Bioimage Analysis

Basics of Imaging

The signal

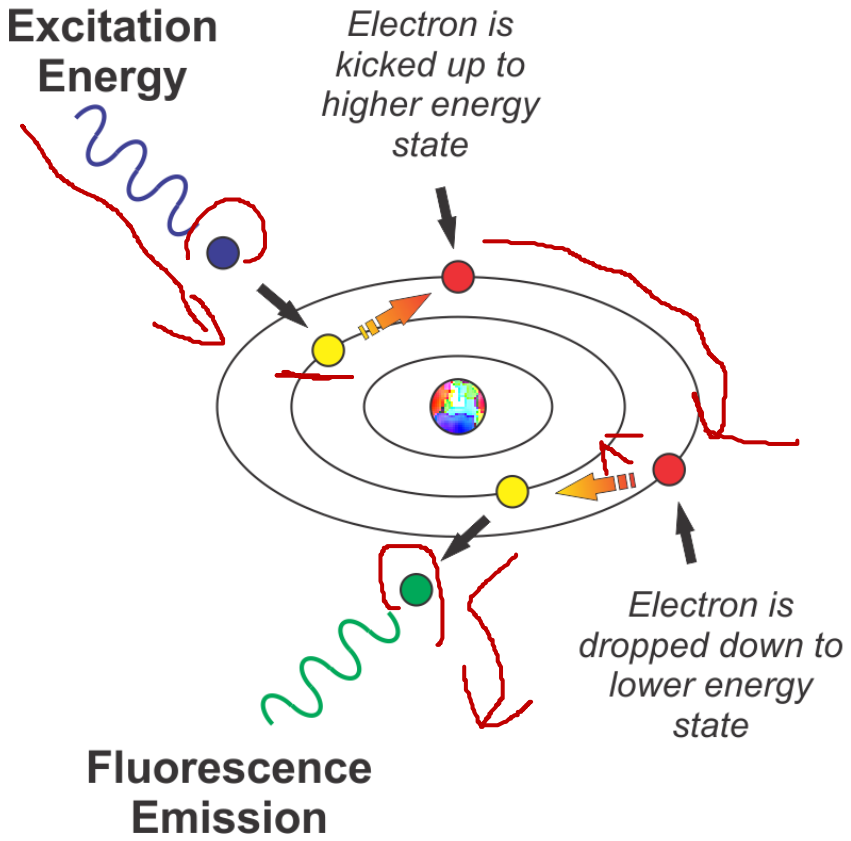
So, now that we have some understanding on what an image actually is, we can start thinking about how images come to be.

To put precisely, we will discuss what the signal is and how we measure it. Remember, most of the time we are not taking pictures, we are doing measurements.

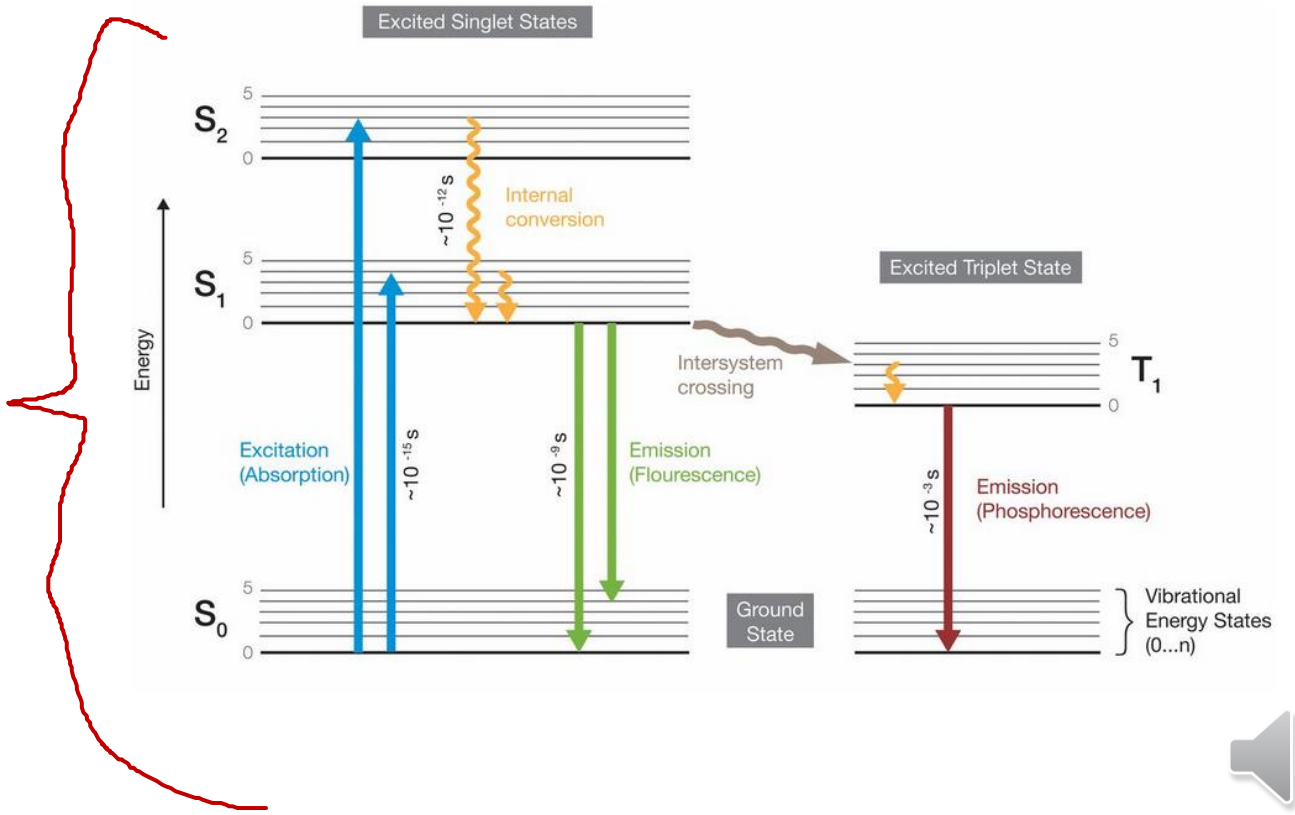
The signal of course depends on what we want to measure, but as we are discussing light microscopy our raw signal will inevitably be composed of photons.

Fluoresence

Works roughly like this.

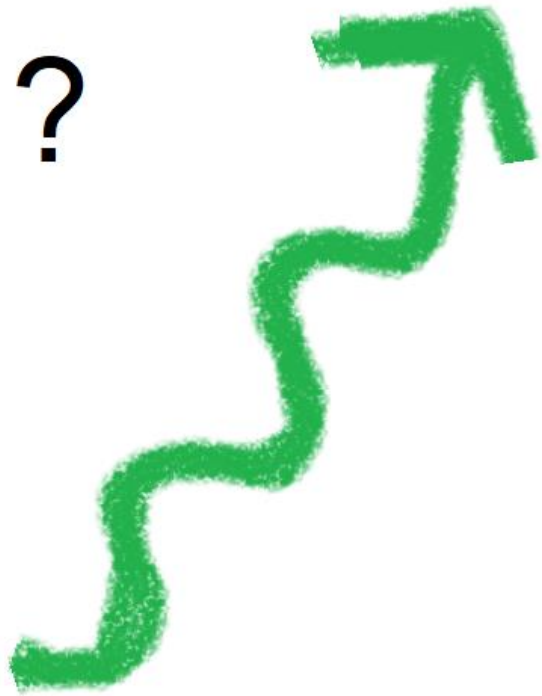


But that is simplified.
This is more realistic.



And that is just the excitation point

Additionally, few other things may happen to the photon before it reaches the fluorophore or the detector.



Refraction
Light can be refracted, i.e. scatter because of collision with something substantial such as electrons. Scattered light might end up where want, or it might not.

Absorption
Light can be absorbed by the sample. So the photons are lost on the way to the sample, or on the way from the sample.

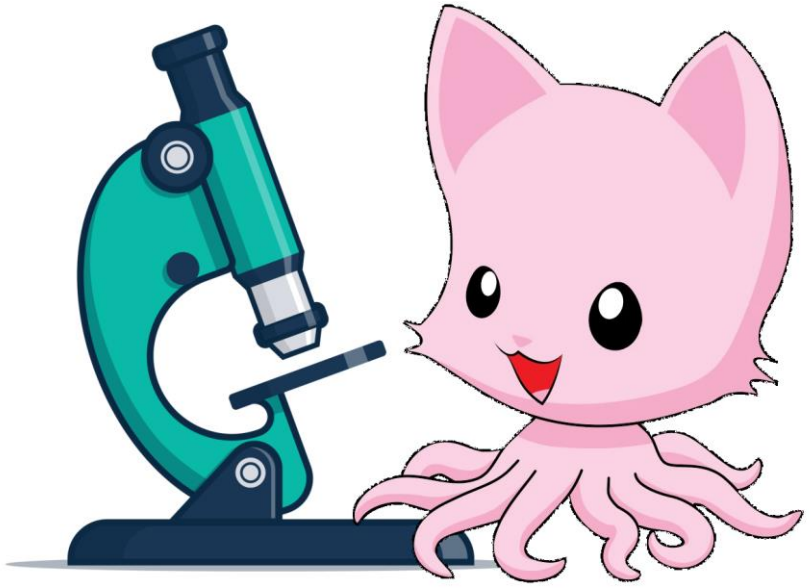
Both these effects happen more often the longer the photon has to travel through the sample. This has implications.

Modalities

Capturing the light

We'll focus on the two modalities bioscientists are most likely to encounter in the laboratory, though let it be noted that this is not the Full Story ™.

First we'll walk through how widefield microscopes work and then we'll walk through how confocal microscopes work.



Widefield Microscopes

Widefield microscopes

Widefield microscope acquires an image of the entire field at once. This is often simple and robust*.

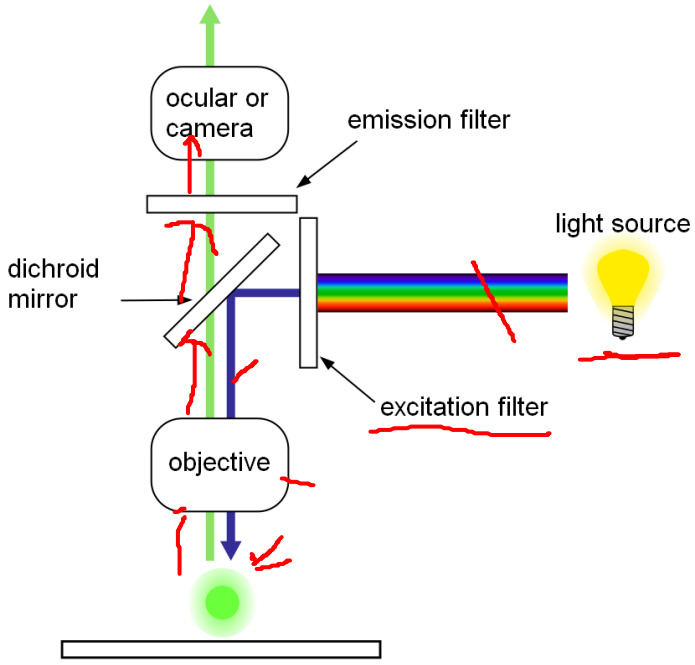


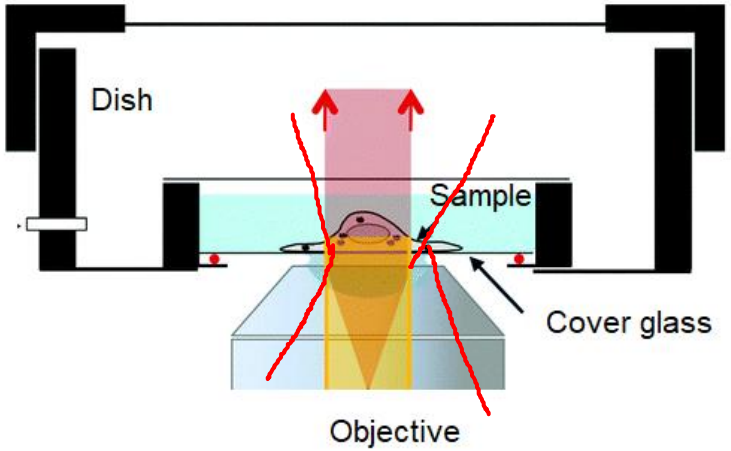
Image aqcuisition time is often called 'exposure time' during which sample is exposed to the light and the emission signal is collected.

The longer the exposure time, the more signal we get.

*Terms and conditions apply.

Widefield microscopes

Widefield microscope bathes the sample in light. Everything in the field of view will be excited. Everything in focus, everything out of focus.*



You really don’t want to imagine more than a single layer of cells.

* Of course we get bit less signal from the out of focus areas, but still if you sample is thick, your image is going to be messy.

Widefield microscopes

Can be pretty darn fast. With **good** dye/stain, exposure times of 100-300 ms are often enough, allowing you to get 3-10 images per second.

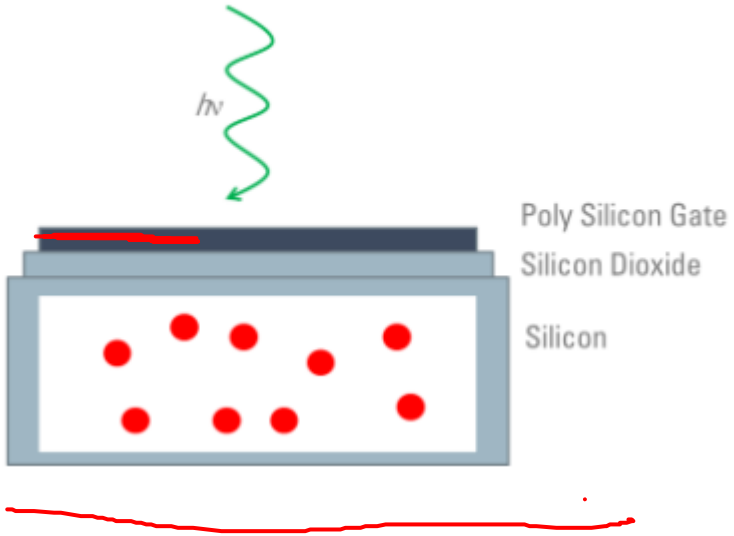
Relatively easy to use – just point at what you want to see, set exposure time´, chose filters and you are ready to go.

Depth resolution is poor / nonexistent. You can take stack and deconvolve but confocal is almost always better.

Widefield microscopes

Detection

Microscopy camera chips consist of millions of photodiodes. Incoming photon energy is used to excite electrons of the silicon which are in turn collected in a storage well and afterwards transferred to an amplifier. This process is almost linear.



Imaging parameters (WF)

Exposure time
The more time you use to expose the image, the better signal you will get. The more time you use, the more you will burn your sample.

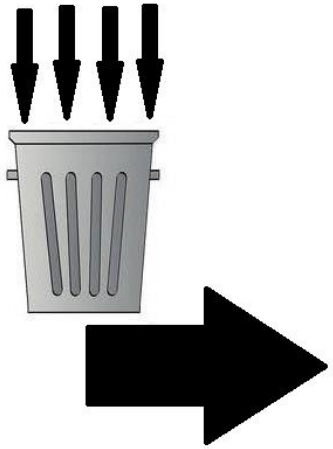
Optical magnification
Objective as well as various lenses in the light path determines the pixel size, effectively determining the resolution.

Binning
Can be used to gain more signal at the cost of resolution.

Binning

Binning means combining pixels so that intensity values become higher, but we get fewer pixels.

This has fringe uses when the sample is dim and/or we want to acquire images quickly.



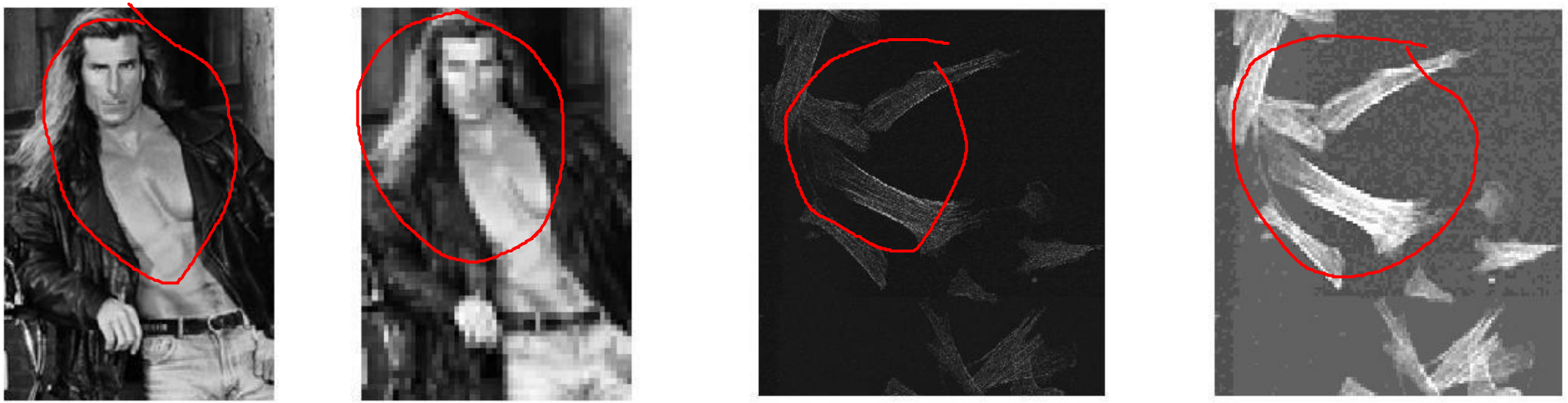
0	10	20	20
0	10	20	40
20	30	40	60
20	40	60	80

2x2 binning

20	100
110	240

Binning

Let’s have a look how it works. So let’s bin poor Fabio and some cells.



With Fabio, there was no need for this operation. This image is shown to explain how binning affects the resolution.

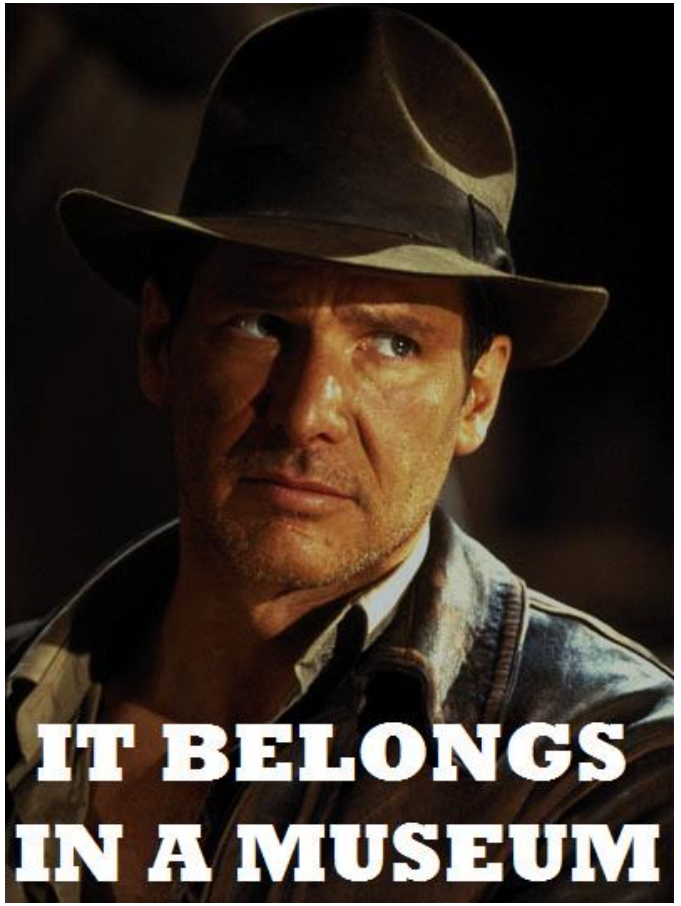
However with the cells, the signal is greatly magnified.

Binning

That being said, you only really need binning if your signal is really poor, or if you are working with a stain that disappears when you look at it.

If you find yourself in such a situation, the first thing you should do is optimize your protocols and get a better label.

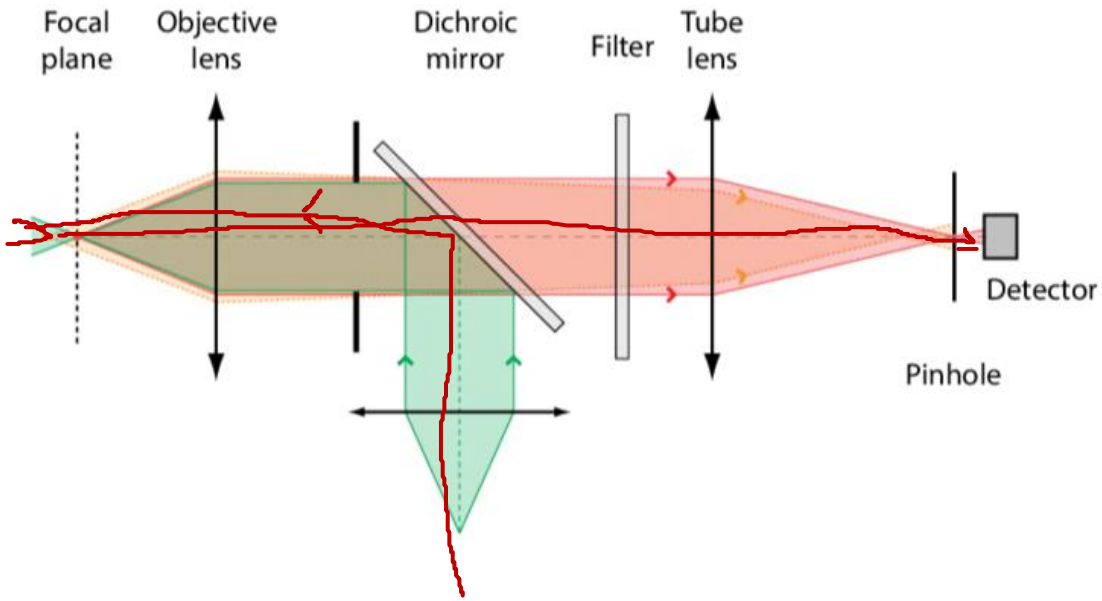
In cases where you need as many pictures as possible quickly as possible (short exposure times), binning might actually help.



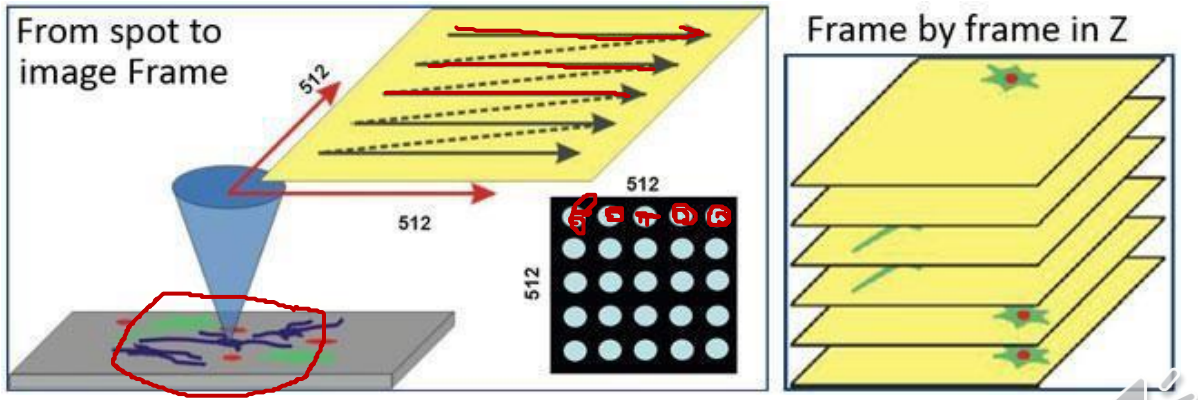
Confocal Microscopes

Scanning confocal microscopes

Magic word here is 'scanning'. Laser is used to excite the fluorophores at each pixel individually. Normal scanning speeds being around 400 Hz, or 400 lines / second, this means that laser spends less than 5 microseconds in one pixel.

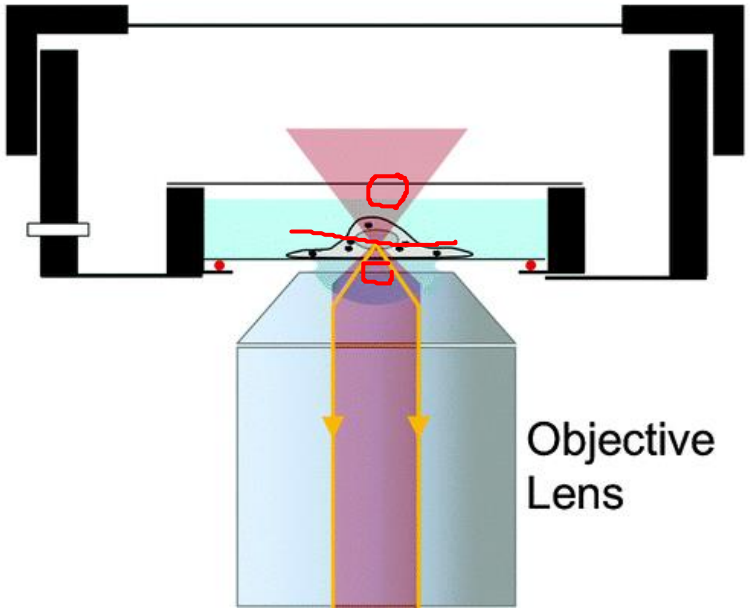


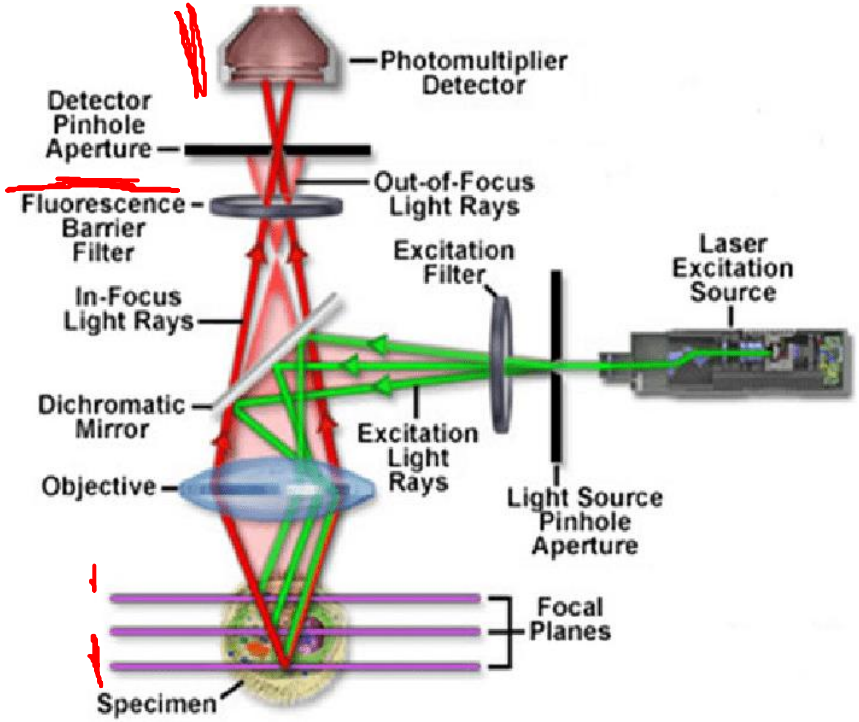
Yes, the sample is literally scanned



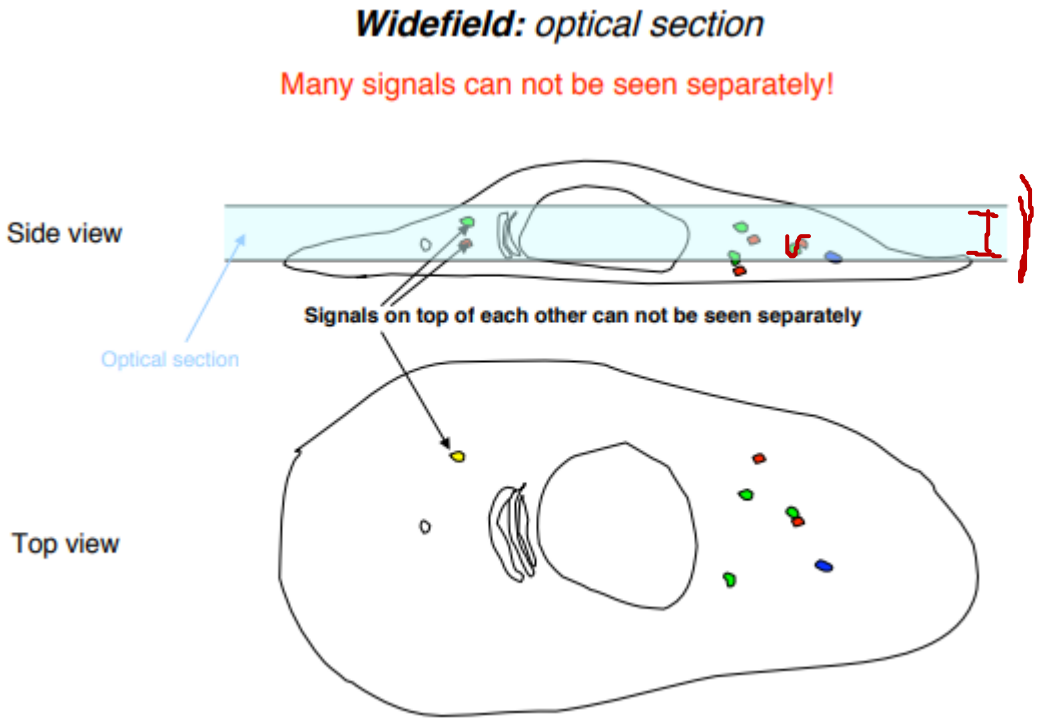
Scanning confocal microscopes

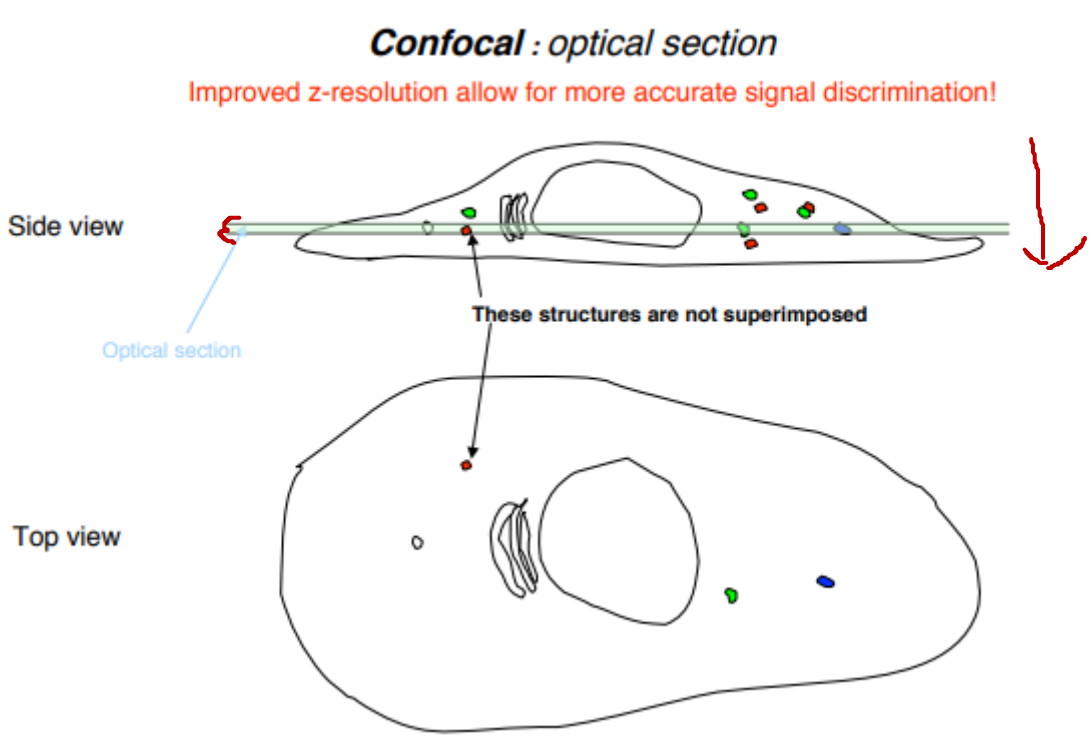
Pinhole us used to remove out of excitation light meaning that while the laser illuminates ‘everything’ we only measure signal from the focal point.





Confocal v.s. widefield





Scanning confocal microscopes

Detection
The first part of a photomultiplier tube is a photocatode that emits electrons when hit by a photon.

The signal is then amplified by a cascade of dynodes and the signal is measured at the anode.

Voltage dropping resistors determine how much amplification is applied – this is the **gain** you can adjust on the panel.

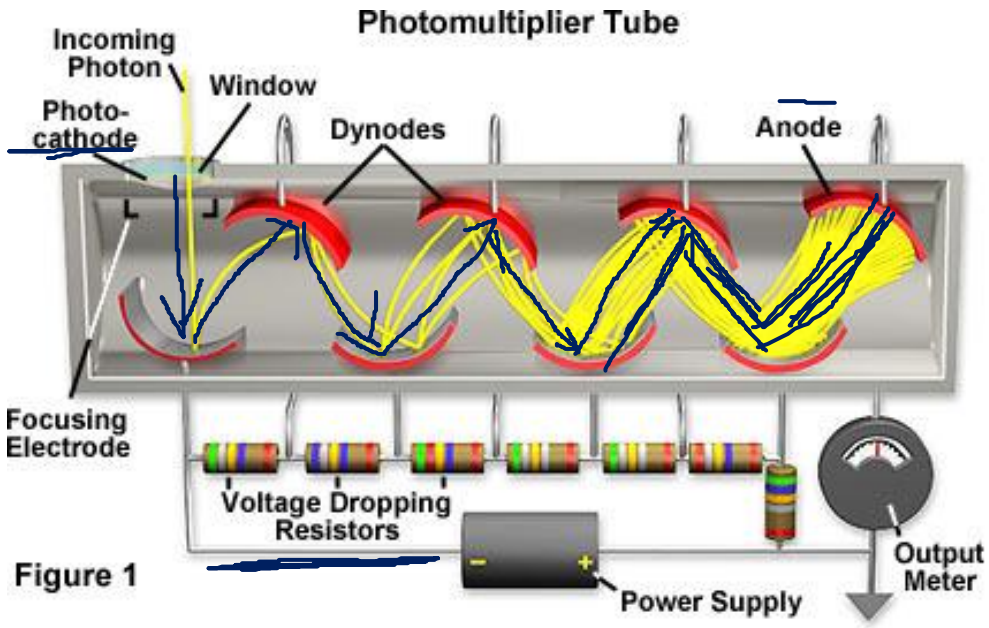
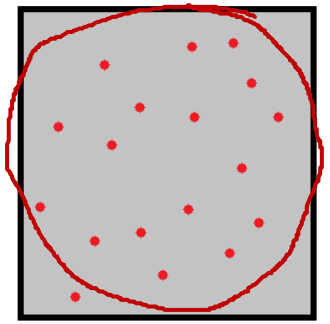


Figure 1

Imaging parameters (confocal)

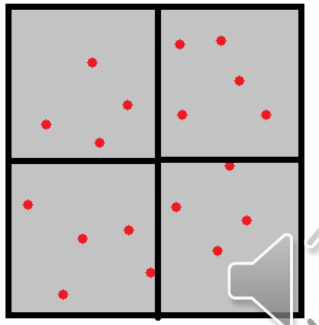
Scanning speed
The faster you scan, the faster you get an image and the less you bleach your sample. However, the faster you scan, the worse signal you get.

Zoom/format (or pixel size)
Boils down to the question, how large part of the sample lays under one pixel. Larger the pixel size, the better the signal. Smaller the pixel, the better the resolution… to a limit.



Gain/Offset

Increased gain increases the signal and also the noise. Offset can be used to reduce noise and signal.



Imaging parameters (confocal)

Scanning speed combined with the **image format** give a parameter called ‘pixel dwell time’.

As you might guess this tells you how long the laser stays on a single pixel before moving onward. **Pixel dwell times** are usually in the range of microseconds.

The longer the laser stays in one place, the less noise we get. On the other hand, the longer the laser stays in one place, the slower the whole thing is.

Gain and offset

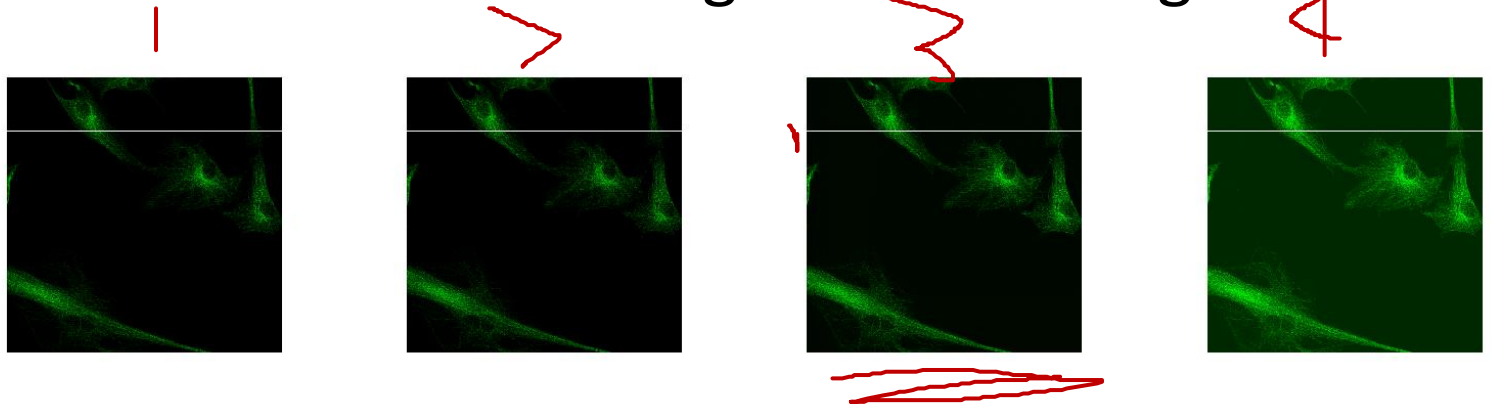
Offset tells us how low signal we regard as zero. This can be used to remove the background noise when it is homogenous.

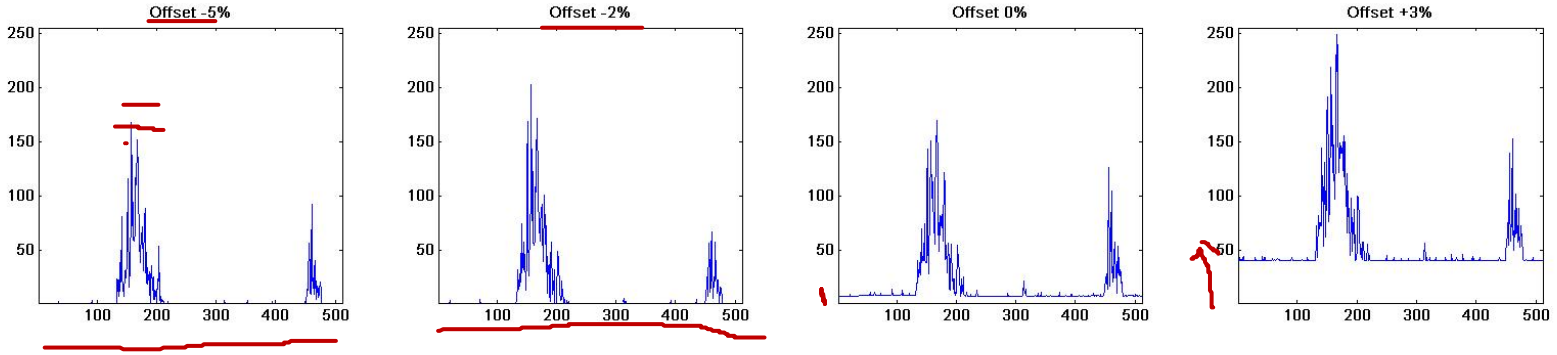
Gain tells us how much the signal is amplified. This parameter is not linear even if Leica tells you it is.

Gain, scanning speed, laser power and resolution should be adjusted to avoid overexposure and optimized according to you needs.

Offset

Offset is user to correct for the background. Poorly adjusted offset introduces additional signal to the image.



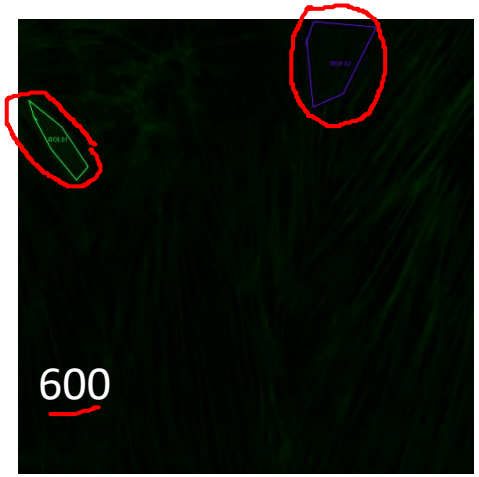


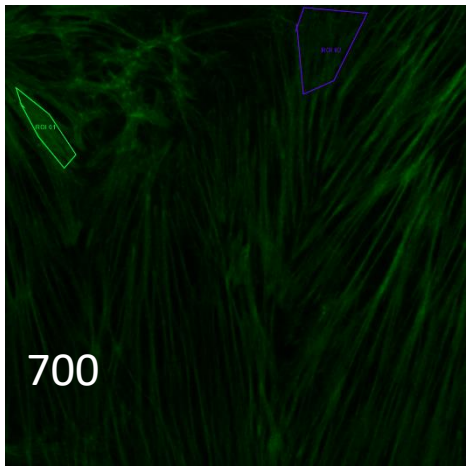
If you wish to quantify structures, you can set offset pretty haphazardly. ‘Good enough’ will do.

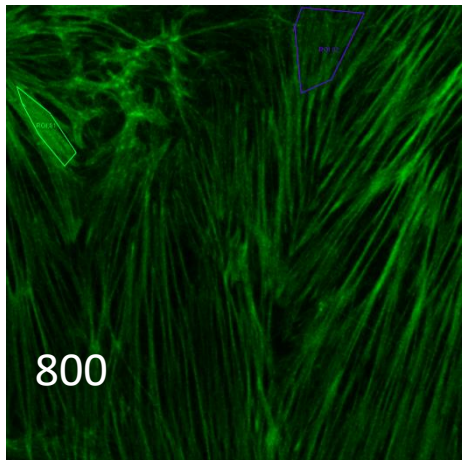
If you want to quantify intensity with a confocal, you need to evaluate your life choices.

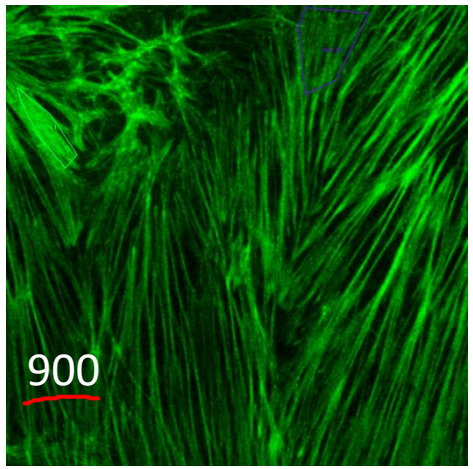
Gain

I took a picture of the same location using same laser power but different gains. The fraction is stable only really in narrow band of gain.









	600	700	800	900
ROI1	20,39	51,4	111,01	182,44
ROI2	9,73	23,26	50,36	85,25
Fraction	2,095581	2,209802	2,204329	2,140059

This has several implications on measuring intensity. You must use the same settings for all samples, but not all samples are visible with the same settings.

Noise

Noise, or unwanted signal, is present in every image. This is mostly ‘physics happens’ kind of a problem.

You can reduce the noise almost always, but this improvement comes at a price. The price you pay is time, either to prepare a better sample or spend more time measuring your data.

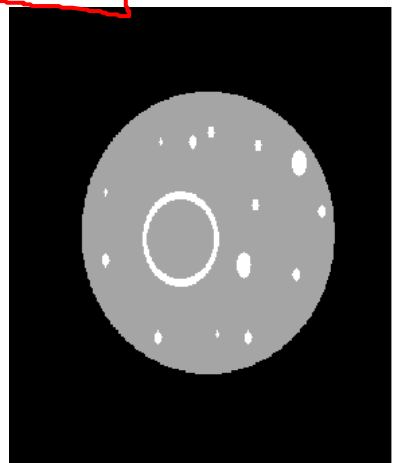
Post-processing only ever alleviates the problem. The problem can be only solved by preparing a better sample or recording better images.

Averaging

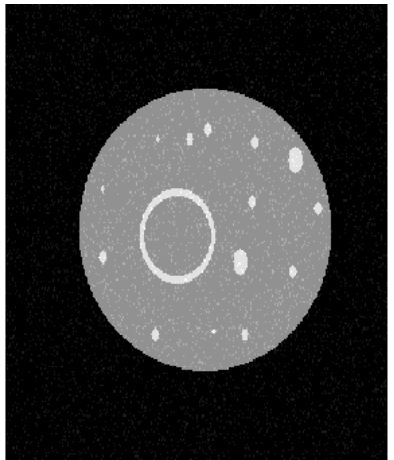
Averaging images is a commonly used procedure with noisy images used to reduce the noise and so increase the signal to noise ration.

Let’s say there is some ’truth’, a cell where we have stained vesicles of some sort. Then let’s say we acquire three pictures of it. All of them are bit grainy because of measurement errors.

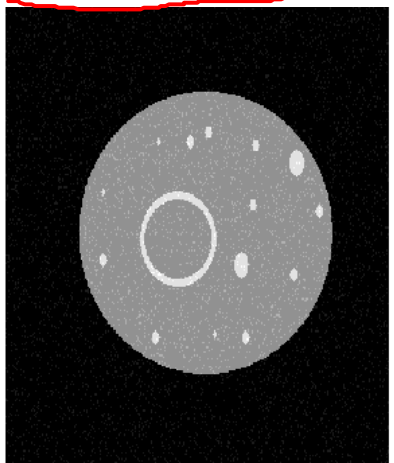
Truth



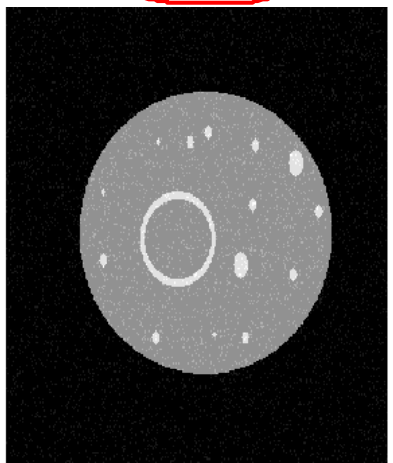
Measurement 1



Measurement 2



Measurement 3

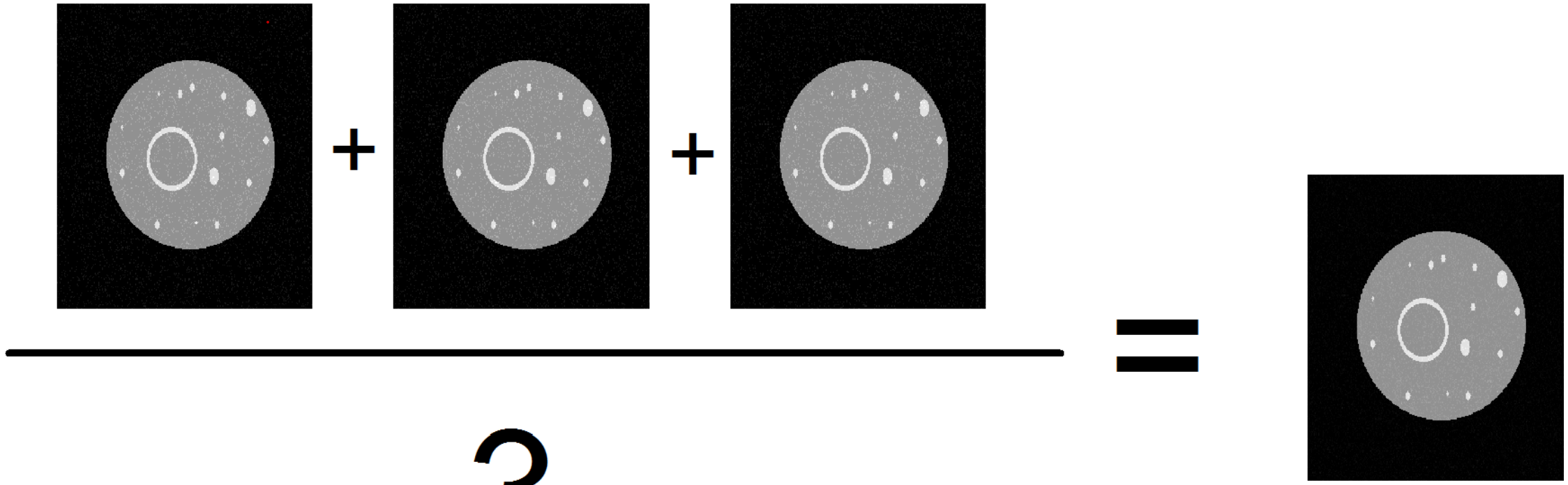


Averaging

$$\frac{6+0+0}{3} = 2$$

Basically works basically like this.

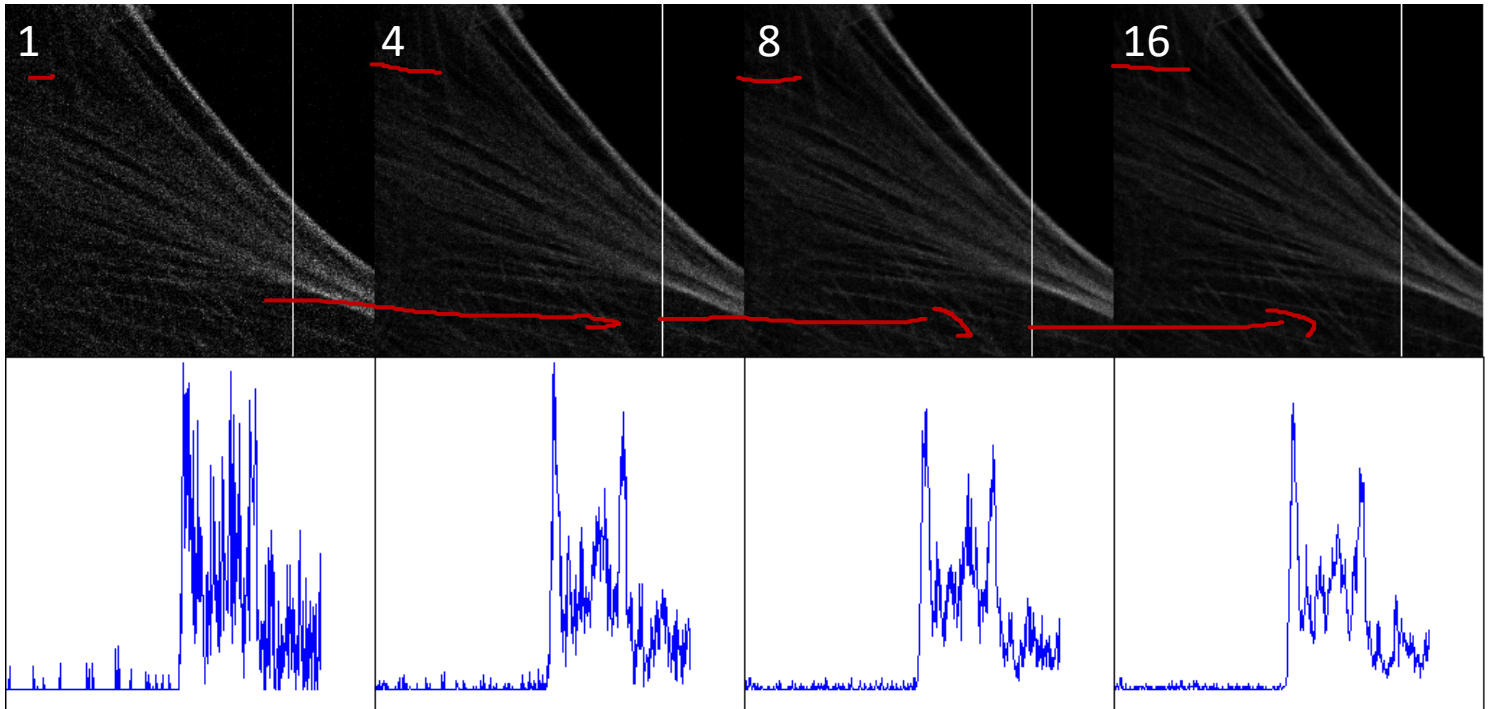
1 2 3



Averaging

You can always increase the time used to record the data.

This is an easy solution, but unfortunately it increases the time needed and sometimes you don't have the time, or your sample cannot take it.



Same data recorded with average of 1,4,8 and 16.

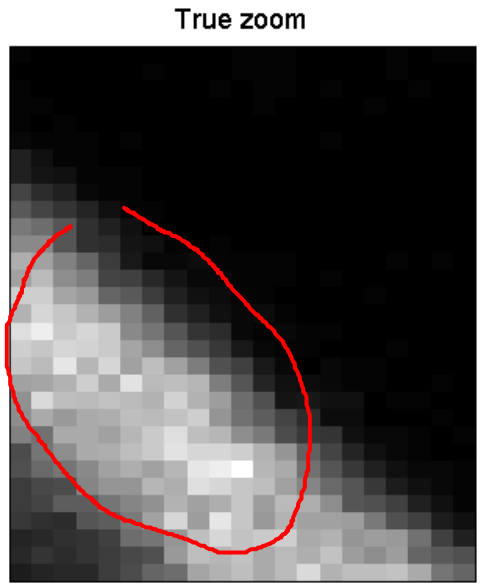
As we increase the averaging, the data becomes less noisy.

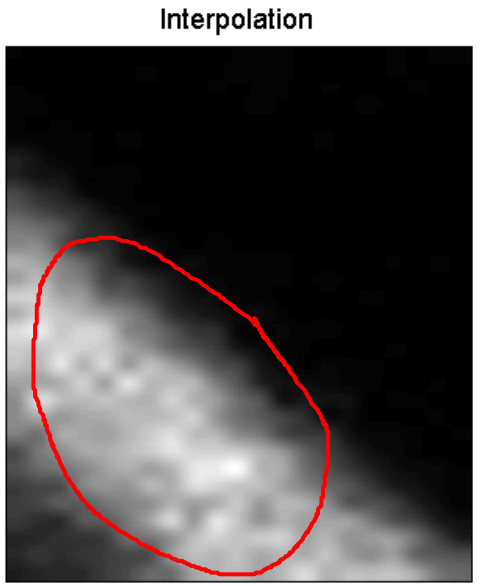
Noise is reduced by the factor $\frac{1}{\sqrt{N}}$ where N = average!

Mind your tools

Care must be taken when viewing images. Many of the tools meant for photography and general image manipulation automatically smooth the images for the user.

This is less of a problem when you are looking at cat pictures, but when dealing with data caution must be taken.

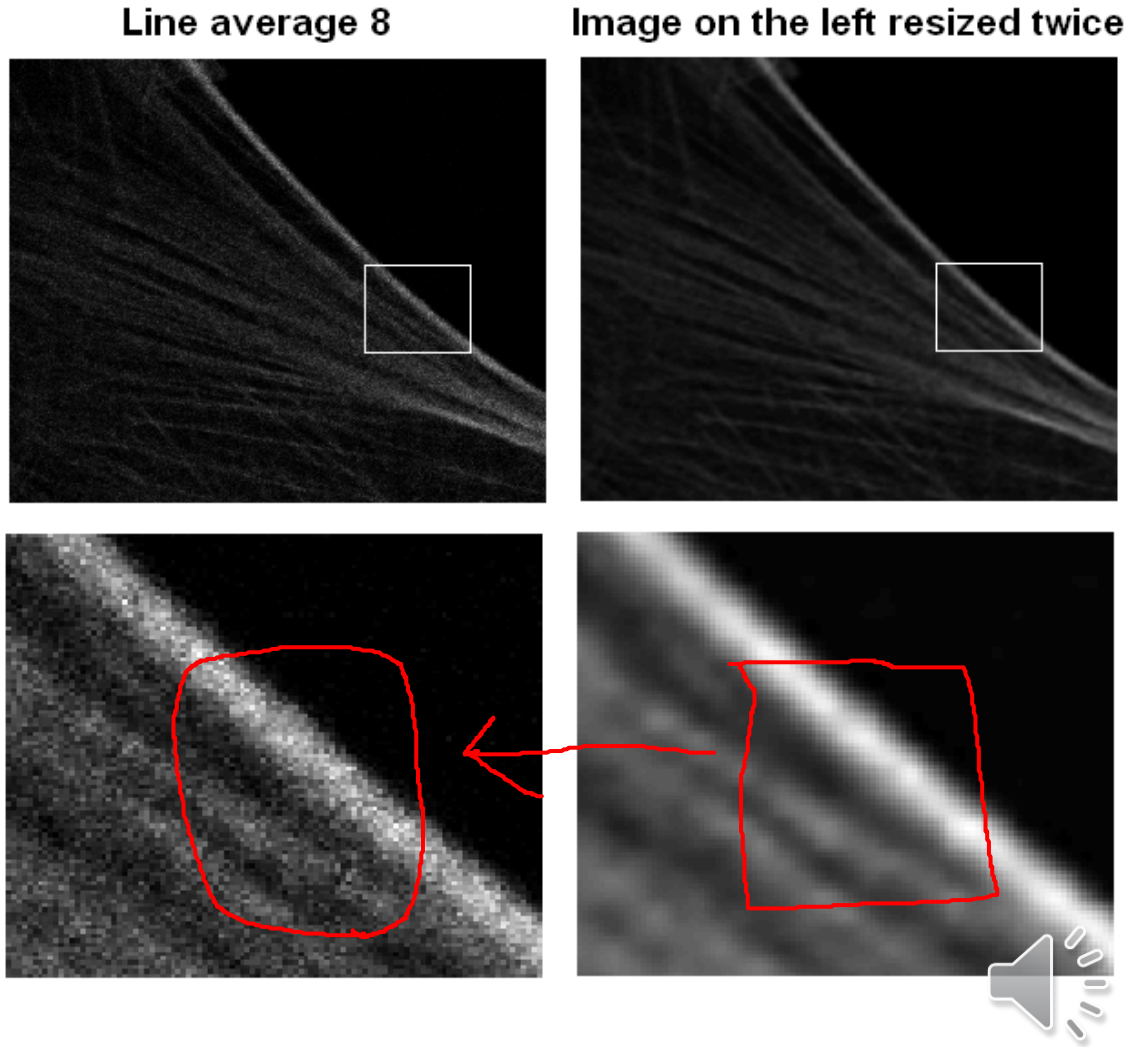




Resizing

If and if you need to resize your images, that is the last thing you do to them before attaching the to a document.

Image on the right has been shrank by factor of four and then expanded again to the same size as before.



To wrap things up

Quick note on post-processing

You often hear claims that image quality can be improved after acquisition. This depends a lot on what you mean by quality.

Any and all attempts to reduce noise in post-prosessing will also reduce resolution. Deconvoluton is an exception to this.

Any and all attempts to increase resolution will also increase noise.

Noise and signal are both defined as ‘sudden change of intensity’, which means that coming up with mathematical tools that affect one without affecting the other are hard to come by.

On optimization

When you start a new imaging project where you need to measure something from the pictures, it is often good to spend one session taking pictures with various settings.

This allows you to figure out how quickly you can record usable data. This is especially crucial when you are working with 3D data (so confocals) as shortening imaging time even few minutes per stack could mean hours of saved time.

Concept of worst usable image

Ideally you do not want to spend any more time at the microscope than absolutely necessary.

If you take better images than you need, you are wasting time and money. If you need lots of data, this cost might be considerable.

You should establish ‘worst usable image’ that gives you the information you need while using as little time as possible. This might require some optimization.

Questions