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MICROSCOPY TRAINING

Microscopy Concepts

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Welcome

MyScope was developed by Microscopy Australia to provide an online learning environment for those who want to learn about microscopy. The platform provides insights into the fundamental science behind different microscopes, explores what can and cannot be measured by different systems and provides a realistic operating experience on high end microscopes.
We sincerely hope you find our website: www.myscope.training an enjoyable environment. In there you can explore the microscopy space and leave ready to undertake your own exciting experiments.

Module Contents

What is Microscopy?

Microscopy is the term that describes the use of lenses to reveal details of an object that are not visible to the unaided eye. A hand-held lens is the simplest way to do this, magnifying the details of a specimen.

In the most basic form, a microscope is made up of a lens, used near the eye and a tube that has another lens set further away from the eye but closer to the object. The object to be studied needs preparation, placement and illumination.

The development of microscopes has not stopped since the 1600s when Dutchman Antonie van Leeuwenhoek first recorded minute details in various natural samples examined with simple single lens microscopes that he had created using his knowledge of instrumentation and manipulating glass. Advances in specimen preparation and illumination have enabled researchers to refine and expand endeavours in the search for answers to the unknown. They have also extended the types of illumination from visible light to X-rays and electron beams so ever greater detail can be observed.

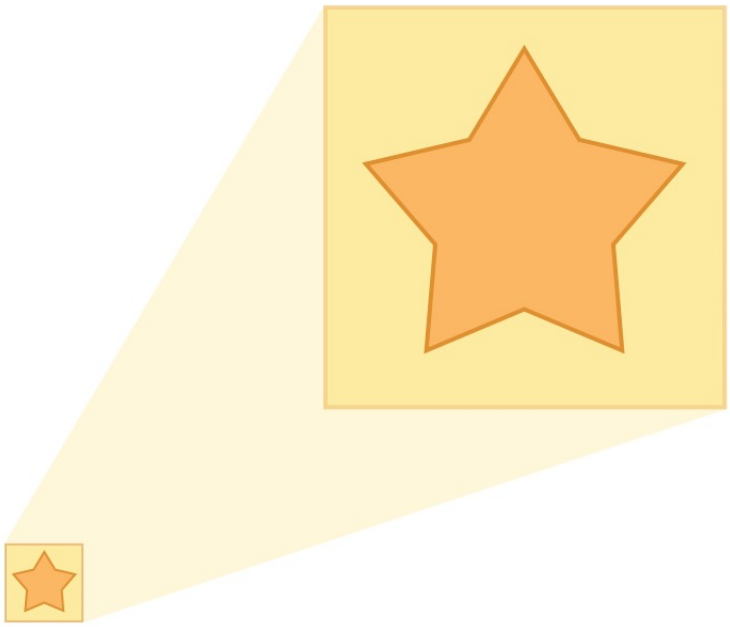
The type of microscope you need to see your features of interest will depend on:
- the size of your sample
- the size of the features

the nature of the information you want to collect eg. do you want to determine:
- what size or shape it is
- where certain molecules are located
- what its chemical composition is
- or maybe the orientation of its crystals?

The availability of the instruments and the time it takes to become proficient in the technique also needs to be taken into account.

Why do microscopy? – Magnification

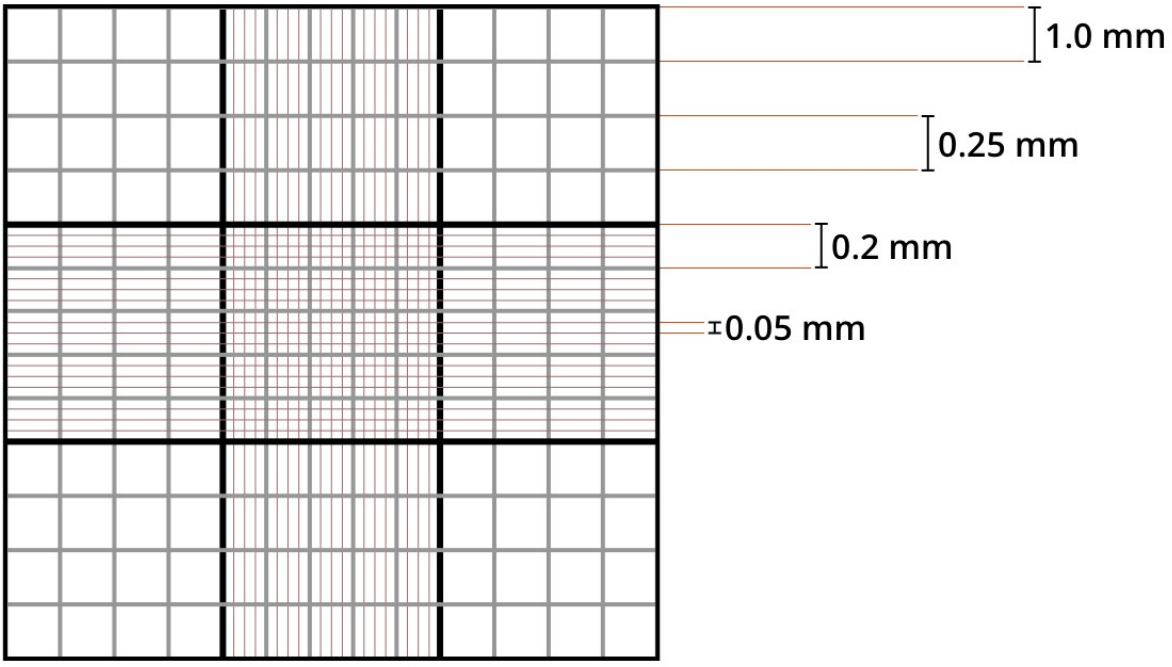
Magnification is the amount that the dimensions of an image are, or appear to be, enlarged compared to the same dimensions in the actual sample. How much you can see either through the eyepiece or on the screen is called the field of view (FOV).



Magnification and measurement

Measuring the size of your sample, or features within your sample, by using microscopy can be done in different ways but all depend on knowing the extent of magnification in one way or another. Magnification was described above and is controlled by the objective lens in the microscope. This is the lens nearest to the sample. The higher the magnification, the smaller the field of view will be.

Describing an image as showing a given magnification only applies to a fixed display size. If an image is enlarged, then so is its magnification. When images were captured onto a fixed-sized photographic film negative, it was clear that the negative showed a certain magnification such as 200x or 50,000x and that any increase in size when an image was printed could easily be calculated and used to increase the magnification factor. Magnification for digital images is usually calibrated in the microscope software. If it hasn't been, or to check the calibration, capture an image of a stage micrometer. You can then determine the pixel size by looking at how many pixels in the image cover a measured distance on the micrometer. In a light microscope, you can also use a haemacytometer for this if you don't have a stage micrometer.

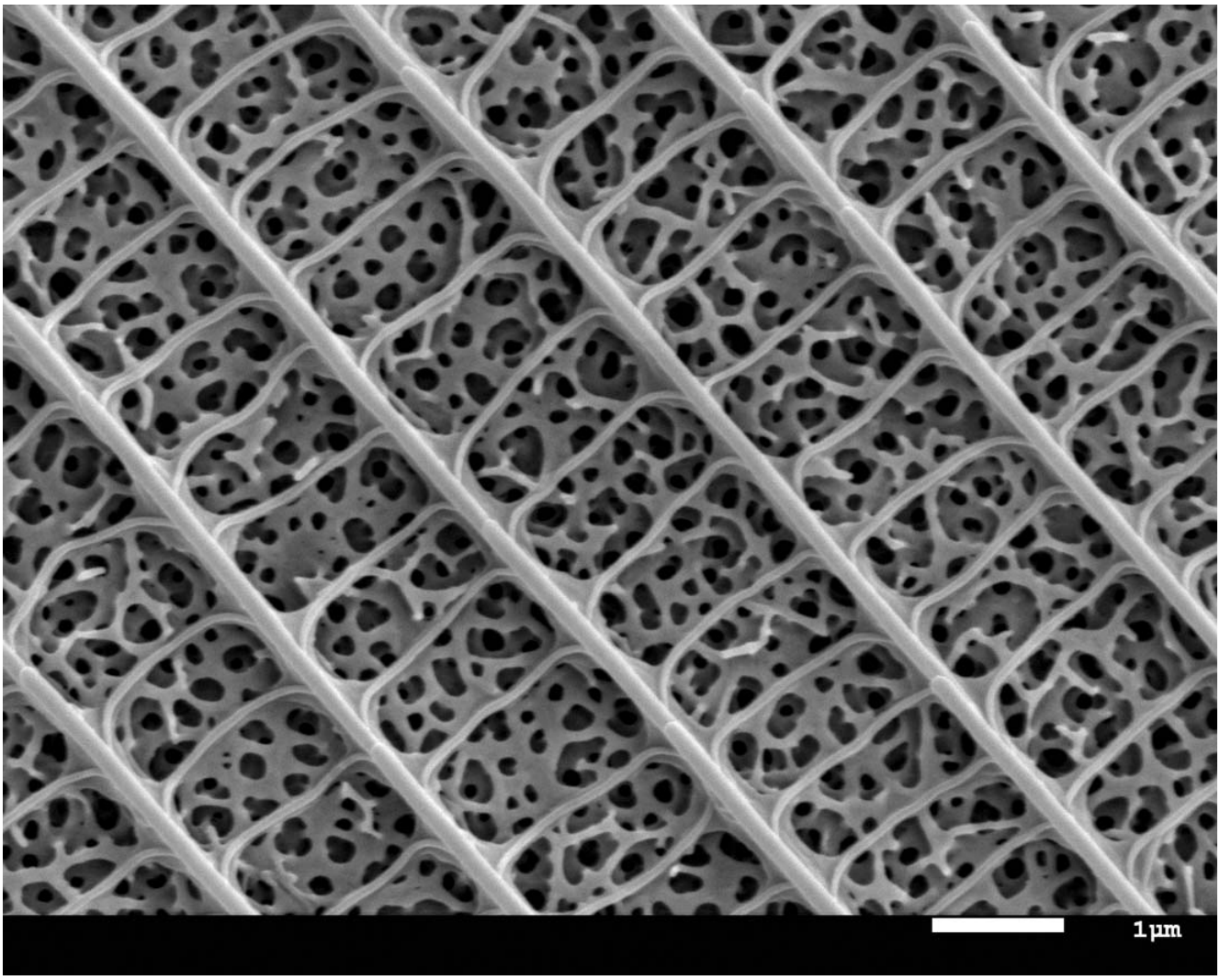


Number of cells in a 1mm^2 square x 10^4 = No. cells/ml.

Other more effective ways to represent the extent of magnification, and therefore the size of features in the image, regardless of display size include:

- describing the horizontal field width (HFW). HFW means the width of the area captured in the image. You can then calculate the proportion of this that is taken up by your feature of interest. Note: micrometre is abbreviated as µm and nanometre as nm.
  For example, if the HFW is 25µm, and your feature of interest takes up 20% of the width of the image, then 25µm x 20% = 5µm. So, your feature is 5µm wide.
- use of a scale bar. These are often added by the instrument software and provide another easy way to calculate the size of different components in the image. When doing this, all measurements must be done on the image at the same dimensions – you can't zoom in further if you have already made one of the measurements.

An example of such a calculation is as follows:



First measure the length of the scale bar (on screen or on a printed page). Say this comes to 11 mm. Then, measure the width of your feature of interest; in this case say it is the distance between the long ridges, which measures about 17.5 mm (on the screen or printed page). Next, note the length that the scale bar represents, which is 1 µm (the number next to the scale bar).

Divide the size of the feature by the measured length of the scale bar and multiply by the length represented by the scale bar. This will give you the real width of the feature: (17.5/11) x 1µm = 1.59µm.

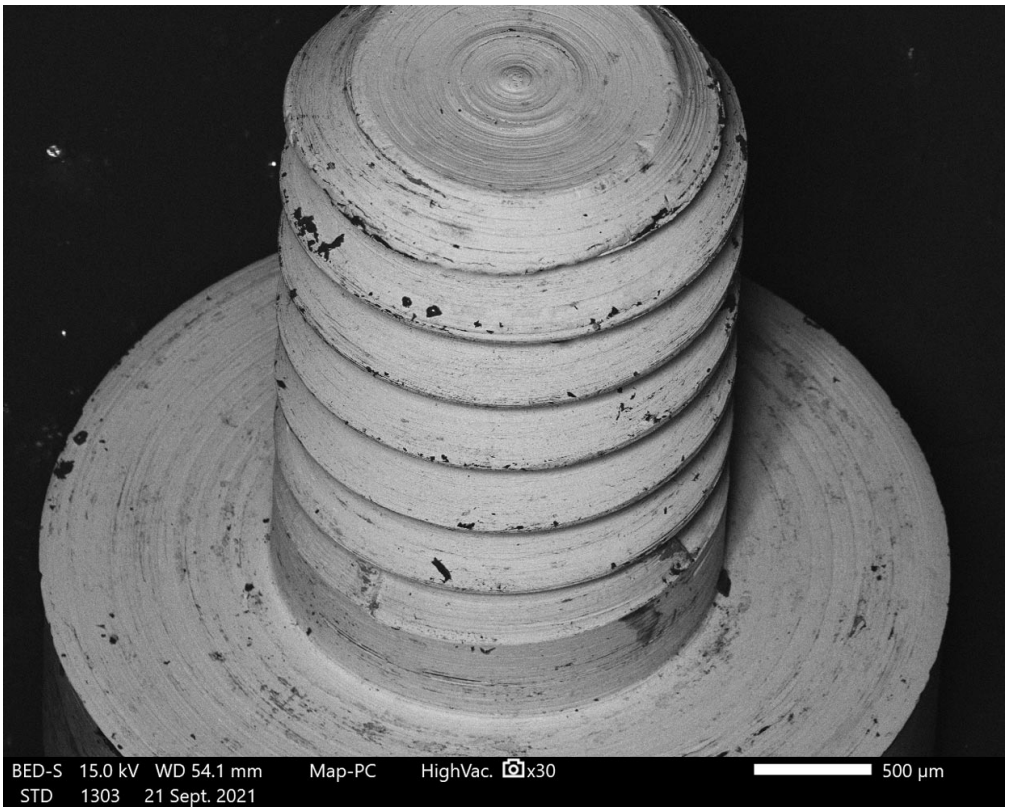
Resolution

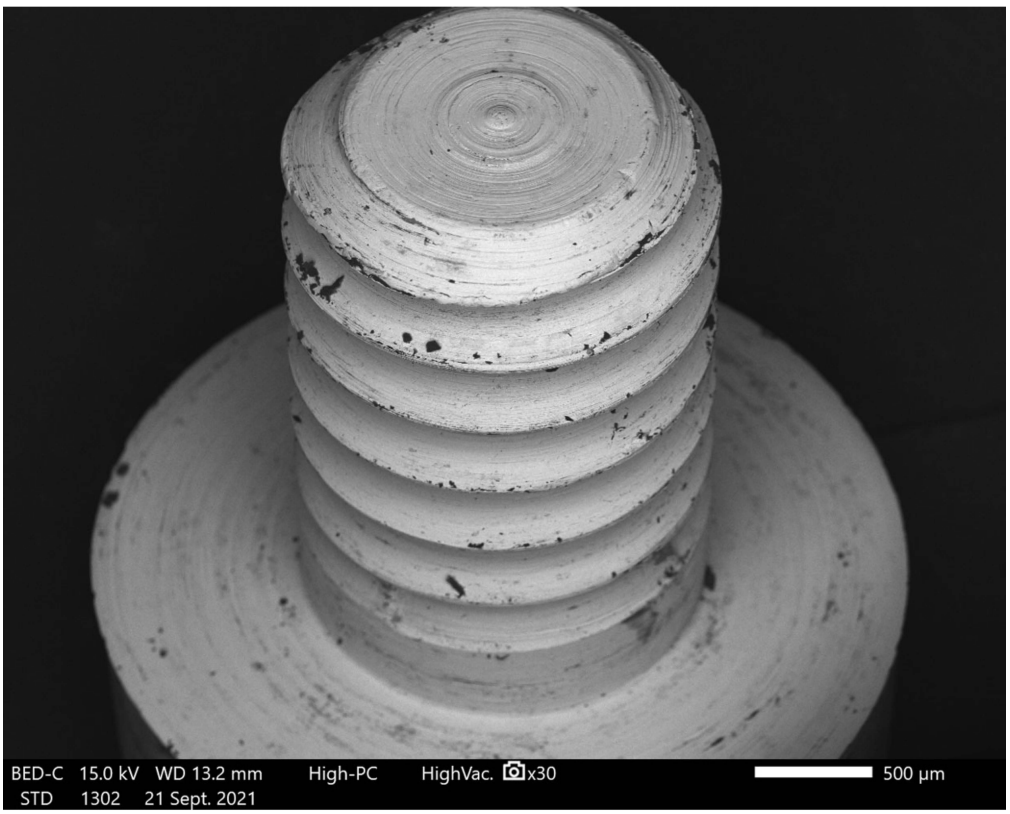
Resolution is the ability to see small, close together objects as distinctly separate.

As an example, in a light microscope, more light can be captured by an objective lens that has a larger numerical aperture. This in turn will give higher resolution. The theoretical limit of resolution on a light microscope is about 200 nanometres (half the wavelength of violet light). The theoretical limit of resolution of electron microscopes is about 250 picometres (0.25nm). More on this in the Wavelength, Frequency and Energy section below.

Depth of field

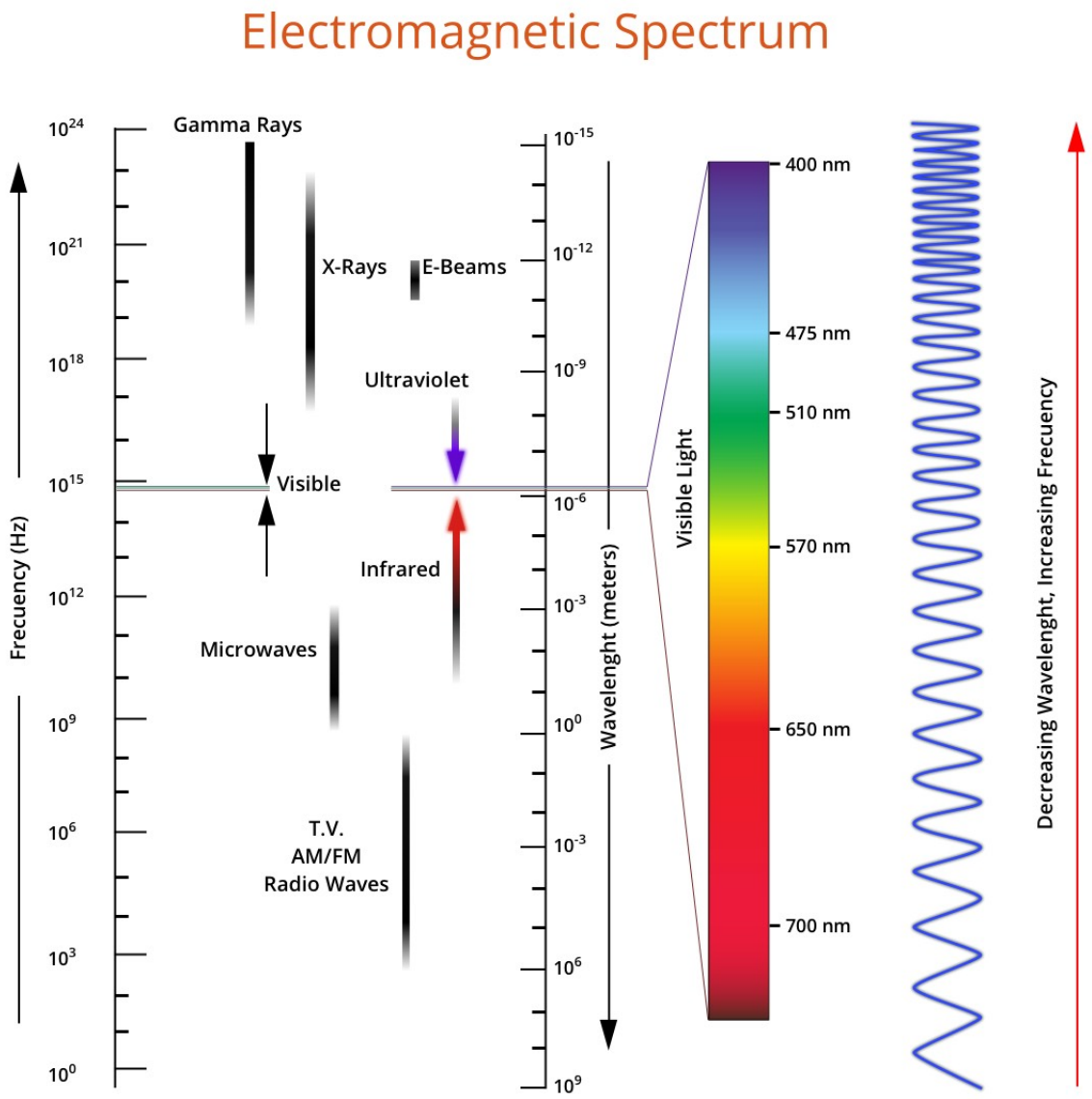
Depth of field tells us how much you can get in focus at the same time. This is particularly important for thick and uneven specimens such as sponges, embryos and insects where we want the top and bottom of the object to both be in focus at the same time. The microscopy technique chosen needs to have the ability to have the depth of field you need to usefully visualise your sample.





Electromagnetic Spectrum –
Introduction to the Electromagnetic Spectrum

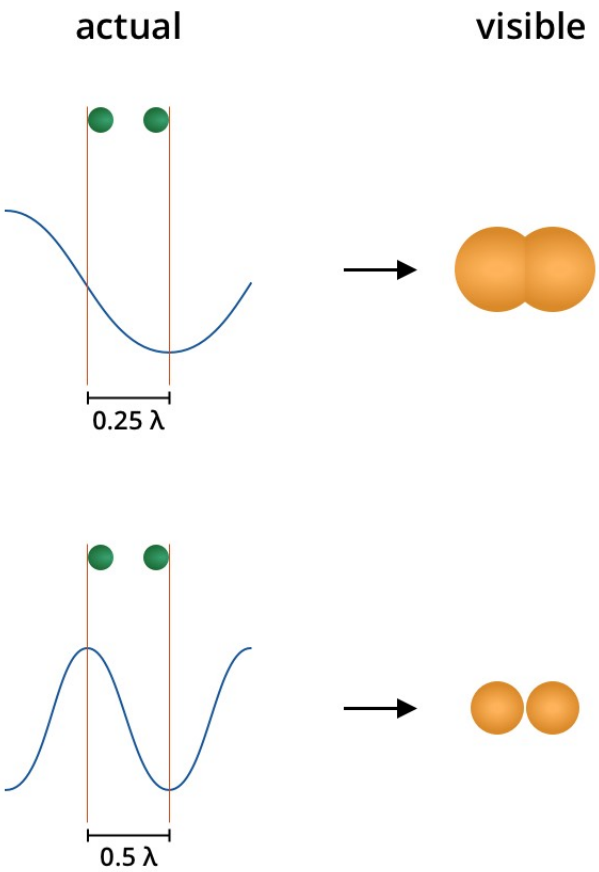
We are most familiar with visible light as this is how we see the world around us in our daily lives. However, this visible light is just a small part of the much broader spectrum of electromagnetic radiation that is represented in the diagram below.



The visible spectrum covers wavelengths from about 400 (blue) to 700 (red) nanometres. All other wavelengths in the electromagnetic spectrum are invisible. Low energy, long wavelengths, at about 3 metres are used to transmit FM Radio signals. X-rays cover a wide part of the spectrum extending out from the UV. Electron beams generated by electron microscopes sit at the high energy, short wavelength end of the spectrum around 2–4 picometres (10-12).

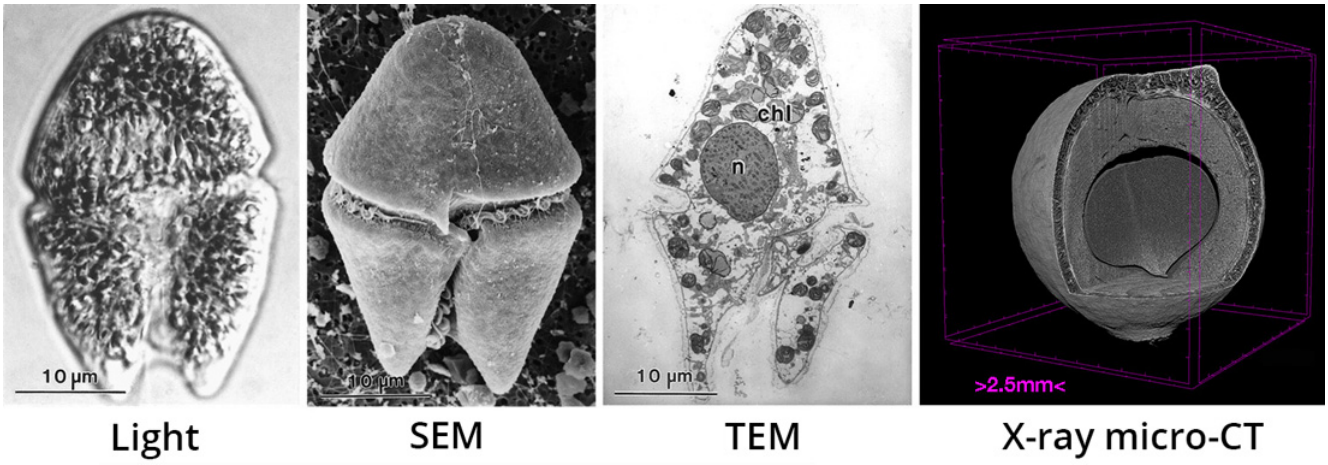
Wavelength, frequency and energy

The frequency of electromagnetic radiation is the number of waves per second. Higher frequency radiation has shorter wavelengths and higher energy. In microscopy, higher frequency radiation gives greater resolution because shorter wavelengths separate at a closer distance than longer wavelengths.



Electromagnetic spectrum and microscopy

Different types of microscopy use different wavelengths to illuminate the samples and deliver different benefits and resolution.



Optical Microscopy

Uses visible light to pass through, or reflect off, the sample.

Advantages:

- Uses visible light to illuminate a thin translucent sample using transmitted light or the surface of an opaque sample using reflected light.
- Magnification max - approx. 2000x.
- Direct imaging with no need of sample pre-treatment.
- The only microscopy for imaging the natural colour of a sample.
- Fast and adaptable to all kinds of sample systems, from gas to liquid and to solid sample systems in any shapes or geometries.
- Easy to be integrated with digital camera systems for data storage and analysis.

Disadvantages:

- Low resolution, usually down to only sub-micrometre or a few hundreds of nanometres, mainly due to the light diffraction limit.

X-ray Microscopy

Uses X-rays to pass through the sample.

Advantages:

- In its basic form, an X-ray microscope operates in a similar way to a light microscope but doesn't need optically transparent samples. It can attain resolution to tens of nanometres and can image in 2D or 2D over time.
- X-rays can also be used in a tomographic set-up to image samples beyond two dimensions to observe surfaces and interiors of samples non-destructively to sub-micrometre resolution. Some specialised instruments can get down to 50nm resolution. Can image in 2D, 3D and over time (4D)
- Most kinds of samples can be imaged but lower atomic number materials work best
- Can differentiate parts of solid samples based on differences in radio-opacity

Disadvantages:

- Higher resolutions require perfectly stationary samples
- Staining may be required for biological samples.
- Maximum sample size depends on composition. The denser the sample, the smaller the sample must be to allow sufficient transmission of the beam.

Scanning Electron Microscopy (SEM)

Uses an electron beam scanning across the surface of the sample.

Advantages:
- Observe surfaces of objects at high resolution
- Magnification approx. 500,000x
- Almost all kinds of samples, conducting and non-conducting (conductive coating needed)
- Based on surface interaction (no requirement of electron-transparent sample)
- Imaging at all directions through x-y-z (3D) rotation of the sample
- Specialised SEM works directly on non-conductive samples

Disadvantages:
- Resolution, usually above a few tens of nanometres
- Usually requires surface metal-coating to make the sample electrically conductive.

Transmission Electron Microscop (TEM)

Uses an electron beam passing through the sample

Advantages:
- Can observe very thin cross-sections of an object
- Magnification approx. 5,000,000x
- High resolution, as small as 0.2nm
- Direct imaging of internal structure of objects after preparation
- Direct imaging of crystal lattices in samples
- Can delineate the defects in a crystalline sample
- Using the electron diffraction technique you can determine the type of crystal structure (phase identification), symmetry determination, lattice parameter measurements, disorder and defect identification.

Disadvantages:
- To prepare an electron-transparent sample from larger pieces of starting material is difficult (due to the conductivity or electron density, and sample thickness).

Interactions with Matter

When a beam hits a sample a number of interactions can occur. Some type of interactions can be observed or measured to provide information about the sample. Many of these interactions occur at the same time but depending on the type of information you need, you can choose which ones to measure.

Absorption

Energy from the beam can be either fully or partially absorbed by the sample. The reduction of beam energy as it emerges from the sample can be measured.
Technique examples: electron energy-loss spectroscopy (EELS), bright field light with stains

Reflection and scattering off surfaces

Beams can bounce back when they encounter a sample in their path. If the surface of that sample is irregular, the beam bounces back at different angles depending on the orientation of the part it hits. This is called scattering. If the surface is flat, the beam can bounce back as a single coherent beam, as light does from a mirror. This is called reflection.
Technique examples: optical darkfield techniques, polarised light microscopy, reflectance microscopy.

Beam scattering within samples

High energy beams such as high energy X-rays and electron beams can penetrate the surface of samples and bounce around the electron cloud. This throws the beam in many directions and we refer to this as scattering. This effect can lead to the beam being dissipated in the sample or emerging, where it can be measured.
Techniques examples: X-ray diffraction, High-angle annular dark-field TEM, small angle scattering.

Transmission and refraction

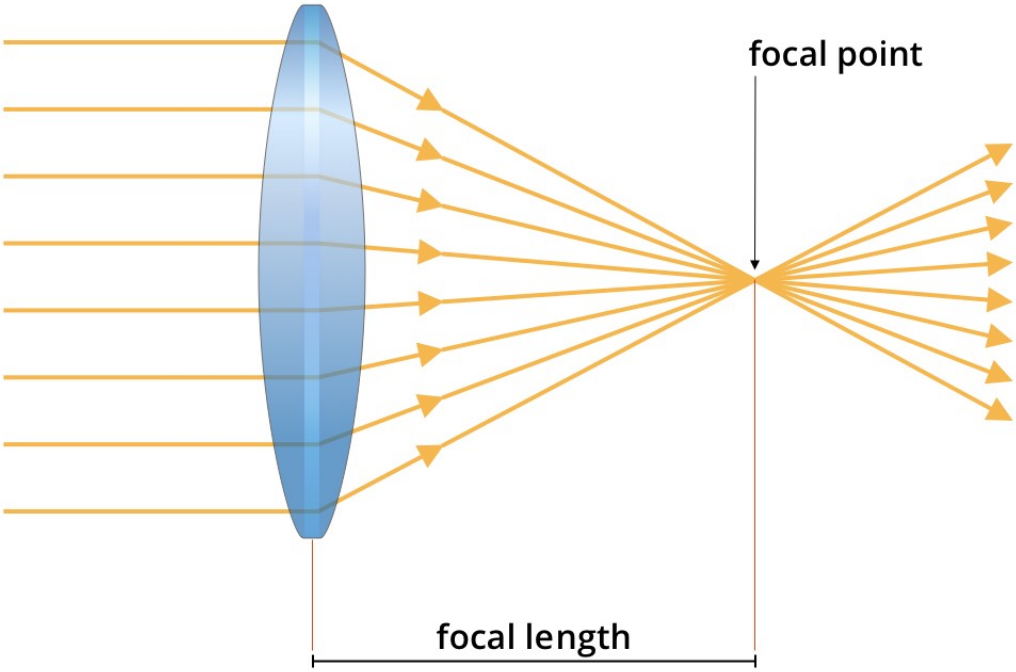
Beams can pass through a sample. The extent to which they do this will depend on the thickness and density of the sample and the wavelength of the beam. Transmission is the basis of many microscopy techniques.

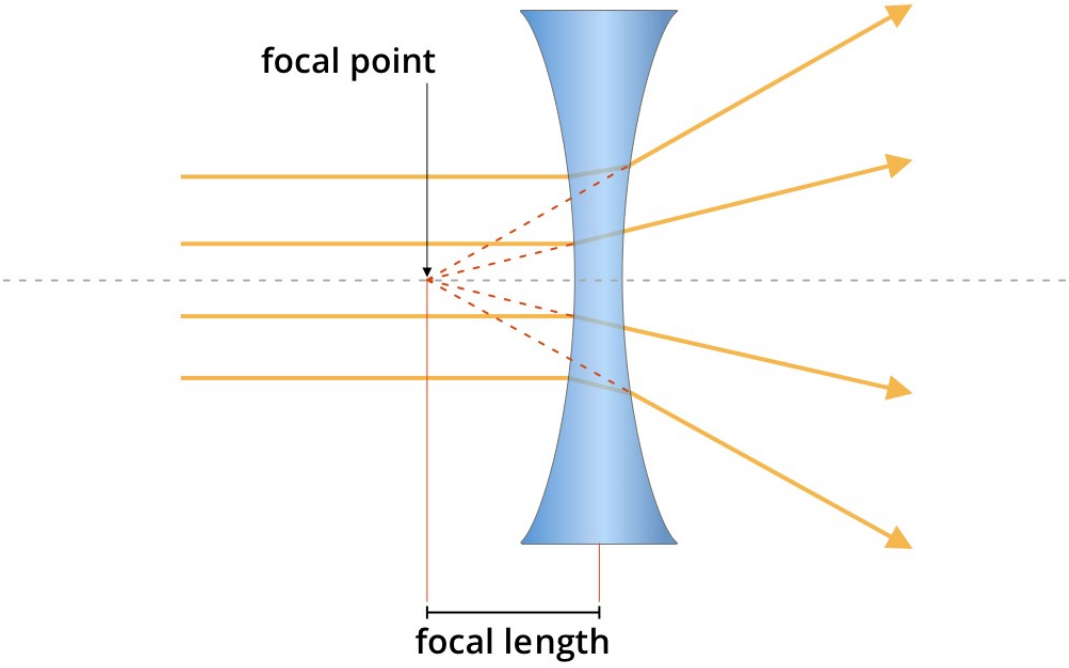
- Depending on the nature of the material, the beam can be bent by the sample as it passes through it. This is called refraction. Some refractive materials are used as lenses – see section on lenses below. A measure of how much a given material bends the light is known as the refractive index. The refractive index affects the resolution you can achieve with a given microscopy technique. Refractive index is also temperature dependent, which is important if your microscopy experiment uses altered temperatures. It can be significant in light microscopy for live cell work where differences even between 23 and 37 degrees Celsius can affect your results.
  Technique examples: bright-field transmitted light microscopy, bright-field TEM, phase contrast, transmitted light differential interference contrast (DIC).
- When a wave hits an opening, it spreads out on the other side. We call this diffraction. When there are many openings close together, the waves run into each other to form bright spots or peaks through constructive interference. These can be used to study materials. In microscopy, diffraction itself is used to study the structure of crystalline materials – the periodic structure of a crystalline solid acts as a diffraction grating, scattering an electron beam in a predictable manner.
  Technique examples: X-ray diffraction, electron diffraction, electron backscatter diffraqction (EBSD).
- Secondary emission occurs when a beam hits a sample and causes other particles to be emitted. These can be collected and used for imaging or analysis, or, ignored where other forms of emission are important. These secondary particles are generally of lower energy than the primary beam, but not always.
  Technique examples: – fluorescence, SEM, Secondary ion mass spectroscopy (SIMS).
- Staining is often used to generate contrast in transmission techniques. With very thin and transparent samples such as cells and tissue sections, some kind of staining or labelling is often necessary to enhance sample interactions with the beam to increase contrast and make features more visible. Stains tend to bind either generally or differentially to chemicals such as proteins, lipids or nucleic acids within the sample to absorb certain wavelengths, or to fluoresce, emitting at distinctive wavelengths. Examples are dyes, heavy metals and fluorescently labelled antibodies.
  Technique examples: histological stains such as haematoxylin and eosin, or toluidine blue for bright-field light microscopy, heavy metal staining such as osmium, or gold-labelled antibodies for TEM, fluorescent labelling for many fluorescence techniques.

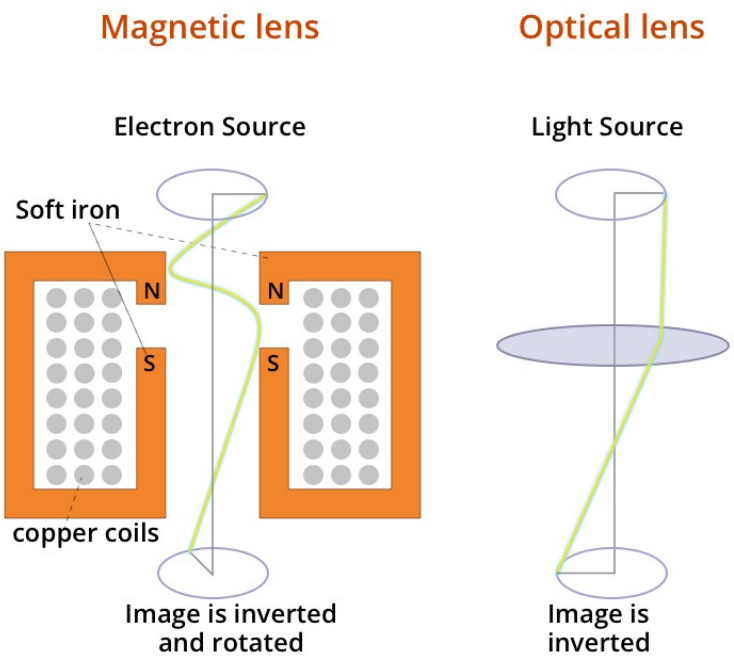
Lenses and Aberrations – Lenses

A lens is a device that bends a beam of electromagnetic radiation either focusing or deflecting it. A lens can focus a beam to form an image, unlike a prism, which refracts a beam without focusing it.
Lenses can be either transparent material, such as the glass or plastic used to focus visible light, or they can consist of rings of magnets for focussing beams of charged particles such as electrons.

Lenses for visible light are made from materials that can be polished or moulded to the desired shape. They can be made to be either convex or concave. Convex lenses bend light in towards a focal point whereas concave lenses diverge light.



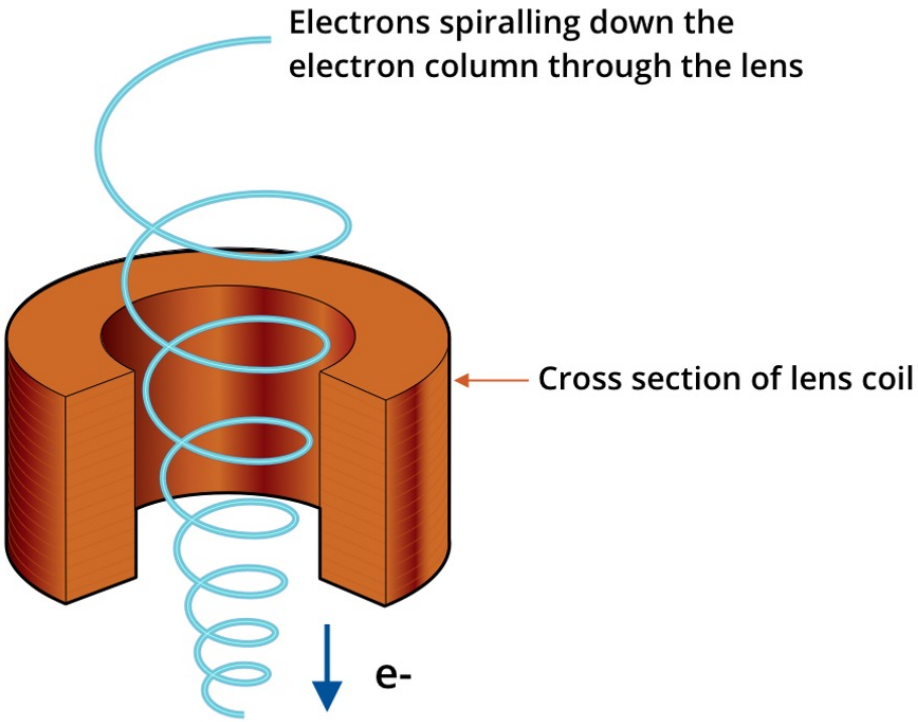




As we have seen, the purpose of a lens in a light microscope is to change the path of the light in a desired direction. Electrons cannot travel through glass or plastic lenses and are therefore not appropriate for use in an electron microscope.

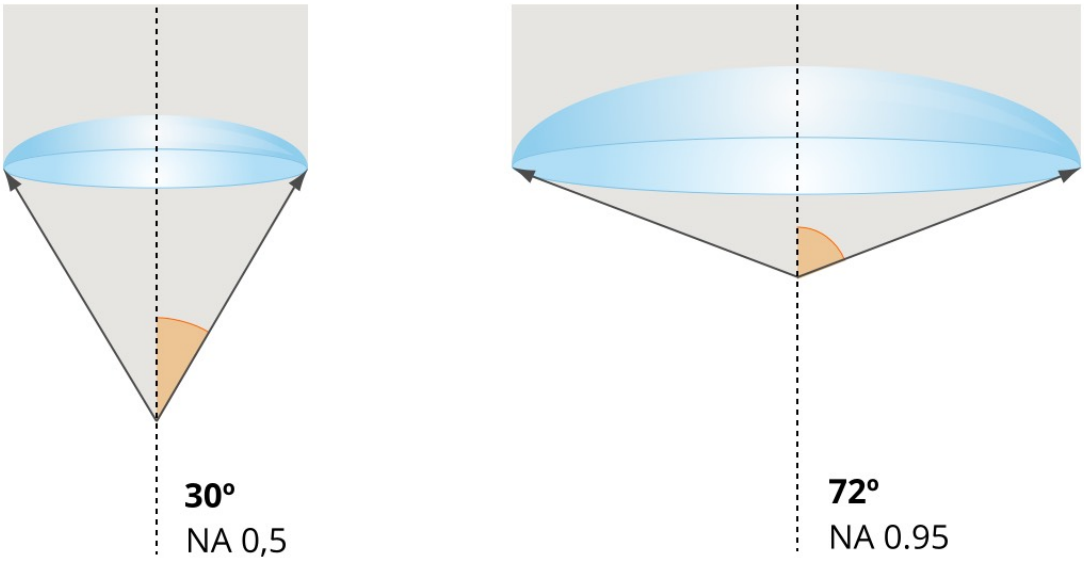
As electrons are charged particles, their path can be bent by a magnetic field. Therefore, in electron microscopes, a series of electromagnetic lenses and apertures are used to reduce the diameter of the electron source and to direct a small, focused beam of electrons (or spot) onto the specimen.

These electromagnetic lenses are constructed with ferromagnetic materials and wound copper wire. They produce a focal length that can be changed by varying the current through the coil. The magnetic field bends the path of electrons in a similar way that solid glass lenses bend light rays. Under the influence of a magnetic field, electrons assume a helical path, spiralling down the column.



Lenses used in X-ray microscopy systems use different methods to deflect the beam. They are very complex and beyond the scope of this introduction.

Different lenses can allow the passage of the illuminating beam to different extents. The more illumination that passes through a lens, the greater the resolution of the system. In different microscopy techniques this is controlled in different ways. In lenses for visible light microscopy, lenses are manufactured so they can gather more or less light. The relative amount of light gathered by a lens is known as its numerical aperture (NA). The greater the NA number, the more light it gathers and the greater the resolution it gives. Higher NA lenses are also more expensive, and their effectiveness is limited by inherent aberrations. This is described below.

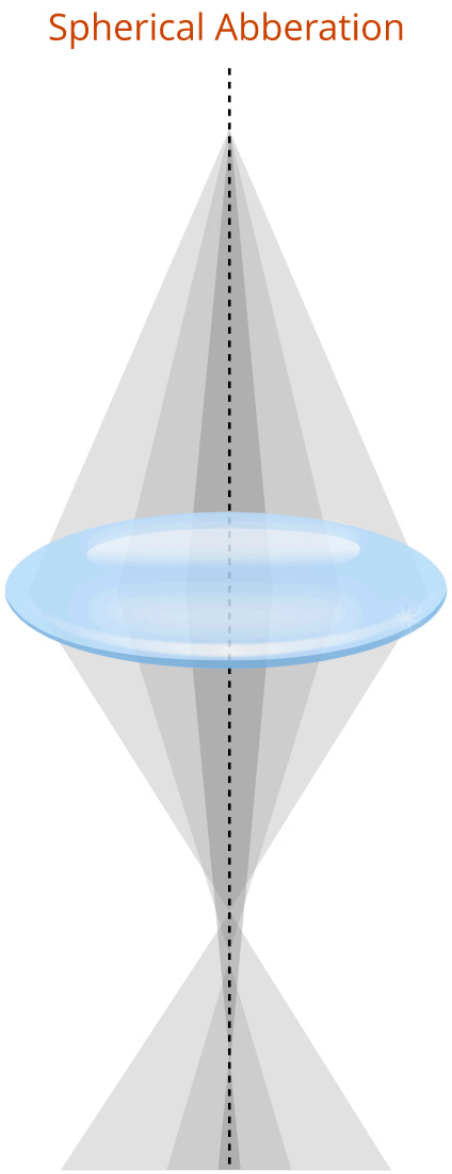


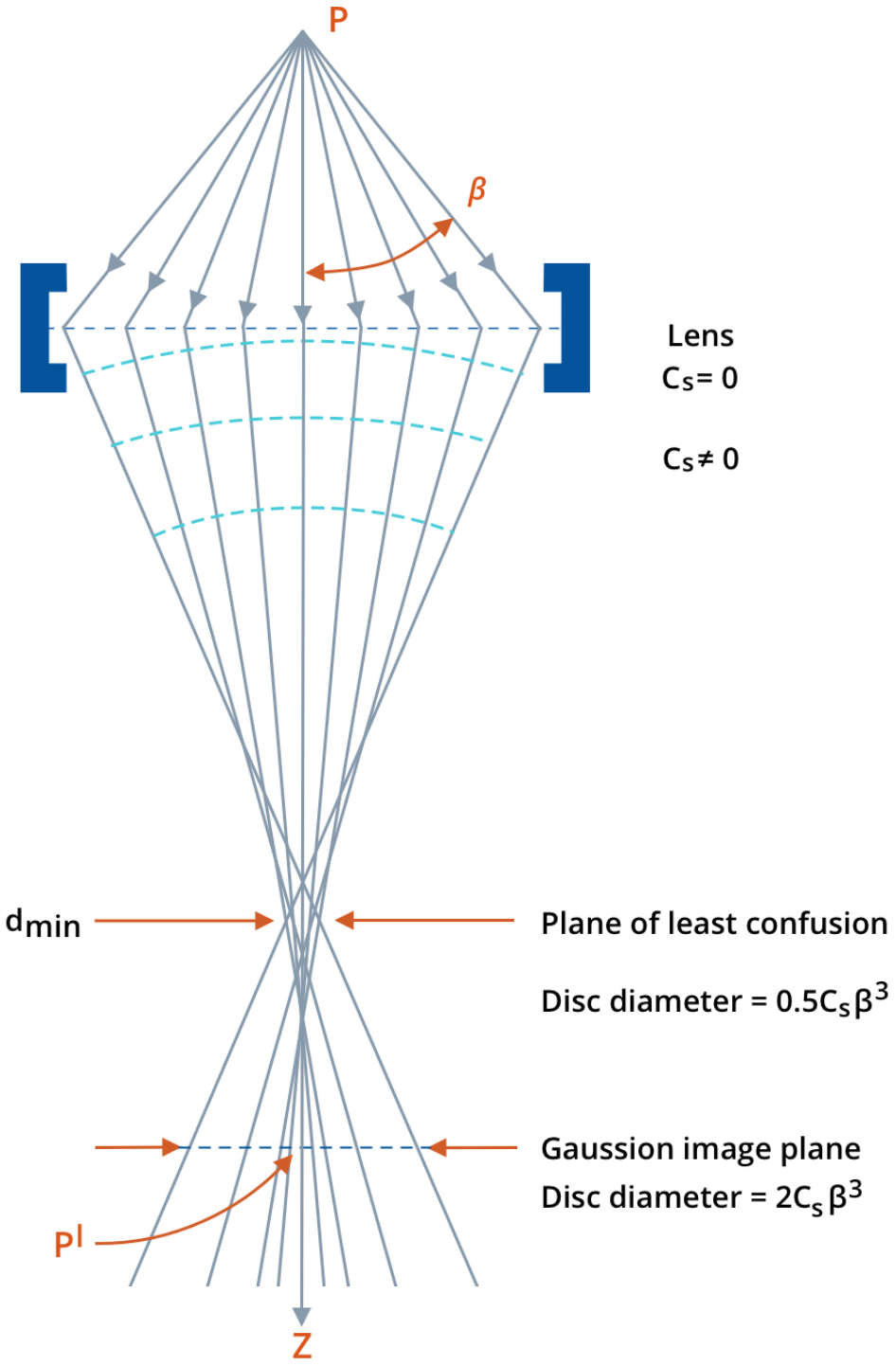
In other microscopy systems that use electromagnetic lenses, one way that the amount of illumination passing through a lens is controlled is by inserting a metal strip with different sized holes (apertures) into the path of the beam. The sized hole is selected to be most compatible with what you need to best visualise the sample.

Aberrations – Types of aberration

Aberrations are imperfections in the way lenses transmit and focus the illuminating beams. Understanding these aberrations is important for choosing the best lens to view your sample.
Over the last 300 years, high-end light microscopy objective lenses have developed to near perfection, while less expensive light microscopy objectives and electromagnetic lenses remain quite imperfect. There are many kinds of lens defects, but we will emphasise only those that limit microscope performance in substantial ways. These are unavoidable and it is necessary to learn how to use the microscope to minimise them. They are:
- Spherical aberration
- Chromatic aberration
- Astigmatism

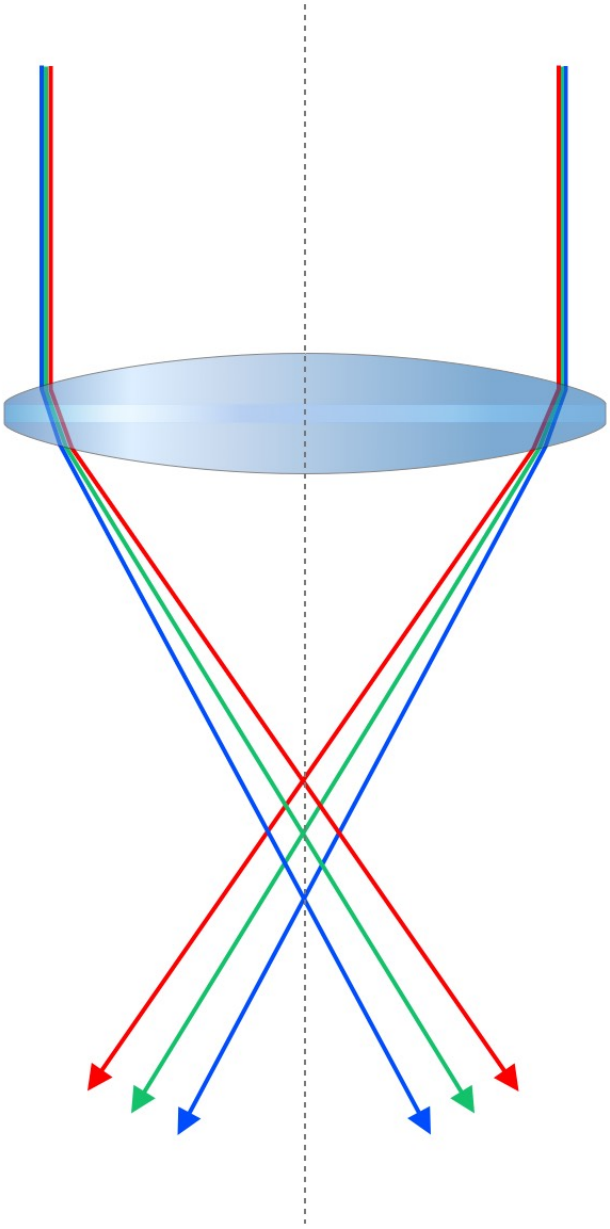
Spherical aberration is the most significant in defining the performance of the objective lens. It is an inherent property of a simple lens and occurs because the lens is more powerful at the edge that in the centre. This results in an image that will not be perfectly sharp.

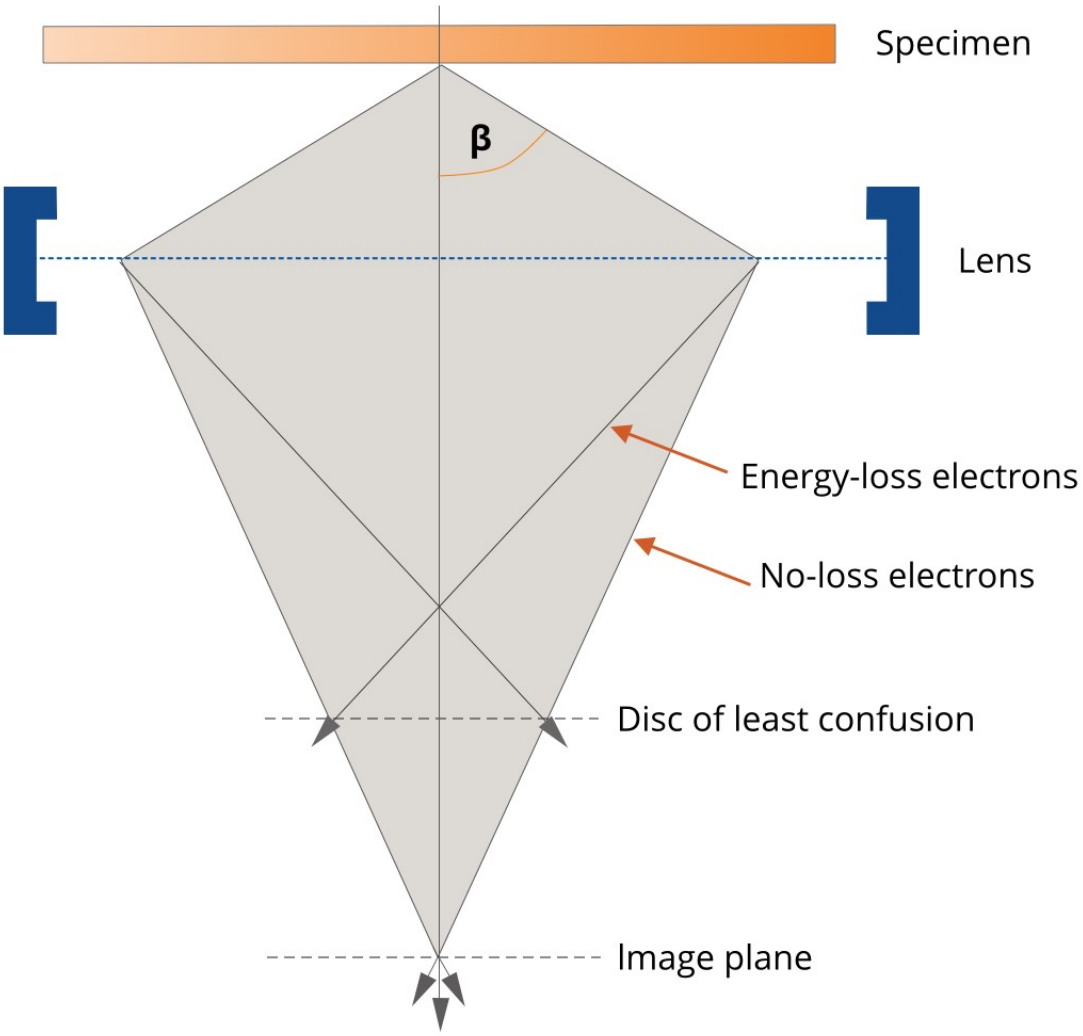




Chromatic aberration is also an inherent property of lenses and occurs because all lenses bend beams of different wavelengths to different extents: higher wavelengths (lower energies) are bent less. This is the same reason that a prism splits white light into a spectrum. White light is composed of the different colours (different wavelengths) of the visible spectrum. Similarly, electron beams emerge from the electron gun with a range of different wavelengths. The presence of chromatic aberration means that we cannot get all wavelengths in focus at the same time.

Chromatic aberration





Astigmatism occurs when the beam is not perfectly round. This can occur when parts of the microscope column are not properly adjusted. In practice, it is more significant for electron microscopy than for light microscopy and is corrected by controllers during the focussing process.

Overcoming aberrations

One way to overcome chromatic aberration is to try and get a beam as close as possible to a single wavelength. In light microscopy you can do this by using just one colour of light, and although this works well, if we always did this, we would be throwing out one of the great advantages of light microscopy - the ability to show different structures or substances in different colours.

In electron microscopy, monochromators are available on high-end electron microscopes that reduce the spread of wavelengths emerging from the electron gun. They can significantly improve the resolution that can be achieved.

Spherical aberration is most pronounced in wide lenses. As wide lenses typically give the best resolution, there is a trade off between spherical aberration and resolution. Spherical aberration becomes worse much more rapidly than the resolution increases.

In light microscopy, these aberrations must be corrected to make a usable high-NA lens. Fortunately, we can correct them, but it requires multiple optical elements, which is why high-NA objectives are expensive.

In electron microscopy, aberration-correctors for spherical aberration are available on high-end electron microscopes and significantly improve the resolution. Aberration-corrected transmission electron microscopes allows users to routinely reaching atomic resolution of crystalline samples.

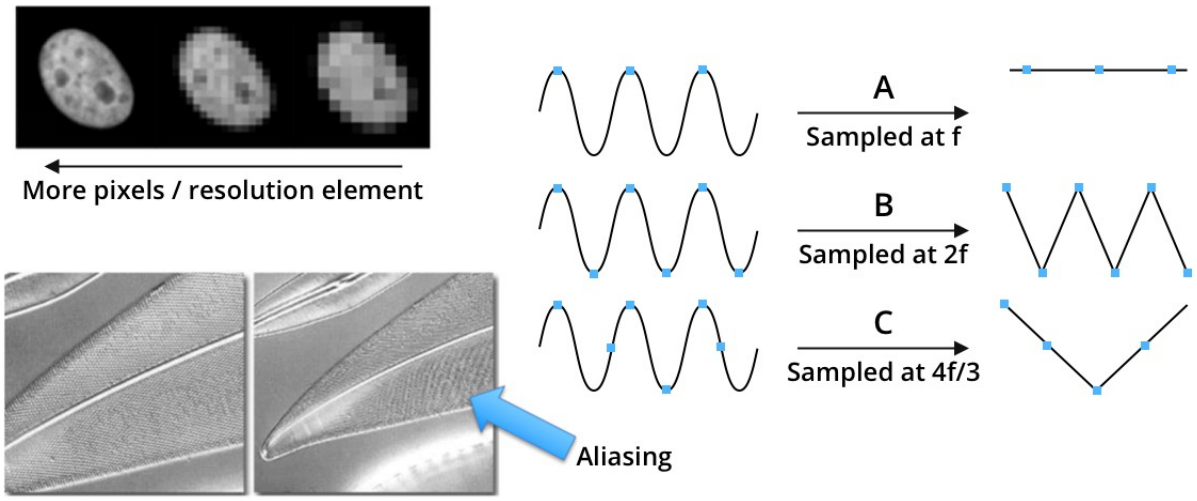
Calibrations

Any measurements made during imaging are only as accurate as the instrument calibration indicates. If absolute accuracy is critical to your research, regular calibrations against a standard reference would become part of your imaging routine. Standards can be grids, meshes, beads or objects with known dimensions and positions in space (for multi-dimensional imaging tools). These standards are produced and provided by such entities as the US National Institute of Standards and Technology (NIST).

Nyquist Sampling

When capturing images with a digital camera, the pixel size you choose relates to resolution of your final image. If you do not choose enough pixels you will not be able to capture enough of the data to accurately see the features you are interested in and artifacts such as aliasing can distort your image.

To work out the pixel size you need to achieve a given degree of resolution, Harry Nyquist calculated that you need at least 2.3 pixels for each unit of resolution that you need. For example, if you need to see a resolution of 1µm, then you need 2.3 pixels/µm to optimally capture an image of your sample.



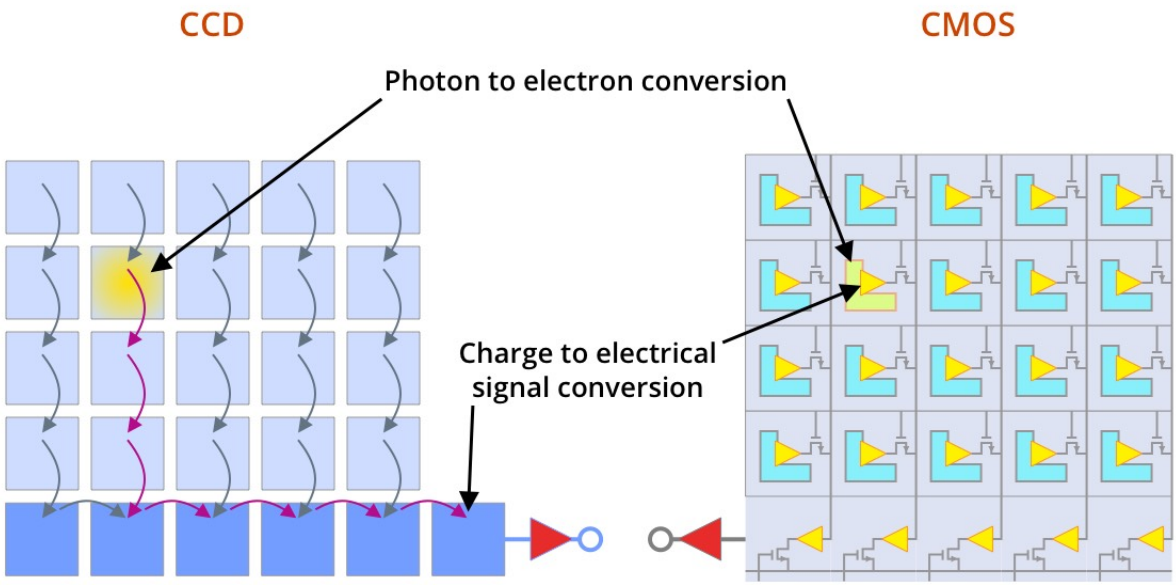
Microscopy can be extremely useful for object tracking over time and observing dynamic processes. When capturing a time course, it is important to select the number of time points that provide experimentally meaningful information, but not so many that you risk damaging your sample through beam–sample interactions or end up collecting vast quantities of unnecessary data that requires processing and storage.

Detectors and Cameras

Nowadays, many microscopes capture images on digital cameras. Cameras allow you to capture a visual representation of what you see down the eyepiece or on the screen. The quality of a digital image depends on the quality of the camera, namely on the number and brightness of the pixels.

There are two main types of cameras used in microscopy: charge-coupled devices (CCD) and complementary metal-oxide semiconductors (CMOS). In transmission electron microscopy these are gradually being replaced with direct-electron cameras, which give a sharper image.

Different types of camera technologies bring different benefits.



CCD	CMOS
Higher power consumption	Lower power consumption
More pixels / better quality low-noise images	Lower sensitivity
Slightly more expensive	Slightly cheaper
Slower, but more consistent readout	Fast readout

In light microscopy, another consideration when choosing cameras is whether or not they capture images in colour. If speed of image capture is important then a colour camera is very useful. If speed is not important, it is possible to capture each of the red, green and blue channels separately and combine them afterwards in image processing software.

The reason for this is that a colour camera uses four pixels to capture the colour information for each point: one for red, one for blue and two for green. This takes up a lot more of the pixels, making an image on a colour camera considerably less information rich than one taken on a monochrome camera of equivalent pixel capacity. A monochrome camera will give more information and a better signal to noise ratio for a given pixel capacity.

Signal-to-noise ratio is an important consideration in collecting all kinds of experimental data and is equally important in microscopy. If you are expecting low levels of signal to noise, camera choice is one area that can help maximise the meaningful signal that can be captured. Image analysis and processing can also help to distinguish your signal from background noise.

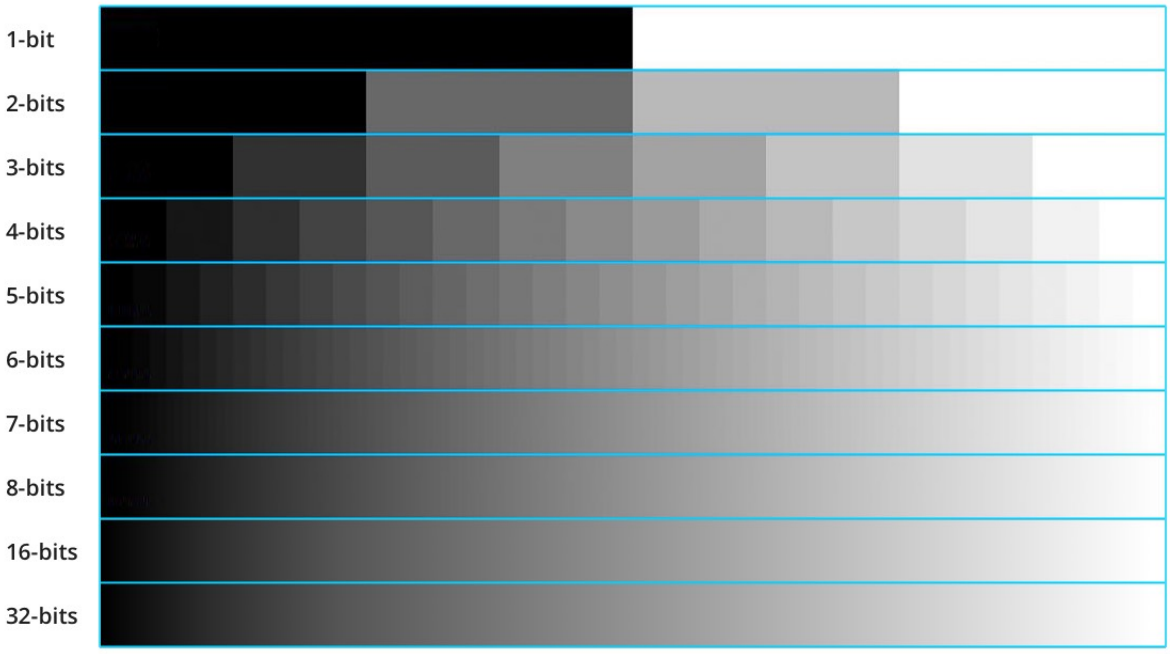
Detectors allow you to capture types of non-visual information from your sample such as those described above in the section on beam–sample interactions. Scanning techniques like scanning electron microscopy and confocal

microscopy use detectors that collect information from each point that encounters the beam as it scans over the sample. These can then be assembled by the image processing software to construct an image of the sample.

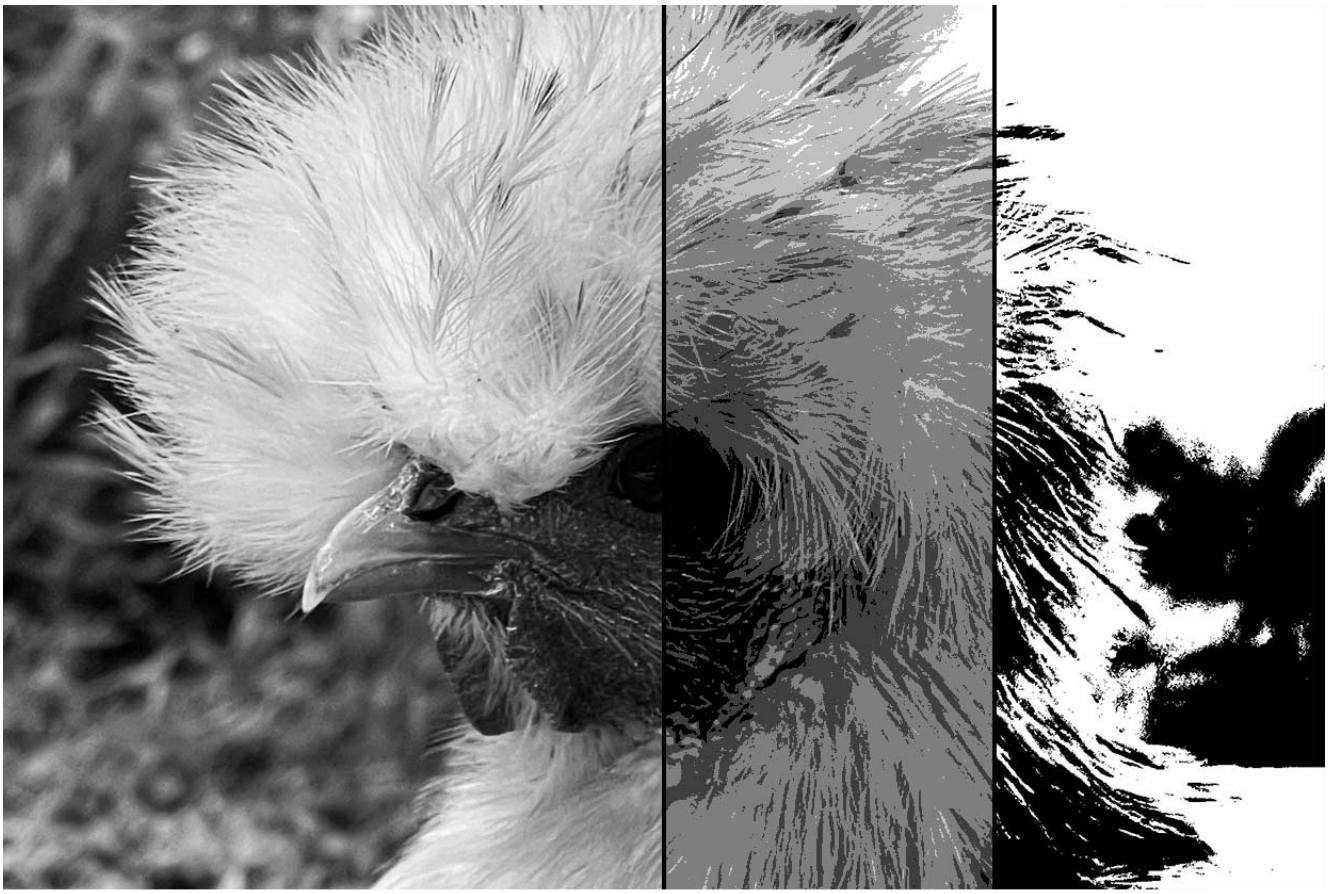
Other types of detector are specifically designed to capture the information generated by many specialised techniques. Information from detectors can be combined with digital images from the camera to provide positional information about the data, correlating it with visible features on the sample.

When capturing digital images, cameras need to be set up so that they capture an appropriate bit depth to adequately collect the range of tones that contribute to the meaningful data you wish to acquire from your sample. The accuracy of a digital image is related to the accuracy of the conversion to its constituent black, white and grey levels. Bit depth determines the number of increments of grey levels in your image. The higher the bit depth the more shades of grey there are in the image and the more continuous the shading appears to the viewer. The trade off in capturing high bit depth image files is that they are much larger than lower bit depth files. This means that they take up more disc space to store and take longer, or are more difficult, to process.

Bits per color	Log formula	Grayscale values	R,G,B Values
1	2^1	2	8
2	2^2	4	64
4	2^4	16	4096
8	2^8	256	16.77 million
10	2^{10}	1024	1.07 billion
12	2^{12}	4096	68.72 billion
16	2^{16}	65536	4722.44 billion



Also keep in mind that if you have a high bit depth, you have more dynamic range to work with. For instance, if you have 12-bit detector and are only collecting signal in the bottom 100 grey levels, you aren't taking advantage of the full dynamic range of the detector (4096 grey levels). If you have very dim and sensitive samples, a high dynamic range (16-bit) camera would be helpful because the signal only falls in the bottom thousand grey levels, which would be the bottom tens of grey levels on an 8-bit camera.


8-bit
4-bit
1-bit

Digital Images – Introducing digital images

Capturing microscopic images digitally provides great advantages in processing and analysis of your data. However, there are many factors that need to be considered to ensure that you get the most from your digital data.

File formats

Image file formats vary, most particularly in whether or not they lose information when saved. This is often described as lossy versus lossless. This is very important, especially if your image goes through repeated cycles of opening and saving, such as when you are editing images – each time an image is saved using a lossy method, more information is lost, leading to cumulative deterioration. Lossy file formats compress the images to make smaller files, usually smaller than lossless methods but this loss is irreversible. The main lossy, but very popular, file format for images is jpg. It has the ability to save with various levels of compression, from none, (lossless) to highly compressed and lossy.

Some commonly used lossless file formats used for images are tiff, png, bmp, raw and pdf.

Proprietary formats that are specific to different microscopes tend to hold the most information, including metadata and should be kept when collecting and organising your images. Copies can be saved in more convenient formats, but the originals should be retained. These proprietary formats can often be opened in software such as ImageJ where much image processing and analysis can be carried out.

Metadata

Metadata is information about the file and the conditions under which it was captured. It is stored within the image file and should be retained as part of the image for future reference. It is often saved in proprietary formats but not in others.

Bit depth

Bit depth (bits, bytes, dynamic range) was described above and should be set on the microscope when capturing your images. You can subsequently adjust the bit depth in image processing software (see above). We can only see about 100 grey levels, which is equivalent to 8-bit. However, if you add colour to the intensity levels using various look-up tables, you may be able to distinguish more details. 8-bit is fine for just looking at images of your sample and generally speaking you only need to choose 16-bit or higher if you need to analyse your data in complex ways that require the finer intensity level steps.

Histograms and Look-up Tables (LUTS)

Digital images are just a display of intensity values across a range of pixels. Here is an image of a dog and the pixel intensity values of a single pixel (f{0,0}) as well as a 16x16 square of pixels within the image (f{332:348, 293:309).

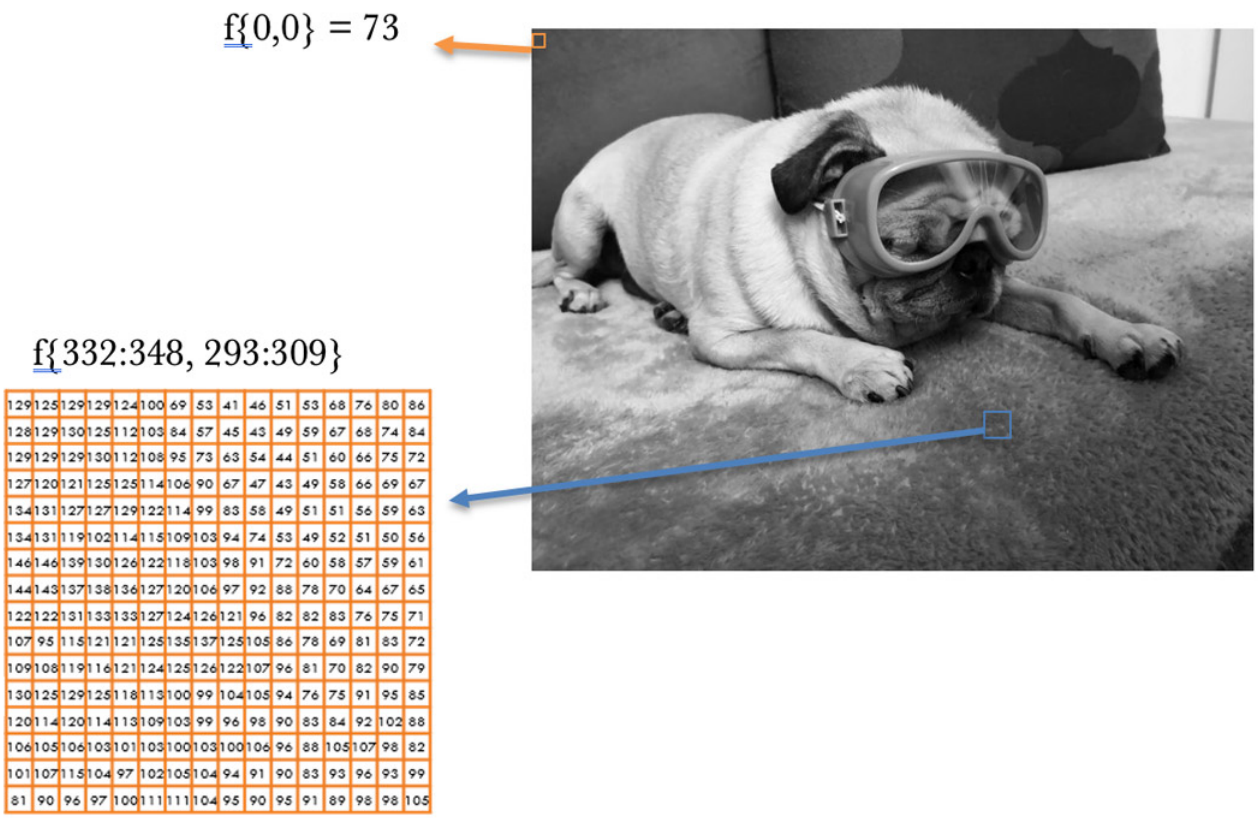
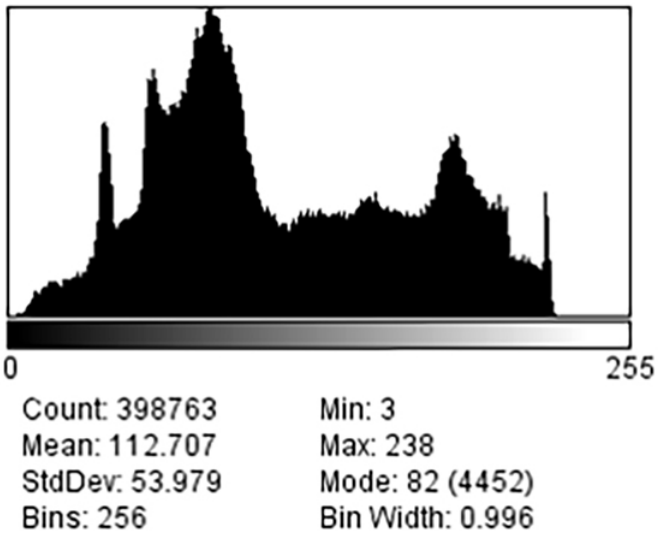


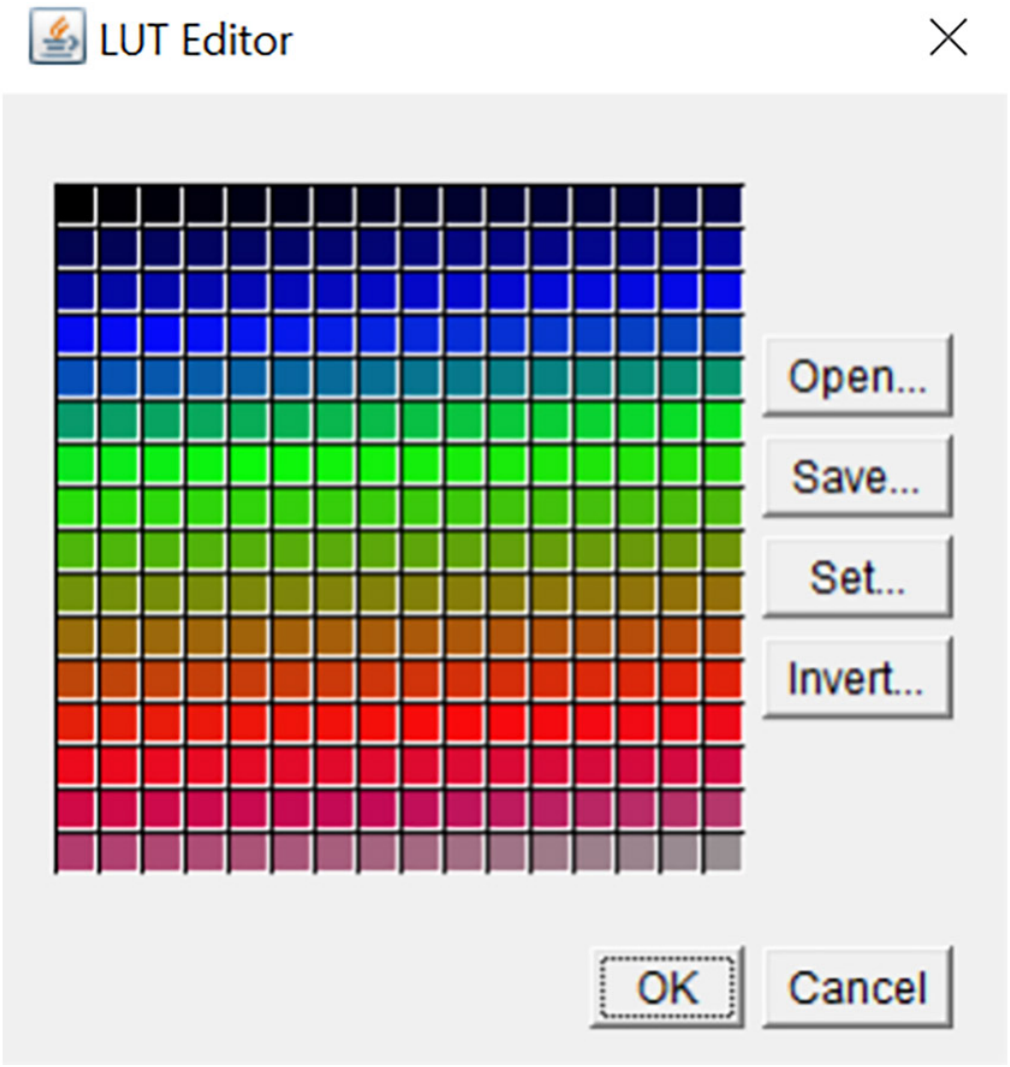
Image data can be displayed as a histogram of intensity values. This is incredibly useful when optimising camera or detector settings.



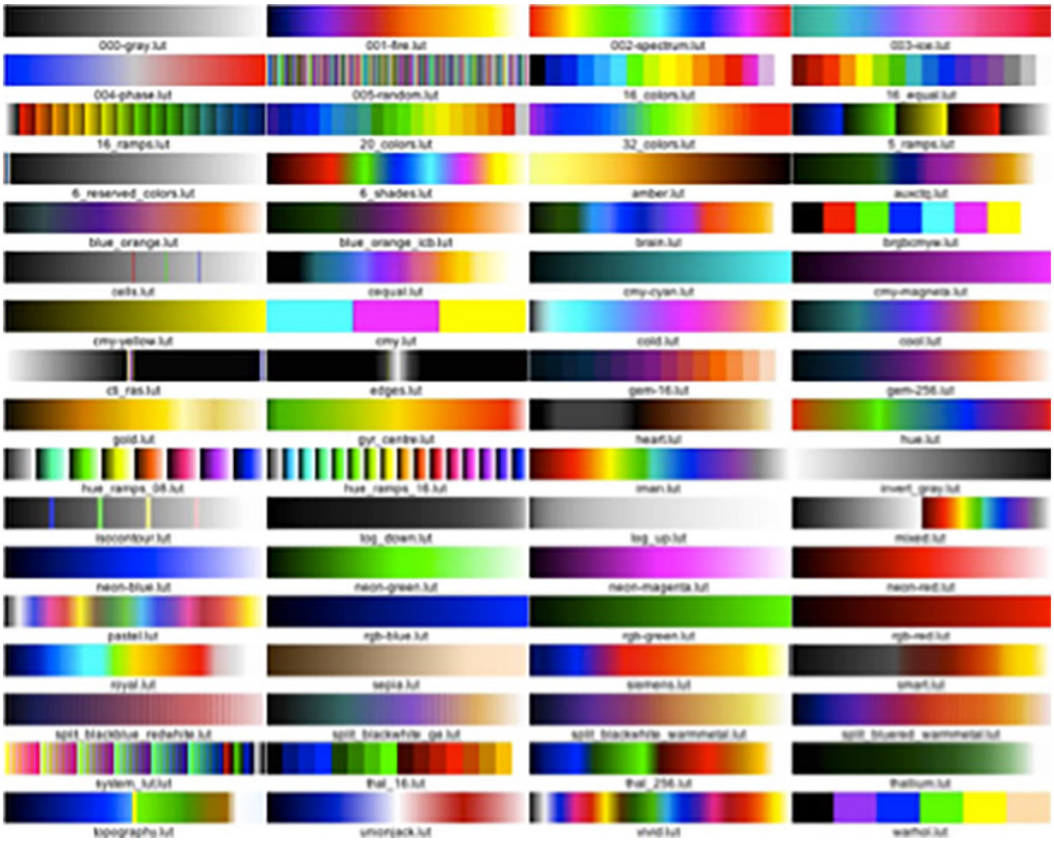
You can apply a Look-Up Table (LUT) to your image to help visualise your data. Here we are displaying the data with a linear magenta LUT. This means as the image intensity goes up, the magenta appears brighter.

Here is the same image with a Rainbow LUT.

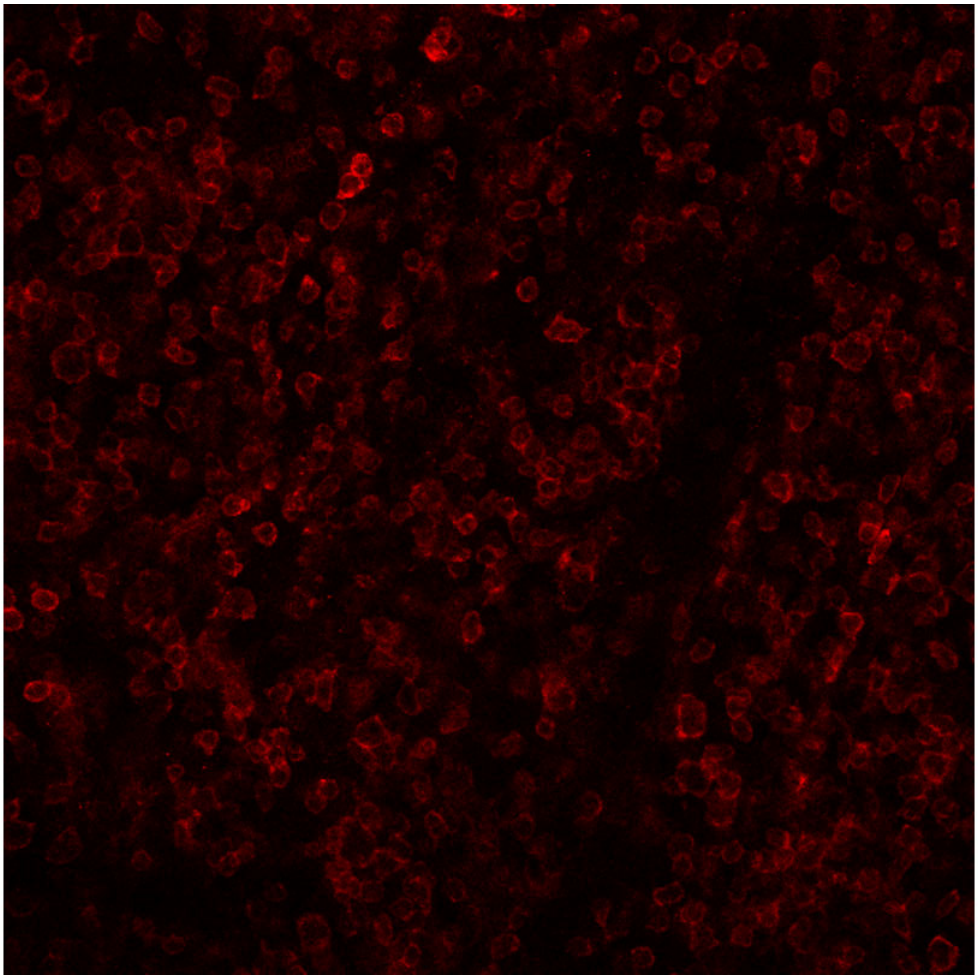
The Rainbow LUT uses different colours across ranges of pixel intensities. The LUT editor shows how the colours change with each pixel intensity from 0 (upper left) to 255 (lower right).



Digital images can be displayed in many different ways using LUTs without actually changing the data. The intensity values haven't changed...just the display. This is still 8-bit data from 0-255. There are lots of different LUTs. Here is a small sample.

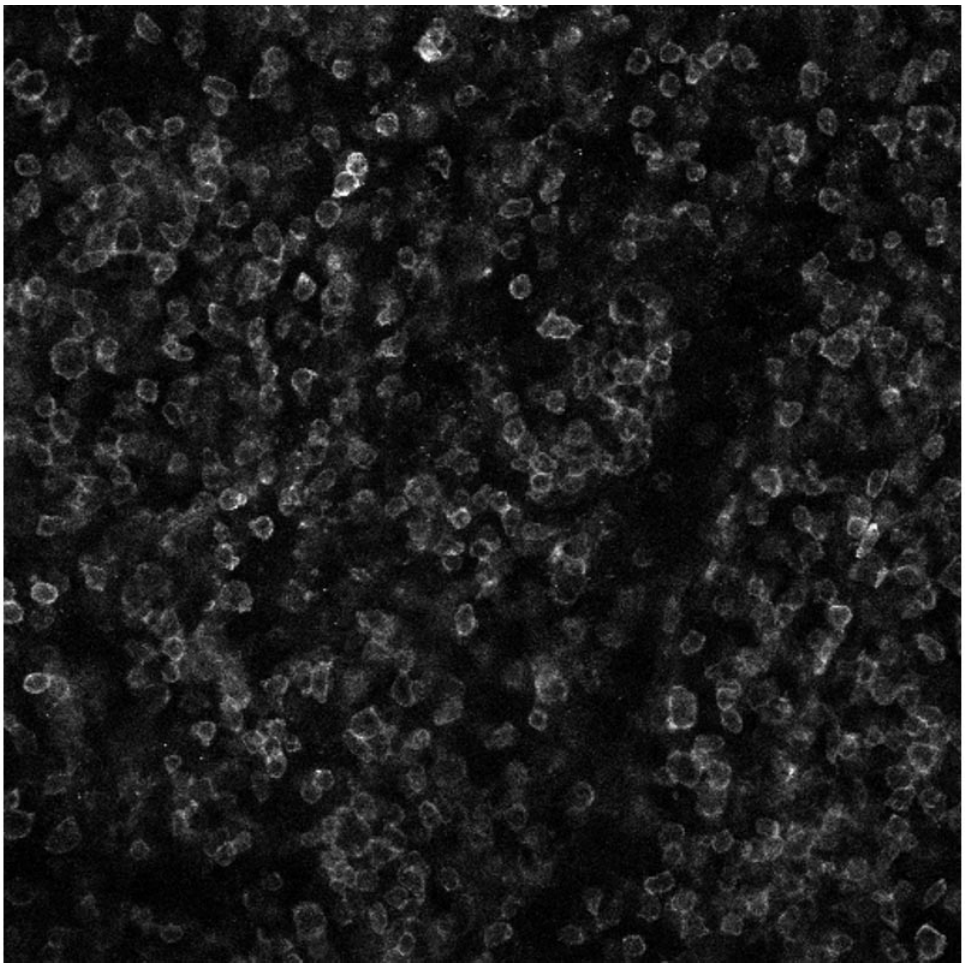


Here is confocal image of Alexa568 labelling tissue. Down the eyepiece the tissue will look red/orange. So, we can colour it with a Red LUT.

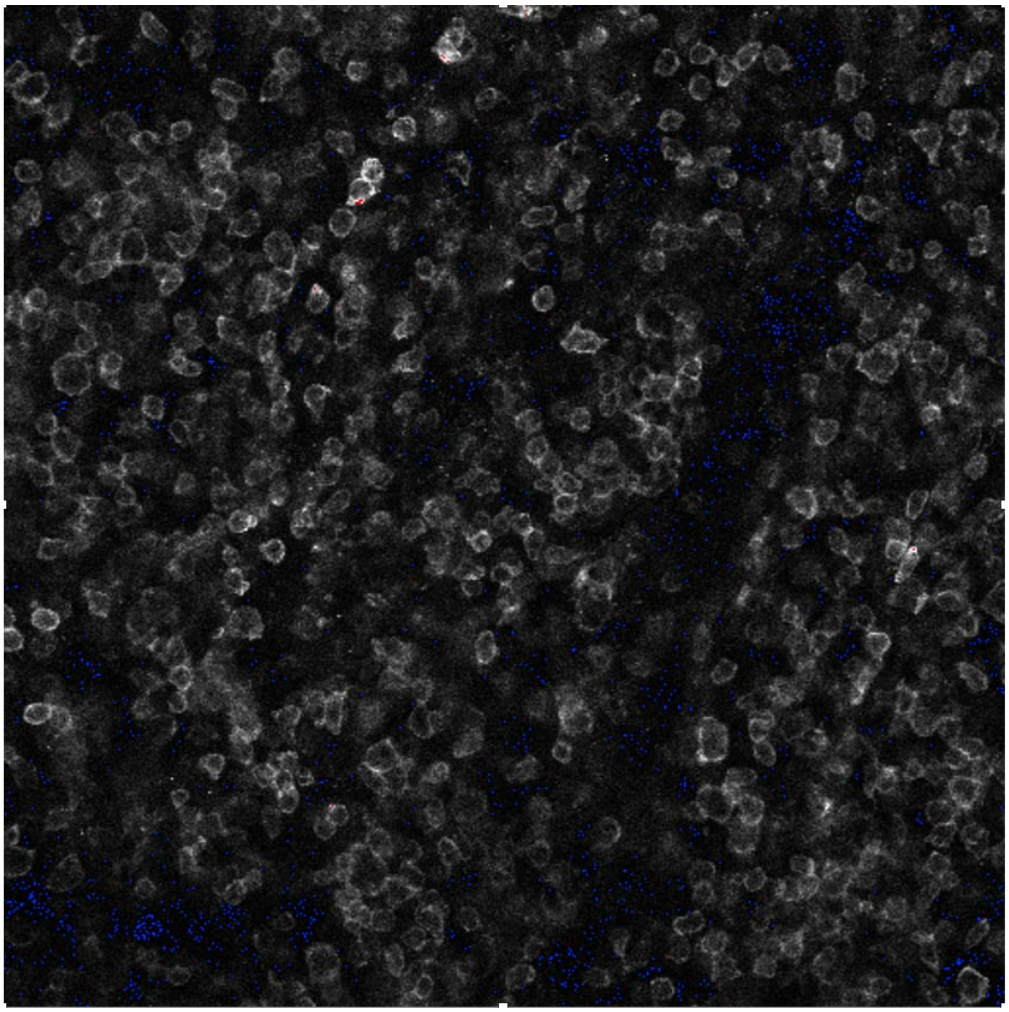


But our eyes don't see Red very well. We can only discern ~40 levels of red. But we captured 255 with an 8-bit

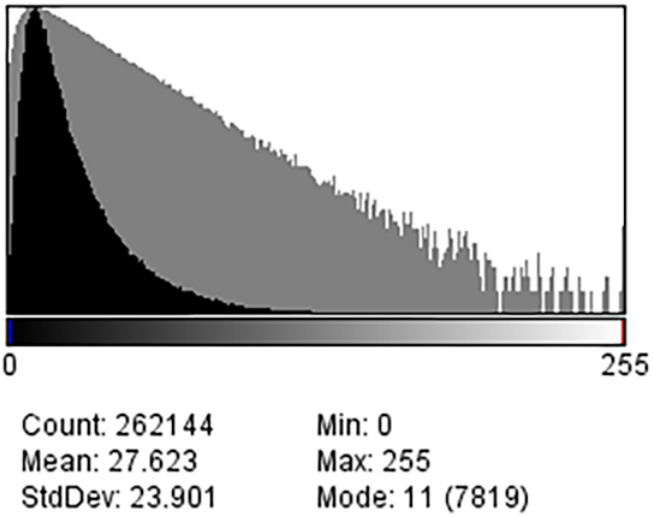
detector. So, to make it clearer, we can use a GRAY LUT, in which our eyes can see over 100 levels.



If we are trying to optimise detector settings, we may want to use a HiLo LUT that colours the undersaturated pixels (those with 0 intensity and therefore below the detection ability) BLUE and the saturated pixels (brighter than the detector can detect and therefore capped at 255) with RED.



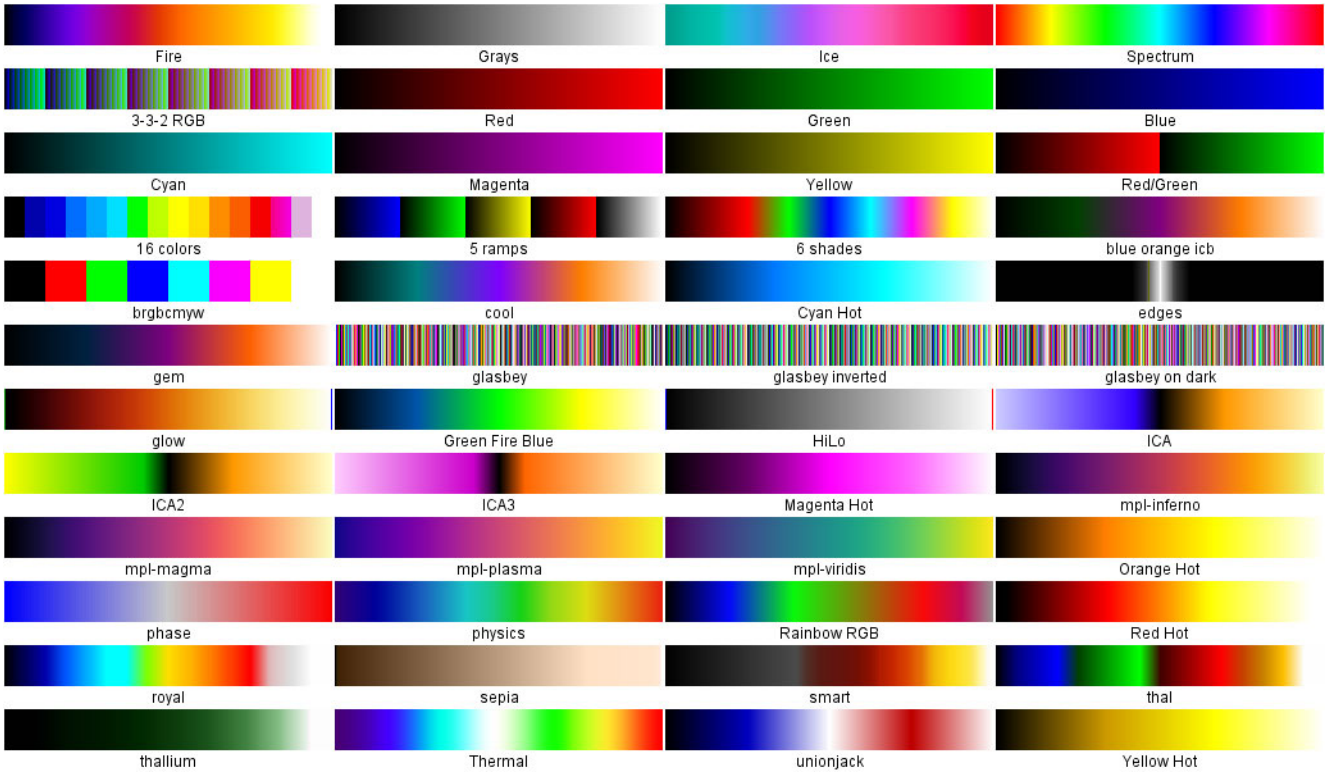
You can see only a few RED (saturated) pixels and a few BLUE (undersaturated) pixels. This LUT makes it easy to optimise the detector settings to ensure you are not missing data from the bright or dim areas in the sample. Here you can see that with the linearly scaled histogram alone, you can't tell if there are any saturated pixels. Even when scaled logarithmically, although you can tell some pixels are saturated, you don't know where they are in the image and whether there is a bit of artifact or if it is actually the sample of interest that is saturated.



In X-ray micro-tomography LUTs can also be used to highlight and distinguish features of interest. Choice of LUTs can make a huge difference to how your data appears and it is possible to create your own LUT that is optimal for your sample, as has been done for the cat skull below.

Scanned cat skull visualised using a custom LUT.

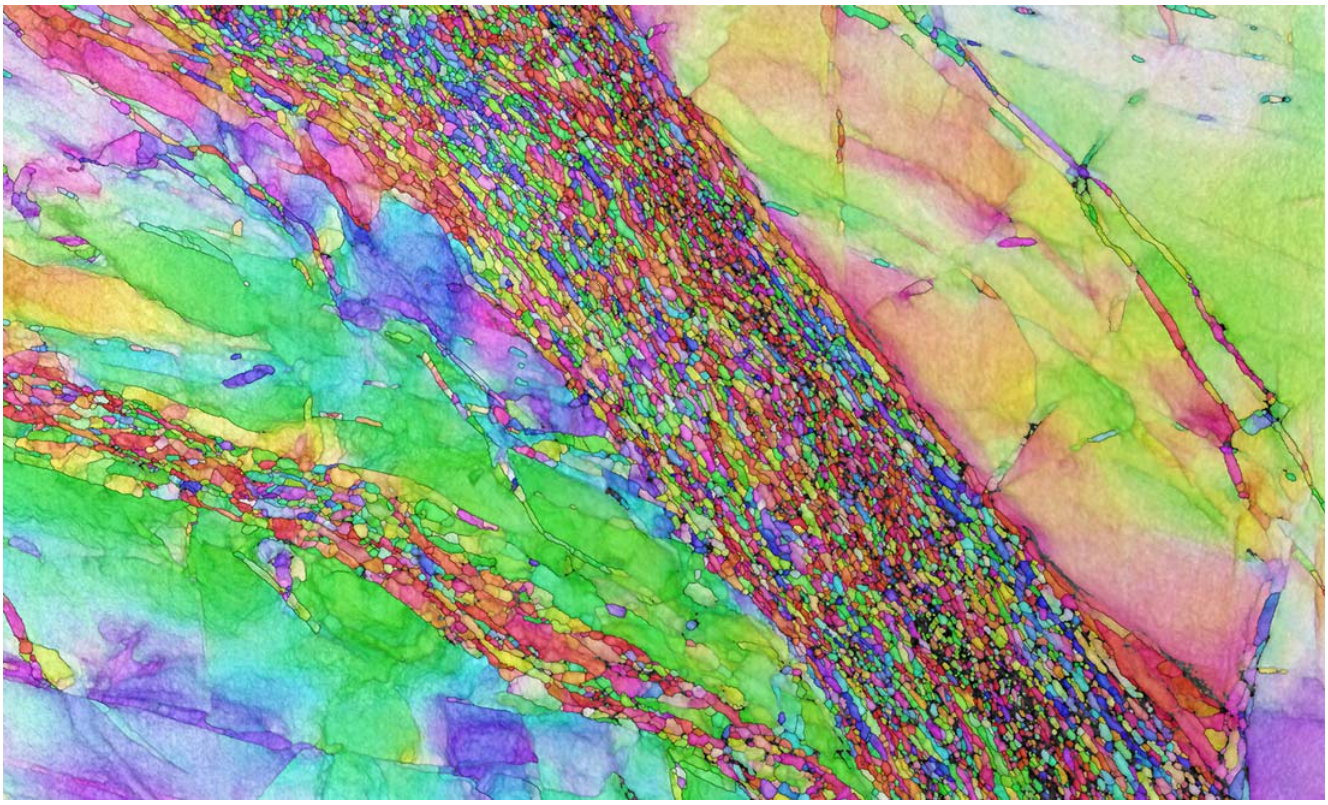
LUTs are often supplied with instrument and image analysis software.

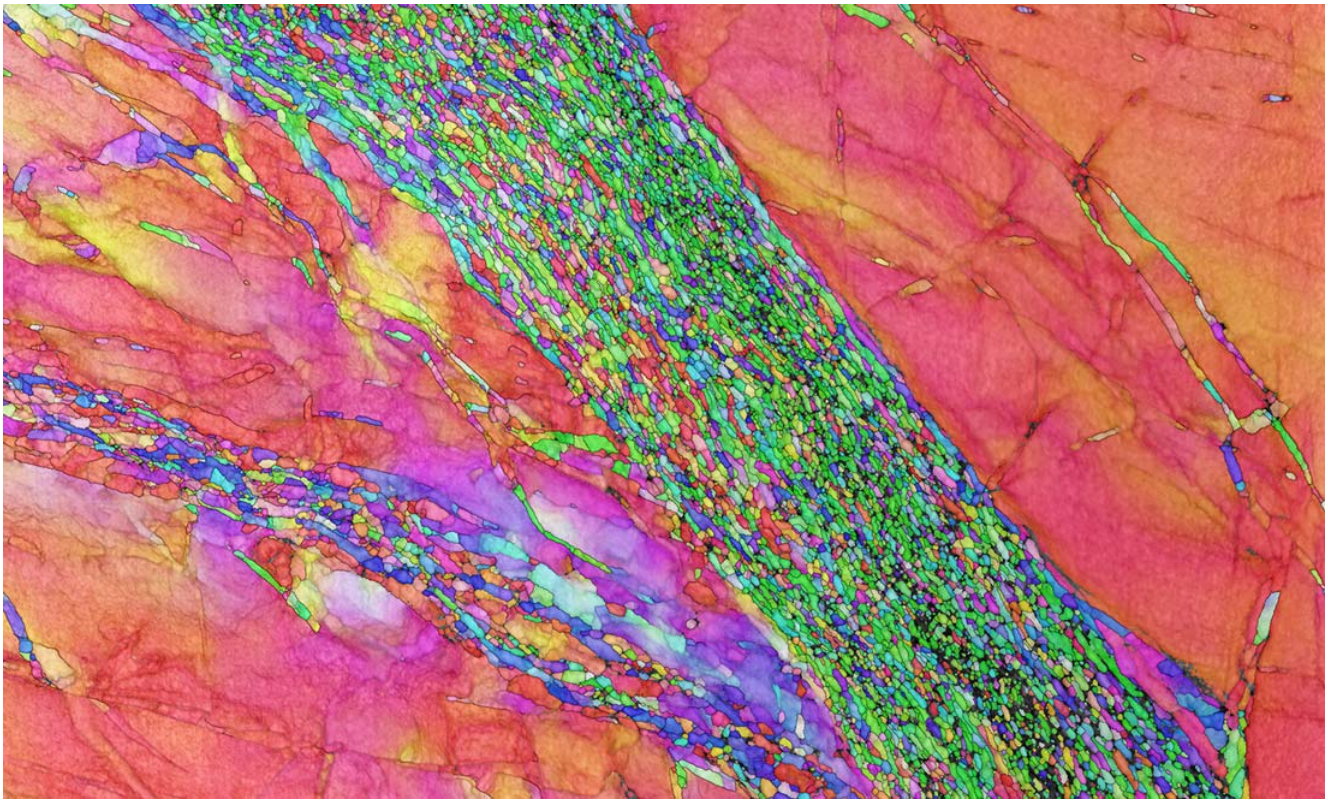


Default LUTs available through the FIJI image analysis software.

The same cat skull data set as above, visualised using the Glasbey on Dark LUT in FIJI.

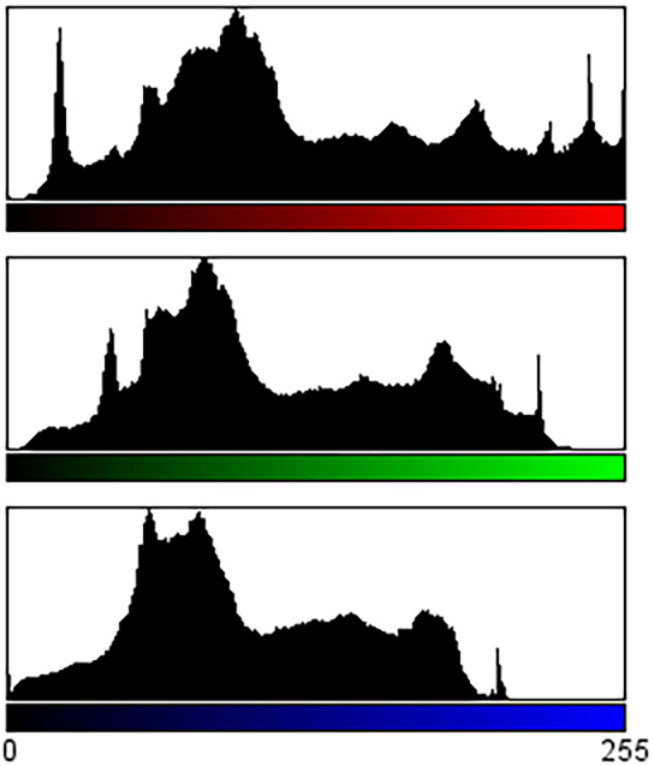
In electron backscatter diffraction (EBSD) and transmission Kikuchi diffraction (TKD) LUTs are used to visualise the crystal orientation data held in each pixel. In the images below, you can see how different LUTs show the data differently.





Colour

Colour cameras collect RGB colour images. Typically, a red image with intensities from 0-255, a green image with intensities from 0-255 and a blue image with intensities from 0-255. The colour histogram will actually be made up of three histograms.

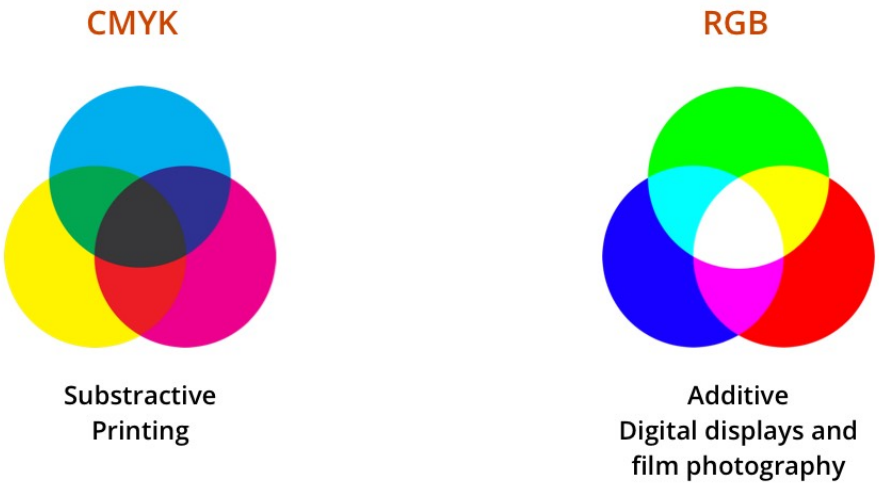


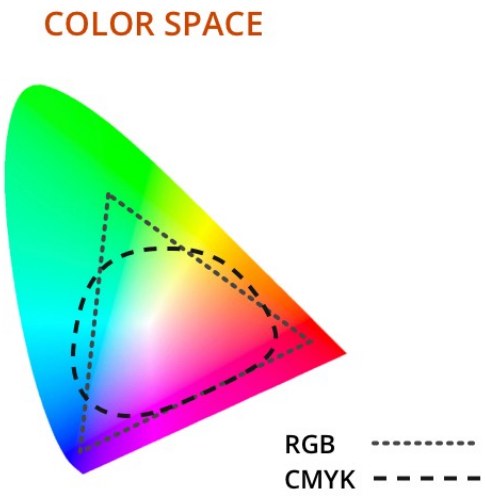
Count: 1108992

rMean: 125.24	rSD: 64.69	rMode: 94
gMean: 113.23	gSD: 54.31	gMode: 80
bMean: 99.77	bSD: 47.06	bMode: 58

Greyscale vs colour

This was discussed above with respect to cameras. If you only need monochrome images, saving them in greyscale makes the file sizes smaller for the same pixel dimensions. If you need colour images for analysis and for displaying on screen, RGB should be chosen. CMYK is only used when preparing images for print and this is usually handled by the graphic designers and printers when they are preparing manuscripts or other content for print. Converting from RGB to CMYK is changing from subtractive colour to additive colour and many fluorescent images can end up looking less vibrant in CMYK.





Images for Print in Journals and Posters

Printed images are usually produced at a much higher pixel density than on-screen images. The standard for print is 300dpi rather than the 72 or 96dpi routinely used for images displayed on screens. When preparing images for print, without resampling the image, convert the pixel density to 300dpi and check the image dimensions to ensure the image will be big enough to be reproduced at the required size on the printed page.

When assembling figures, always keep a copy of the original images and keep versions of the figure with any annotations on separate layers so they can easily be altered if required.

Images for Presentation

Images for presentation can be displayed at screen resolution but remember that they are enlarged significantly when projected. Make sure you do not 'stretch' a 72dpi image further once you've placed it into a slide, otherwise will look pixelated and blurry once projected.

Projectors are really bad at displaying images, so if possible, try out your presentation first. It may be helpful to adjust colours to make the images project better.

Colour enhancement

You may choose to colour enhance some greyscale images to make your presentation and other science communication activities more engaging. Colour-enhanced images can also be used by journals on their covers with the added benefit of drawing attention to your paper. However, it is always advisable to also present the uncoloured image when it comes to showing your results. This allows viewers to see the original data that you actually captured and to draw their own objective conclusions.
Ethical issues must also be considered, and these are addressed below.
There is a very useful YouTube video: **Beginner's Guide to Colouring Electron Microscope Images** by Microscopy Australia's Dr Jenny Whiting, which provides practical guidance on why and how to colour enhance greyscale images.

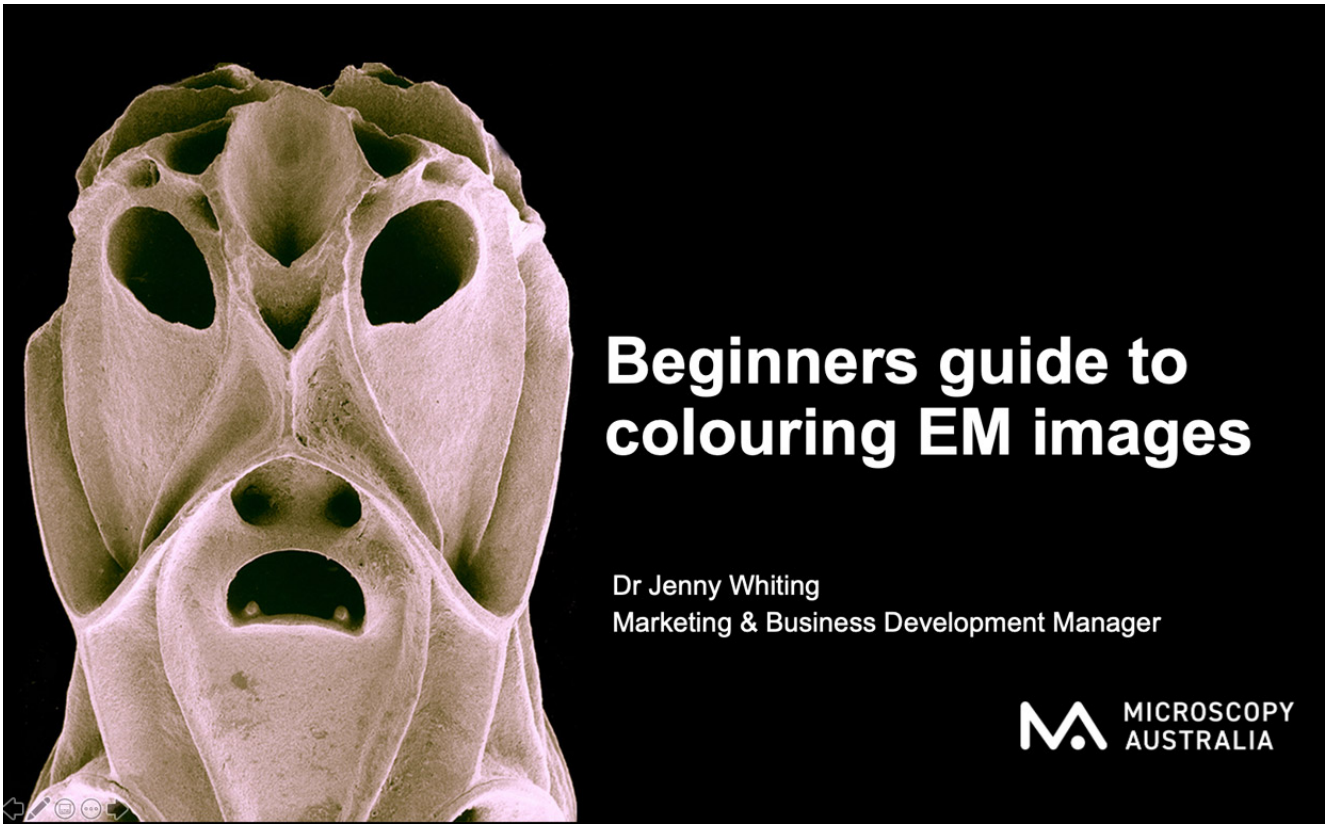
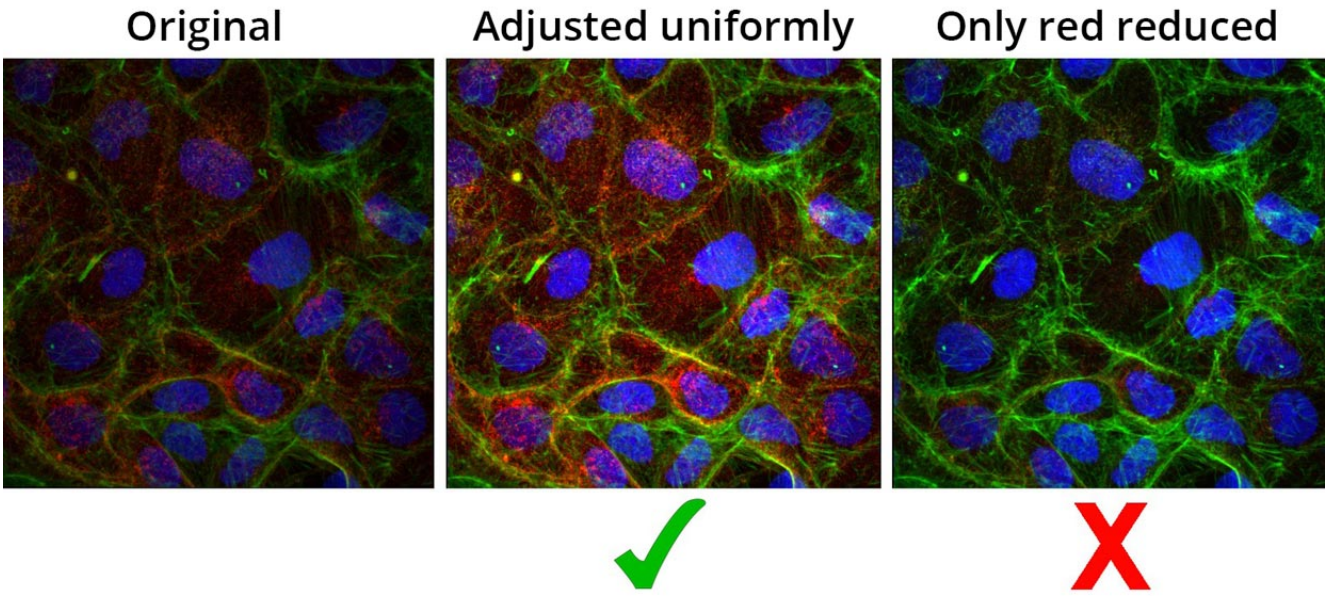


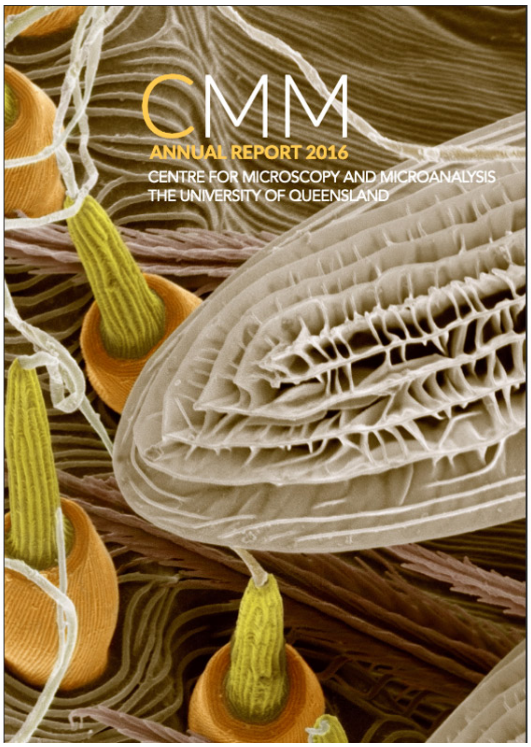
Image Ethics

Digital images are very easy to manipulate using various image processing software packages. This can lead to the temptation to selectively 'improve' your data 'just to get rid of some of that background'. This, of course, is completely unacceptable as the image no longer faithfully reflects the raw data gathered, making such manipulation scientific fraud. Any adjustment to the data should not be selective or biased, but must be uniform across the data and purely for the purposes of making the dataset, as a whole, clearer. It is certainly possible and acceptable to improve how your data looks by applying processing uniformly and by clearly stating what you did and why you did it.



When you publish your data in research papers, it is important to explain in your methods section and figure legends exactly what you did, both in capturing your image, and in post processing.

Other adjustments, such as colour enhancement, must be declared in order to present an accurate reflection of the raw data. Even here, there may be circumstances where data integrity is compromised and colour enhanced images, such as those from electron microscopes are best kept for presentations, science communication and journal covers.



A number of resources exist to assist with maintaining academic integrity. They are often available from the various journal publishers or from your university or institution's ethics office.

Credits

Microscopy Australia acknowledges the huge input of time and expertise by the many staff members and associates who have contributed to the development of MyScope over the years.

For the Microscopy Concepts module we thank: Paul Rigby, Pam Young, Trevor Hinwood, Luke Wensing, Louise Cole, Jenny Whiting, Nikki Stanford.