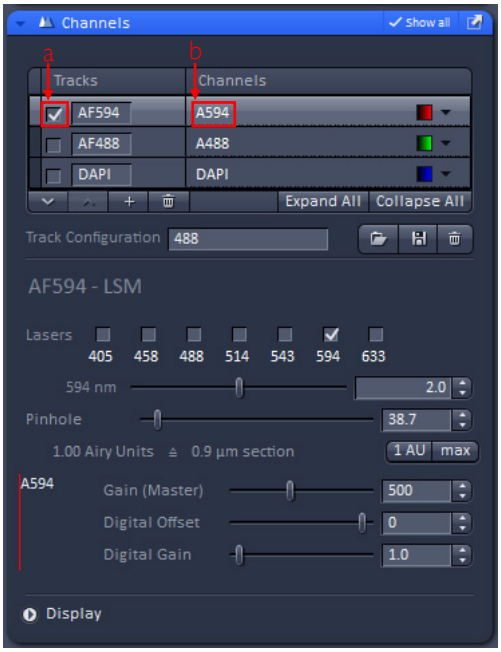
3. Adjust Pinhole Size

3.1) In the channels section, 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].

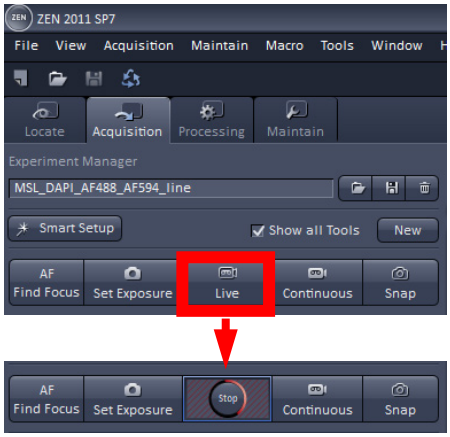
3.2) Verify that the pinhole size is the same in other channels. If not, type in the correct number [c], copying whatever the number is for the longest wavelength fluorophore. Note that the Airy units can be different between channels. That is fine, what you need is for all channels to have the same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

4. Begin live imaging with the confocal

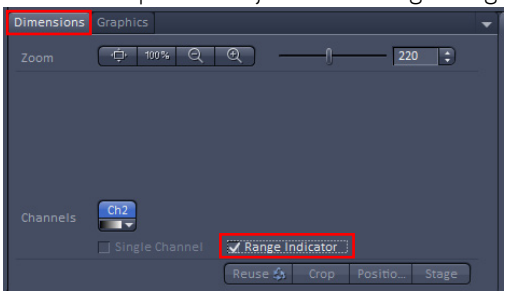
4.1) Adjust settings for channels one at a time. Select the channel you want to adjust first by leaving only that channel with a checkmark [a], and by clicking on it in the channel list so that it is highlighted in gray [b]. Channels that have a checkmark will be scanned by the confocal. The channel highlighted in gray is the one whose parameters you will be able to adjust in the bottom of the channels section.



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) Make sure the display settings in the panel below the image are reset. If not, you will not be able to correctly evaluate which pixels are saturated and which are at zero intensity.

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.
- 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, Gain (Master) and Laser Power

What are you trying to do?

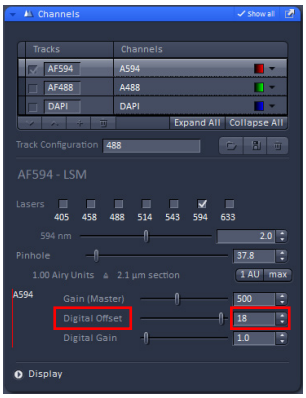
Your goal is to obtain an image with good enough contrast for your analysis, without saturated pixels, with minimal bleaching. To do so, you will balance the Gain (Master) and Laser Power settings while keeping track of pixel intensities, bleaching and image quality using the Histo display mode.

The following adjustments should be performed one channel at a time [4.1].

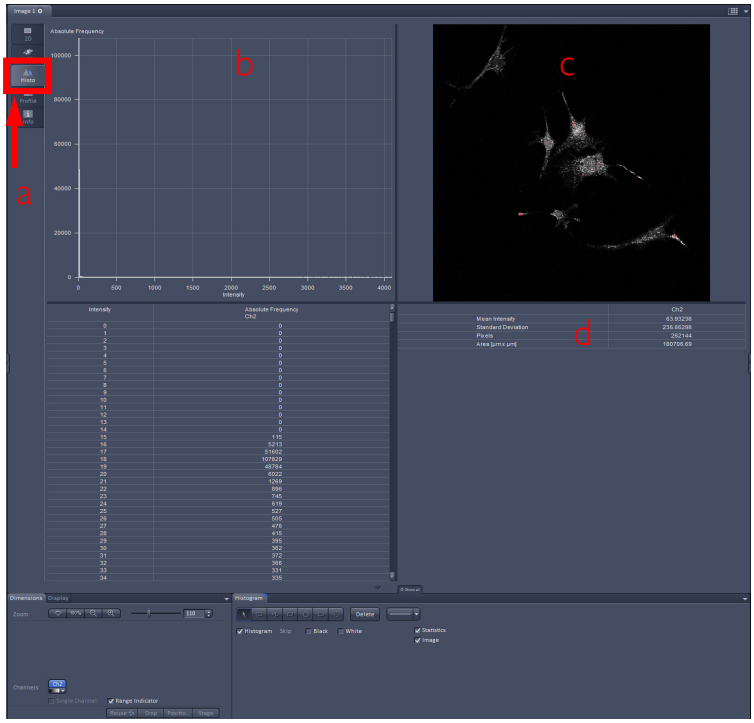
5.1) Find an area of your sample that is bright, but within the range of fluorophore levels you are interested in. Do not optimize your settings in a dim part of the sample as your pixel intensities will likely be saturated in brighter regions.

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 the display settings are reset [4.4]

5.3) In the Channels section, increase the Digital Offset one unit at a time by using small buttons 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 between 5-20.

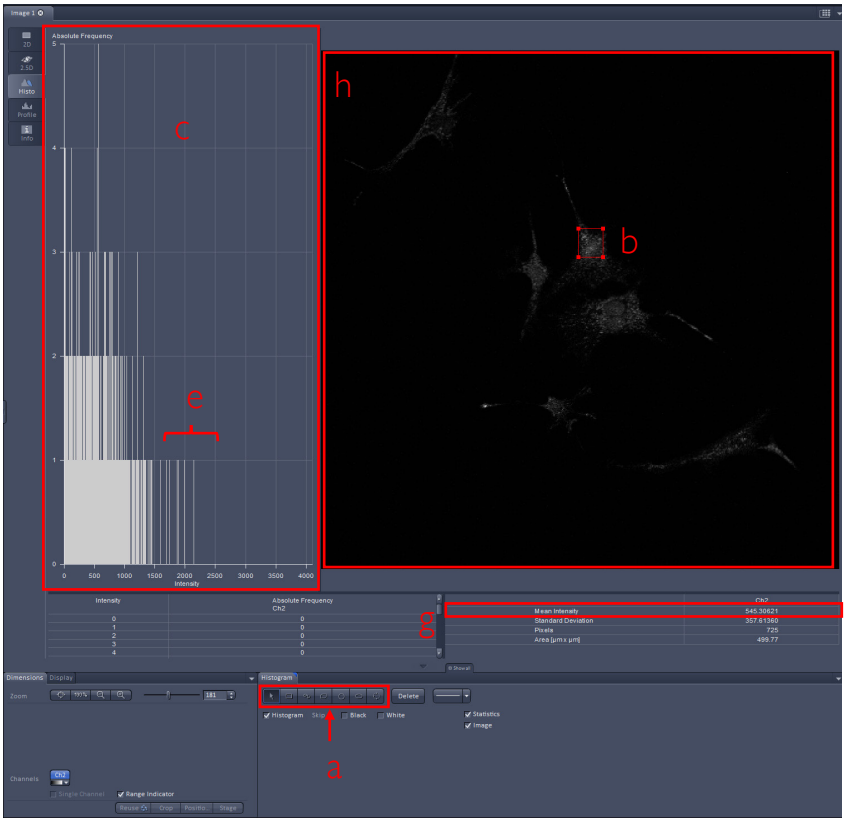


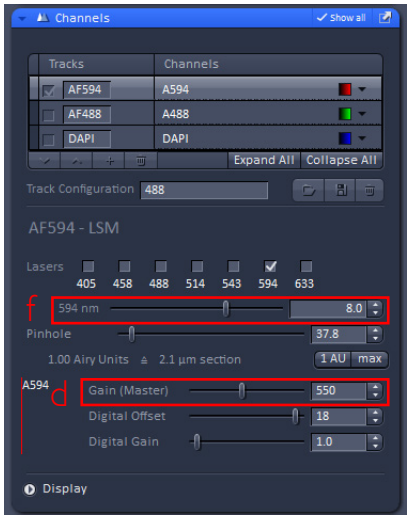
5.4) Switch to the Histo display mode [a]. In this mode, the top left will be a histogram of pixel intensities [b], the top right will be the image [c] and the bottom right will be summary statistics [d]. You can adjust the size of the panels by dragging on the lines that divide them to make the size of the image larger, for more comfortable viewing.



5.5) In the graphics tab at the bottom select an appropriate shape [a] and draw a region around the brightest object in the field of view [b]. The histogram of pixel intensities will now correspond to the pixels inside the box you drew [c].

5.6) Adjust the Gain (Master) [d] until the brightest pixels in the histogram (the right edge of the histogram) are between 2000-2500 [e]. Typical Gain (Master) values are 450-850.





5.7) Adjust Gain (Master) [d] and Laser Power [f] while keeping track of three things:

a. Pixel intensities, by monitoring the histogram panel [c]. The brightest pixels should be between 2000-2500 [e].
b. Bleaching, by monitoring the Mean intensity in the summary statistics section [g]. You should not see a consistent decrease in this number while imaging in Live mode. The number will oscillate; if it is not trending down consistently, you are fine.
c. Image quality, by monitoring the image panel [h].

If you want higher image quality, lower Gain (Master) and increase Laser Power, while keeping brightest pixels between 2000 and 2500 [e]. If you want lower bleaching, increase Gain (Master) and lower Laser Power.

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

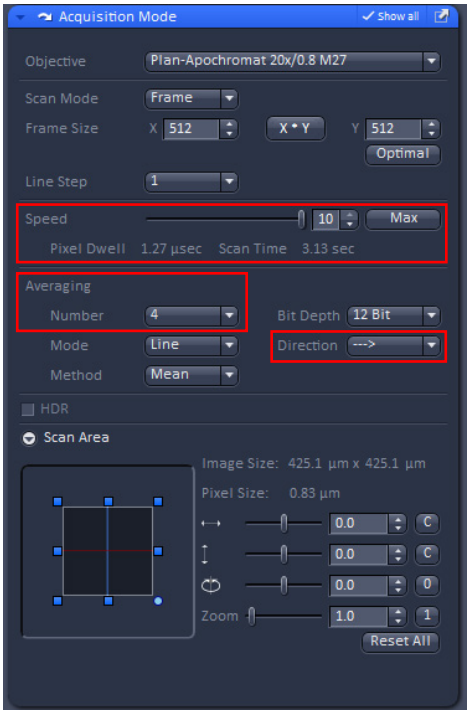
Tips when adjusting laser power

Some lasers are much weaker than others and may require you to increase power high as 100%. This includes the 543 nm and 594 nm lasers on the LSM710. Do not worry if the laser power for those laser lines is very high; if you are not bleaching, you are fine.

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.

A good way to adjust the laser power is by factors of 2. Lower your gain until your brightest pixels are around 1000. Then, double your laser power. If your laser power is above 1%, the intensities of your brightest pixels should go to around 2000. Now, check the image quality and bleaching to decide whether the change was worth it. If so, try the same trick again. If the brightest pixel intensities are significantly lower than 2000, that means your laser power is too high, even if you do not see bleaching. Below 1% laser power, the linearity of the laser power parameter is not as accurate, so if pixel intensities do not double when you double your laser power, do not worry.

6. Further contrast adjustments: Averaging, Speed, Direction



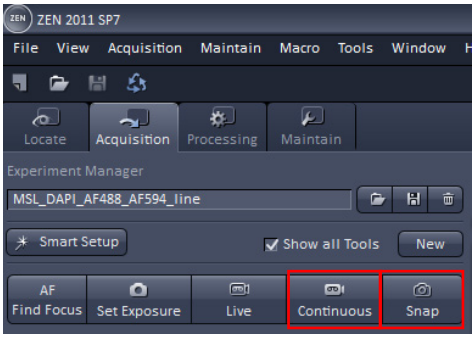
6.1) In the Acquisition Mode section, adjust Averaging Number until you obtain an image of acceptable quality, time to acquire and bleaching. Keep in mind the following:

a. Increasing averaging number improves image quality but there are diminishing returns: the more you average, the less noticeable the improvements.

b. Very noisy features in images improve more noticeably than features that already have high contrast.

c. Averaging is applied to all channels. Consider reducing laser power and increasing gain if another channel looked good without averaging.

d. To see the effect of averaging you will need to either take a Snap or use Continuous imaging mode. Live imaging mode does not do averaging. To evaluate bleaching with increased averaging you will need to use Continuous imaging mode.



6.2) Speed should almost always be set at Max, for reasons provided in the Averaging vs Speed box below.

6.3) Use Snap to acquire images with Direction set to --> or <-->. If the images look the same, use the <--> setting. This will reduce the Scan Time. Leave Corr X and Corr Y settings on 0.00. If the images look different (for example, edges of certain structures seem fuzzy with <-->), set Direction to the --> setting.

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, there may not be room to increase the laser power further. For example, when using the 543nm or 594nm lasers on the LSM710, 100% laser power may be needed, which obviously cannot be increased further.

Averaging vs 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 two scenarios where lowering the speed may be convenient.

The first is 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.

The second is 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 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 scan time.

Cellular vs Subcellular resolution

If you only need to distinguish cells from each other, you will typically use an air objective, like the PlanApo 20X/0.8, and the default Frame Size and Zoom settings are usually fine. At most, you might consider increasing the Frame Size to 1024 x 1024 to get more detail.

If you need subcellular information, you will typically need to use a high NA oil objectives: PlanApo 63X/1.4 or 40X/1.4 on the LSM710, PlanApo 63X/1.4 on the LSM700. To obtain images with the highest possible detail, you will need to carefully set the pixel size, as described below.

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 um by Y um) 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.

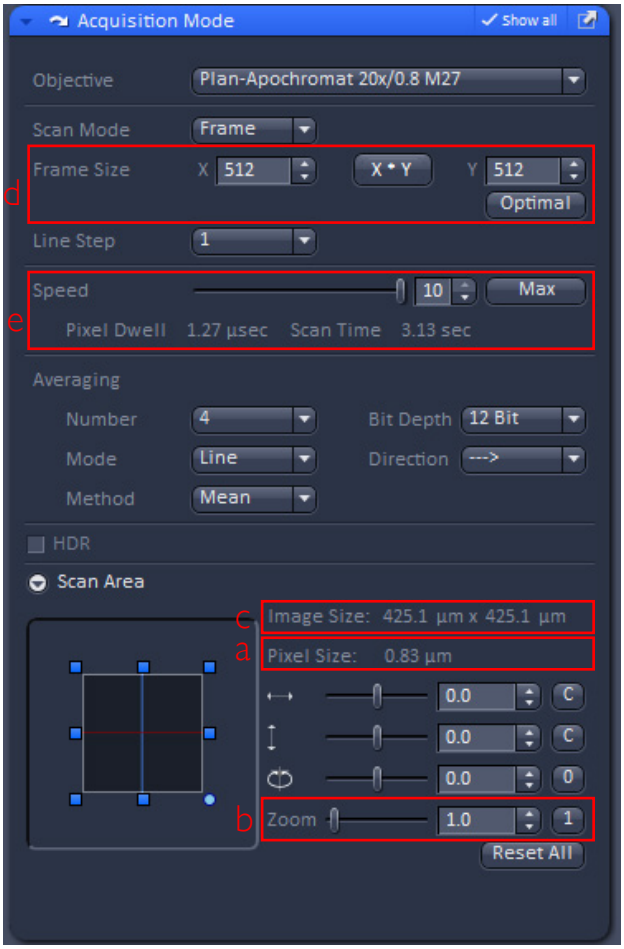
How small should the Pixel Size be?

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. Setting the Pixel Size unnecessarily small can make imaging slower, reduce your field of view, and/or bleach your sample more. The following tables have examples of recommended pixel sizes to obtain high resolution or the highest possible resolution, assuming you are using a PlanApo 63X/1.4 or 40X/1.4 objective, for different fluorophores. 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 um) for different fluorophores

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

	DAPI	GFP/AF488	AF546/AF555/Cy3/Rhod	AF568/AF594/mCherry/TxRed	AF633	Cy5/AF647
High resolution	0.099	0.110	0.122	0.131	0.139	0.144
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 smaller than the highest resolution option in the Recommended Pixel Sizes table above.

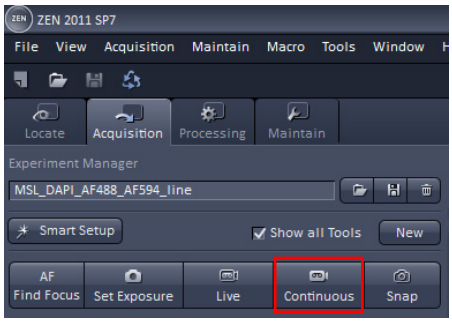
7.2) Decide what Image Size [c] you need, based on the size of structures you need to include in a single image (one cell, one group of cells, one part of cell, etc.). You can explore different image sizes by adjusting Zoom [b].

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

7.4) Adjust Frame Size [d] to get desired Pixel Size [a]. You can select from typical Frame Size options (512 x 512, 1024 x 1024, etc.) by pressing the X * Y button or you can type numbers in the X and Y boxes to do this more precisely. Do not press the Optimal button.

7.5) Set Speed to Max [e], to minimize bleaching. Changing Zoom and Frame Size can lead to much slower imaging. Since Speed is a suboptimal way to adjust image quality (see Averaging vs Speed box above), it is better to set this parameter to the maximum.

7.6) Verify that bleaching and image quality are acceptable using the Continuous imaging 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.



What if it takes too long to take an image?

- 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?

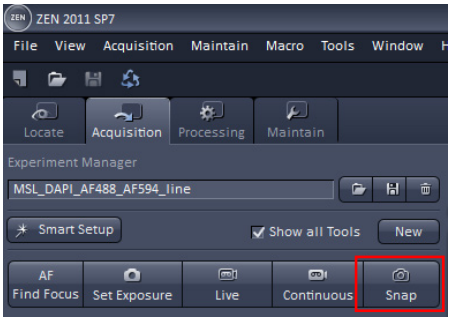
Determine what you are willing to trade off to reduce bleaching:

- 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

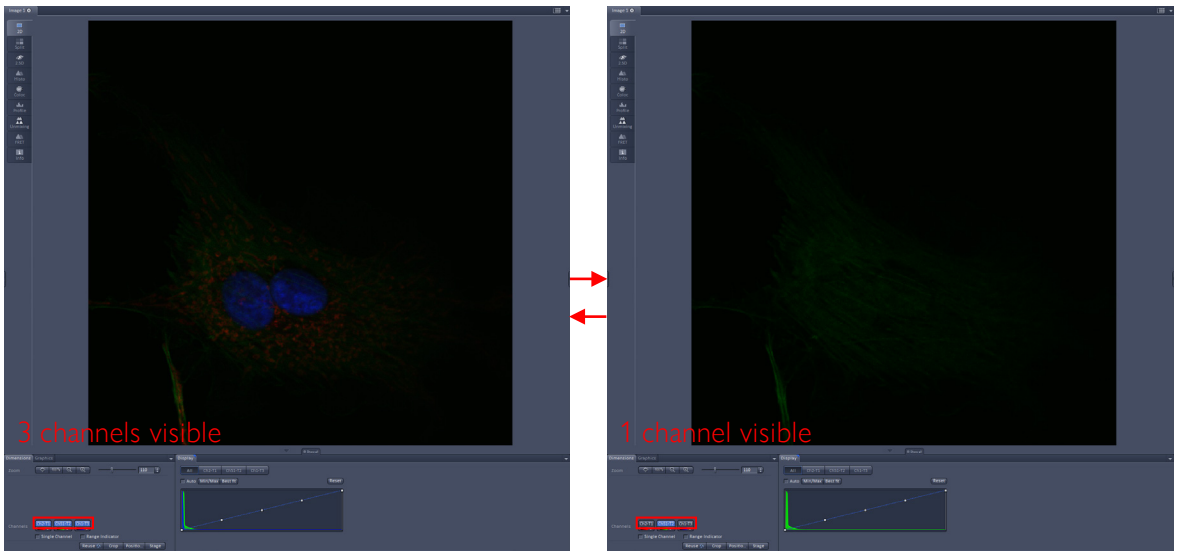


8.1) Once you are satisfied with your settings, acquire an image with the Snap button.

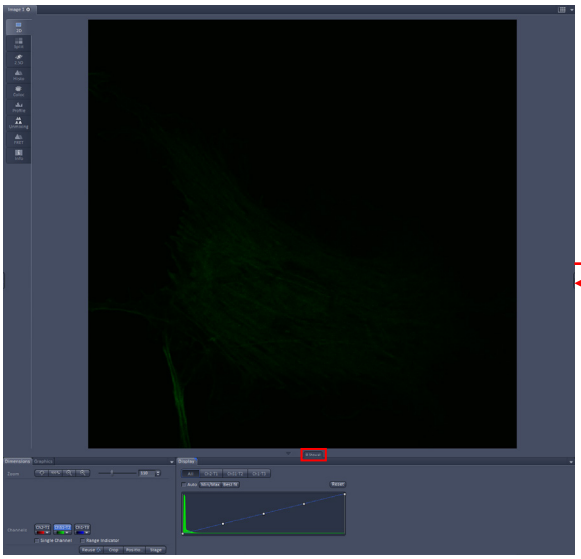
8.2) Images can be saved with File/Save. Make sure you save in .czi format so you retain image metadata. This will allow you to properly report your acquisition settings and reload them for future imaging.

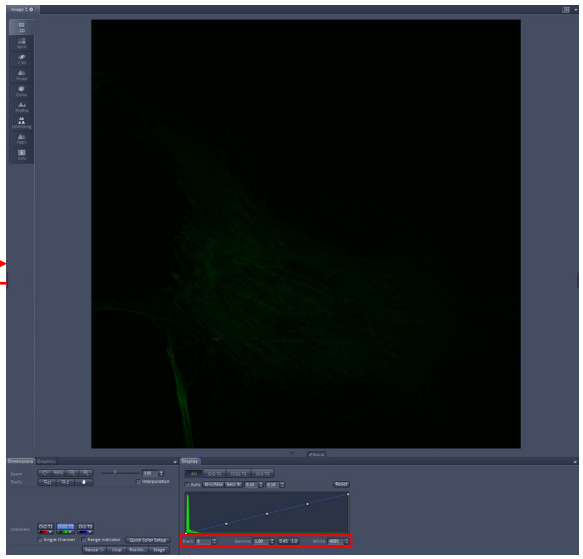
9. Visualization options and adjustments

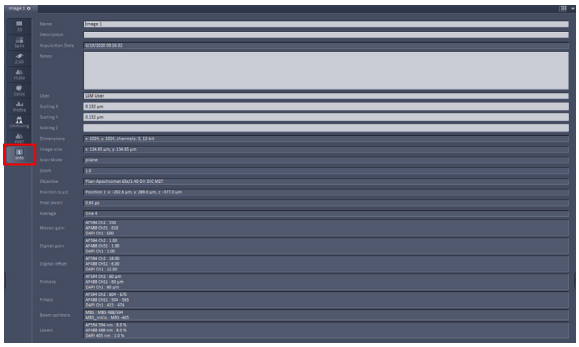
9.1) You can visualize one channel at a time by clicking the buttons with the channel name in the channels section of the Dimensions tab. Channel will be visible if their name is highlighted in light blue



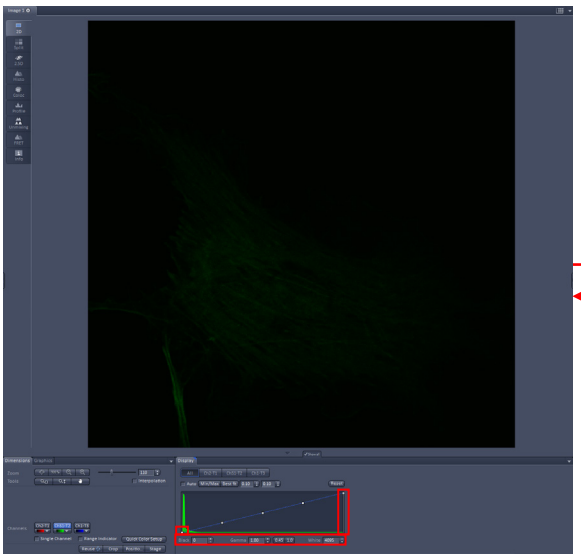
9.3) Clicking Show All will give you more display options. If you want to compare images, they must be acquired with the same settings and displayed with the same settings. To verify the former, you can use click on the info tab. To ensure the latter, all parameters in the display panel (Black, Gamma, White) must have the same values.

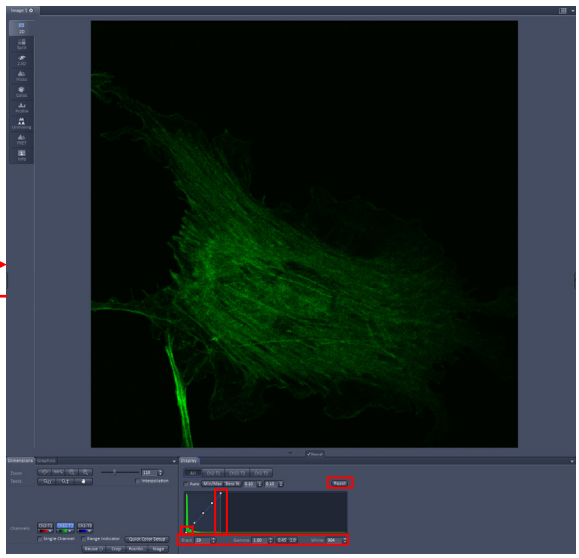




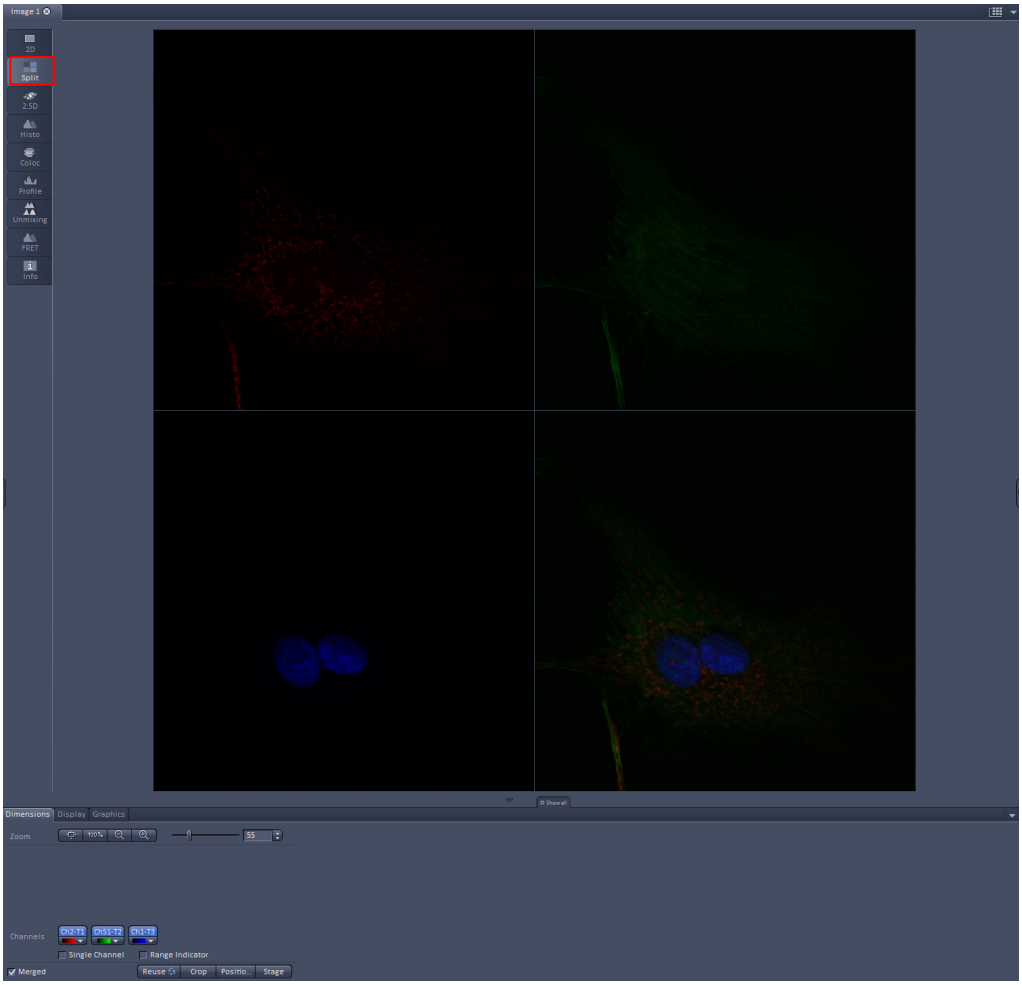


9.2) You can adjust the contrast in each channel by adjusting the Display curve. Dragging the right side of curve to the left will increase the brightness of the image, which can be very helpful in visualizing dim structures. You can also decrease the value in the "White" box to do the same thing. If you increase the value in the "Black" box, you will make dim pixels darker, and shift the left side of the curve to the right. We recommend you do not adjust the value in the "Gamma" box.





9.4) The Split display mode allows visualization of individual channels side-by-side



Things to be careful with when making display adjustments

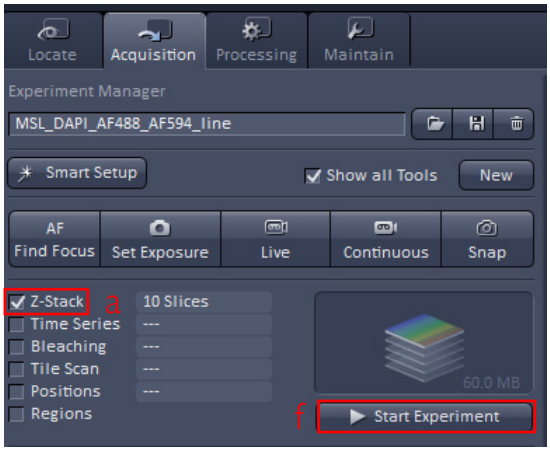
- If you are still optimizing your *acquisition* settings and have adjusted your *display* settings, be careful when interpreting red pixels in the range indicator display. Pixels at or above the "White" value will show up in red, even if they are not saturated. So, even if pixels are below 4095 (the maximum, saturation intensity) they will show up in red if they are at or above the value in the "White" box. This can lead you to incorrect decisions when you are adjusting Laser power or Gain (Master). We recommend you click on the Reset button above the display curve to set "White" back to 4095, "Black" to 0 and Gamma to 1.00 to avoid this issue when adjusting laser power or gain.
- Even if images were acquired with the same settings, it is critical to make the same display adjustments to all of them when comparing them. To ensure this is the case, make sure all image you are comparing have the same "Black", "Gamma" (should be 1.00) and "White" values.

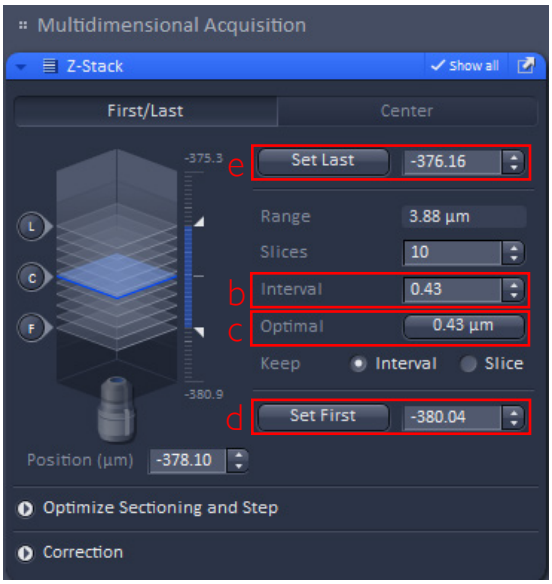
10. Z stacks

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" resolution (see table in section 7), use an interval of 0.2um.
- If you were imaging with "high" resolution (see table in section 7) use the optimal interval recommended by the software (see below).
- If you are not using "high" or "highest" or are using an air objective, 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 um in diameter use an interval of 1-1.5um
- Do not use interval values below 0.2um as this will only bleach your sample, slow down your imaging and not provide any significant additional details in the Z dimension. If you are not doing "highest" resolution imaging, do not use intervals smaller than the "optimal" recommended by the software.





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 um, if using high resolution settings click on optimal [c], if using other settings see Recommended spacing of slices in a Z stack.

10.3) Consider switching to a single channel [4.1], to make the process of finding the start and end of the Z stack faster, and to reduce bleaching. This is particularly useful if you have 3 fluorescent channels or more on the LSM700 or 4 fluorescent channels on the LSM710, as in these cases channels get scanned one at a time over the entire frame, leading to confusing images when changing the focus during the scan.

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.

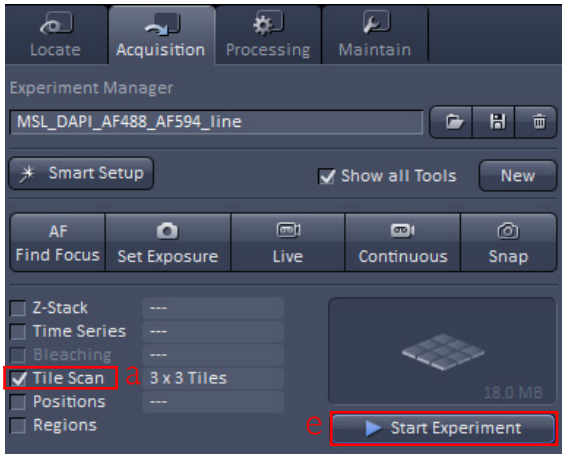
10.4) Still in Live acquisition mode move up in Z, by moving the focus knob away from you, until you are just above your region of interest, and click Set last [e]. This will be where the Z-stack ends.

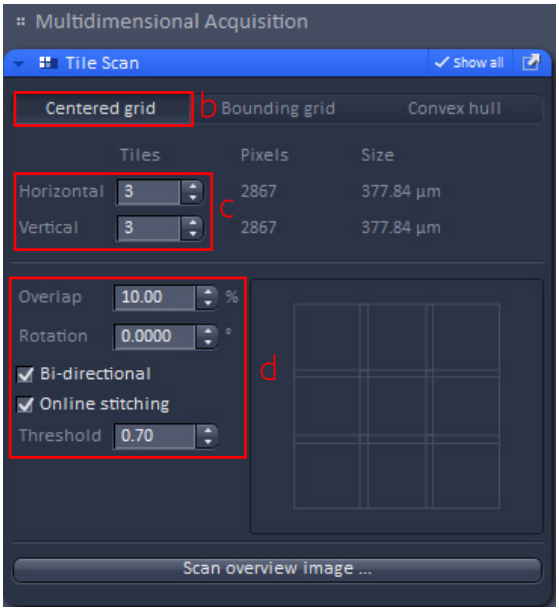
10.5) Stop Live Acquisition.

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

11. Tile scan

Tile scan will allow you to acquire multiple adjacent images; the software will stitch them together into a bigger image. This is useful for imaging large areas that do not fit in a single field of view.





11.1) Click on the Tile Scan checkbox in the top left corner of the Zen interface [a], to activate the Tile Scan window

11.2) Click on Centered Grid [b]

11.3) Put in the number of tiles desired in the horizontal and vertical directions [c]. The mosaic of tiles will be centered on the current position.

11.4) Set overlap to 10%, leave rotation at 0, click on Bi-directional, click on Online stitching and leave threshold at 0.70 [d].

10.6) Press the Start Experiment button [e] to acquire the Tile scan.

What if tiling takes too long?

Check whether you have too many tiles, particularly empty tiles on the edge of your sample. If so, try reducing the number of tiles to better match your sample dimensions.

If you are having trouble sizing the mosaic of tiles properly in Centered Grid mode, you can try the Bounding Grid option, which allows you to mark positions you want to include, and draws the smallest rectangle of tiles that includes all of them.

If your sample has an irregular shape, such that by fitting it into a rectangle you have a lot of empty tiles (for example, in the corners), try using the Convex Hull tiling mode. This works similar to the Bounding Grid mode in that you mark positions, but the software then draws the smallest polygon of tiles that covers the area of interest. This often discards empty corners and is a useful way of reducing the number of tiles you image.