

# AX Image Resolution

Confocal microscopes captures more image detail than a widefield microscope, which can provide higher resolution images.

Here are some **concepts** to help you understand what changes image resolution and what you need to change on the microscope and in this software to get the **best possible image resolution**.

But keep in mind, this is just a demonstration and when you come to do your own imaging, you'll have to consider what analysis you want to do and decide on **how much image detail or resolution you need**. Because the higher the resolution generally the more bleaching and the longer it takes to image.

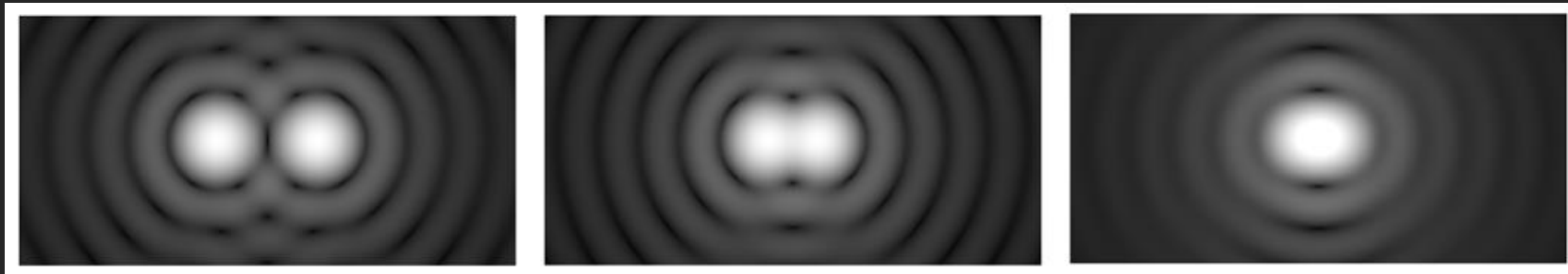
To optimise image resolution you have 3 decisions to make...

1. Choosing a suitable lens and immersion medium
2. Set pinhole size (recommended: equal to or smaller than  $1.2 \text{ AU}$ )
3. Set sampling frequency (Zoom and Z stack step size)

# Resolution is...

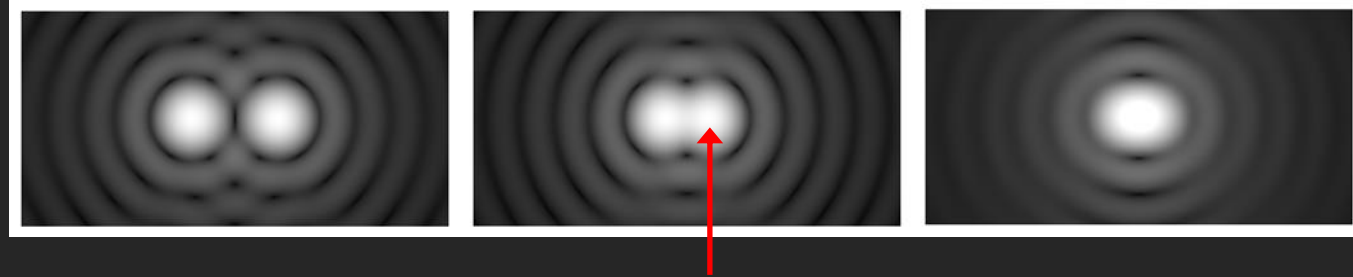
The ability to distinguish separate objects.

The highest resolution you can achieve is called the resolution limit, basically it's as close as 2 objects can get and still be visualised as 2 separate objects.



This limit exists because...

# Light coming from a very small point spreads out in Airy Disks



When another object overlaps the first peak intensity ring, the drop in intensity between the two objects is enough for us to categorise them as 2 different objects.

$$\text{Lateral (XY) Resolution} = 0.61 \lambda / \text{NA}$$

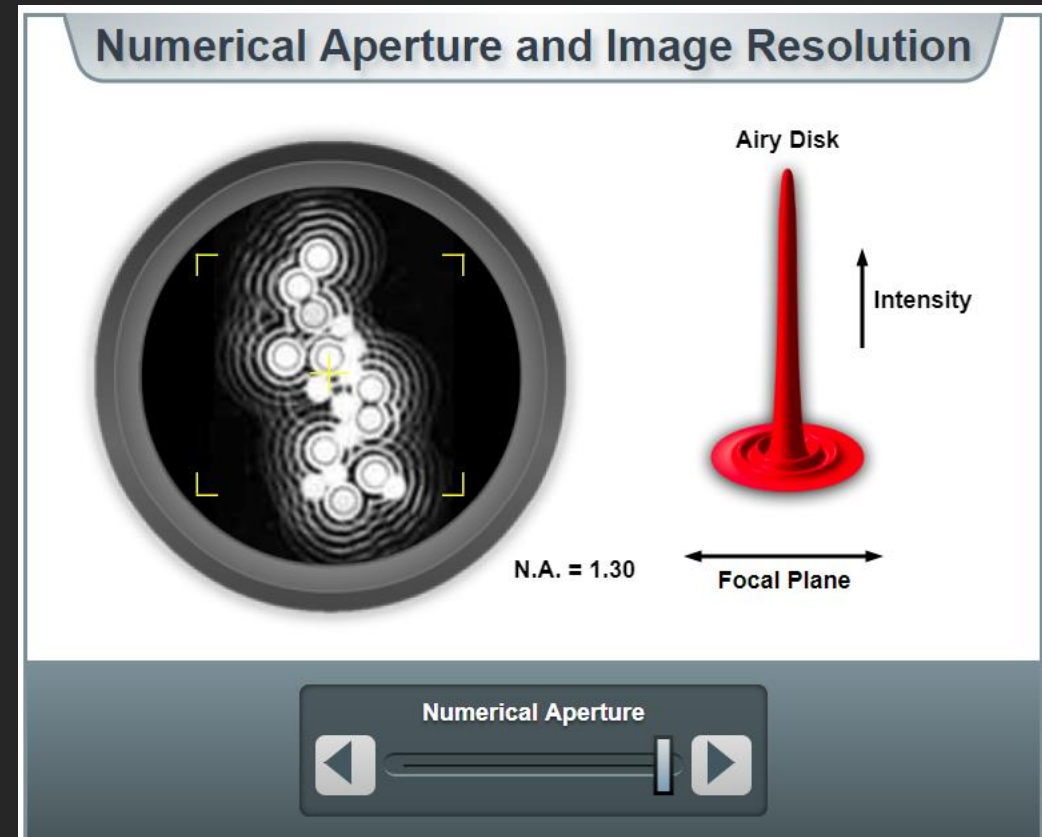
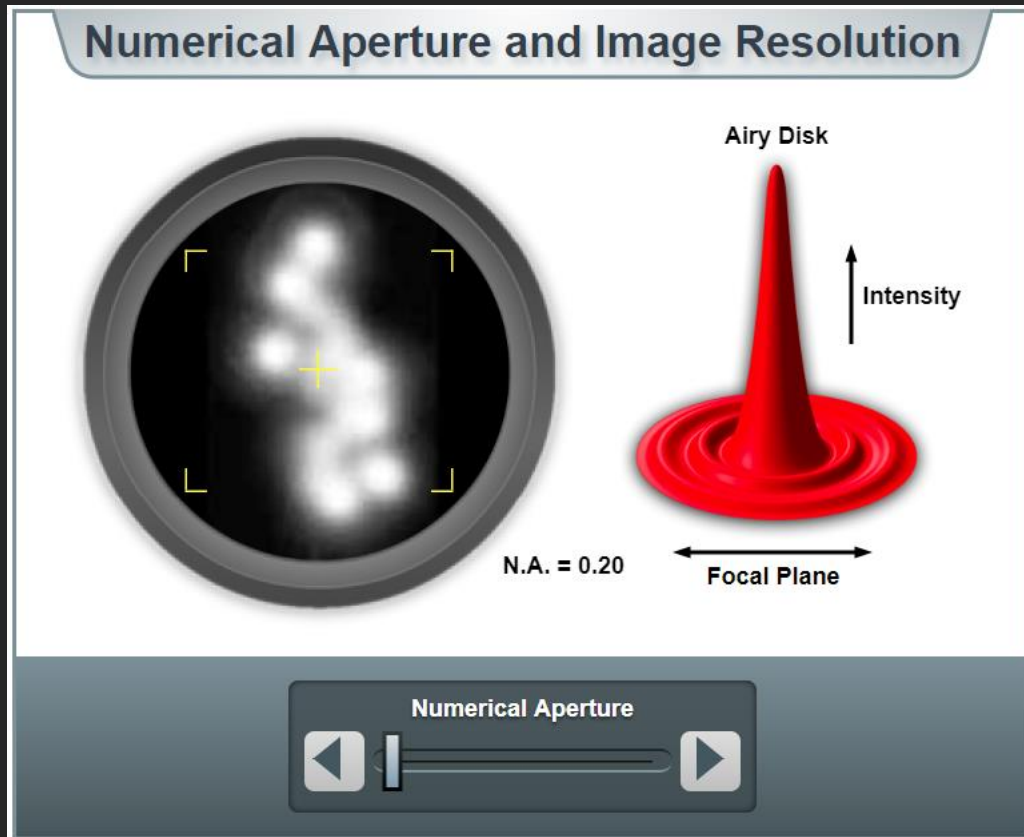
$$\text{Axial (XZ) Resolution} = 2 n \lambda / \text{NA}^2$$

## Rayleigh Criterion

$$\text{Lateral (XY) Resolution} = 0.61 \lambda / \text{NA}$$

E.g.  
 $0.61 \times 480\text{nm} / 0.8 = 366\text{nm}$   
 $0.61 \times 480\text{nm} / 1.4 = 209\text{nm}$

$$\text{Axial (XZ) Resolution} = 2 n \lambda / \text{NA}^2$$

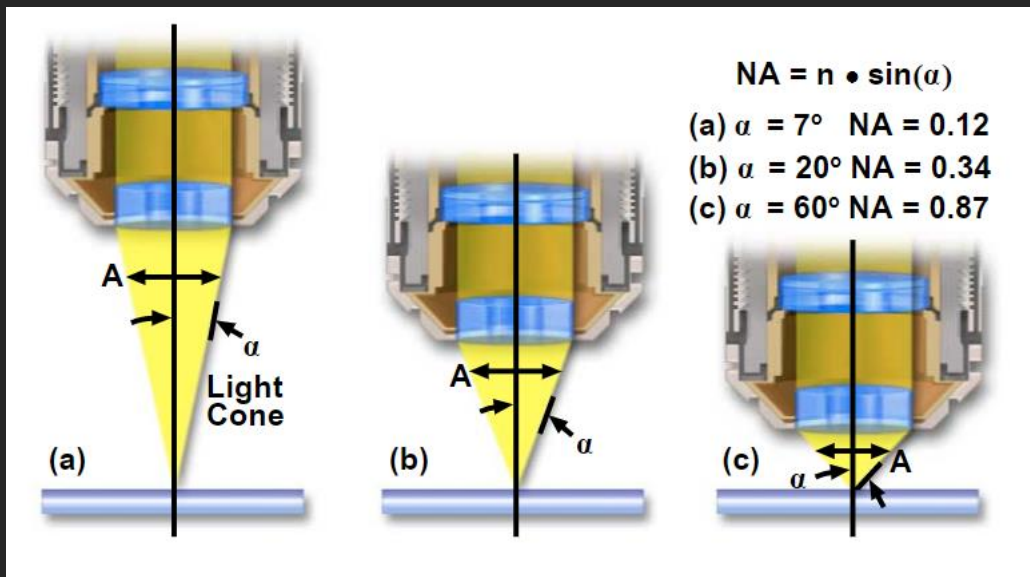


Changing the Numerical Aperture changes resolution!

# Numerical aperture (lens dependent)

NA is a measure of a lens' ability to gather light and detail.

Different lenses have different Numerical apertures



The closer the lens is to your sample and the better matched your immersion medium means you'll have a higher NA and can gather more detail from your sample.

Higher NA = better resolution

$$\text{Numerical Aperture (NA)} = n \times \sin(\alpha)$$

The immersion medium's refractive index:

$$\text{Air} = 1$$

$$\text{Water} = 1.33$$

$$\text{Glycerine} = 1.47$$

$$\text{Immersion Oil} = 1.51 = \text{Glass (coverslip)}$$

Z Resolution =  $2 n \lambda / NA^2$  is affected further by refractive index.

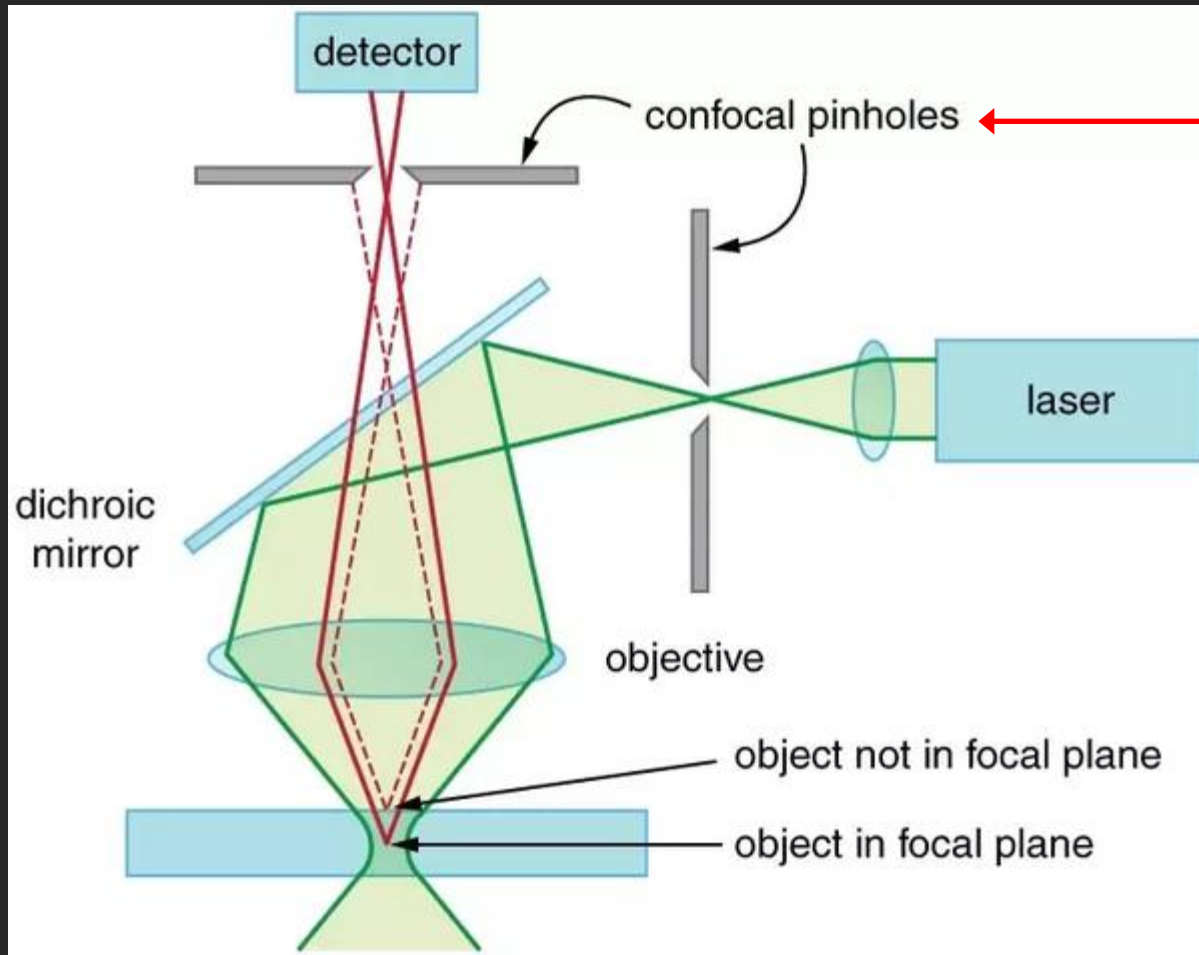
Angle between lens and sample.

# Optimise your image resolution by...

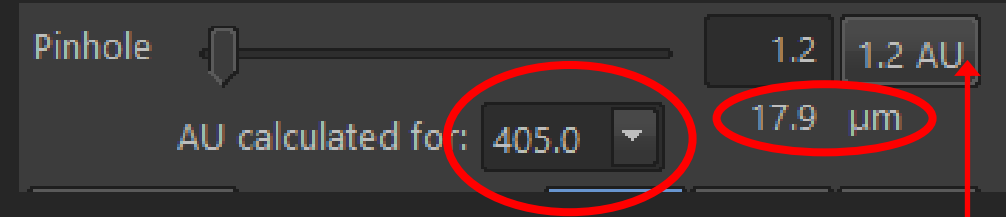
1. Choosing a suitable lens and imaging medium
2. Set pinhole size (recommended: equal to or smaller than 1.2 AU)
3. Set sampling frequency (Zoom and Z stack step size)



Airy disk and numerical aperture affect all microscope machines so what makes confocal systems higher resolution than widefield?



Pinholes : minimises the detection of out of focus signal.



Recommended Pinhole size is 1.2AU

Be aware 1.2AU for different wavelength results in different pinhole sizes.

# Optimise your image resolution by...

1. Choosing a suitable lens and imaging medium
2. Set pinhole size (recommended: equal to or smaller than  $1.2 \text{ AU}$ )
3. Set sampling frequency (Zoom and Z stack step size)

After optimizing the NA and pinhole size, the next thing to consider is how to actually take the image...

You can have the most powerful lens and smallest pinhole but if you don't set up sampling frequency correctly, then you won't capture enough detail to end up with a high resolution image.

# Sampling frequency

(pixel and step size dependent)

- When your specimen emit fluorescent light, it is picked up by the camera and gets translated into digital pixels.
- When we decide on sampling frequency we are telling the microscope how many pixels we want to record within a fixed distance.
  - For optimal resolution, that fixed distance would be the smallest resolution limit you can achieve (lens and pinhole).
- If you only sample once, that is unreliable, like if you ran an experiment without any repeats.
- So you need to sample more than once, within your resolution limit.

A standard sampling frequency can be worked out using the Nyquist Limit (N) equation.

$$N = 0.3 \lambda / NA$$

Most researchers uses the Nyquist limit equation to works out a sampling frequency of 2.2, which means you need to sample 2.2 times within your resolution limit ( $0.61 \lambda / NA$ ) to be sure the signal you are sampling is real.

So how do you apply this 2.2 times sampling frequency...

So how do you apply this 2.2 times sampling frequency...

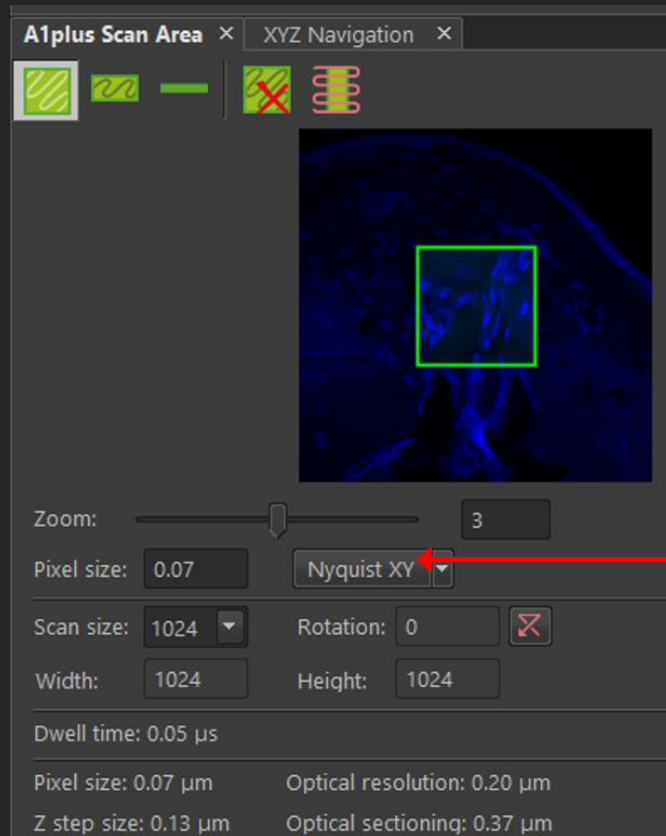
$$\begin{aligned} \text{Lateral (XY) Resolution} \\ = 0.61 \lambda / \text{NA} \end{aligned}$$

Scan area / Zoom

$$\begin{aligned} \text{Axial (XZ) Resolution} \\ = 2 n \lambda / \text{NA}^2 \end{aligned}$$

Z stack step size

# Lateral (XY) sampling frequency can be changed using the ZOOM option.



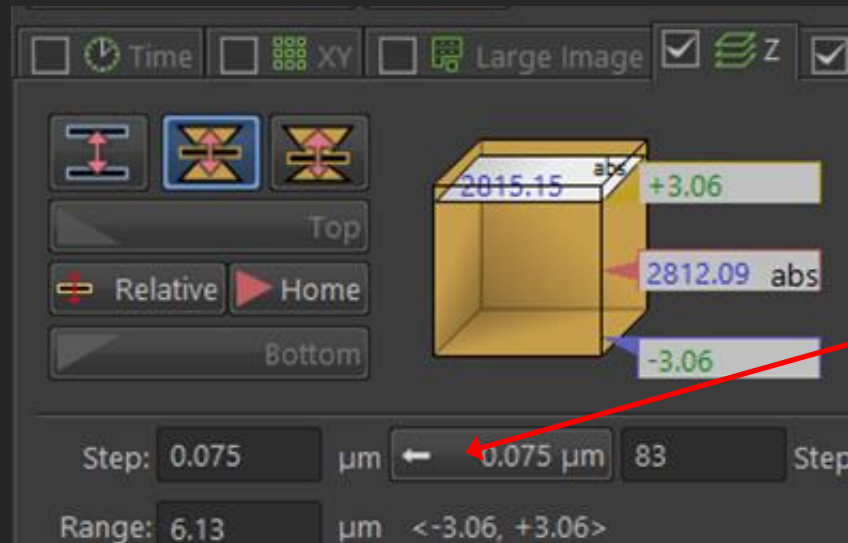
The software knows the lens and pinhole size you are using, so it calculates your resolution limit and Nyquist limit for you.

All you need to do is click on Nyquist XY for recommended pixel size. The correct Zoom will be automatically applied.

You are at your resolution limit, so even if you zoom in even further, you won't be able to distinguish any more detail from your specimen.

Axial (Z) sampling frequency can be changed using the Z stack step size option.

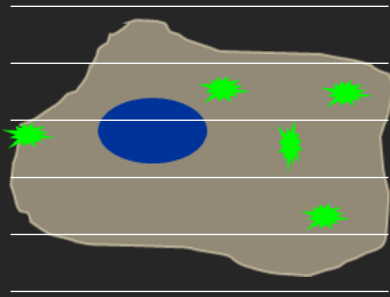
You apply this during your acquisitions set up.



The software again calculated your resolution limit for you. All you need to do is click here to apply the recommended step size during Z stacking.

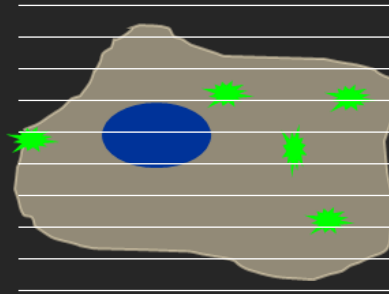
# You don't have to use the recommended step size...

Under-sample



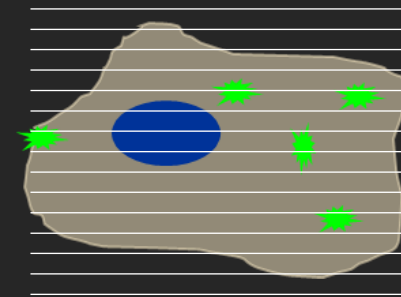
Make step size bigger  
Step size e.g.  $0.5\mu\text{m}$

Recommended  
Step Size



Step size e.g.  $0.3\mu\text{m}$

Oversample



Decrease by  $1/3$   
Step size e.g.  $0.2\mu\text{m}$

Faster acquisition  
Less bleaching

Slower acquisition  
More bleaching



# What happens when you under-sample

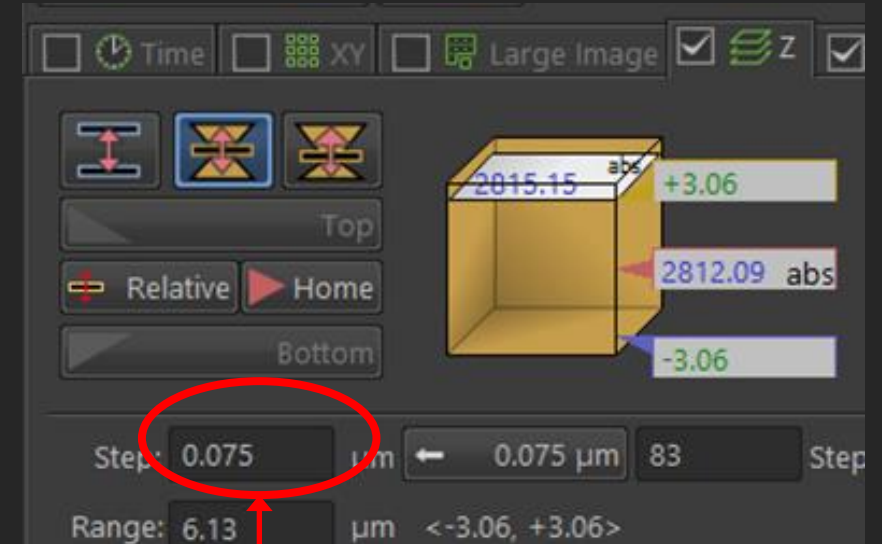
Actual Image



Under-sampled Image

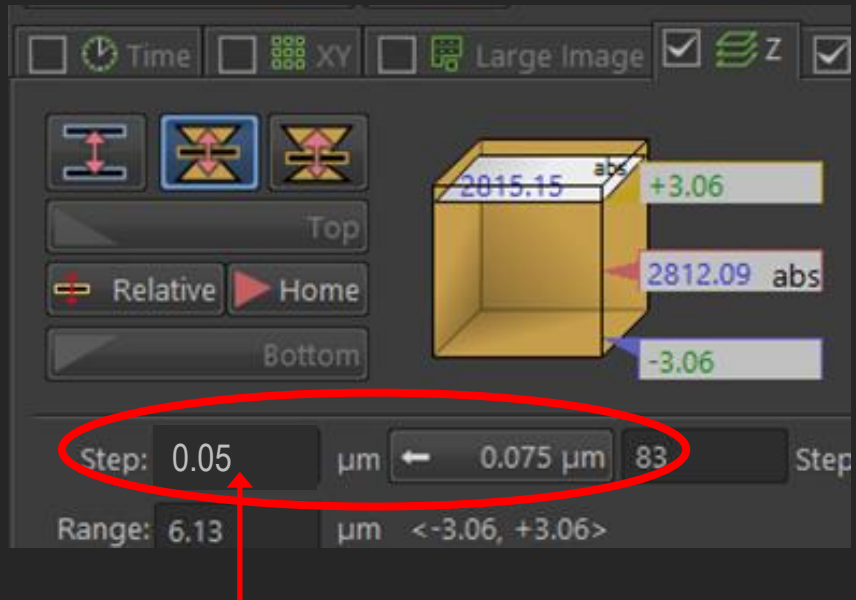


Aliasing occurs when signal becomes indistinguishable and create distortions.

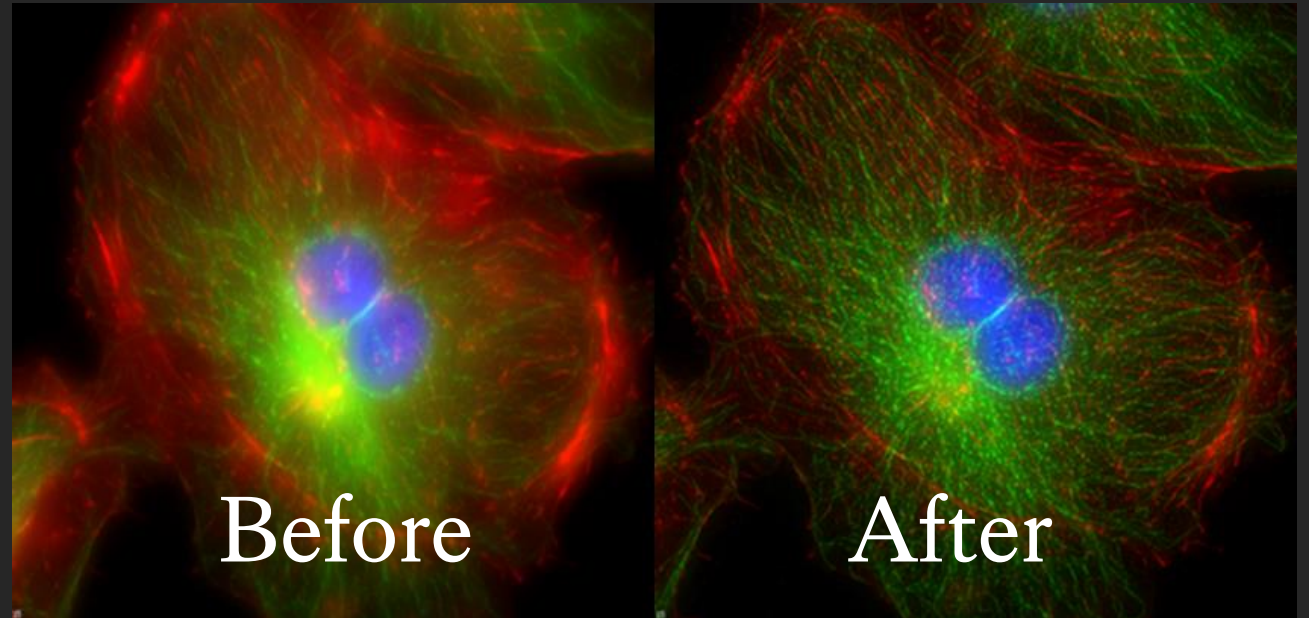


- Under-sampling can save time and minimise bleaching but you do loose information.
- To under-sample, type in a step size bigger than the recommended, E.g. in this case  $0.1\mu\text{m}$

# What happens when you oversample



- You are capturing more information than you need when you oversample.
- But computer algorithms can use this information to 3D Deconvolve, digitally relocate signals for higher resolution images.
- To oversample, type in a step size smaller than the recommended, E.g. in this case  $0.05\mu\text{m}$



## 3D Deconvolution

Actual results may vary...

Image copied from <http://www.biology.wustl.edu/imaging-facility/specs-deltavision.php>

If you need any help, please contact:

George Chennell (07771926760) via WhatsApp

or

Chen Liang (07883166321) via WhatsApp

Happy Imaging!