Introduction to Light Microscopy

January 2013

Today's Workshop

- Intro to Dunn School Facilities
- Fixation and Sample Preparation (30mins)
- Light Microscopes (30mins)
  - Tea (15mins)
- Data Capture and Manipulation (30mins)
  - Image capture in Facility (30mins)

Workshop – chat and introcuce – they can interprupt at any point!
Physics to a minimum – but some of it is necessay

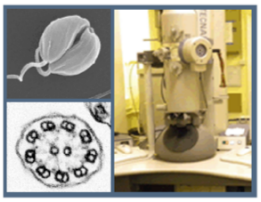
Dunn School Light Microscopy Facilities

January 2013

Dunn School Bioimaging Facility

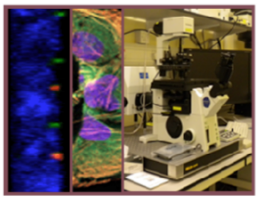
http://web.path.ox.ac.uk/~bioimaging//BioimagingHome.html

Electron Microscopes



EM Workshop
Wednesday 6h
February

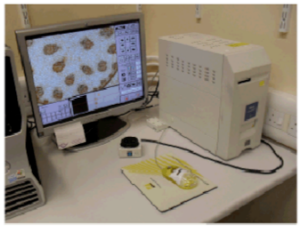
Light Microscopes



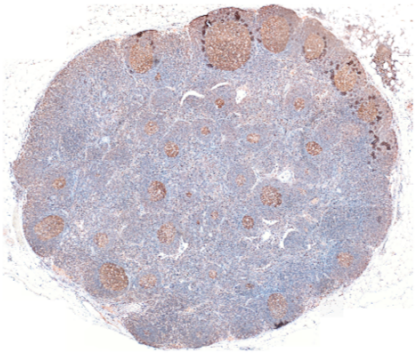
slides scanner
microscope CCD cameras
confocal microscopes

Slide Scanning for Histology

Nikon Slide Scanner 'DSS1'



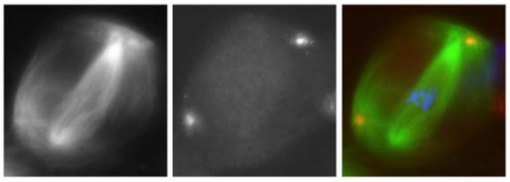
Essentially a digital camera, with a lens in a box



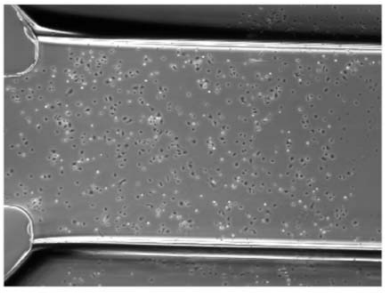
Klinerman Lab (Zoology)

Fluorescence Microscopes

CCD2 and CCD3 (live)



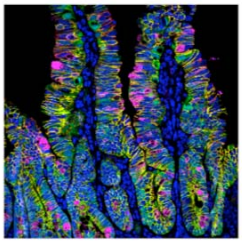
Raff Lab



Greaves Lab

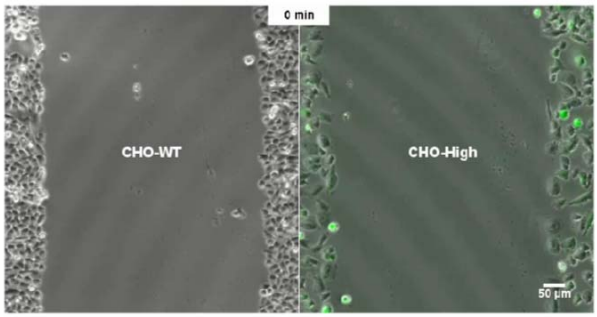
Confocal Microscopes

Olympus FV1000



Hassan Lab

Zeiss LSM510 (live)



Vaux Lab

Micron

The Dunn School microscopy facility is associated with a wider grouping of 'sister' microscopy facilities across the University – this is known as Micron.

The idea behind Micron enables users access to advanced microscopy facilities across the University irrespective of the Department in which they are housed.

Details of some of these facilities are described on the micron website

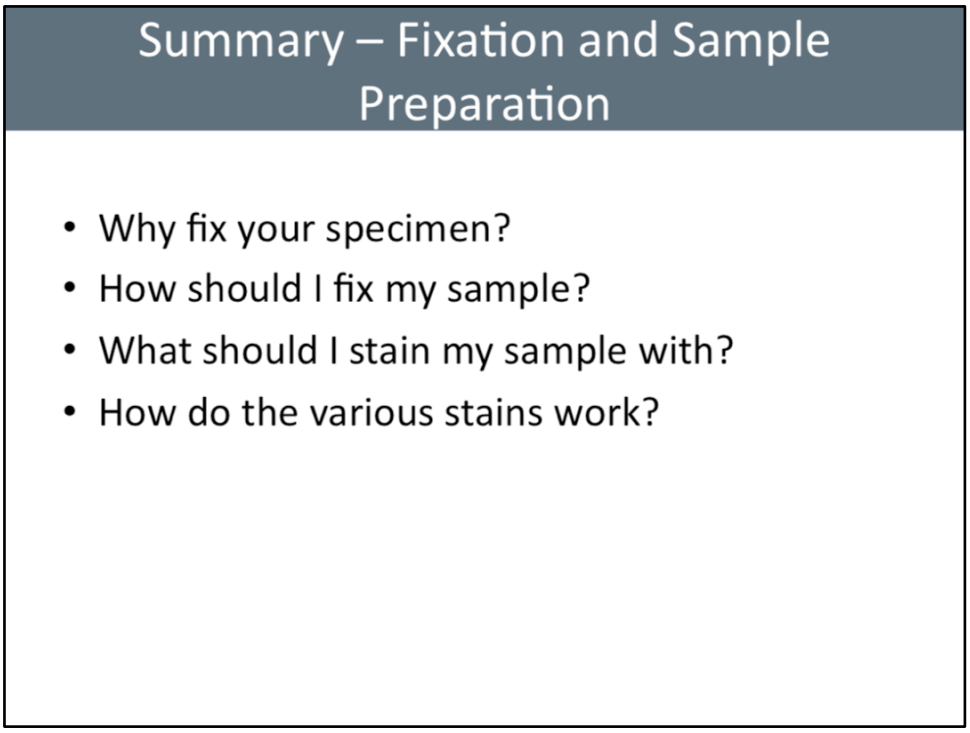
http://www.micron.ox.ac.uk/microngroup/micron_home.php

in addition micron runs an advanced microscopy course in March, which will give far greater detail than this introduction. Any member of the University can attend, as long as they've registered online:

http://www.medsci.ox.ac.uk/skillstraining/coursecatalogue/allcourses/128

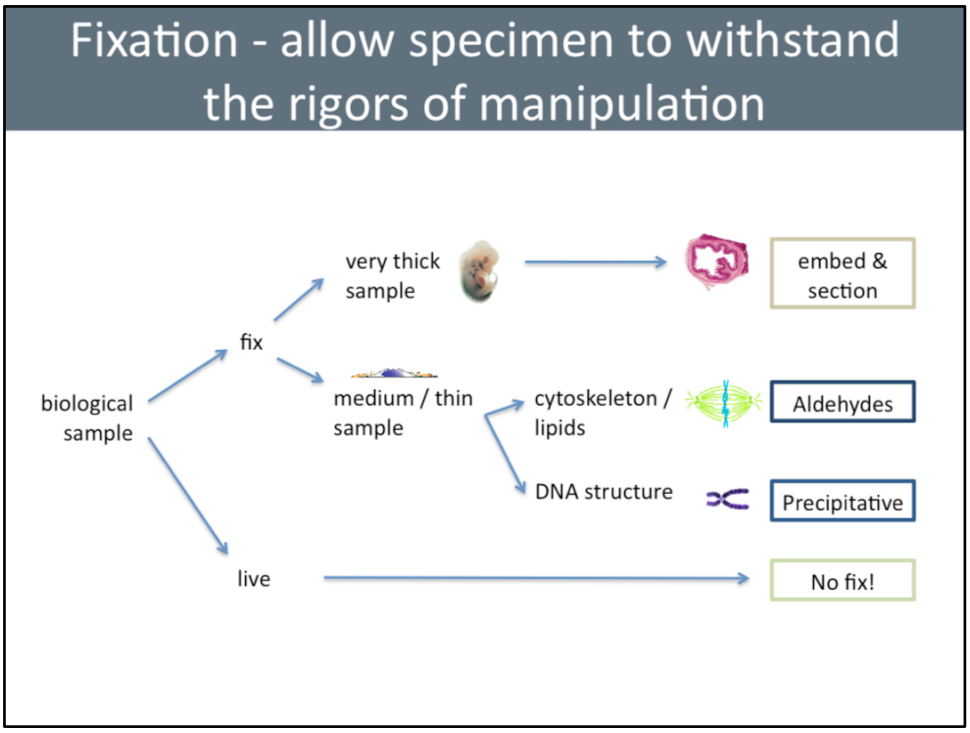
Sample Preparation and Fixation

January 2013



Summary

In this talk I would like to cover how biological samples are fixed and, then how you should treat them in order to observe what you need to answer your particular biological question



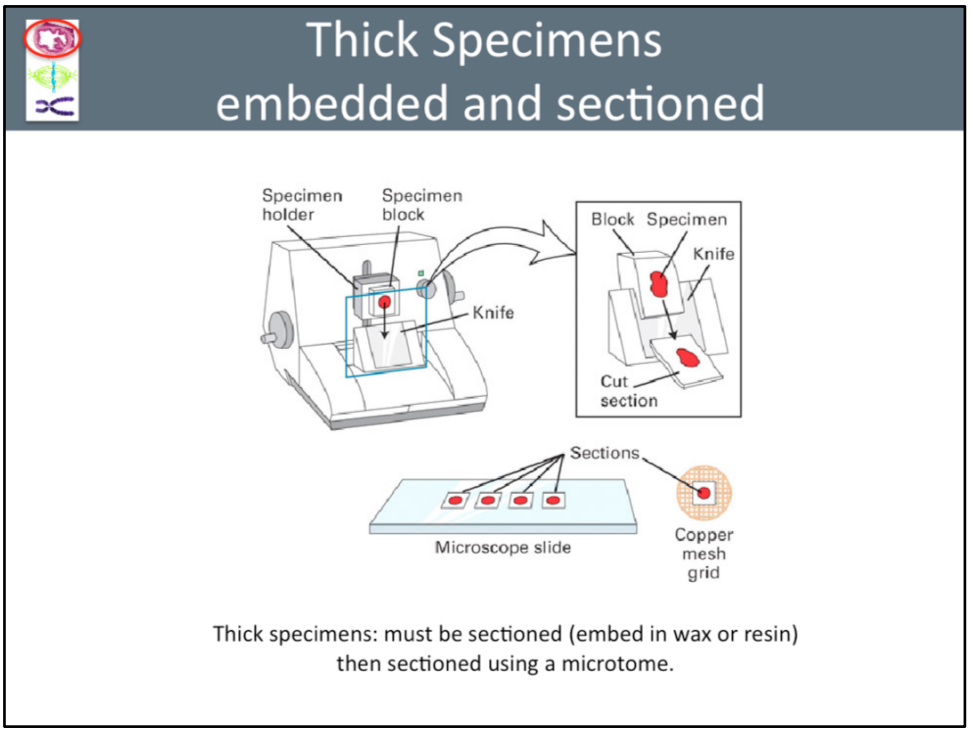
Fixation

The fixation technique to use depends partly on the type of biological sample you have and partly on what you want to see in your sample.

Very thick samples need to be sectioned as they are too thick to visualize using light microscopes. Once fixed (typically in aldehyde fixatives) the specimen is embedded in wax or resin, and once hard, sectioned using a microtome.

Thinner samples such as smaller embryos and tissue culture cells can be fixed in one of a couple of ways. Either using aldehydes, which is ideal for fixation of cytoskeletal components , or precipitative fixatives, best for DNA structure preservation.

Of course you may want to look at you sample live – whereby there's no need to fix!

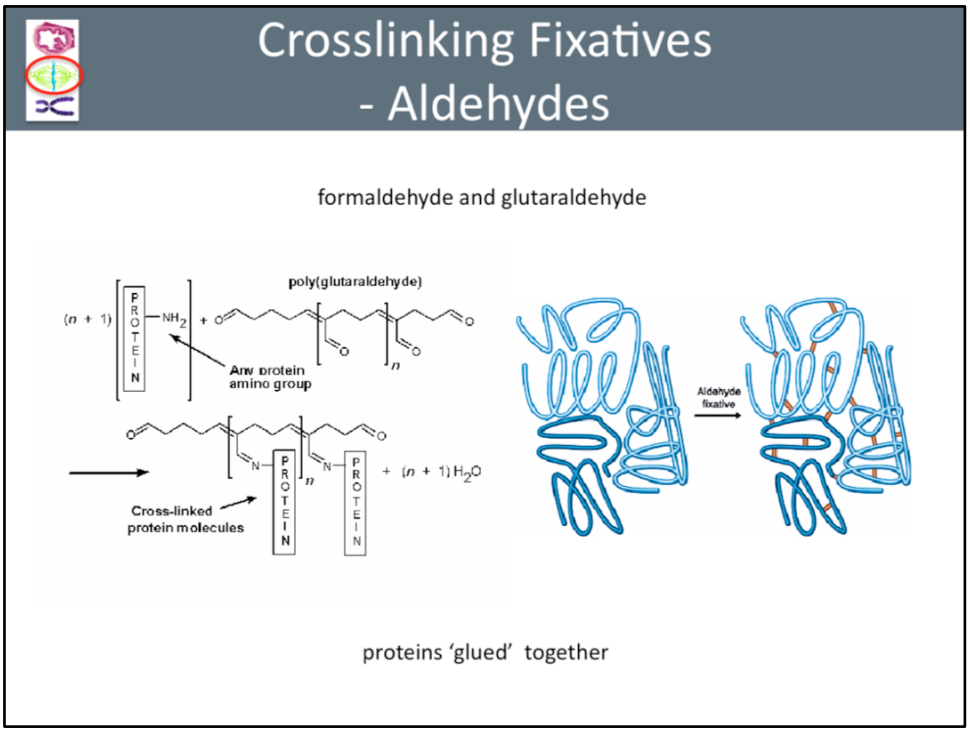


Embedding And Sectioning

This is mainly the realm of the Tissue and Molecular Service in the Dunn School, run by Richard Stillion. For much further information please visit the website of this service

http://www.path.ox.ac.uk/Facilities/facilities_new/histology

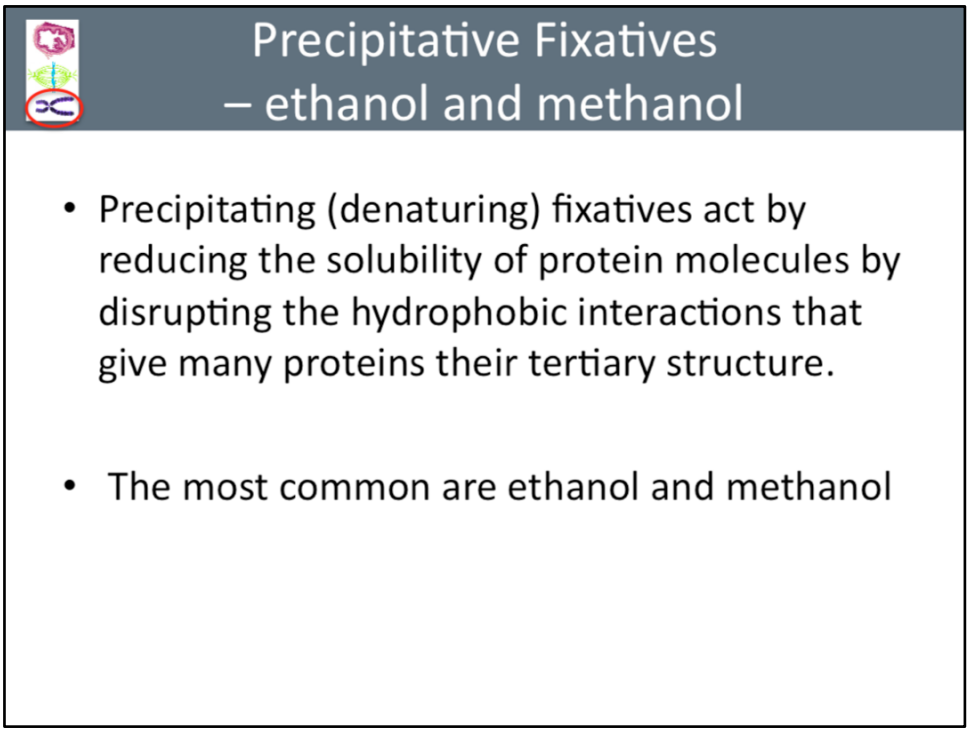
Once fixed (typically in aldehyde fixatives) the specimen is embedded in wax or resin, and once hard, sectioned using a microtome. These sections can then be placed on a slide an stained, either with H and E staining or with fluorescently conjugated antibodies, more of which later .



Cross linking Fixatives – The Aldehydes

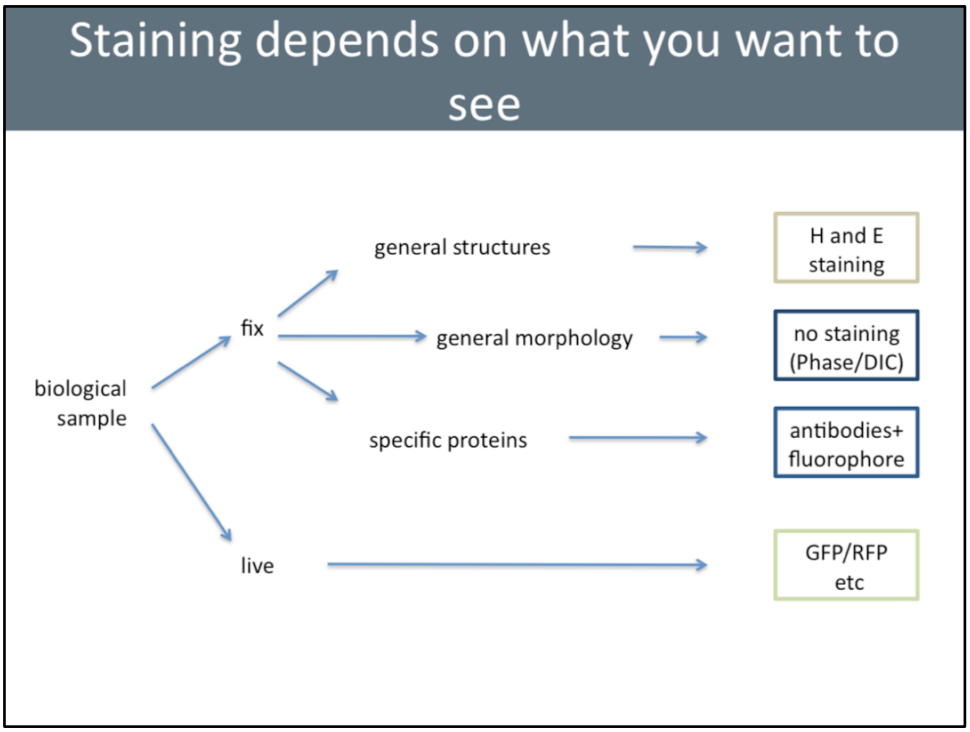
As their name suggests the aldehydes chemically crosslink the amino acid side chains of proteins, preventing movement and therefore preserving structure. Formaldehyde and glutaraldehyde are typical cross-linking fixatives are polymers, with a reactive group every monomer. These reactive groups covalently bind amino groups of amino acid side chains – binding them together.

As a result, proteins in a cell are essentially glued together in place preserving the overall organisation of the specimen.



Precipitative Fixatives

Precipitating (denaturing) fixatives act by reducing the solubility of protein molecules by disrupting the hydrophobic interactions that give many proteins their tertiary structure.



Staining Biological samples

If you have sectioned and embedded thick sections then it is typical to carry out H and E staining which labels general structures.

With thinner sections you needn't actually stain them, as you can observe then using either Phase contrast or DIC Differential Interference contrast microscopy.

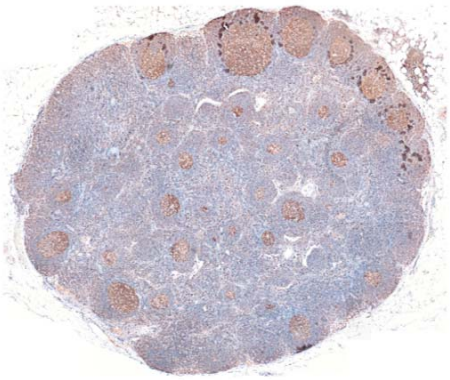
If you would like to know the localisation of specific proteins then it is typical to use antibodies conjugated.

Of course these two techniques are not mutually exclusive and can give images where by the localisation of certain proteins are placed in context using contrast mircoscopy.

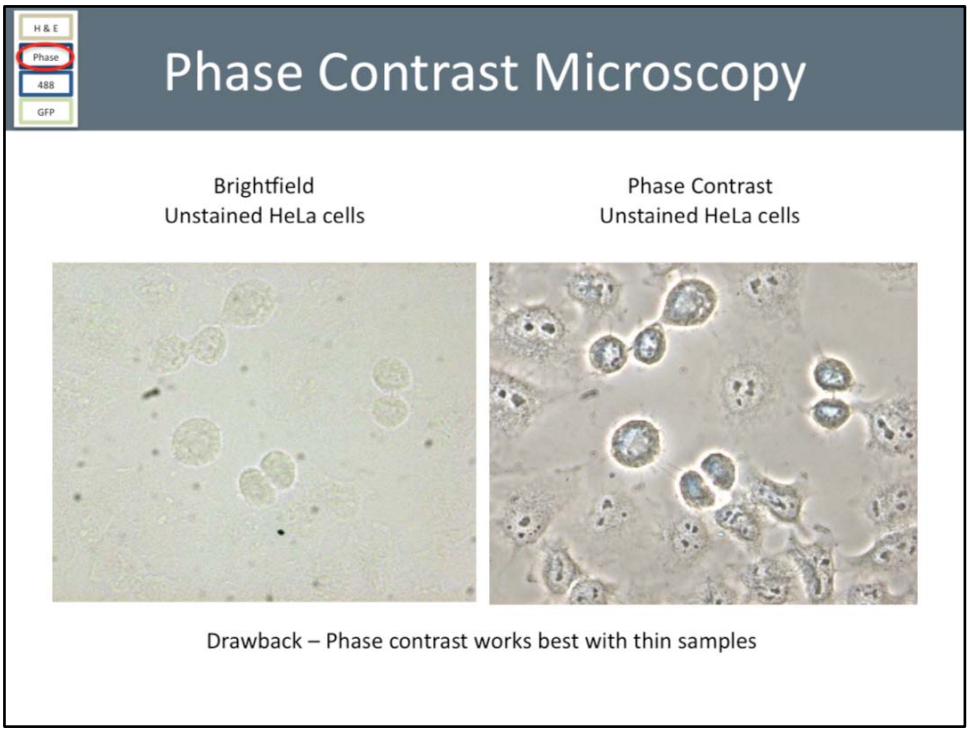
The localisation of certain proteins can be seen in unfixed samples using fluroescent tagging (GFP) technology.

Histology (H and E staining)

- Structures stained by various chemicals.

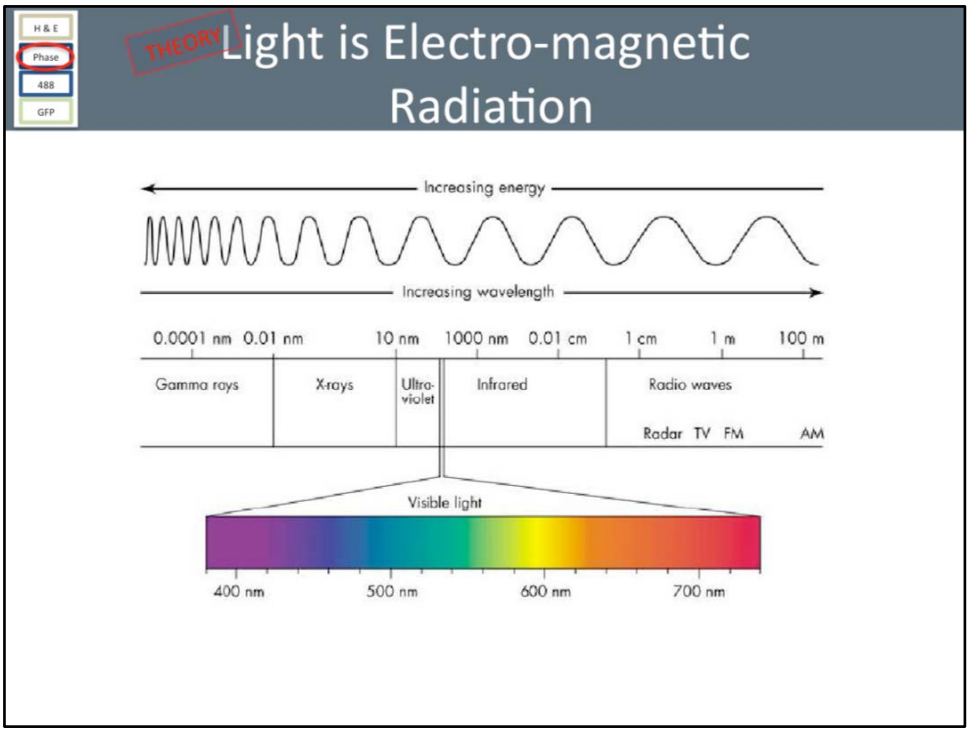


Staining: general stains to visualise transparent structure. For example Giemsa,
toluidine blue and many others.



Phase Contrast Microscopy

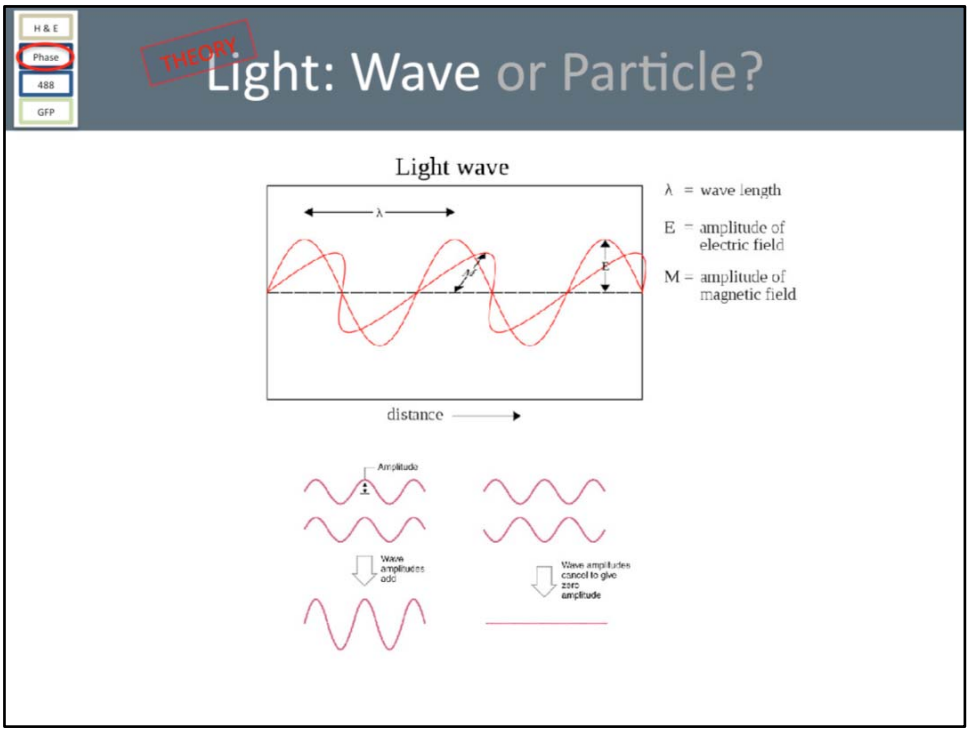
This technique takes advantage of the fact that different structures within a cell (typically) affect light passing through them to varying extents, giving an image with greater contrast than observed in a brightfield image.



Light is electro-magnetic radiation

Light is just part of the electro-magnetic spectrum, from gamma-rays at the very shortest of wavelengths, through to FM, AM and LW radio (where you can listen to the cricket!)

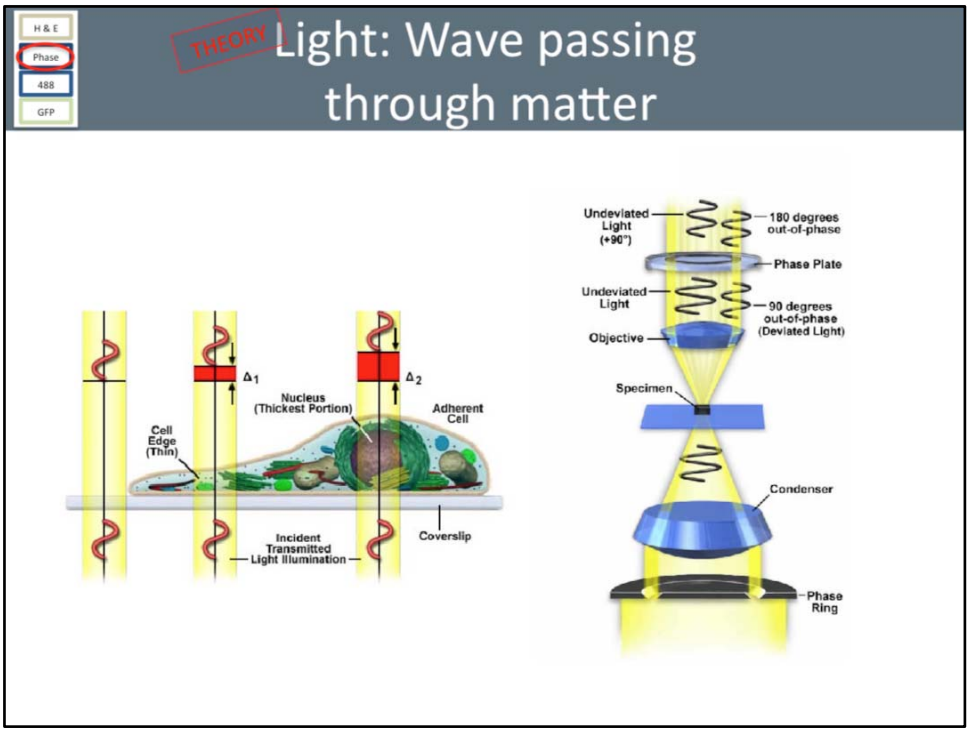
Visible light makes up a tiny fraction of this spectrum and stretches from roughly 400nm (near UV light) to around 700nm (near infra-red light).



Light acts as a wave

One consequence of light being part of the electro-magnetic spectrum is that it has 'wave-like' properties. Light therefore has a certain wavelength (the distance between two peaks or troughs) and these waves also have an amplitude (The height of the peaks/troughs)

In addition these waves can combine in either constructive (whereby the addition of two light waves with the **same** phase generate a combined wave with twice the amplitude) or destructive interference (where two waves **180° out of phase** 'cancel each other out' leaving no wave)



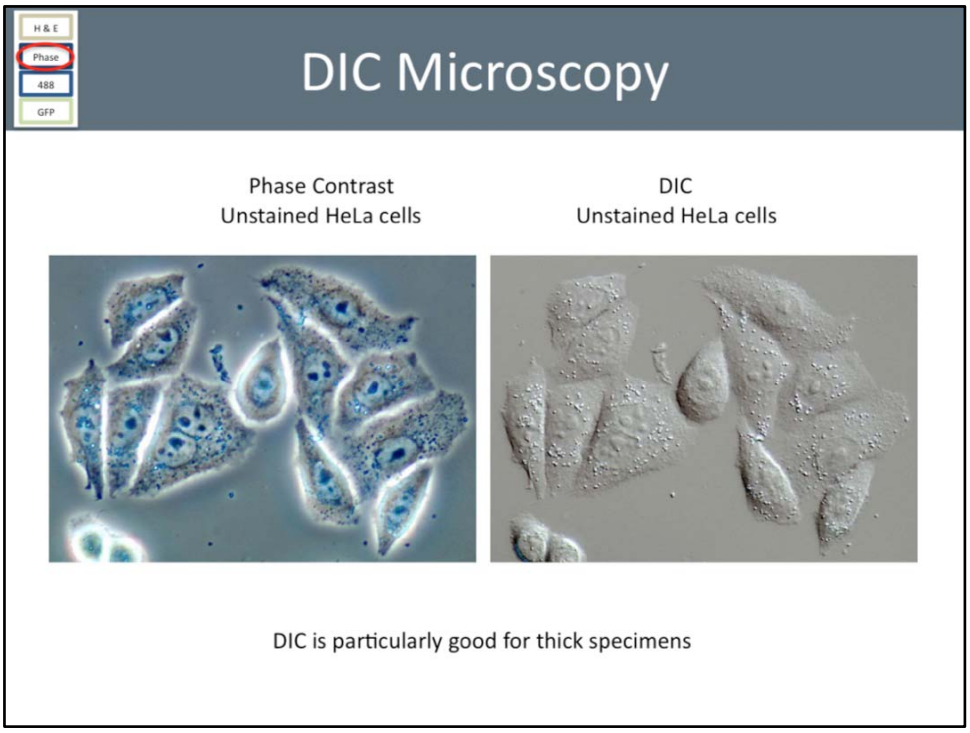
Phase Contrast Microscopy

Phase contrast microscopy uses this constructive or destructive interference property of light waves to generate contrast within an image.

As light passes through a sample its phase is shifted by a certain amount depending on the 'density' of the structures it passes through. Typically light that passes through the nucleus is shifted more than light passing through the cytoplasm.

The basic principle is to separate the illuminating background light from the specimen scattered light. The condenser helps to focus light on the specimen, and the Phase ring (condenser annulus) ensure the illumination light is all in the same phase. As light passes through the specimen some is scattered and is typically shift by 90°. The rest of the light passes straight through the specimen and is unshifted in phase.

Both deviated and undeviated light is collected in the objective lens. The phase of the background light is then shifted by + 90° by the phase plate in the objective lens. The background light is therefore 180° out of phase with the specimen scattered light, giving maximal destructive interference and therefore maximal contrast in the image.

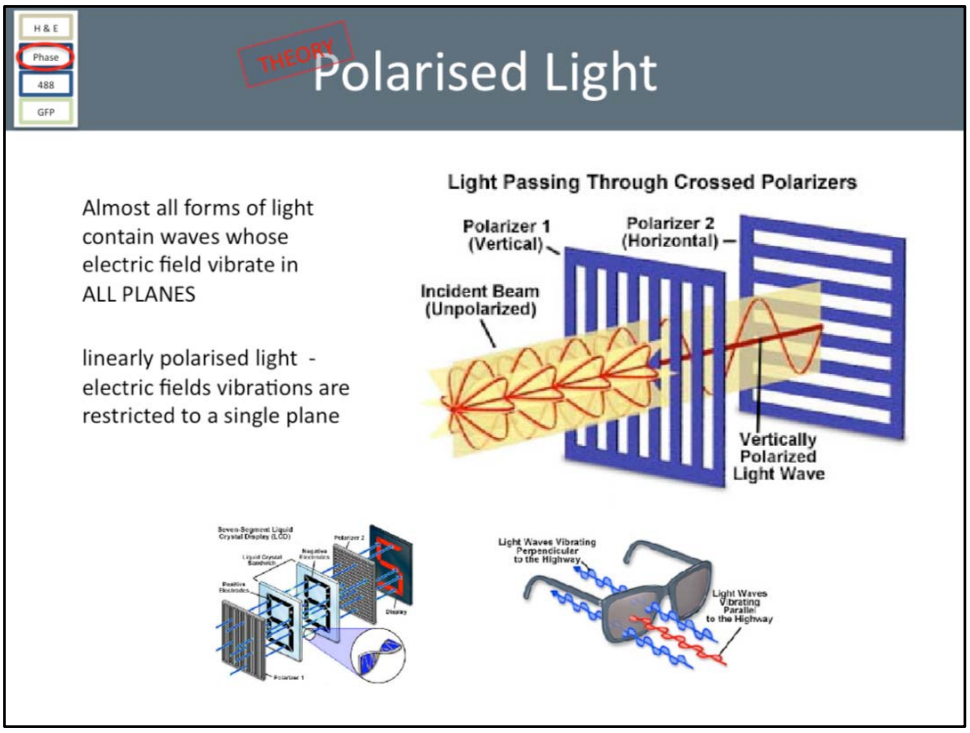


DIC – Differential Interference Contrast Microscopy

This is a second contrast method generates a 'moonscape' image of even unstained samples.

DIC augments tiny differences in thickness gradients (*eg: at **boundaries**) and refractive indices (eg: aqueous cytoplasm vs lipid bilayer) within the specimen. These differences are visible as relief.*

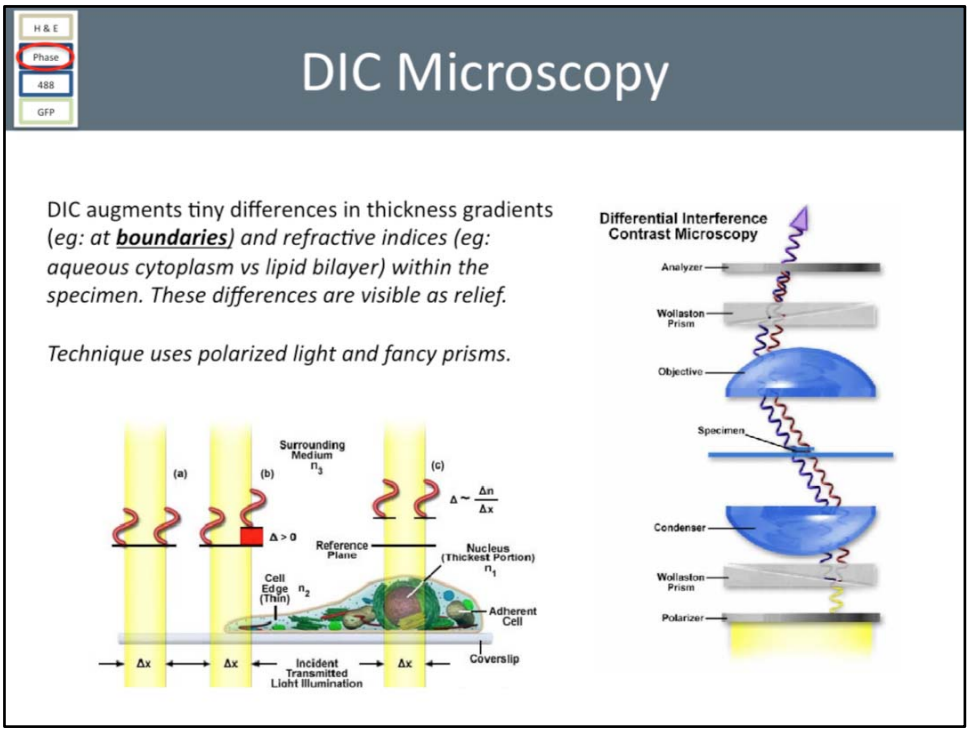
DIC has the advantage over Phase contrast microscopy that it can image slightly thicker specimens.



Polarised Light

Almost all forms of light contain waves whose electric field vibrate in ALL PLANES linearly polarised light - electric fields vibrations are restricted to a single plane.

Importantly, light has to be of the same polarity in order to undergo constructive or destructive interference.



1. Unpolarised light enters the microscope and is polarised at 45°.
Polarised light is required for the technique to work.

2. The polarised light enters the first Wollaston prism and is separated into two rays polarised at 90° to each other.

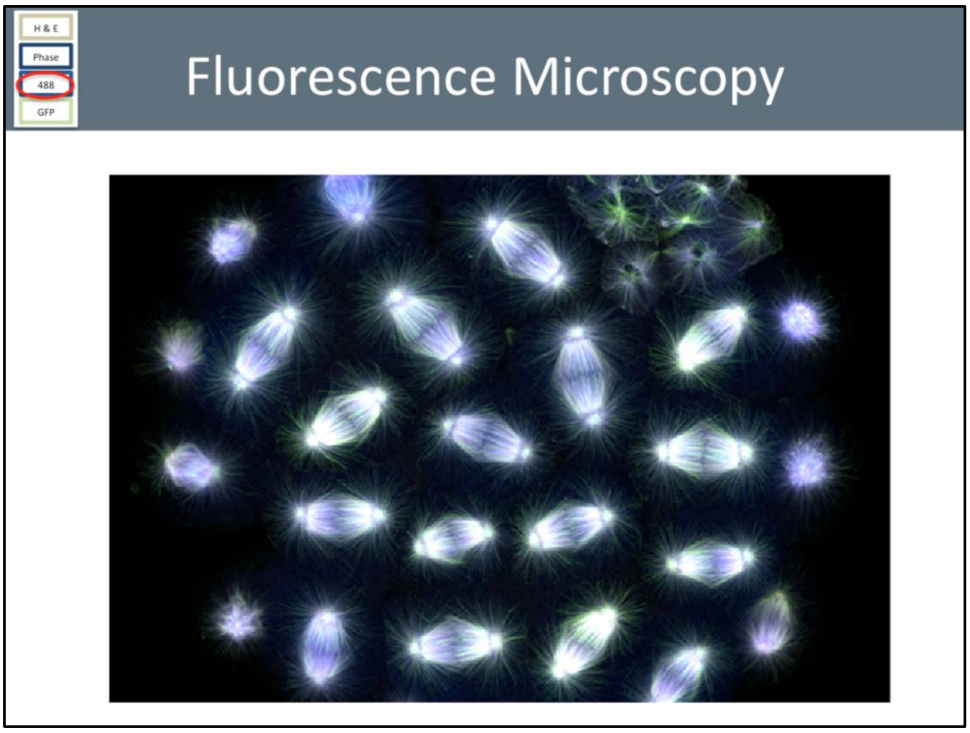
3. The two rays are focused by the condenser for passage through the sample. These two rays are focused so they will pass through two adjacent points in the sample, around 0.2 µm apart.

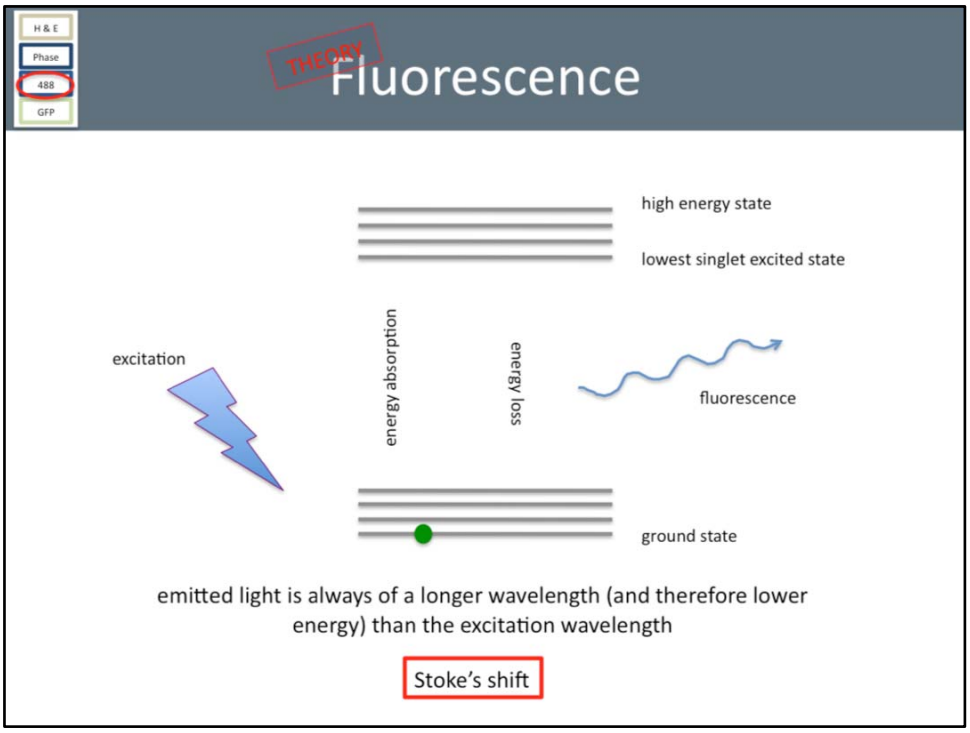
The sample is effectively illuminated by two light sources, one with 0° polarisation and the other with 90° polarisation. These two illuminations are, however, not quite aligned, with one lying slightly offset with respect to the other (by 0.2µm)

4. The rays travel through adjacent areas of the sample. Areas differing in refractive index or thickness will This causes a change in phase of one ray relative to the other due to the delay experienced by the wave in the more optically dense material.
The different polarisations prevent interference between these two images at this point.

5. The rays travel through the objective lens and are focused for the Wollaston prism.

6. The second prism recombines the two rays into one polarised at 135°. The combination of the rays leads to interference, brightening or darkening the image at that point according to the optical path difference.


H & E
Phase
488
GFP
Fluorescence Microscopy



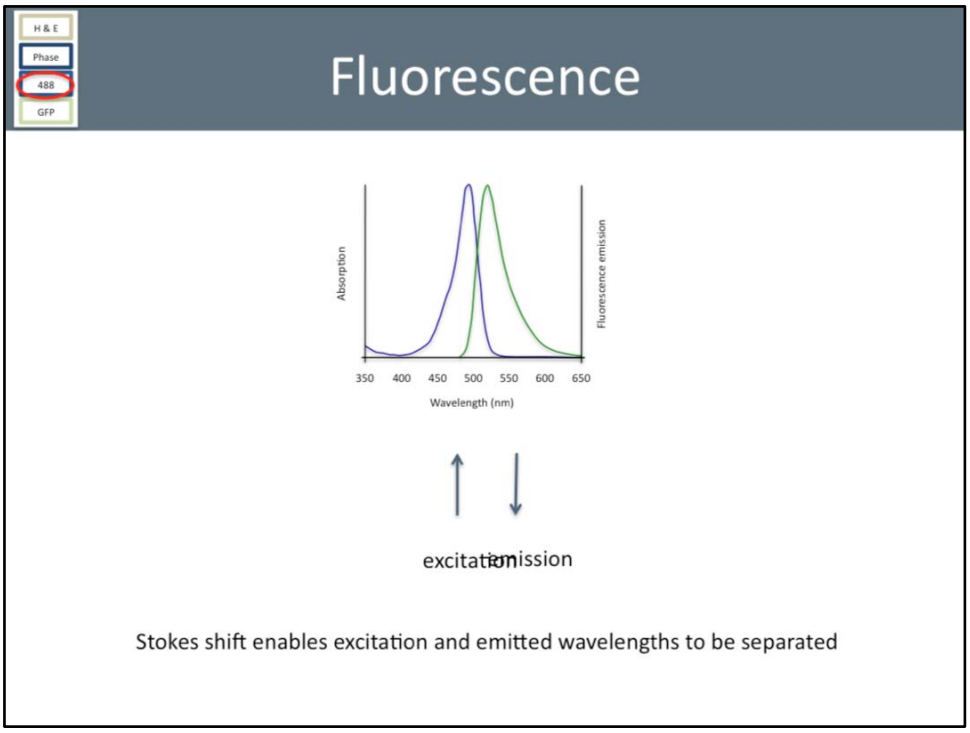
Fluorescence

This is known as a Jablonski diagram and represents the energy levels of an electron .

Electrons normally exist in a ground state energy level, but, within the context of a fluorophore molecule, upon excitation by light, the electron is shifted to a high energy state. At these high energy level state, the excited electron loses some energy in the form of heat and movement such that is shifts slightly to the 'lowest singlet excited state'. After a short period of time the electron then returns to it's ground energy state emitting energy as fluorescent light.

Importantly, due to the energy loss of the electron whilst at these high energy states, the energy emitted as fluorescence is less than that which was originally absorbed by the electron. This manifests itself as light of a longer wavelength compared to that exciting the fluorophore.

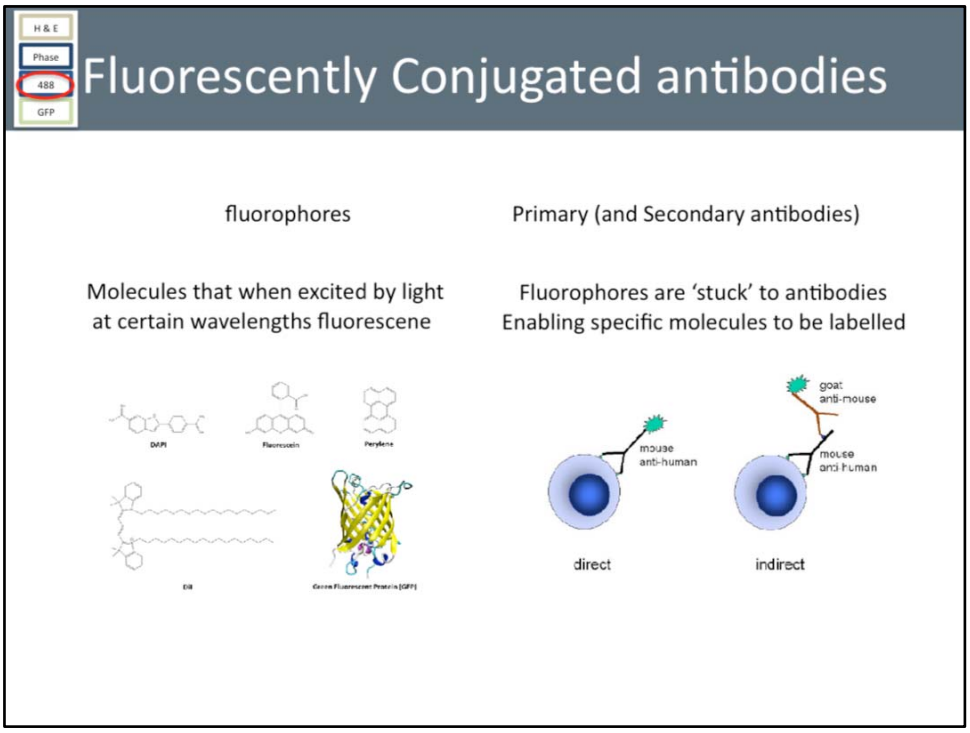
This difference is known as the 'Stoke's Shift'.



Fluorescence

The Stoke's shift property enables the excitation and emission wavelengths of light to be separated.

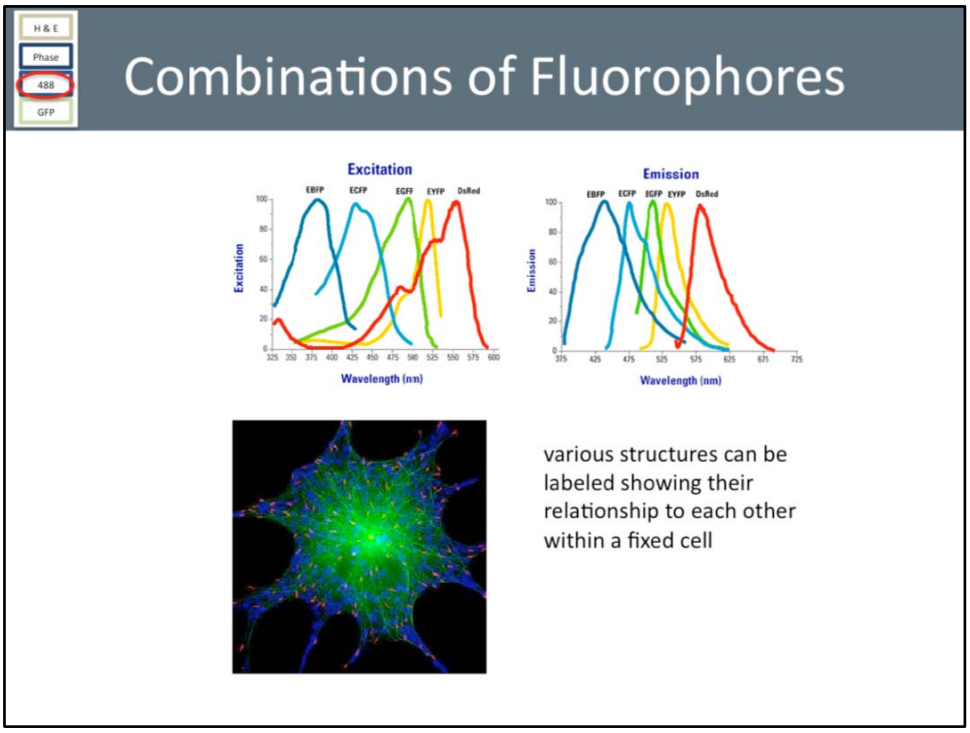
It is this property that the fundamental basis of fluorescence microscopy



Fluorescently Conjugated Antibodies

The Stoke's shift property of fluorophores is only part of the story that enables you to analyze the localisation of different proteins within a biological sample. The second technology that enables this is the development of fluorophore molecules and (typically) their coupling to antibodies.

Fluorophores can either be directly conjugated to an antibody of choice, or indirectly label a protein via a secondary antibody. Here an antibody raised in one species is recognised by a second antibody raised in a different species. This secondary antibody is then directly conjugated to a fluorophore molecule. This enables increased flexibility to the experimenter (as you can easily use different colored fluorophores labeling the same protein), and also there is an element of amplification as multiple secondary antibodies (with conjugated fluorophore molecules) can recognise one primary antibody.



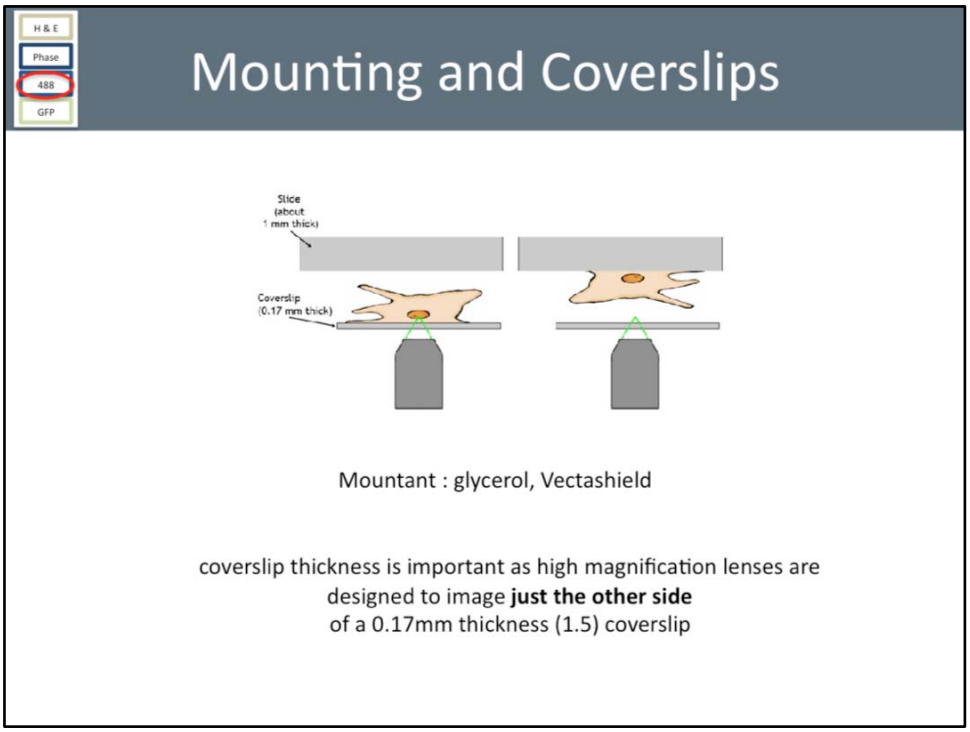
Combinations of Fluorophores

Various fluorophores can be used in combination with each other – each conjugated to an antibody raised in a species raised to recognise a different species of primary antibody.

a typical combination would be

DAPI labeling of DNA (ex 405)
alexa 488 labeling of microtubules (ex488)
alexa 594 labeling protein of interest (ex594)

In this way various structures can be labeled showing their relationship to each other within a fixed cell.

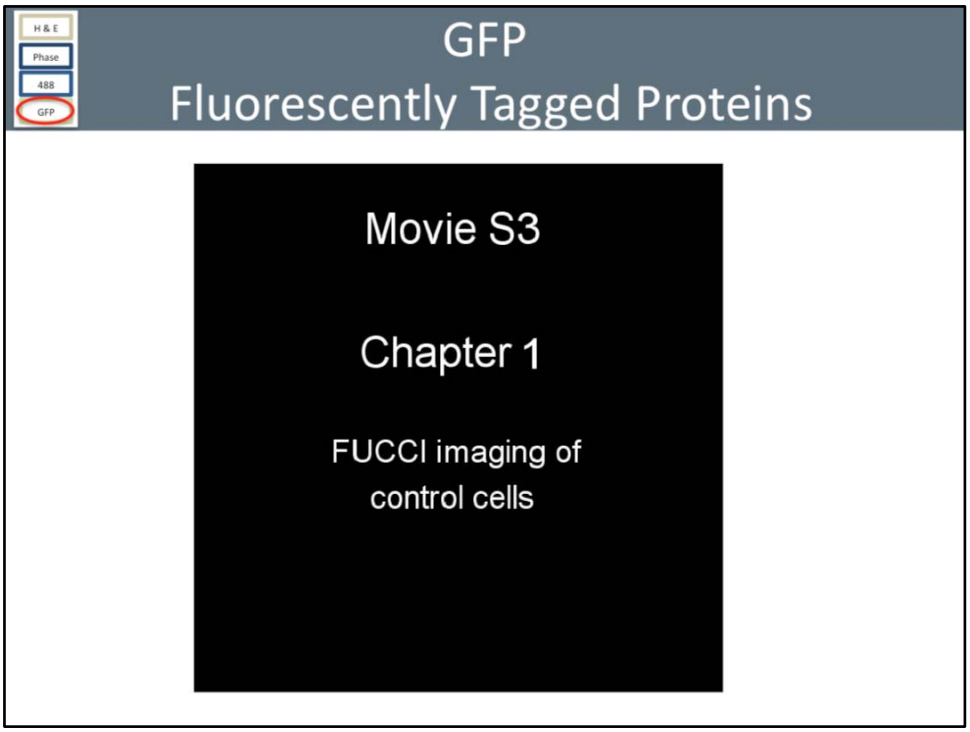


Mounting and Coverslips

Once your sample has been fixed and stained it must lastly be mounted on a slide and (typically) covered by a coverslip.

The mounting in media such as glycerol (or vectashield) provides extra structural support and can help protect the fluorophores from bleaching and photo-damage (as is the case with vectashield)

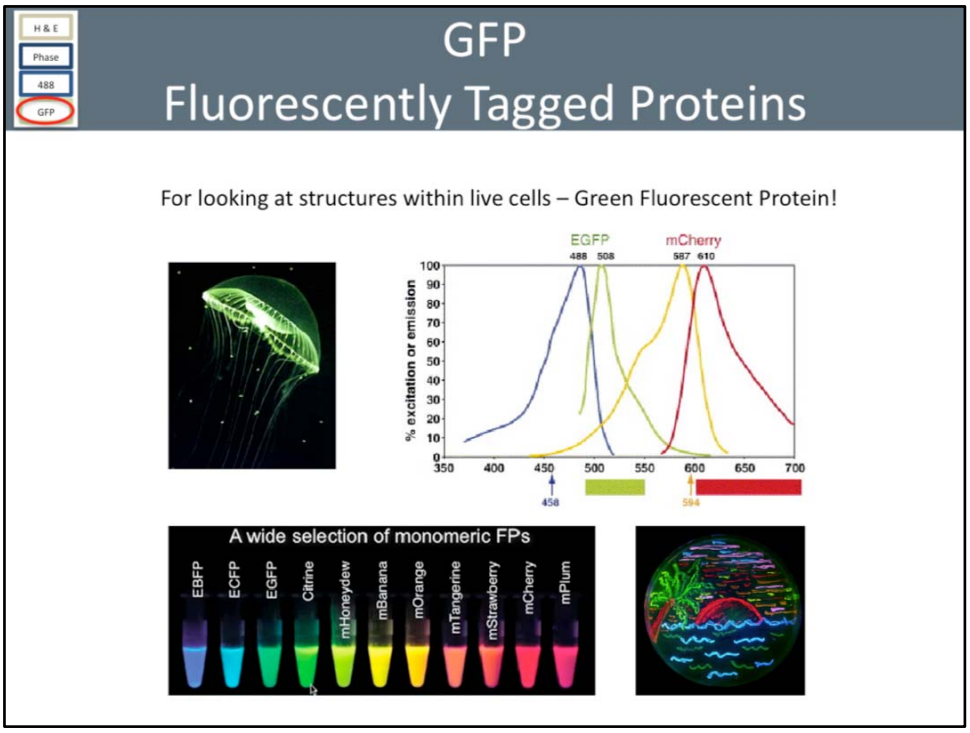
The Specimen is then covered by a coverslip, the thickness of which is highly accurate and designed such that high magnification lenses (60x) image just the other side of the 0.15mm thickness in the specimen.



GFPs and fluorescently tagged Proteins

The last 'staining technology' is that of using fluorescently tagged proteins in live (unfixed) cells, which can be useful to analyze dynamic behavior of a protein or process of interest.

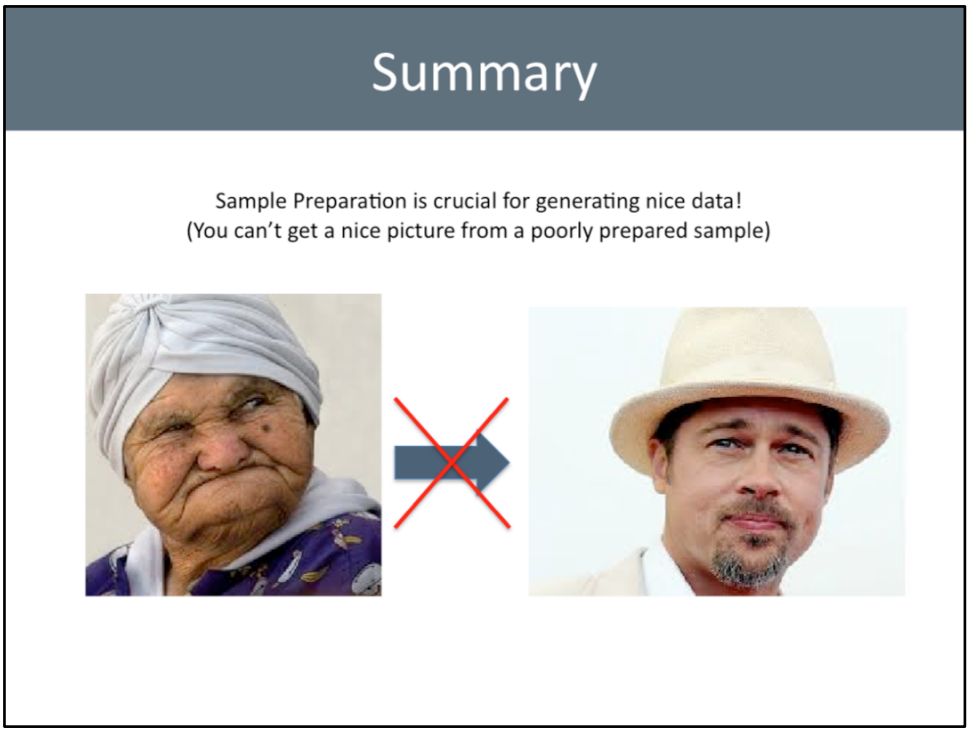
Here is an example where the nuclei of cells are labeled with two proteins; one fluoresces red in G1 (just after mitosis) and the other which fluoresces green in G2 (just before mitosis) (S phase is therefore yellow as both are still expressed).



GFPs and fluorescently tagged Proteins

GFP protein was first identified in the jellyfish *Aequorea victoria.* It is a protein of roughly 30KDa and as it is encoded by a sequence of DNA it can been used to tag your protein of interest.

Subsequently many different mutants of GFP have been engineered to give a series of fluorescent tags which cover most of the visible spectrum. These are named after fruits with the appropriate colors.



Summary

Specimen preparation is crucial to obtaining good quality data. There's no real substitute for it – no matter how good your microscopes!