University of Glasgow | Institute of Cancer Sciences

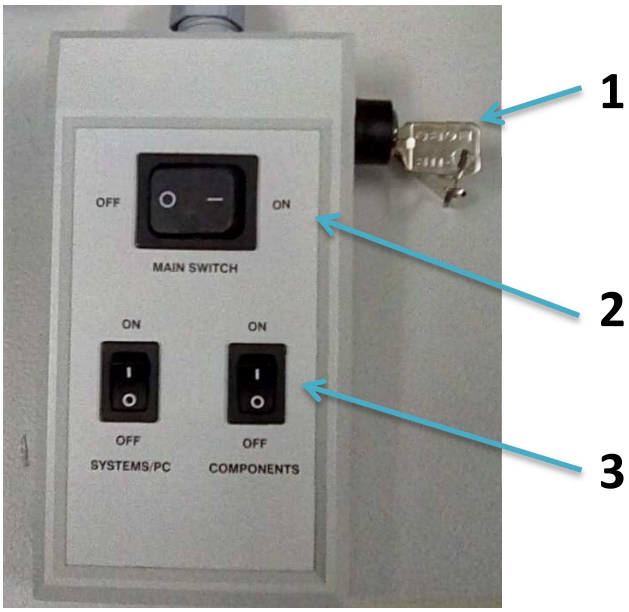
College of Medical, Veterinary and Life Sciences, Garscube Estate.

ZEISS LSM 780 Confocal Microscope SOP

START UP

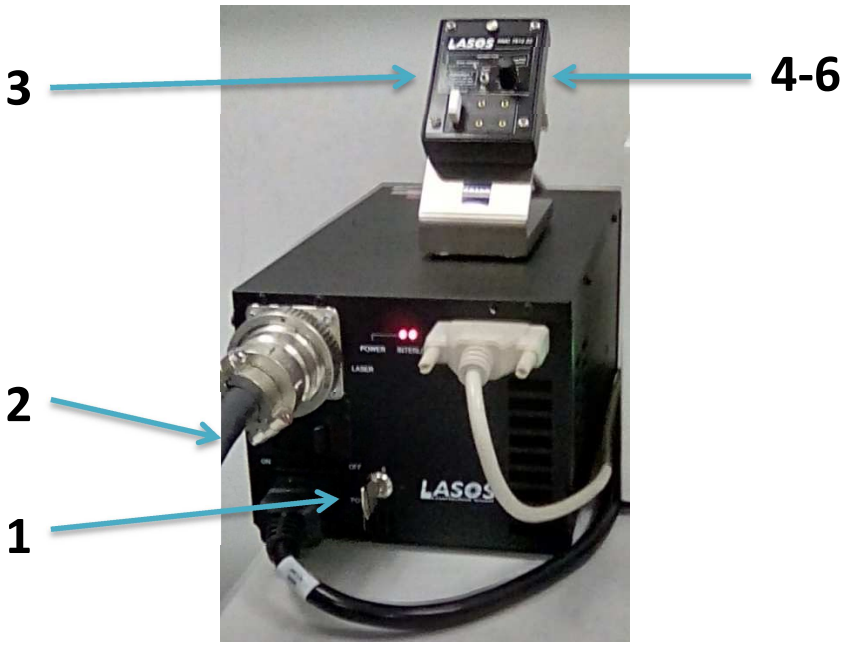
Switch on main power unit

1. Ensure key on side of box is turned in clockwise position to 3'clock.
2. Switch the **main switch** ON.
3. Switch the **system/PC** and **components** ON (turning on the system/PC on its own allows you to use the computer only, without the LSM).



Switch on Argon Laser (LASOS)

1. Switch key on front black box ON (12 to 3 o'clock).
2. Check main power box is ON (should always be).
3. After ~5 mins switch the laser controller from **idle** to **run** (small black box).
4. Wait ~5 mins until the red light goes out and the green light comes on.
5. If red and green lights are on at the same time then turn the laser power down until the red light goes out and the green light stays on.
6. **Do not run the system if the red light is on**.



Switch on HXP 120V FL Lamp

1. Switch the **power** switch on.
2. Leave the intensity level at 2/3 (can turn down intensity if sample is sensitive or bleaches easily).
3. Check the blue shutter open light is on, if not press the shutter open switch.

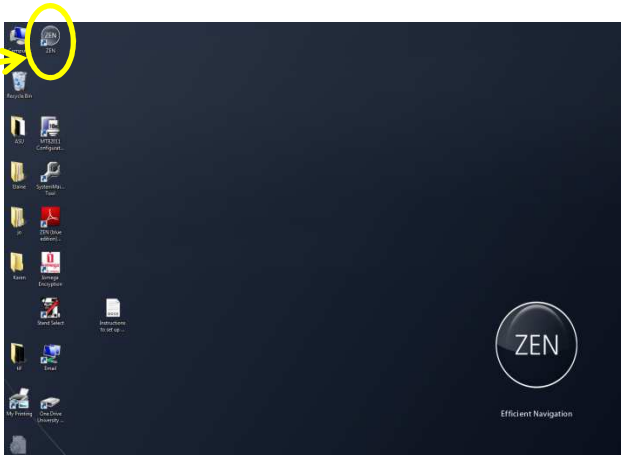
Switch on PC

- Switch on the PC and login as .\LSM User (no password required) or login with your GUID and password.

Running software

Start

Open the **Zen BLACK** software to run the confocal (ZEN blue is for processing images only).



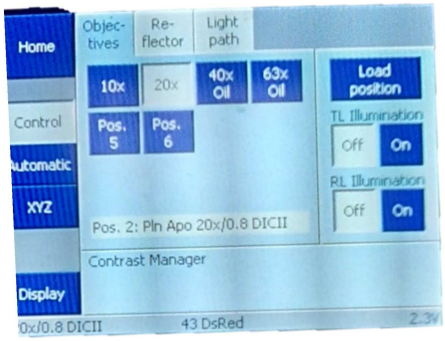
Select **start system** and the system will initialise (you will hear it switching filters etc).

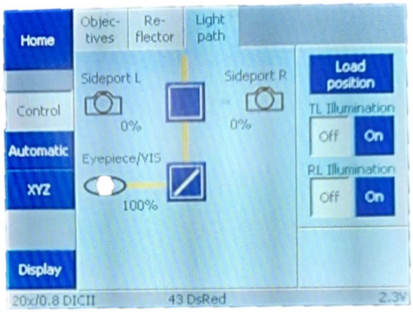
Locate Image

- Open the **locate** window.
- Either
    - Use the software
    - Or use the TFT on the microscope
- Select the appropriate objective. (The X10 and X20 lenses are dry, but the X40 and the X63 need oil. A message will pop up to prompt you. Click ‘done’ when you have added the oil and the objective will move back into position for viewing)
- Select light path (100% to eye pieces)
- Select light source
    - Select **transmitted light (TL) on** and **reflected light (RL) off** to look at **brightfield** images. (make sure that the filter is set to an empty slot)
    - Select **reflected light (RL) on** and **transmitted light (TL) off** and then select the appropriate filter to look at **fluorescence** images.

Software

TFT





Acquire Images

Open the **acquisition** tab.

New – opens a new image

Smart setup

AF - Auto focus

Set Exposure – Computer sets gain

Live – fast scan for previewing image

Continuous – scans using selected settings

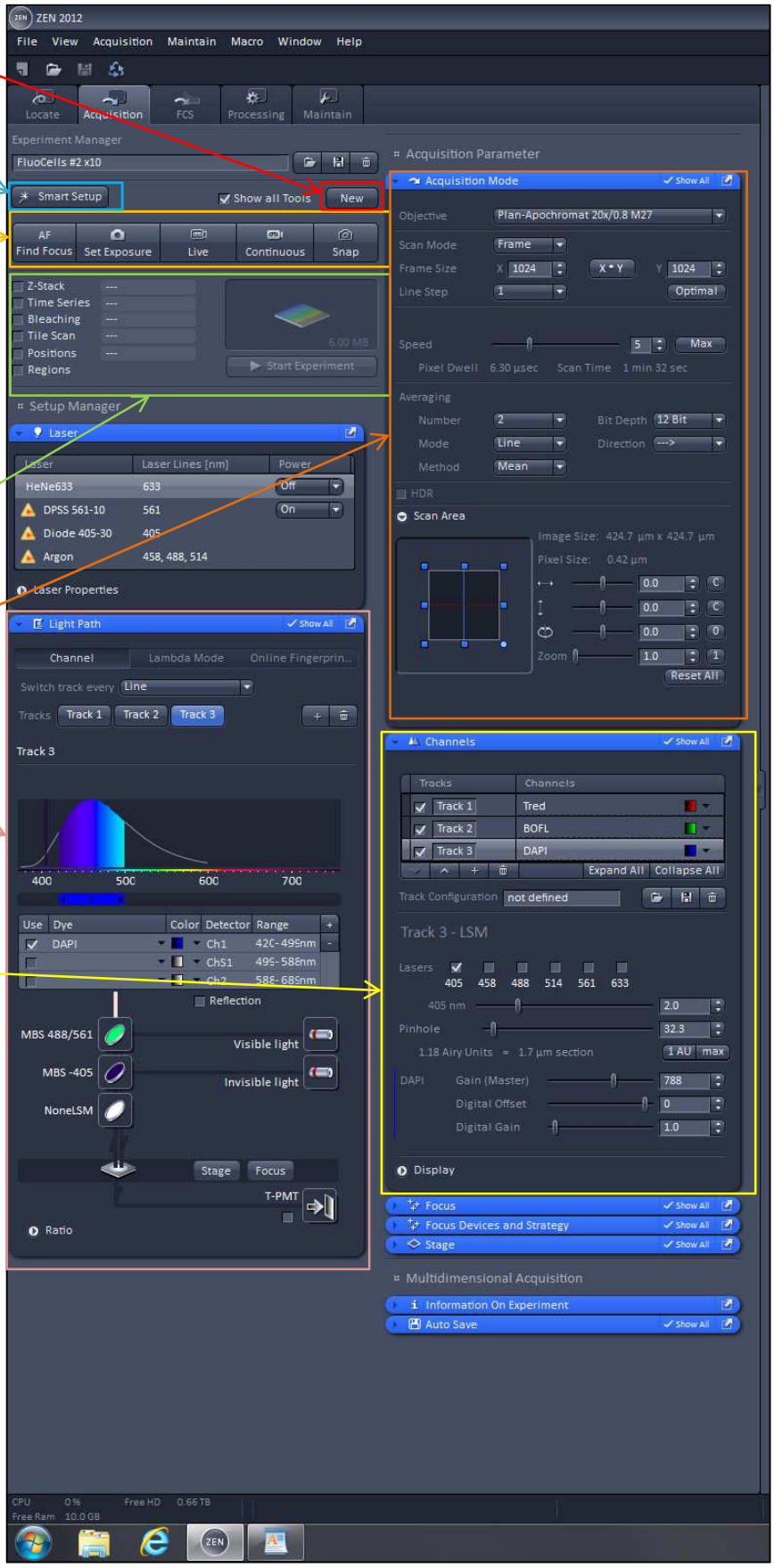
Snap – captures image at selected settings

Advanced Features

Acquisition Mode – selects parameters for the scan

Light path – shows the emission spectra and filters for each track

Channels – shows the probes/dyes you are using, the track they are in and settings for the laser, pinhole and gain for each track



Transmitted light setup

If you are using transmitted light- brightfield, phase or DIC you need to check that the Koehler illumination is correct for the objective you are using for your images. By correctly setting up the correct Koehler illumination you are making sure that your sample is illuminated by a good even light source. (see separate SOP on light microscopy). The quality of transmitted light images that can be obtained with the confocal is poor compared with those that can be obtained with the Axio Observer, and therefore the Axio Observer is the recommended microscope for capturing transmitted light images.

- Select the appropriate objective, condenser, and slider (you only need this for DIC) for your requirements.

Objective	Condenser	Slider
X10	H/ Ph1	
X20	H/ DIC II	DIC I/II
X40 (oil)	H/ DIC III	DIC II/III
X63 (oil)	H/ DIC III	DIC II/III

- For DIC, a corresponding slider must also be used, which fits under the objective lens with the screw facing upwards.
- Brightfield/ Ph/ DIC images can be collected in the detector labelled T-PMT in ZEN (see below).
- Turn the screw in the DIC slider to adjust the shear on the Wollaston prism to make your cells appear concave/ convex as desired.
- Narrowing the diameter of the luminous-field diaphragm can help to increase contrast if your specimen is very dark/ strongly scatters light, but it will reduce the area of the field of view.

Kohler illumination - http://zeiss-campus.magnet.fsu.edu/articles/basics/kohler.html

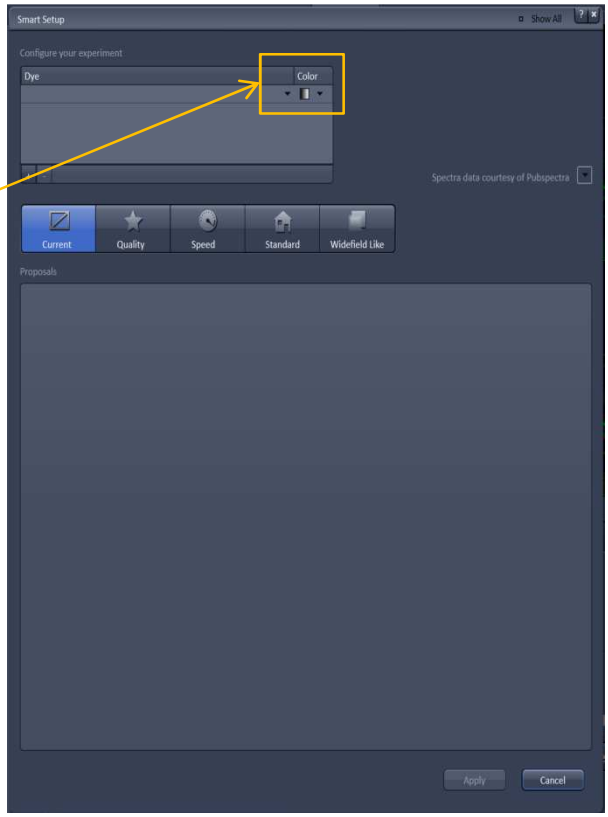
Improving contrast - http://zeiss-campus.magnet.fsu.edu/articles/basics/contrast.html

Fluorescent light setup

Smart Setup

The first time you use a combination of fluorescent probes/dyes you need to setup a configuration that will set up the hardware for your combination of probes/dyes. Click on **Smart Setup**

Select the probes /dyes from the drop down list in the top box in the window. The software will give a pseudo-colour to each of your probes/dyes this can be changed using the drop down menu to the right of the colour box.



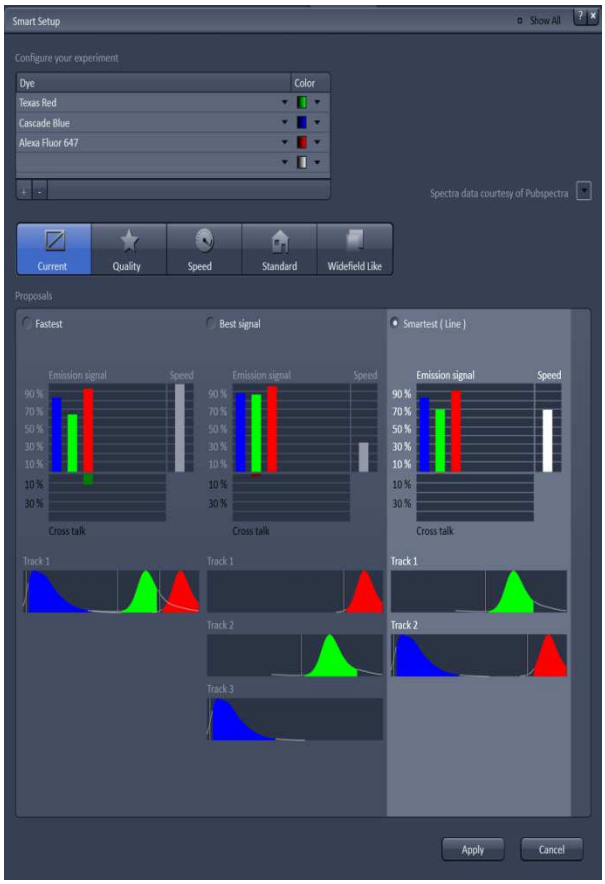
The smart setup will take the selection of probes/dyes you have selected and suggest three options for configuring the software/hardware.

Fastest – all channels collected on the same track. This can lead to bleed through between the different channels 9 (where emissions from one dye overlap with the emissions of another dye)

Best signal – collects each channels on a separate track. This avoids bleed through, but takes the longest. As the laser passes over the sample multiple times it is more likely to bleach the sample

Smartest – collects only compatible channels at the same time. This avoids bleed through with fewer tracks this is slightly quicker than best signal

Select the configuration that best suits what you require by clicking the appropriate radio button and clicking on apply at the bottom of the screen. This gives a basic configuration that you can now fine tune.



Light Path

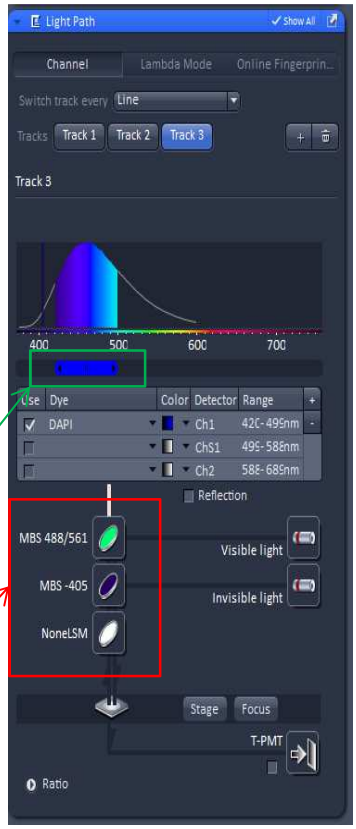
In the Light Path window you can make fine adjustments to the band width for each track. You can also check that the appropriate filters and detectors have been selected for each track.

If you are using the fastest / smartest setup or line capture mode you will need to change the filters so that they are compatible with all the fluorophores, you are using.

GaAsP detectors have greatest sensitivity for emission spectra from 500-650nm [superior to PMT over this range] PMT are just as good as GaAsP detectors for 400nm ie DAPI and have greater sensitivity than GaAsP for 700+nm.

Adjust bandwidth by sliding arrows

Filters : click on them to show available options



Acquisition Mode

Scan Mode:	Frame: captures a whole frame then switches track
	Line: captures a single line then switches track
Frame Size:	The size of the frame in pixels – use optimal size
Speed:	Slower laser spends more time on single point more time more bleaching (max good starting point)
Averaging:	Averaging improves signal to noise ratio. Effects scan time. Use line and mean.
Bit Depth:	Selects dynamic range, 8 bit gives 256 grey scales, 12 bit gives 4096 grey scales (publication quality is at least 12 bit also use 12 for doing quantitative measurements or imaging low fluorescence intensities)
Direction:	Direction the laser scans.
Binning:	Binning is used to aggregate pixel intensities. The higher the binning the higher the pixel intensity but the lower the resolution. Do not perform binning.
Interpolation:	Smooths pixels (makes the image appear less grainy when you zoom in)
Scan Area:	Controls to zoom into an area of interest.

Channels

Shows the channels on each track along with the pseudo-colour assigned to each channel, you can change the colours here.

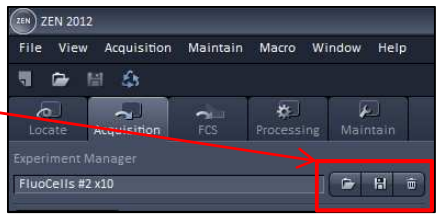
Shows information on the track highlighted above.

Lasers:	Shows the lasers switched on in each track.
Laser power:	Ideally 1%, higher power more bleaching.
Pinhole:	Shows size of pinhole for this track. Ideally should be set to 1Airy Unit (AU). 1AU is the best compromise between resolution and signal; the pinhole size needs to be the same for all channels; the pinhole size can be increased if insufficient fluorescent light is emitted from the sample making the visualisation of the image noisy; increasing the pinhole size increases the optical slice thickness, but decreases the lateral and axial resolution)
Gain (Master):	Regulates brightness of the image. Should be between **500-700**, if outside this range the laser power can be adjusted.
Digital offset:	Sets background/threshold level of image, leave at 0,used when looking at images with DIC
Digital Gain:	Post acquisition enhancement leave at 1

Save Acquisition configuration

You can save the configuration you have created in the Acquisition tab under Experimental manager

Load and save configurations for the confocal



The acquisition configuration can also be set up by opening a previously saved .czi files. Open a previous image and select **reuse**.

Capture an image

Set up the following starting parameters

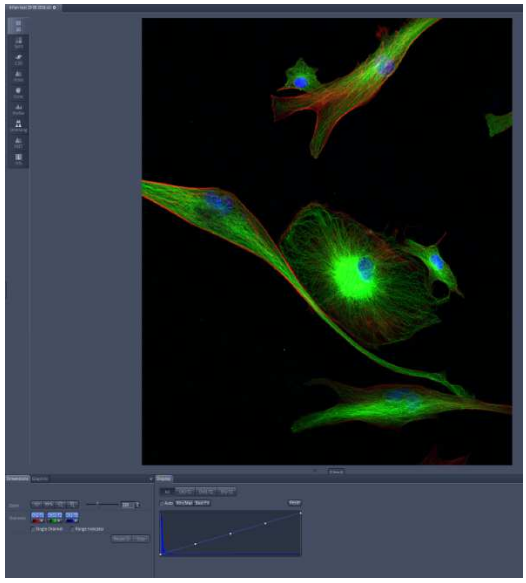
Select Scan Mode:	Frame	Mode:	Line
Resolution:	512x512	Method:`	Mean
Speed:	Max	Bit Depth:	8
Average:	1	Zoom:	1
Laser Power:	1%	Pin Hole:	1AU

These are basic parameters that give you a fast scan rate initially. Many will need to be changed to improve quality.

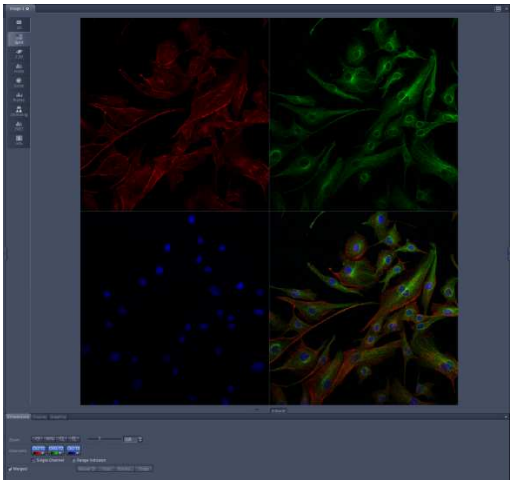
Place your sample on the microscope stage, go to the **Locate tab** and focus on your sample and find an area of interest on the slide, then return to the **Acquisition tab**. Click on **Set Exposure** this will attempt to set the correct gain (master) to show you an image. Then click on **Live** to see a live image captured at the lowest resolution and fastest speed

You will now see an image on the right of the screen, you will need to refocus the image on the screen at this point.

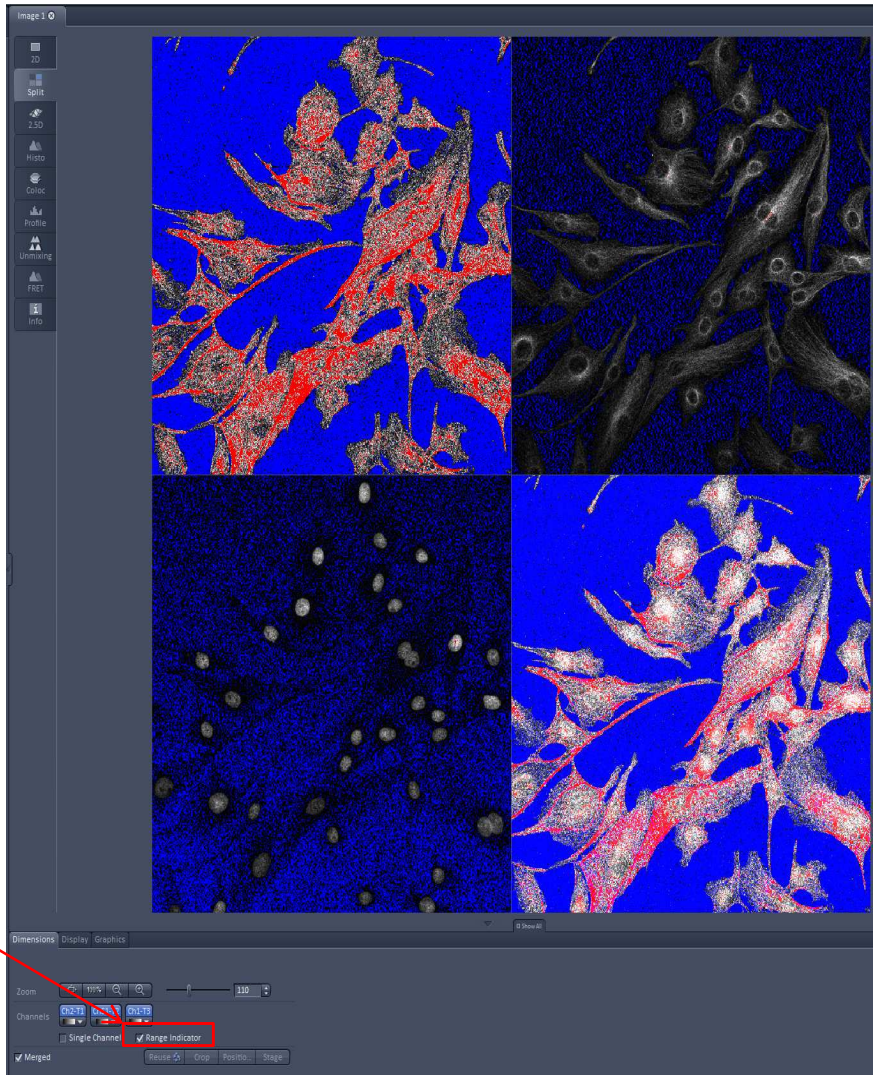
The 2D tab shows the live image, which allows you to optimise the image, adjust focus etc.



The split tab shows the individual channels along with a combined image.



Optimise Exposure



Select the **Range Indicator** box

When an image is acquired you want to be within the dynamic range of the detector. When you click on the range indicator it takes each channel and shows it as a grey scale image with a pseudo colour image overlaid showing over and under saturated pixels.

The red pixels indicate over saturated and the blue zero saturation

Adjust the **Gain** and **Laser power** for each channel to optimise the image, increase the **Gain** to remove the red pixels, remember the gain should be between 500 and 700, if it goes out with this range adjust the laser power.

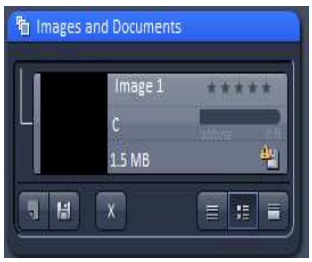
Capture image

Once you are happy with the optimisation select **Snap**.The captured image will appear in a window on the right of the screen.

Save Image

You can save the image by using the **Image and Documents** window on the right of the screen.

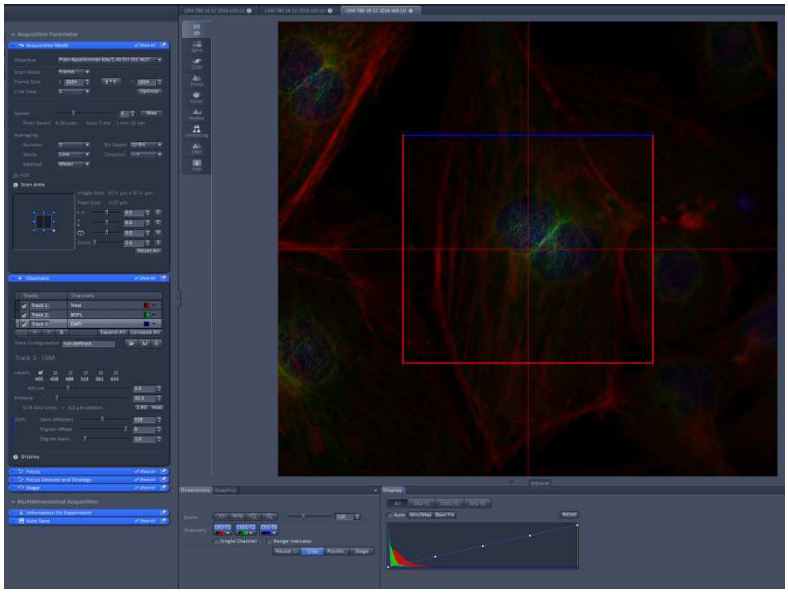
You should save your data as a **.czi** file as this stores all the information on the image. You can reopen this file and export **.tiff** or **.jpg** files at a later date.



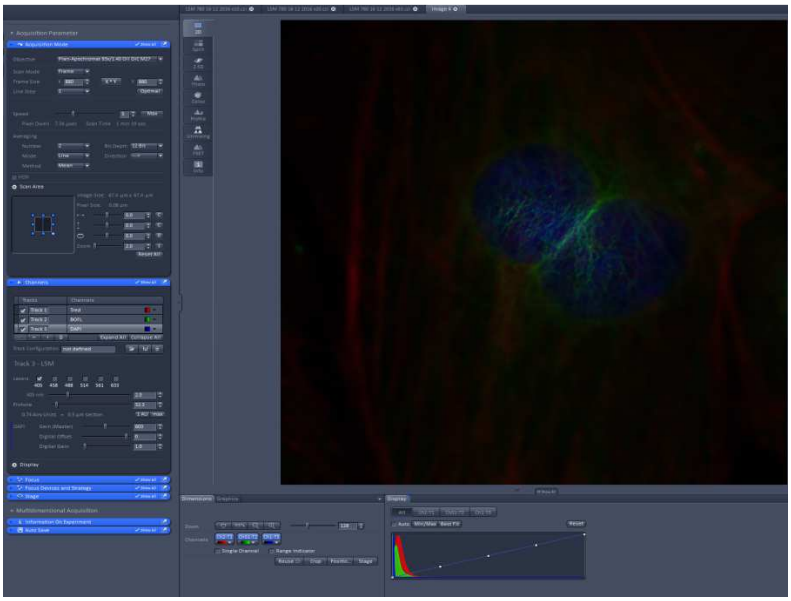
Zoom into area on image

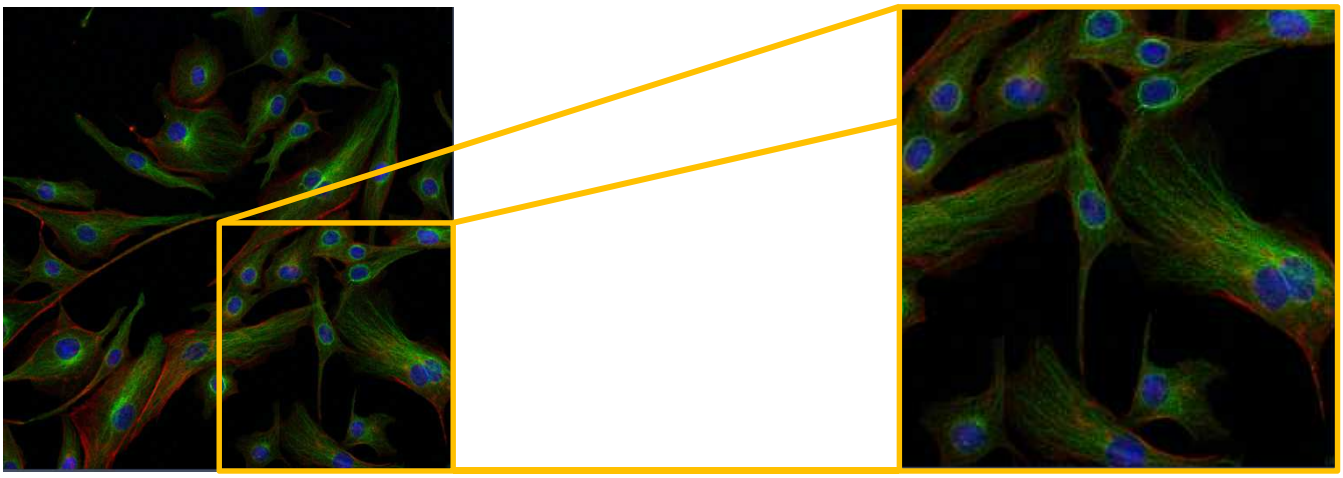
Click on the crop tool at the bottom of the image window. You can use the red square to zoom into an area of interest this can be set to any shape and rotated, but make sure that the blue box side is along the longest axis of the shape, as this gives you the best scan speed.

Once you have the box in the right location click snap and it will zoom into the area that has been selected.



By zooming into an area of interest you are scanning a smaller area with the same number of points.This means that the field of view is reduced, the pixel size is smaller, details are shown magnified with better resolution





512 x 512 pixels

Pixel size 2um

512 x 512 pixels

Pixel size 1um

Z stack

Set up your sample as above for a single plane. Then select Z Stack from the main Acquisition tab.

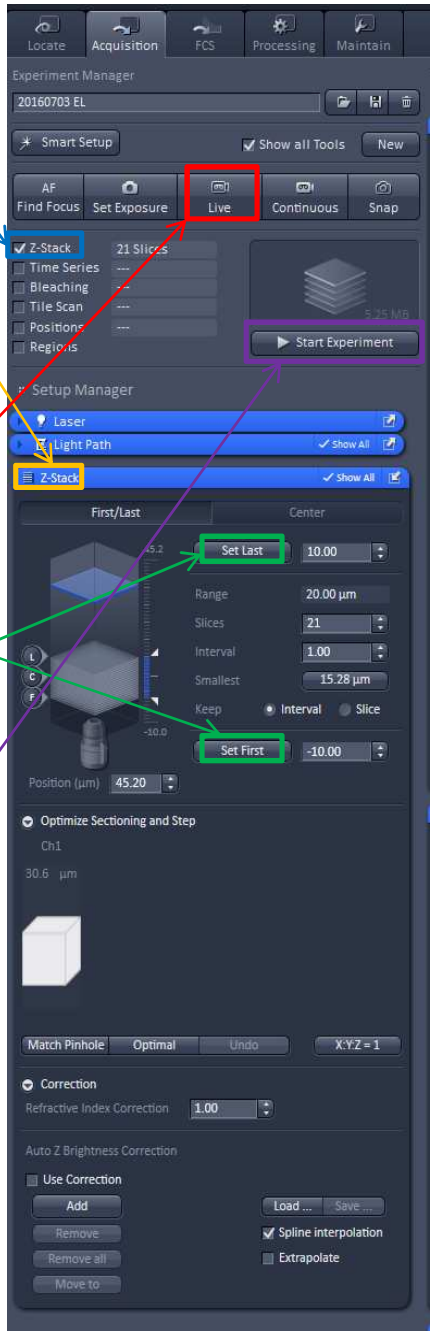
Open up the Z-Stack window.

Select Live scanning.

Adjust the microscope focus to reach the desired starting point for the z-stack. Then select Set First.

Adjust the focus to the desired end point. Then select Set Last

Select Start Experiment and the Z stack will be captured



Slices: Shows how many slices will make up the stack.

Interval: The distance between each slice.

Optimal: Calculates each slice to be 0.5 of the z-resolution for proper Nyquist sampling.

Keep: Uses either interval or number to determine the stack.

Match Pinhole: For multiple wavelengths this ensures that the pinholes are yielding the same section thicknesses.

X:Y:Z=1 : matches the settings in the Z to the 2D XY.

The Gallery tab will display each individual slice of the z-Stack.

Save the z-Stack as a czi file format

To review a Z stack: Select **ortho** view to view the Z information for a particular plane within your stacked tiles.

To analyse a Z stack: Compress it to a 2D flat image by selecting **maximum intensity projection**.

Then add annotations.

Tile scan

Set up your sample as above for a single plane. Then select Tile Scan from the main Acquisition tab.

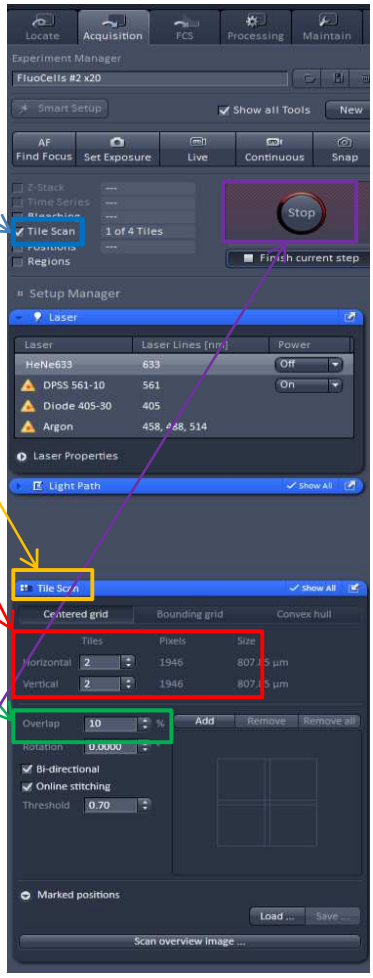
Open up the Tile Scan window.

Select the number of Tiles.

Select overlap 10% and stitching during acquisition (this can also be applied after acquisition if not applied during).

Select Start Experiment and the Tile Scan will be captured

Using the other tabs you can choose your tile scan area by indicating 2 points of interest. It will then automatically scan these points and the area between them.



SHUT DOWN

1. Exit the software.
2. Shut down the computer.
3. Switch the Argon laser power back to idle.
4. Turn the main laser power unit key to 12 **o'clock but leave the main power switch on so the fan can cool the laser.**
5. Remove the slide and clean the microscope lens…
6. Switch off the HXP 120C FL lamp.
7. Once the fan has turned itself off, switch off the main laser power unit.

Troubleshooting

The software will not start eg will not load past 4% → first try restarting the computer and software, if this does not work , shut down everything as above and retart everything from scratch..

Notes

Pinhole

The pinhole is used to produce a thin optical section, at 1 Airy Unit (AU) the light from above and below the focal plane is stopped from reaching the detector. This gives a thin optical section and gives a high Z resolution. However it does also reduce the signal strength at the detector.

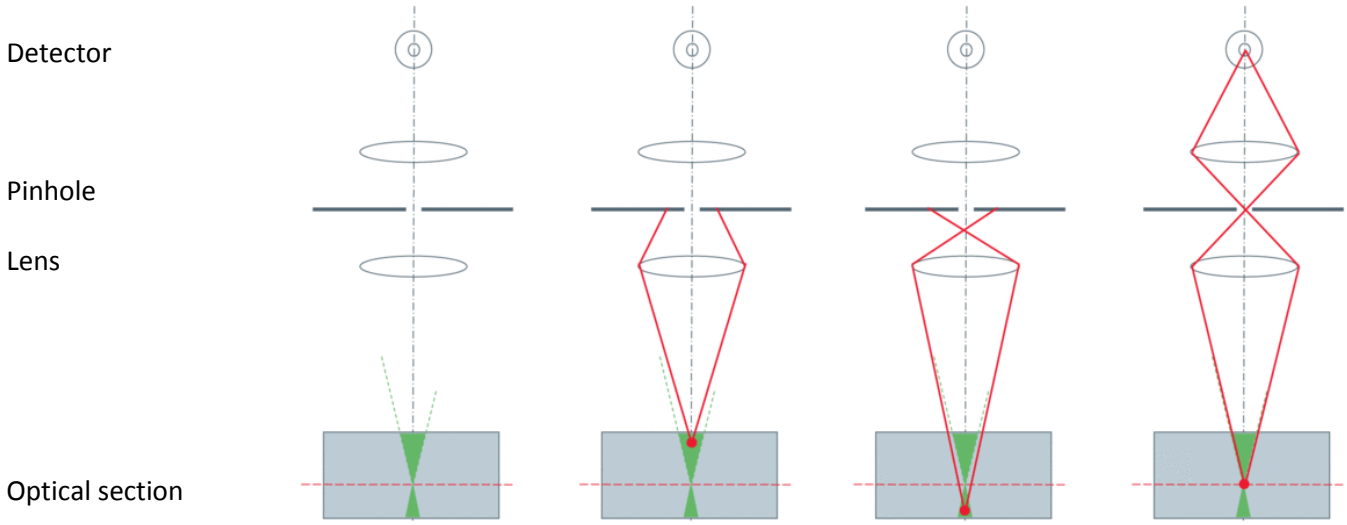
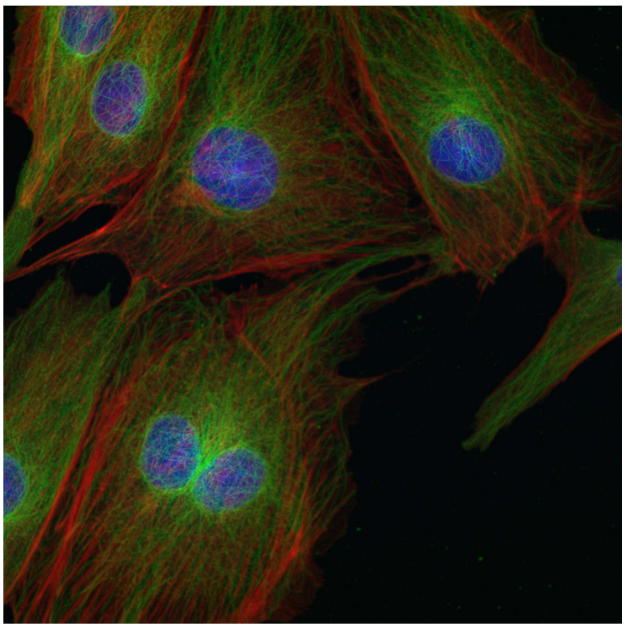
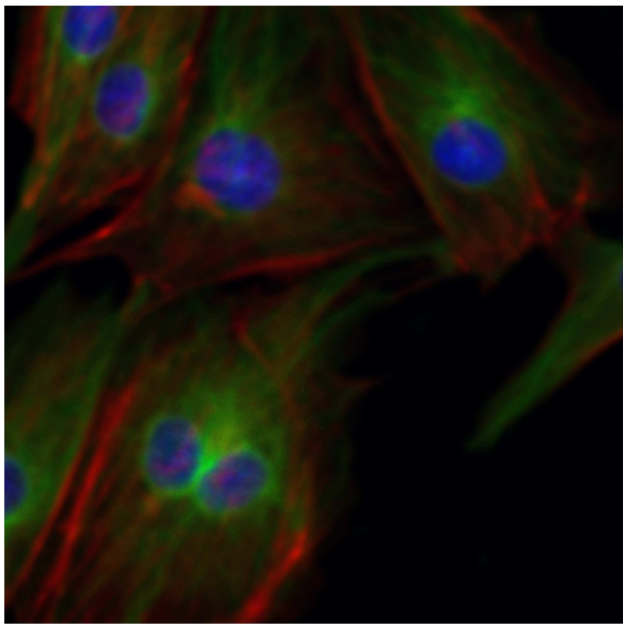


Image from http://www.leica-microsystems.com/science-lab/confocal-microscopy/

The pictures below show the same image captured with the pinhole set at 1AU and at the maximum setting, The picture taken at 1AU shows more detail due to higher z-resolution and a reduced out of focus blur



Pinhole 1AU



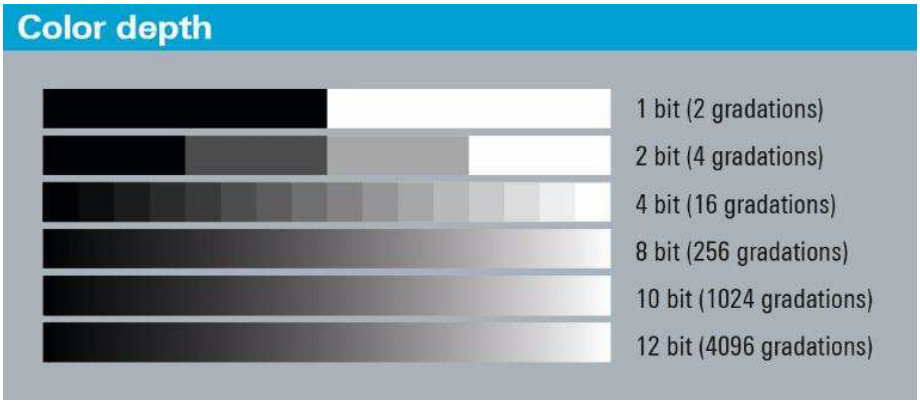
Pinhole max

Bit Depth

Remember even though we see the images on the screen in colour the photo multiplier tube (PMT) on the confocal is capturing each channel independently and recording the intensity value of each pixel and then applying a false colour to that channel. The human eye can only discriminate around 60 gray levels or around 6bit.

You can set the bit depth in the **Acquisition Mode** the higher the bit number the more graduations the signal from the PMT is split in too.

The higher the bit rate the larger the data file.



From: http://www.root6.com/broadcast-engineering/ultra-high-definition-with-high-dynamic-range-uhd-hdr/

In depth - http://zeiss-campus.magnet.fsu.edu/articles/basics/digitalimaging.html#bitdepth

Fluorescence Microscopy and Fluorescent proteins

http://zeiss-campus.magnet.fsu.edu/articles/basics/fluorescence.html

http://zeiss-campus.magnet.fsu.edu/articles/probes/index.html

http://zeiss-campus.magnet.fsu.edu/referencelibrary/basics/fluorescence.html

http://zeiss-campus.magnet.fsu.edu/referencelibrary/fluorescentproteins/fpindex.html

Frame size / number of Pixels

How many points do you need to sample, to get the best resolution of your image?

The number of points you sample on your image can be changed using the **Frame size,** there are standard frame sizes listed starting at 128x128. (That is the image is split into a grid with 128 sample points by 128 sample points or pixels, a total of 16,384 pixels) up to 6144x6144 pixels (37,748,736 total).

The more points you use however the longer the time required to scan the image and also the larger the data file for the image.

The table below show this info for an image that was 66.9x66.9um in size.

Frame Size	Pixel size (um)	Scan Time (s)	Data size (Mb)
128x128	0.53	1.45	0.096
256x256	0.26	5.81	0.38
512x512	0.13	23.23	1.5
1024x1024	0.07	92	6
2048x2048	0.03	269	24
4096x4096	0.02	855	96
6144x6144	0.01	2220	210

However more is not better - there is a limit to the resolution

The limits to resolution are to do with the Numerical Aperture of the objective, Point Spread Function, Rayleigh Criterion, Airy Disk, which can be further reading.

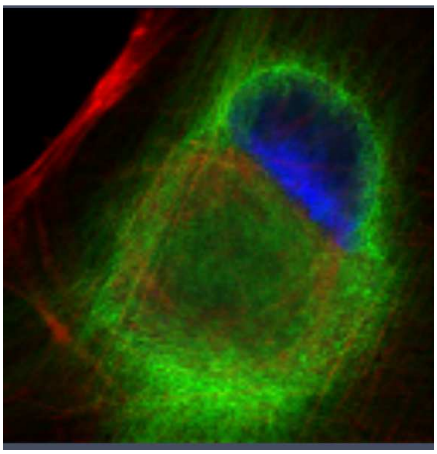
http://zeiss-campus.magnet.fsu.edu/articles/basics/psf.html

http://zeiss-campus.magnet.fsu.edu/tutorials/basics/airydiskbasics/indexflash.html

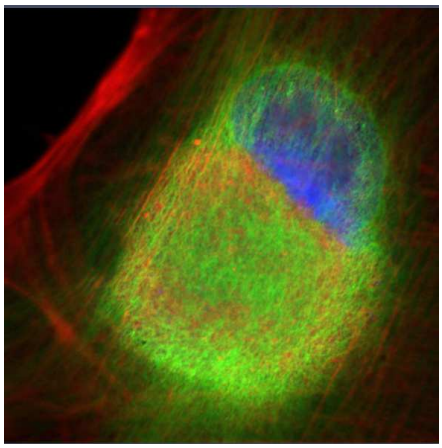
The software will do the calculations for you if you click on **Optimal.**

For the above image clicking on optimal gives

Frame Size	Pixel size (um)	Scan Time (s)	Data size (Mb)
880 x 880	0.08	79	4.4



128 x 128pixel



880 x 880 pixel

You can also increase the number of pixels by zooming into the image keeping the frame size the same.

Photo bleaching

The image below shows a time lapse experiment where each pixel is sampled 16 times for 6.3us per frame (0.1ms/ frame). This series of images shows the effect of over exposure to the laser light on the fluorophores, they bleach with more exposure to the laser.

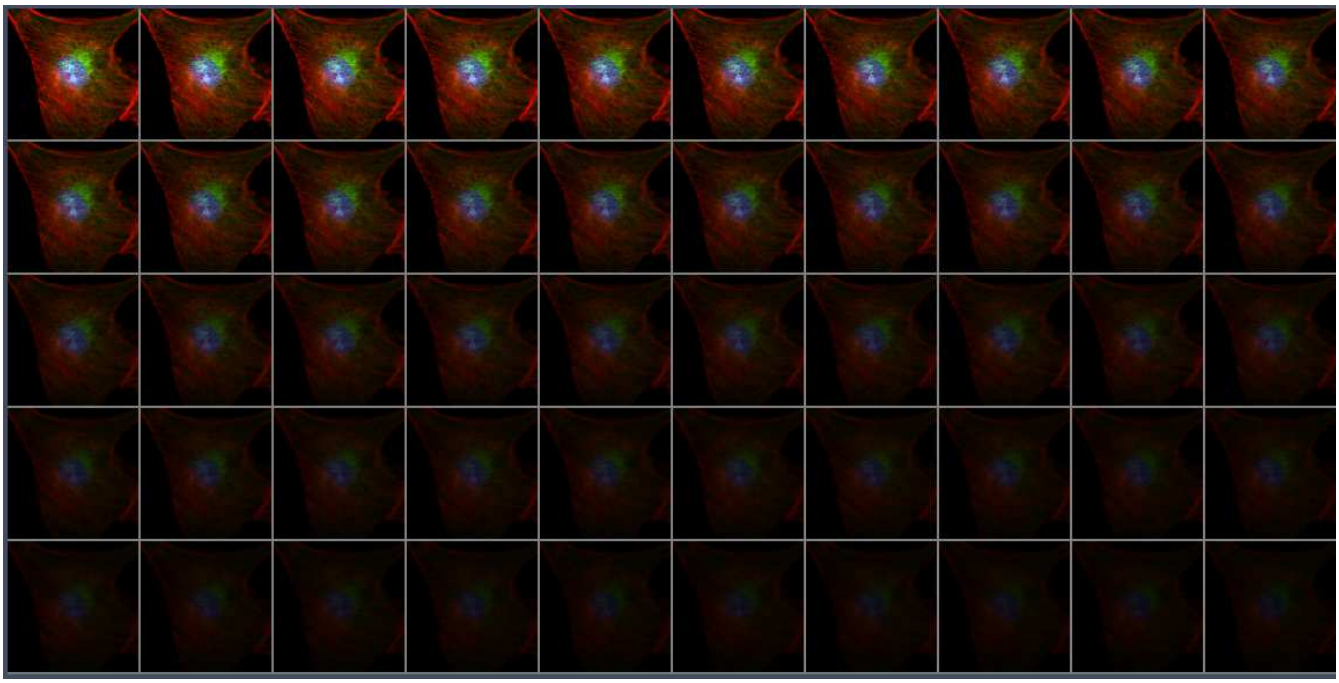


Image storage and manipulation

All images should be saved in the .czi format, any manipulation of images should be carried out on a copy of the original image and the manipulated image should be stored with the original image. The .czi stores a large amount of meta data regarding the image.

You can also open a saved image within Zen and re-use the settings which sets up the microscope to all the settings of the original image

A couple of articles on data manipulation

Cromey D.W. Avoiding Twisted Pixels: Ethical Guidelines for the Appropriate
Use and Manipulation of Scientific Digital Images, *Sci Eng Ethics*. 2010 December ; 16(4): 639–667.
doi:10.1007/s11948-010-9201-y.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4114110/pdf/nihms575182.pdf

Abstract
Digital imaging has provided scientists with new opportunities to acquire and manipulate data using techniques that were difficult or impossible to employ in the past. Because digital images are easier to manipulate than film images, new problems have emerged. One growing concern in the scientific community is that digital images are not being handled with sufficient care. The problem is twofold: (1) the very small, yet troubling, number of intentional falsifications that have been identified, and (2) the more common unintentional, inappropriate manipulation of images for publication. Journals and professional societies have begun to address the issue with specific digital imaging guidelines. Unfortunately, the guidelines provided often do not come with instructions to explain their importance. Thus they deal with what should or should not be done, but not the associated 'why' that is required for understanding the rules. This article proposes 12 guidelines for scientific digital image manipulation and discusses the technical reasons behind these guidelines. These guidelines can be incorporated into lab meetings and graduate student training in order to provoke discussion and begin to bring an end to the culture of "data beautification".
Rossner M, Yamada KM. What's in a picture? The temptation of image manipulation. Journal of Cell Biology. 2004; 166(1):11–15. [PubMed: 15240566]
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2172141/

From Natures guidelines for submissions http://www.nature.com/authors/policies/image.html

Microscopy

Authors should be prepared to supply the editors with original data on request, at the resolution collected, from which their images were generated. Cells from multiple fields should not be juxtaposed in a single field; instead multiple supporting fields of cells should be shown as Supplementary Information.

Specific guidelines: Adjustments should be applied to the entire image. Threshold manipulation, expansion or contraction of signal ranges and the altering of high signals should be avoided. If "Pseudo-colouring" and nonlinear adjustment (for example "gamma changes") are used, this must be disclosed. Adjustments of individual colour channels are sometimes necessary on "merged" images, but this should be noted in the figure legend.

We encourage inclusion of the following with the final revised version of the manuscript for publication:

- In the Methods, specify the type of equipment (microscopes/objective lenses, cameras, detectors, filter model and batch number) and acquisition software used. Although we appreciate that there is some variation between instruments, equipment settings for critical measurements should also be listed.
- A single Supplementary Methods file (or part of a larger Methods section) titled "equipment and settings" should list for each image: acquisition information, including time and space resolution data (xyzt and pixel dimensions); image bit depth; experimental conditions such as temperature and imaging medium; and fluorochromes (excitation and emission wavelengths or ranges, filters, dichroic beamsplitters, if any).
- The display lookup table (LUT) and the quantitative map between the LUT and the bitmap should be provided, especially when rainbow pseudocolor is used. If the LUT is linear and covers the full range of the data, that should be stated.
- Processing software should be named and manipulations indicated (such as type of deconvolution, three-dimensional reconstructions, surface and volume rendering, "gamma changes," filtering, thresholding and projection).
- Authors should state the measured resolution at which an image was acquired and any downstream processing or averaging that enhances the resolution of the image.

Editorials providing more detail for these policies:

All scientists should find the time to understand the software packages that they use to collect, analyse and display their data, and share this knowledge with new researchers. *Nature Nanotechnology*. Image rights and wrongs, September 2007.

In response to recent cases of image manipulation, the Nature family of journals is now "spot-checking" images from randomly chosen papers. *Nature Immunology*. Spot checks, March 2007.

Nature journals have prepared new guidelines in an attempt to clarify boundaries of acceptability in preparing images for publication. *Nature Methods*. A picture worth a thousand words (of explanation), April 2006.

Guidelines for acceptable image presentation. *Nature Cell Biology*. Appreciating data: warts, wrinkles and all, March 2006.

Data beautification is unacceptable in published research. *Nature Cell Biology*. Beautification and fraud, February 2006.

Nature's new guidelines for digital images encourage openness about the way data are manipulated. *Nature*. Not picture perfect, 23 February 2006.

What constitutes best practice in data display? *Nature Cell Biology*. Gel slicing and dicing: a recipe for disaster, April 2004.