Bioimage Analysis

Basics of Image - anatomy of an image

What is an image

Image is an array of information.

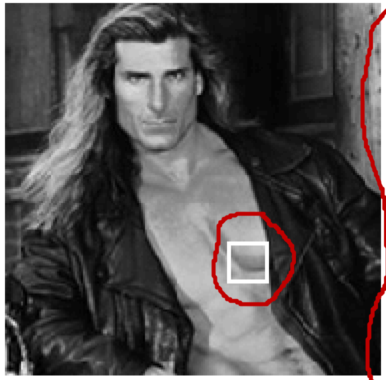
Let’s have a look at Fabio. He’ll be with us for for a while as our ’example’ image.

Fabio is just numbers

Image is an intuitive way to show 2D data.

This data could be for example, intensity of fluorophore at given part of the cell, or it could be Leonardo’s Mona Lisa.

Or it could be Fabio.



<=>

158	169	178	185	183	177	174	177	181	179	184	187	188	189	193	195	195	193	204	186	103
147	165	173	180	181	177	175	176	178	179	183	187	190	192	193	195	195	190	200	191	116
140	155	164	175	178	177	174	173	173	177	179	185	190	192	193	193	194	187	192	187	123
135	138	154	170	176	172	168	169	172	175	177	182	187	189	189	189	189	188	187	183	129
127	130	144	159	166	168	167	168	169	173	176	179	183	185	185	185	184	184	180	176	131
124	130	136	144	152	158	163	163	162	167	170	173	174	177	180	180	178	173	168	163	124
127	124	131	138	141	144	149	154	157	157	161	164	164	168	173	175	172	163	159	153	117
141	127	129	131	134	137	141	145	148	147	150	154	157	159	163	164	159	151	145	141	111
163	146	130	119	123	132	136	138	142	141	144	148	149	147	151	153	151	146	139	133	103
179	163	141	122	121	128	132	134	135	133	137	142	142	139	143	148	147	138	132	127	94
183	173	163	145	128	122	126	130	130	134	136	141	141	137	138	141	139	129	127	123	85
178	181	179	166	143	125	119	121	123	124	126	130	133	133	135	135	132	128	125	113	71
165	180	181	178	166	147	128	118	116	120	119	122	126	128	130	129	125	119	117	98	59
156	168	174	180	180	170	152	134	122	117	112	109	108	105	104	102	98	93	96	79	49
157	157	168	176	177	175	170	155	139	128	121	117	114	111	112	113	111	118	125	107	75
152	147	156	165	175	181	175	162	154	155	147	146	150	150	149	156	164	170	171	167	122
143	143	153	163	171	178	176	168	162	165	165	171	175	175	175	177	178	177	175	173	132
143	151	162	170	174	177	176	170	166	163	166	171	170	169	174	177	175	170	167	170	138
154	165	176	181	179	177	175	170	168	167	168	168	166	165	172	175	172	170	164	168	143
163	168	179	183	181	179	177	174	174	172	170	172	174	175	178	179	175	174	166	166	145
172	171	178	182	182	181	180	179	181	174	170	170	173	175	178	180	179	174	166	162	143

50 Shades of Gray (and more)

We saw that Fabio’s pectorialis major was made from numbers between 80 and 200~ish.

These numbers are actually arbitrary and have to do with the **bit depth** of the image. **Bit depth** explains how many values we have between black and white. We will discuss this later.

The values between black and white are greys, forming a greyscale.

50 Shades of Gray

Black and white image (also known as greyscale image) has some white, some black and many kinds of gray in it.

When people began creating first computer screens and printers, there were to things to be considered.

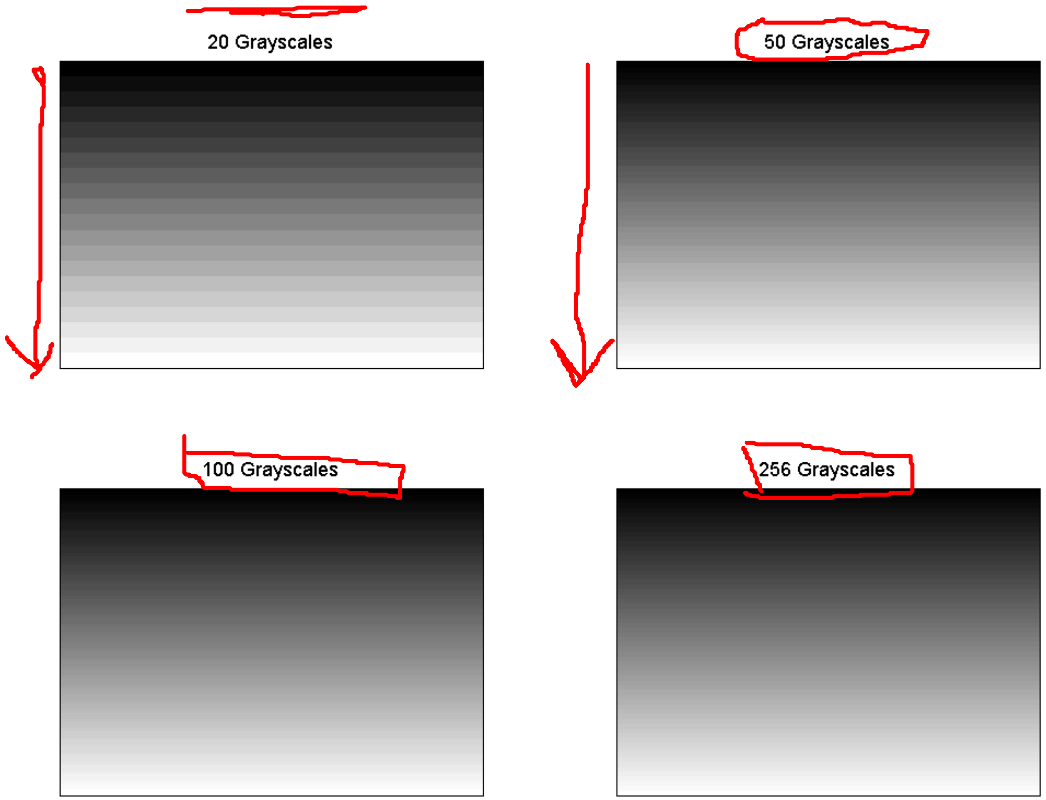
1) How many different greys we can show?
2) How many different greys humans can see?

50 Shades of Gray

Let's take black and add white until we get pure white. Now, let's do these additions in x steps.

Let x be 20, 50, 100 and 256.

As you can see, the transitions become smooth between 50 and 100.



Should we worry about the shades of gray?

Sometimes there is little need to worry. Human eye can only see so much, and if all you want to show is Fabio (or his biological equivalent to him) you can go with whatever the machine does.

On the other hand, if you plan to **quantify** your data, say compare intensities or do colocalization there is all the reason to worry. Because now, the different values are measurements and not just an image.

Whiter shade of pale

An image on a microscope can be acquired with various **bit depths**. This is to say, we get to decide how many numbers there are between black and white.

Bit depth represents the accuracy of division between white and black. The more bits we have, the more shades of grey we have and the more miniscule differences we can show.

8 bits -> 2^8 = 256 grayscales

12 bits -> 2^{12} = 4096 grayscales

16 bits -> 2^{16} = 65536 grayscales

You might recall that already 8 bits will give you a ‘smooth’ image. The additional bits only matter if we want to know how much signal we are getting out.

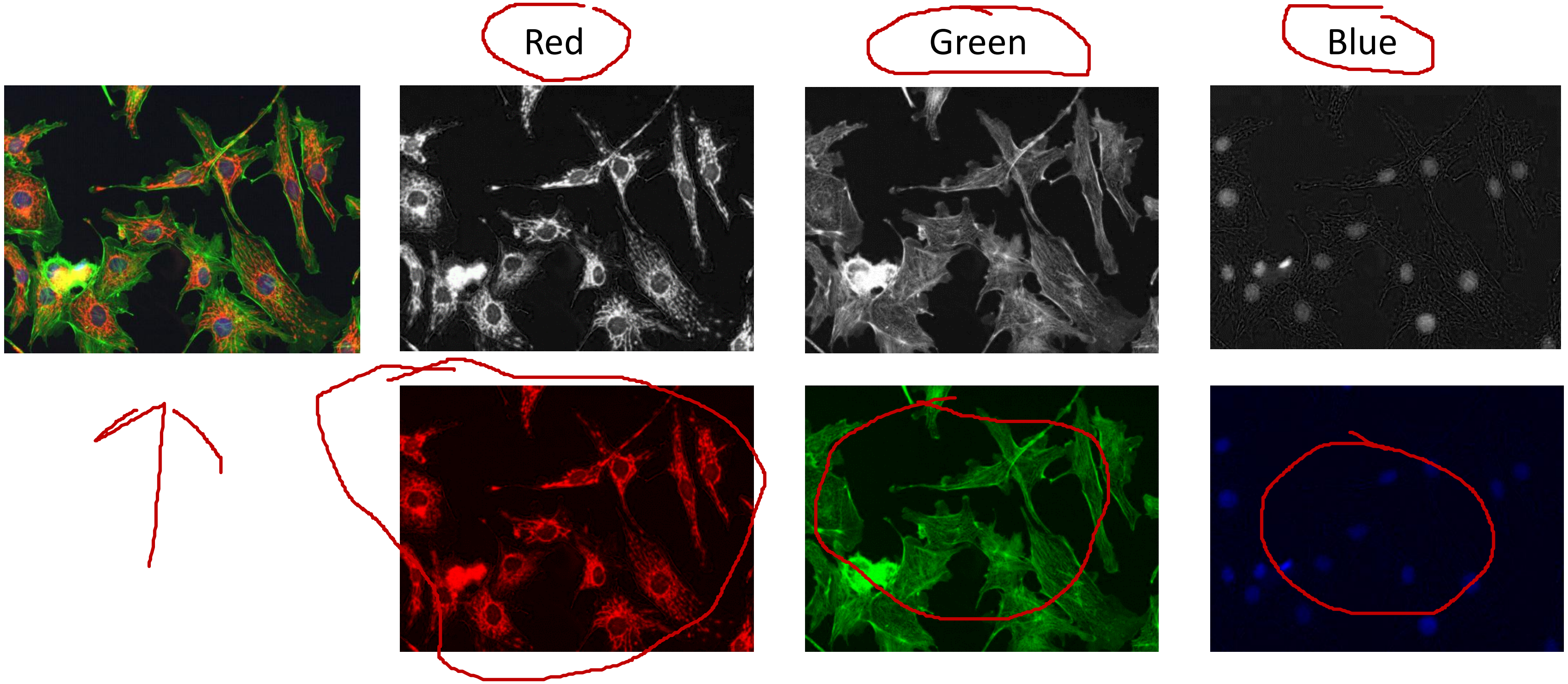
Color images!

RGB stands for red/green/blue and RGB images are basically three greyscale images layered on each other, so that each pixel has three values. This is why we tend to talk about 'green' or 'blue' channel.   Let Fabio demonstrate.

You'll notice that it is hard to see any detail on the blue image.

Microscopy images are not color images

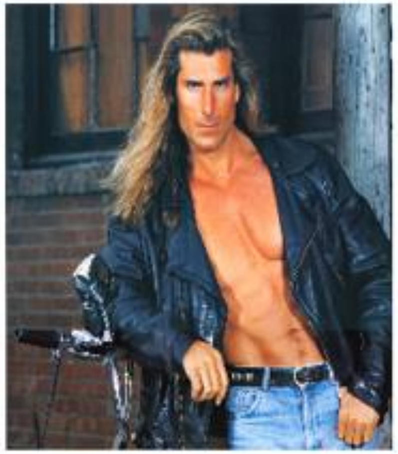
You might think they are, but they really are not.



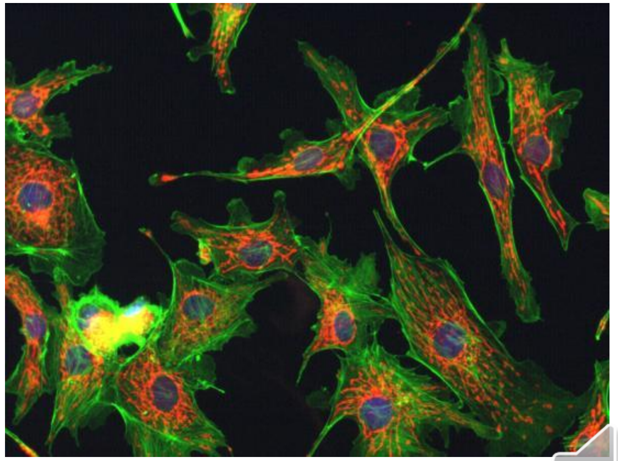
Each channel is greyscale image. In the final product these images tinted to a chosen colour and overlaid.

Picture v.s data

You might be inclined to thing that these are both images and in everyday language this is the case.



However the image below is actually data while the image above is a picture.



While you can take pictures haphazardly, a great care should be taken when acquiring data.

Picture v.s data

The same rules do not apply to pictures and data.

Picture where you cannot see anything is pretty much useless.

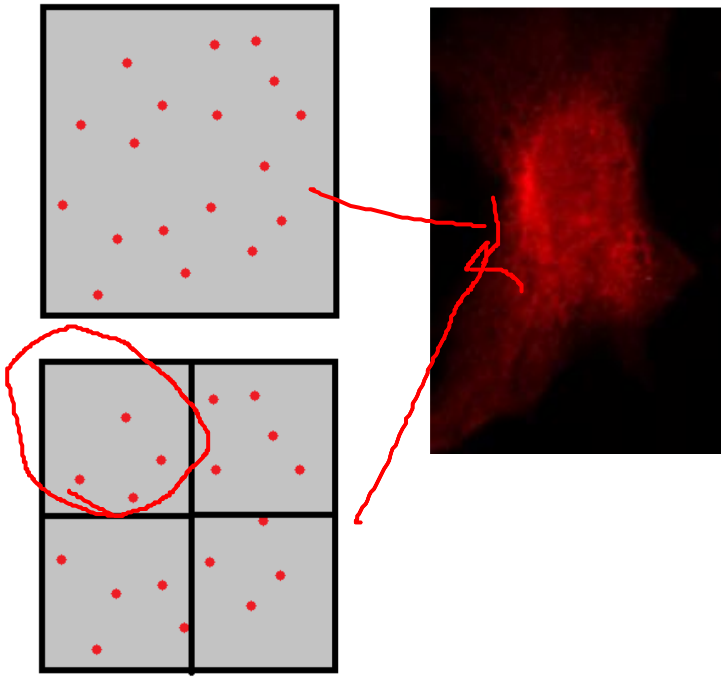
Measurement where the computer can see things, even if you cannot see them can still be useful.

Don’t judge your data on the criteria you might apply to pictures.

Resolution and sampling

Resolution

The physical size of your pixels affects the quality of your signal.



The bigger the pixels, the more signal you get However, bigger pixels mean also having worse resolution.

This brings us to the matters of **sampling** and **resolution**.

Sampling

Sampling or in layman's terms 'how big pixels you take'.

Optimal sampling = using the optimal pixel size.

Oversampling = using smaller pixels than physically possible.

Undersampling = using larger pixels than the optimal.

Undersampling might sound bad, but there are a lot of cases where it is a valid strategy!

Sampling

In simplest terms it means 'how many pixels we are using to portray information'. Higher the sampling, higher the resolution.

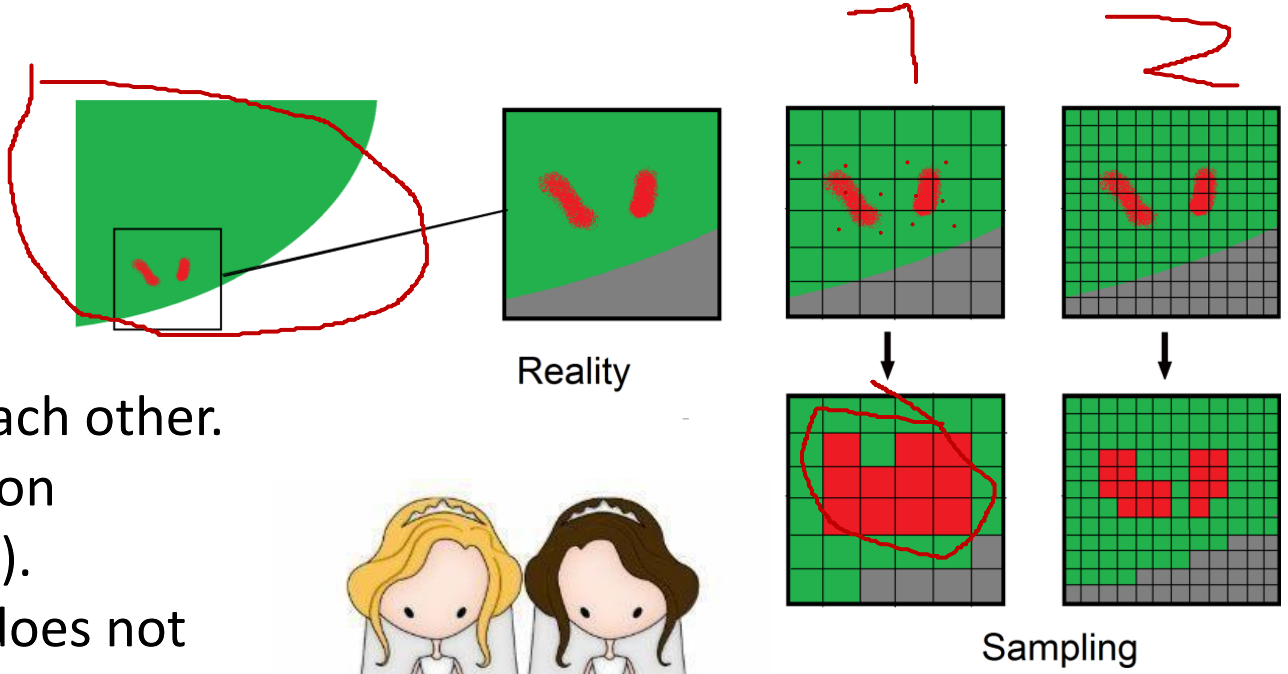
High resolution Fabio Low resolution Fabio

As you can see, here the low resolution Fabio is just as fabulous as his high resolution counterpart.

Resolution

Image size (e.g. 512x512) ≠ resolution

Zoom ≠ resolution



Pixel size and resolution are married to each other. You cannot achieve best possible resolution without best possible pixel size (sampling). However smaller than optimal pixel size does not improve resolution.

Ability to differentiate objects from others, that is ability to resolve structures = **resolution**

Mrs. Pixelsize & Mrs.Resolution

Resolution = ability to resolve structures

Resolution

Your pixel size might be for example 70 nm. But that doesn't mean your ability to resolve things is 70 nm. The rough equation* for **lateral** (xy) resolution reads:

$$r = \frac{\lambda}{NA}$$

where

r = shortest distance between two points on a specimen that can still be distinguished by the observer

λ = excitation wavelength for confocals and emission wavelength for widefield systems

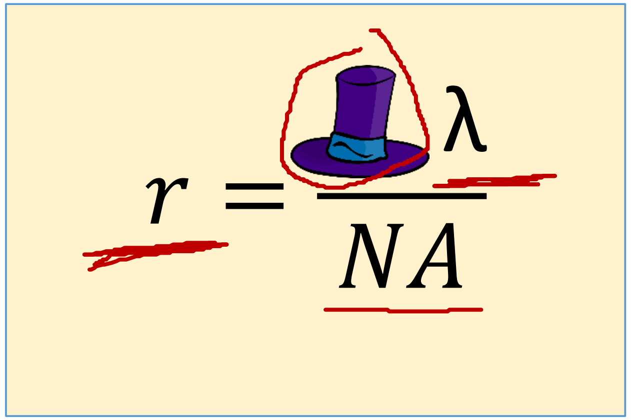
NA = numerical aperture of your objective

 = multiplication factor that depends on the modality of the microscope

* This is the rough equation. The exact equation we leave for the physicists.

Resolution

The equation on the previous page has few immediate consequences.



Firstly, bigger the NA, the better the resolution.

Secondly, smaller the hat, the better the resolution.

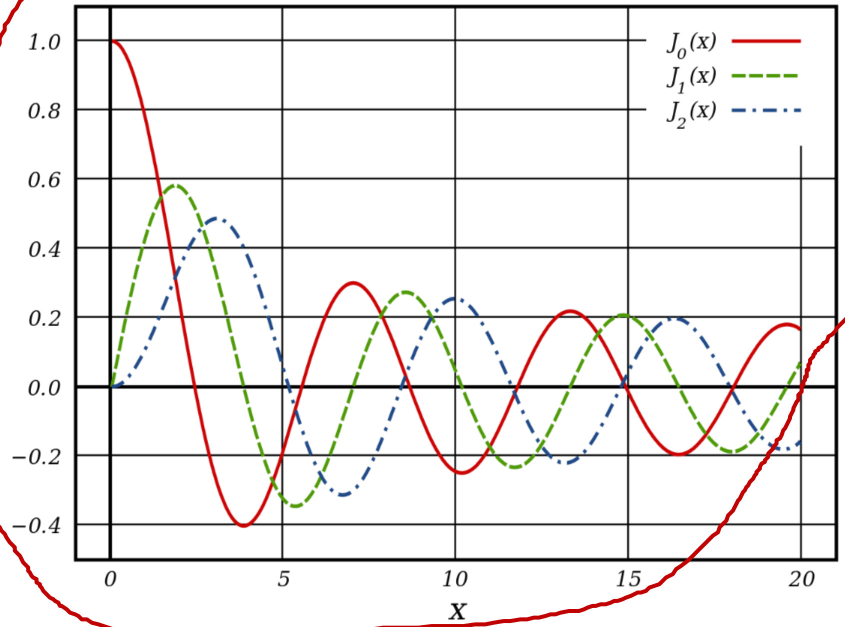
Usually given values for the  are 0.4, 0.5 and 0.61 depending on the modality.

Resolution

Those different values for  come from theoretical models based on Bessel functions used to model the propagation of light and which are way beyond the scope of this course.

It's not magic, it's physics.

For confocal the  is about 0.4 while for the widefield it's about 0.61



$$r = \frac{\square\lambda}{NA}$$

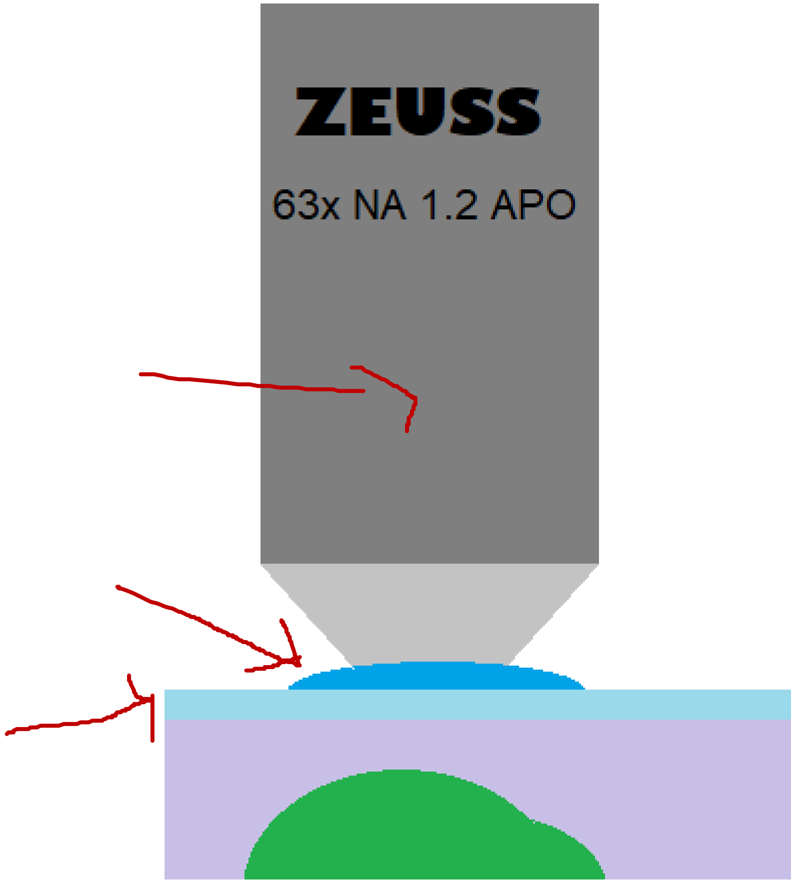
Resolution

For the z-resolution the equation reads

$$r = \frac{\lambda n}{NA^2},$$

where n Refractive Index of the medium between the lens and the specimen.

For confocals  is about 1.4.



Resolution

Recalling our equations

$$r_z = \frac{\lambda n}{NA^2}, \quad r_{xy} = \frac{\lambda}{NA}$$

Assuming green 488 nm excitation laser, we can tabulate the following.

	XY resolution	z resolution
Oil 63x 1.4 NA	140 nm	530 nm
Glycerol 63x 1.3 NA	150 nm	600 nm
Glycerol 20x 0.75 NA	260 nm	1800 nm

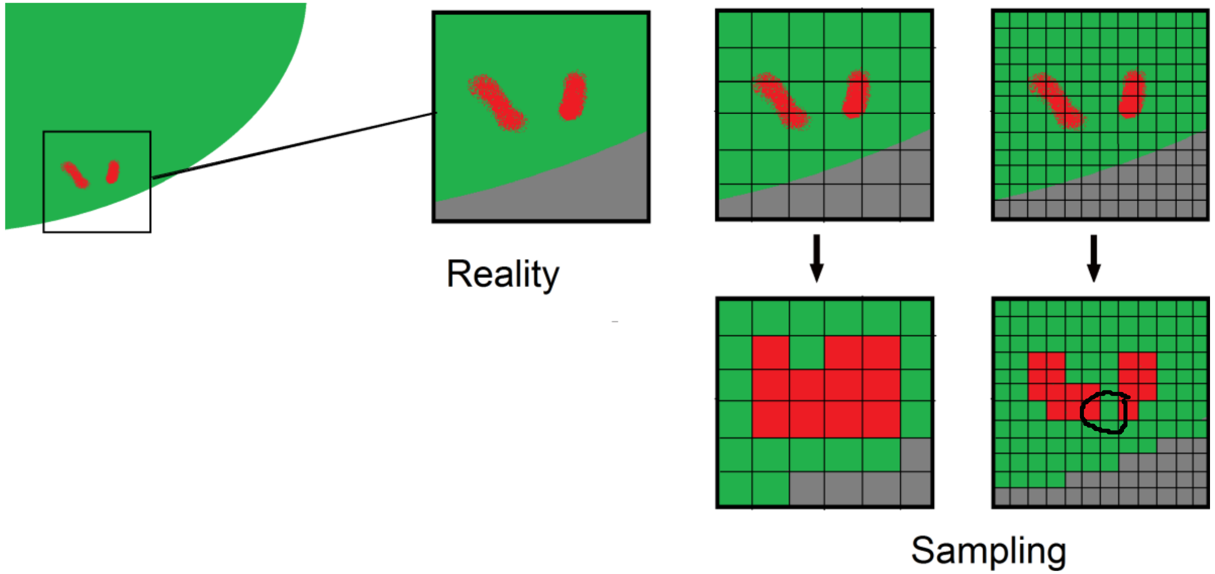
While oil objectives give nominally better resolution than glycerol objectives, aberrations caused by refractive index mismatch will likely undo any potential gains as long as you are using conventional mounting media.

Prolong glass with refractive index 1.52 is notable exception.

Optimal sampling

However as noted earlier, resolution and sampling are not the same.

You often find a recommendation that your pixel size should be 2.3 times smaller than the optimal resolution if you want to resolve your image as accurately as possible.



	XY sampling	z sampling
Oil 63x 1.4 NA	61 nm	230 nm
Glycerol 63x 1.3 NA	65 nm	243 nm
Glycerol 20x 0.75 NA	113 nm	780 nm

Questions?