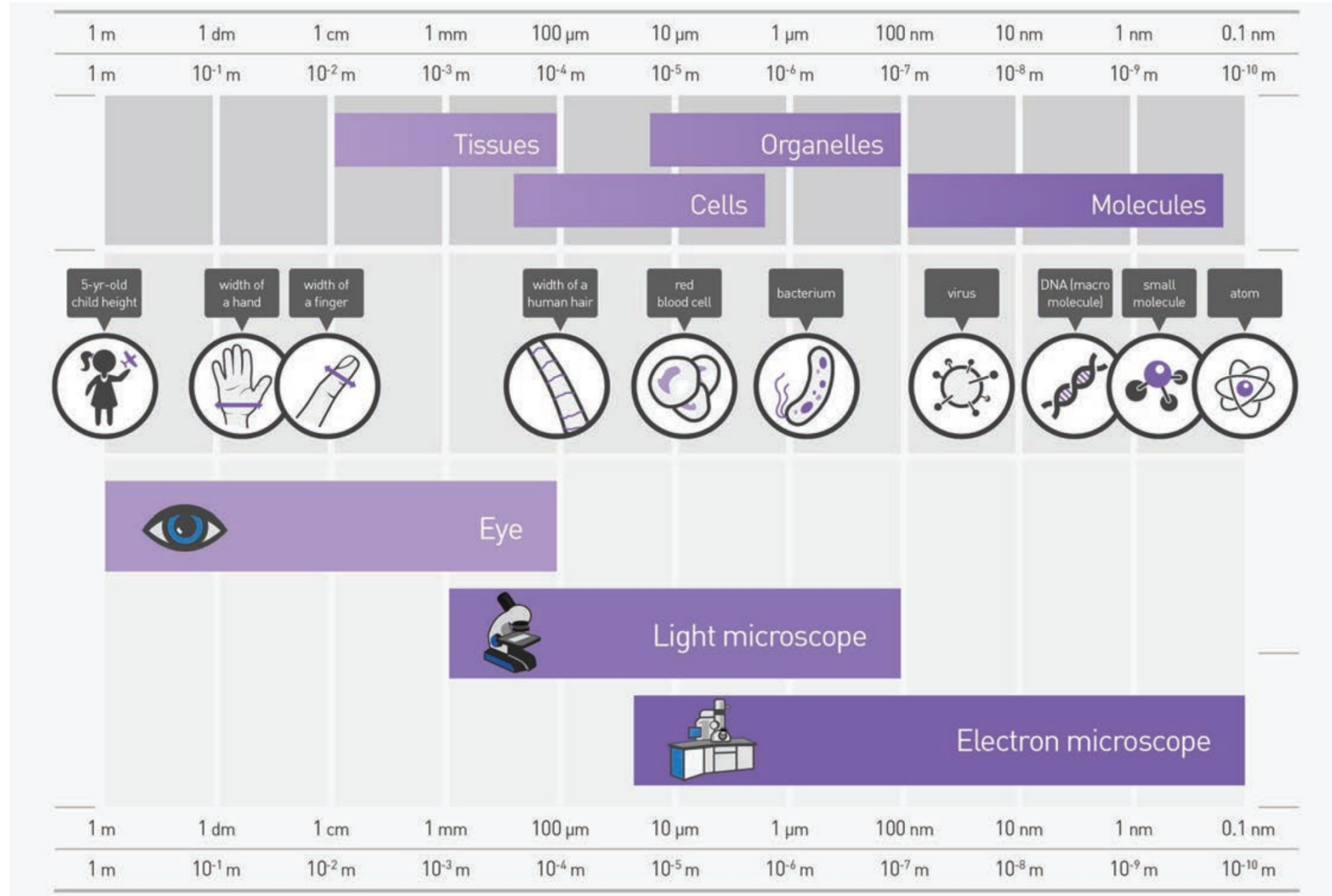


Introduction to light-microscopy

Illustrated with widefield microscopy

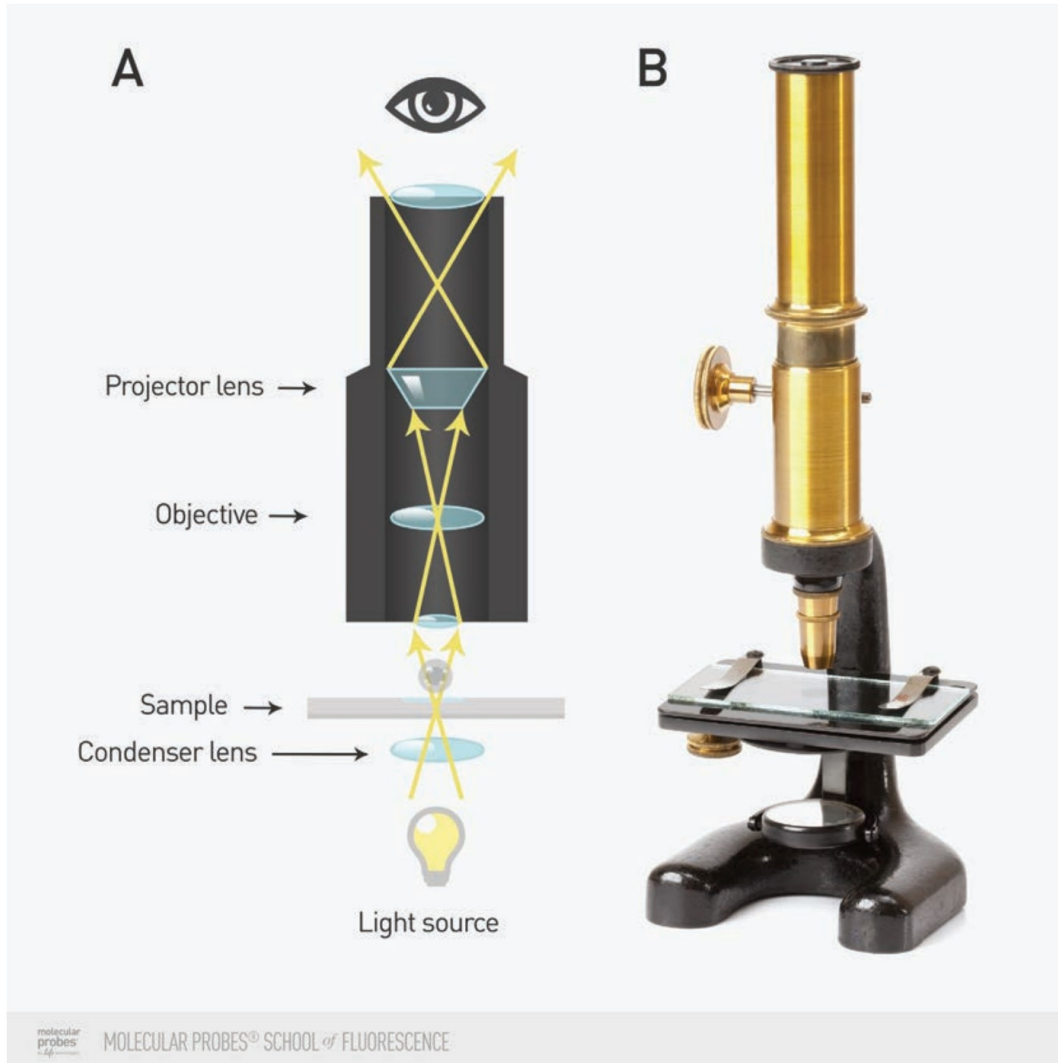
Scale of observable objects

Figure 3.6. The resolving power of various microscopes, with representative objects within range for both light microscopes and electron microscopes.



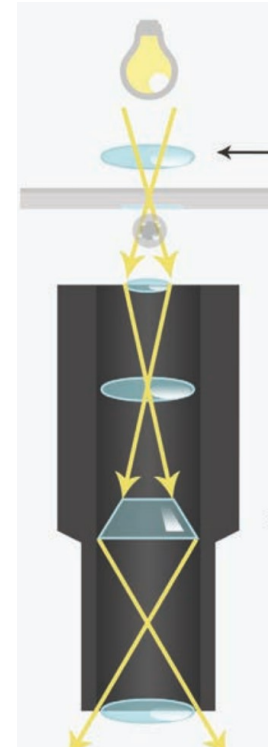
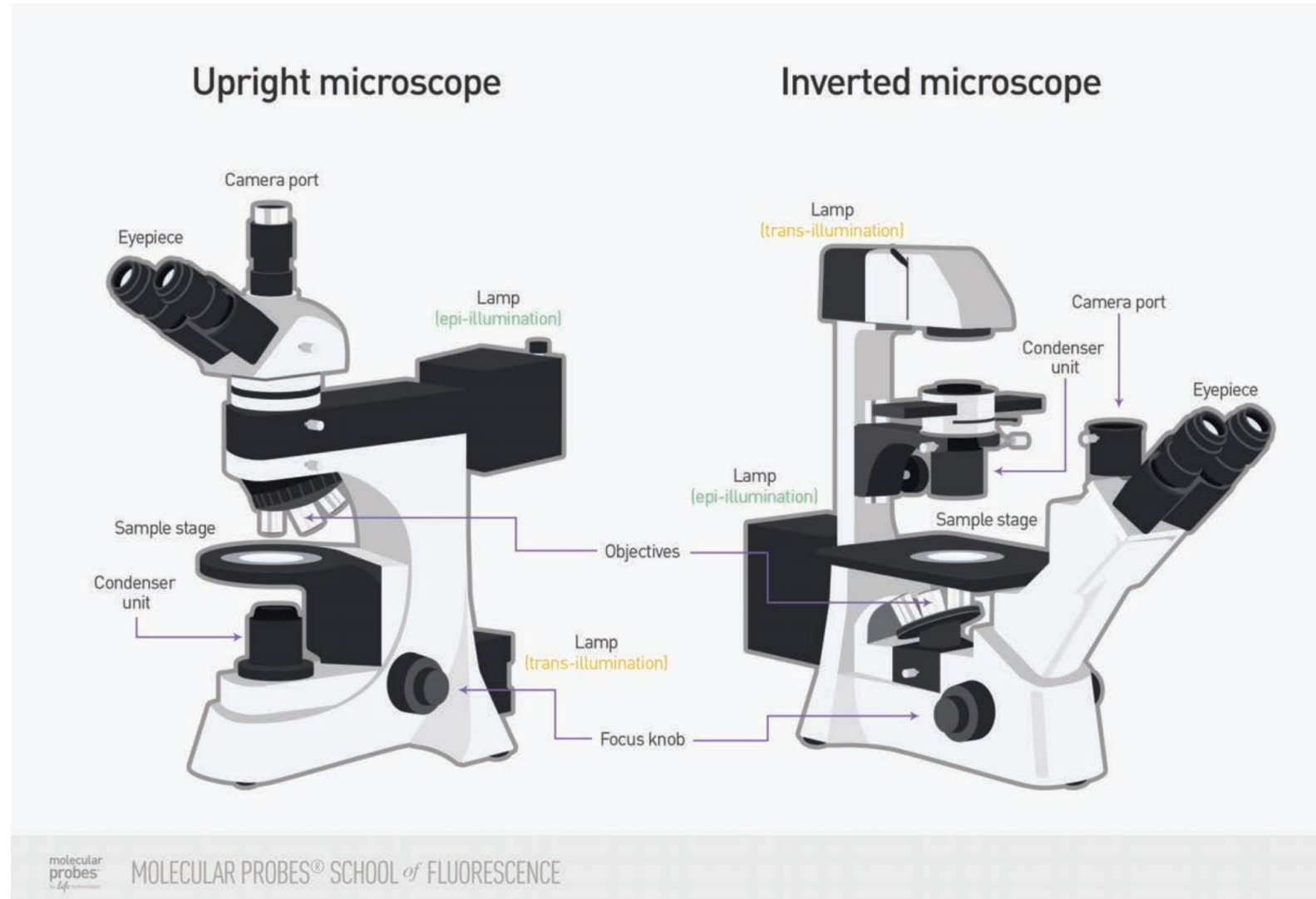
Widefield microscope – brightfield illumination / transmitted light

Figure 2.2. The light path through lenses and sample in basic brightfield microscopy (A). Antique 19th century drum-style compound microscope (B).



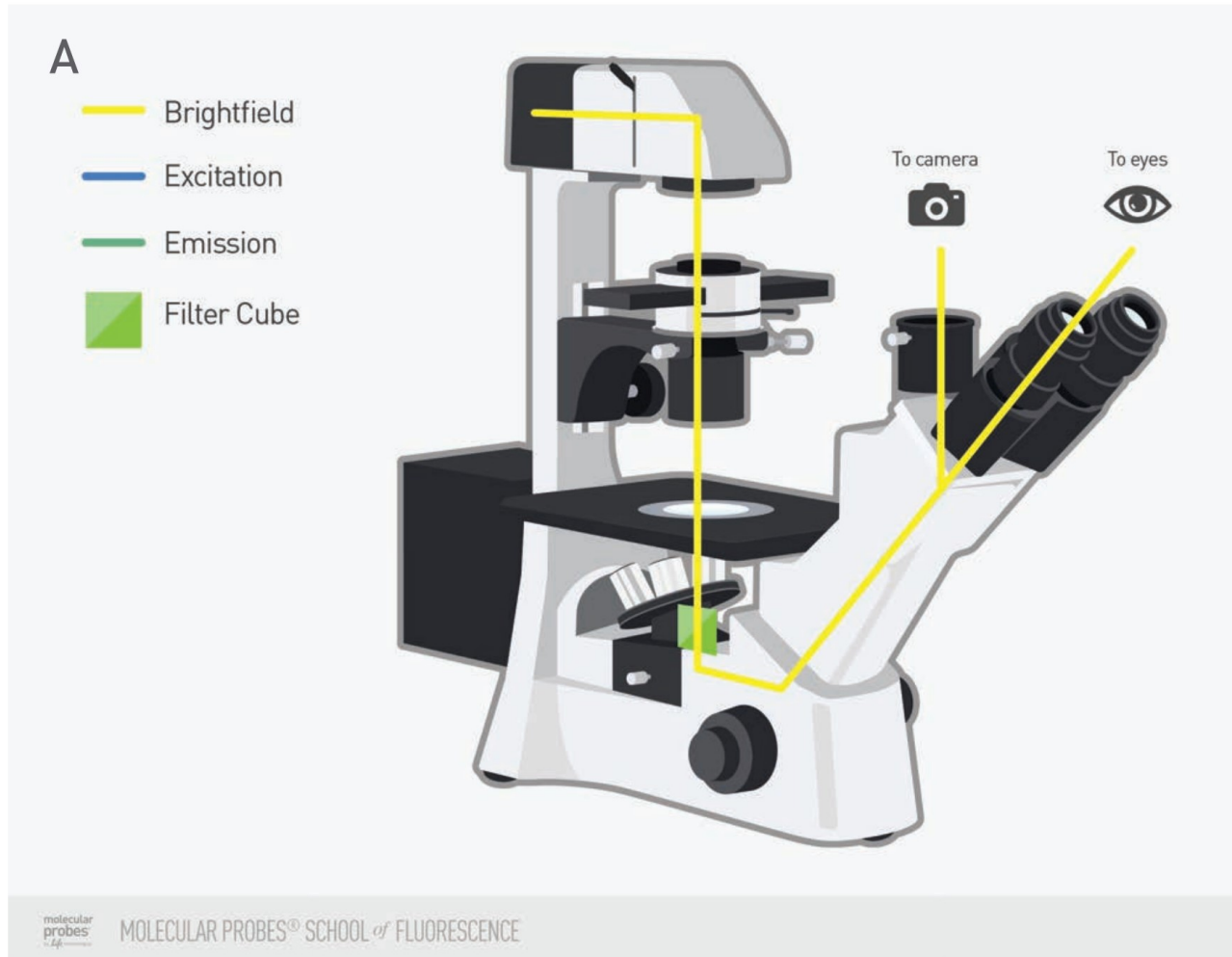
Up-right / Inverted microscopes

Figure 3.2. Inverted and upright microscopes both utilize epifluorescent illumination: the main difference is the location of the objectives relative to the stage where the sample is placed.



Inverted microscope – transmitted-light path

Figure 3.7A. The yellow line represents the light path for brightfield illumination. All of the illumination light does not travel through the objective, only the light that is transmitted through the sample. For this reason, images acquired using brightfield illumination are sometimes referred to as "transmitted" images.



Widefield microscopy – Contrast methods

Biological samples are often **transparent**

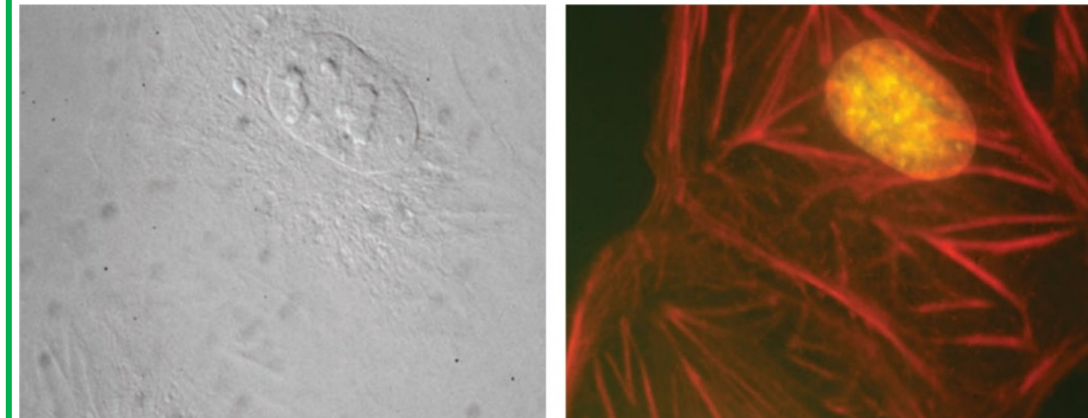
AND/OR

Substructures are not distinguishable

Contrast methods are used to reveal these structures, organelles...

Sample contrasting by fluorescence labelling

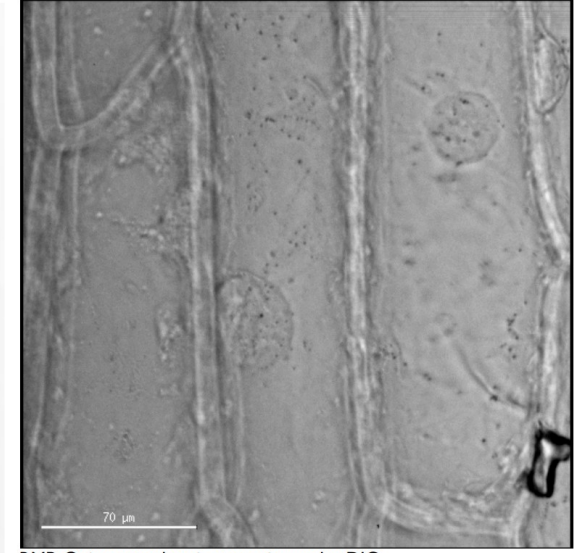
Figure 2.3. An image of the same field of BPAE cells captured using brightfield (left) and fluorescence (right) microscopy. Fluorescent labeling of the nucleus (yellow) and actin (red) makes it possible to see much more detailed cell structure.



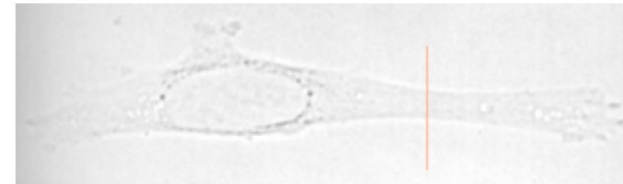
From ThermoFisher – MPSEF



RMP: Onion epidermis bright field



RMP: Onion cytoplasmic streaming under DIC

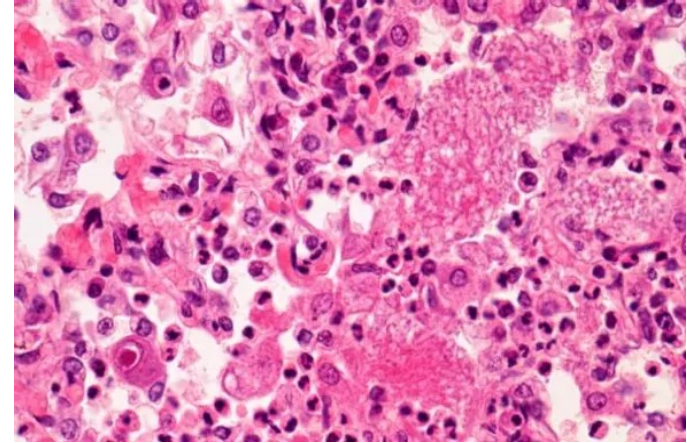


From Micron Course 2017 Oxford

Optical contrasting with transmitted light (DIC, Phase contrast...)

Contrasting methods – transmitted light

- **Chemical staining** with classical brightfield illumination
Ex : H&E staining in histochemistry
-



H&E staining from wellcomecollection.org

- **Using a filter to “color” the transmitted light** can help emphasizing absorbing structures
principle of light-absorption as in Beer-Lambert law

- **Darkfield**
only light deviated/diffracted by the sample is collected

- **Phase-contrast**
Transform the phase shift of the light (from tissues with different refraction indexes) to a difference of intensity

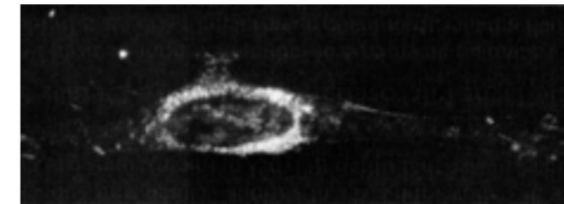
- **DIC**
turns the difference of optical path into a difference of polarization to create contrast

- **Polarised illumination** (ex: for geology)

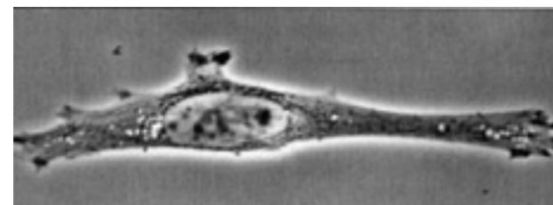
Brightfield



Darkfield



Phase contrast



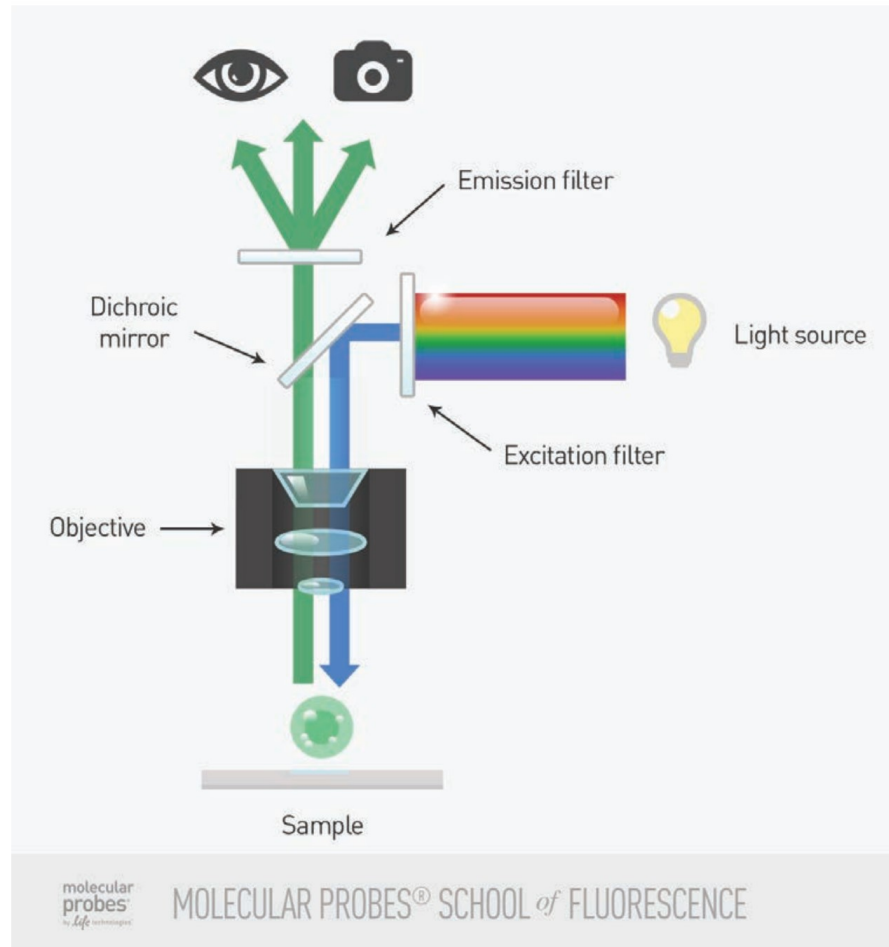
Differential interference contrast (DIC)



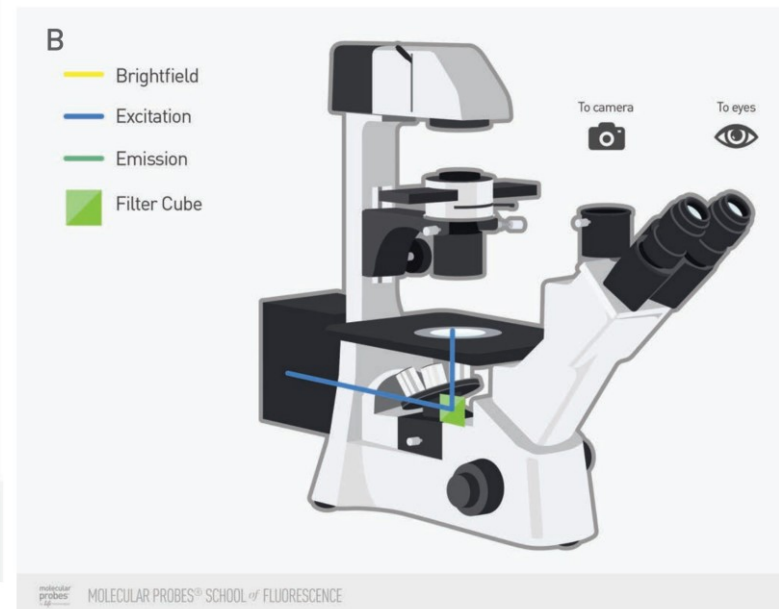
Contrast method – Fluorescence emission

The **sample is labelled with a fluorescent molecule** (protein, dye...),
contrast is achieved by **exciting the fluorophores** (with light), and **imaging the light they emit**

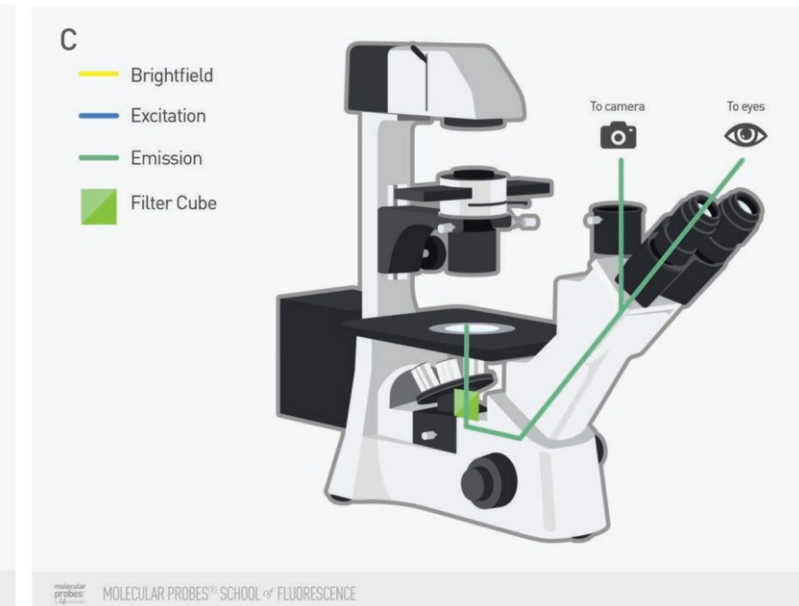
Figure 3.3 Typical light path in an epifluorescence microscope. Notice that the both excitation and emission are controlled by the dichroic, which reflects excitation light (shorter wavelengths) onto the sample and passes the resulting emission light (longer wavelengths) through the filter and on to the detector (the viewer or the camera).



Both excitation/emission happens simultaneously
(at the sample level, not molecular)
i.e emitted fluorescence light is imaged while exciting



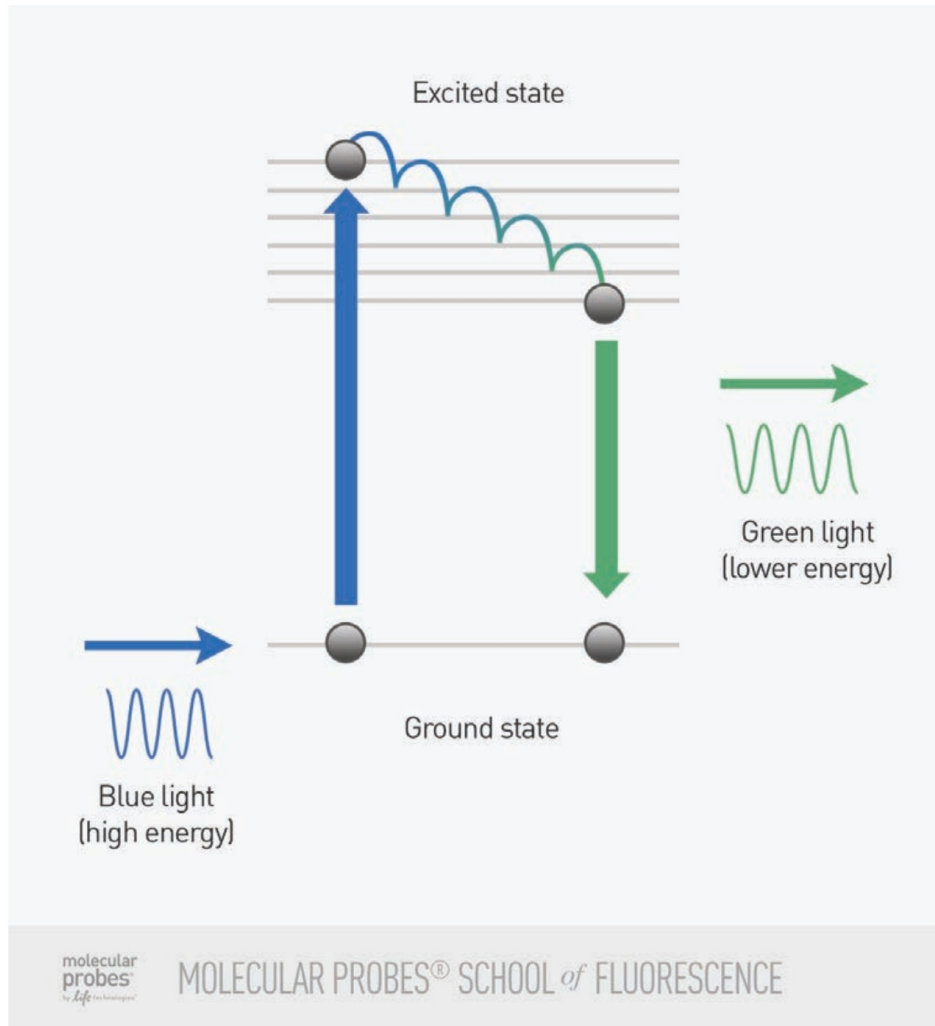
3.7C The green line illustrates the path for light emitted from the fluorescent sample upon excitation. It simultaneously travels through the objective and filter cube and onto the detectors. In epifluorescence microscopy, both the excitation and emission light travel through the same objective.



Fluorescence excitation/emission

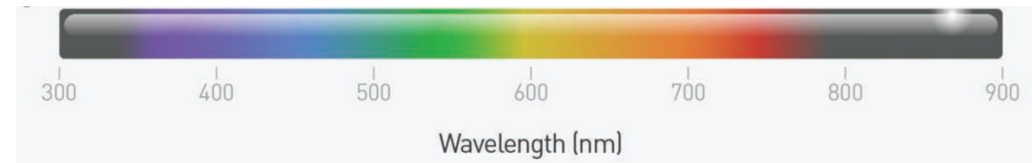
Perrin-Jablonski diagram

Figure 1.4. Simplified Jablonski diagram showing the energy state change of a fluorophore's electron as it undergoes fluorescence, with the corresponding change in the color of light.



$$E = \frac{h \cdot c}{\lambda} \text{ constant}$$

As the energy E decreases, lambda increases
i.e shift from blue toward red, so-called “Stokes-shift”

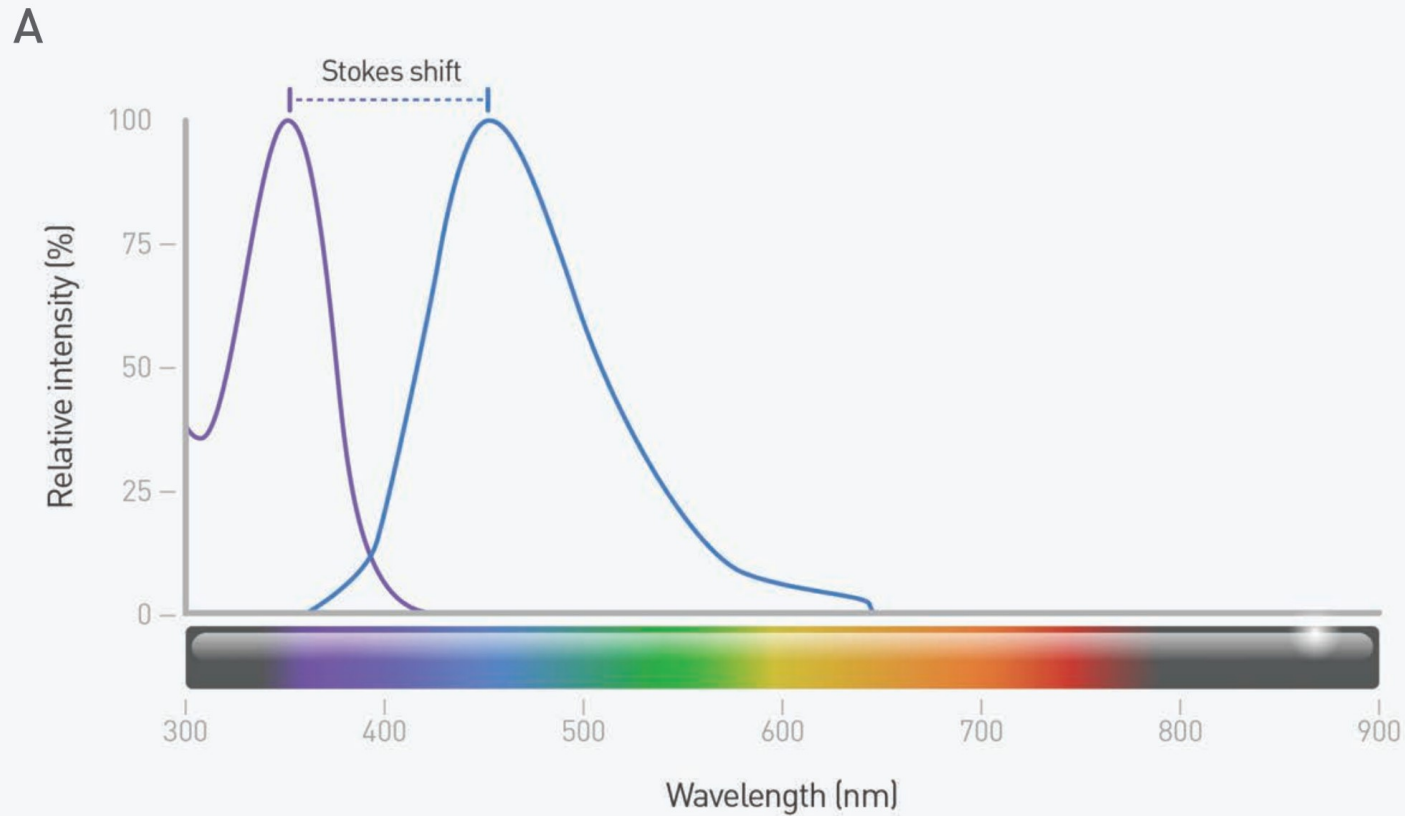


The **cycle absorption/emission is very fast** (ns range) and **is repeated for a given number of cycles**, after which the fluorophore is “bleached”

Absorption/emission spectrum

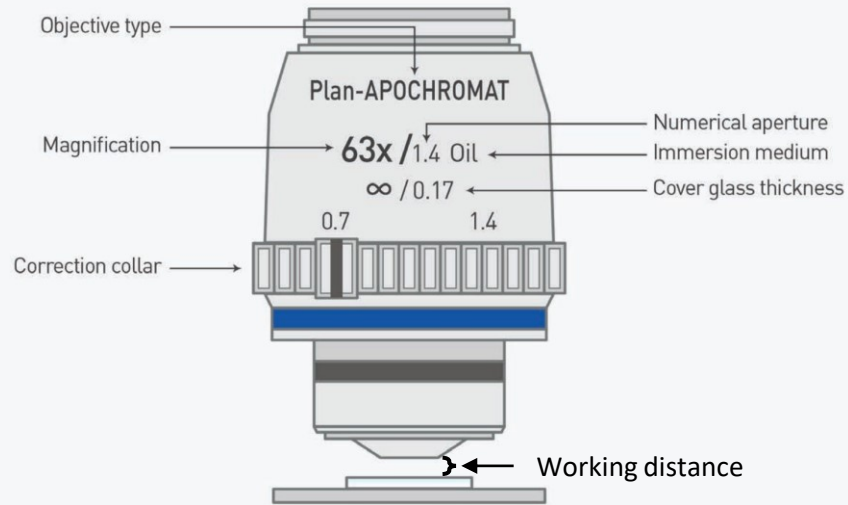
Figure 1.6A. A fluorophore with good separation between the excitation and emission maxima typically results in more reliable detection than a fluorophore with little separation. Compare the fluorophore with a large Stokes shift (A, purple and blue maximum peaks) to that of a fluorophore with a small Stokes shift (B, orange and red peaks).

— Absorption spectrum (for excitation)
— Emission spectrum



Objective and resolution

Figure 5.1. Common notations found on objectives and what they mean.



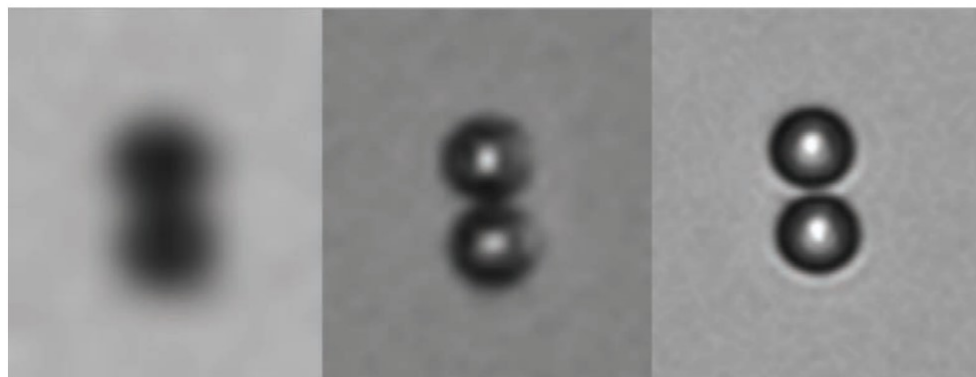
The **objective numerical aperture (NA)** influences the **resolution** and the **size of the field of view**.

The higher the numerical aperture, the higher the resolution, but the smaller the field of view.

Abbe's diffraction law, for lateral (XY) resolution
$$d_{min} = \frac{\lambda}{2.NA}$$

With d_{min} the smallest distance which can be distinguished (ex : between 2 points)

Super-resolution methods exist to work around this limit, using image-reconstruction...



Two 6 μm beads imaged at 4, 10 and 40X magnification
From ThermoFisher - Molecular Probes School of Fluorescence

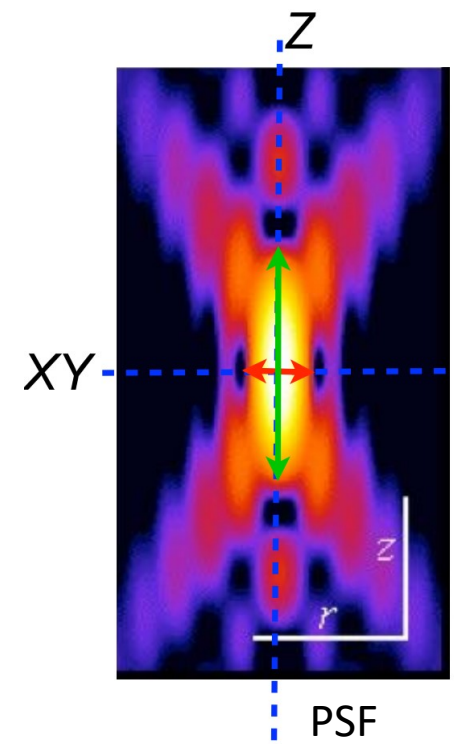
Resolution and diffraction limit

All optical systems are limited by the diffraction of light.

The image of a spot is a diffraction-limited spot.

The image formation is obtained by convolution of the object with the **Point-Spread Function (PSF)** of the system

$$image = object \otimes PSF$$

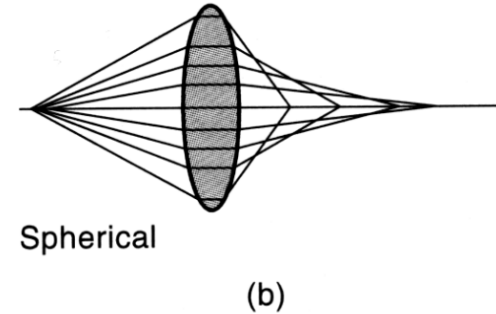
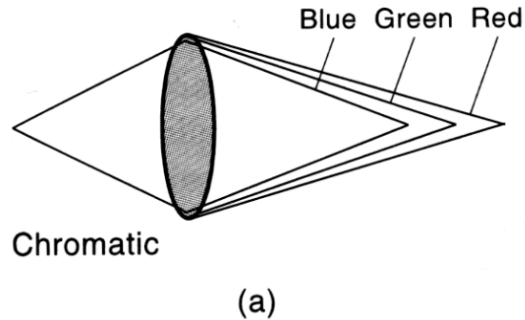


From Micron course 2017
Oxford

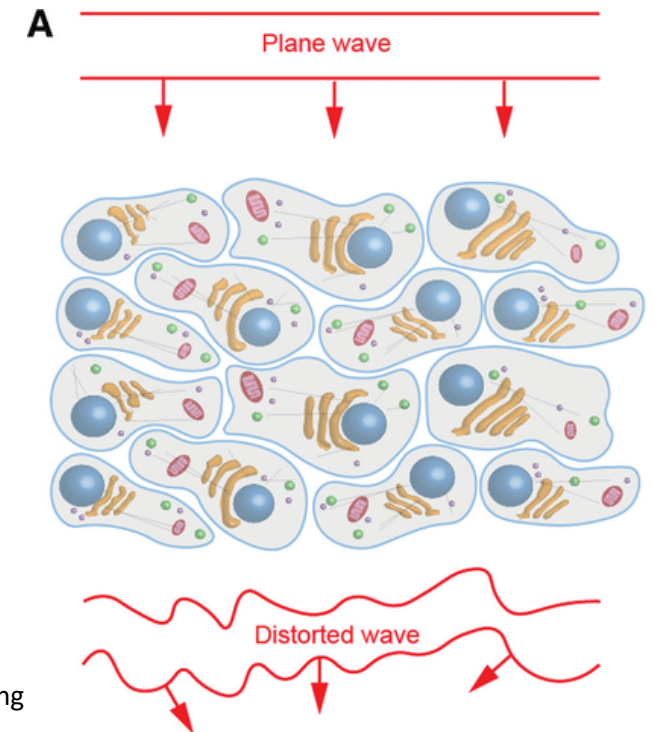
Optical aberrations

- **Optical components are not perfect**

On-axis aberrations



From Micron Course 2017 Oxford



Chromatic aberrations are “systematic” and can/should be corrected (using multi-color beads), especially for multi-colour imaging/localization

- **Photon diffusion/scattering (thick specimen)**

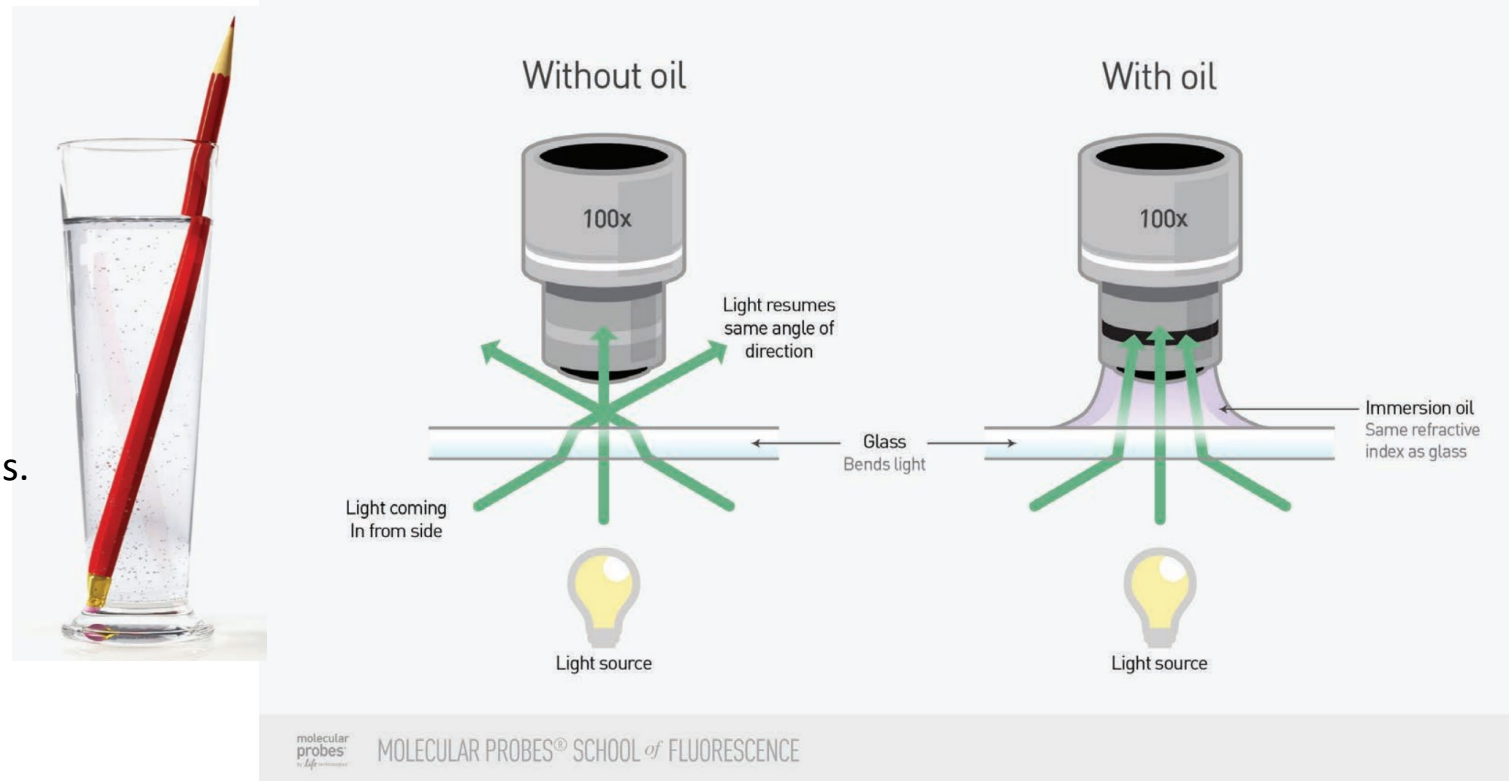
Large specimen are composed of complex heterogeneous tissues which cause diffusion/scattering of the photons within the sample, degrading the image-quality (blur).

Immersion objectives (water/oil)

Figure 5.3. Use of immersion media matched to the objective can minimize the refractive index differences between the objective and the sample.

Oil immersion is used to remove one interface, preventing refractive index mismatch causing aberrations.

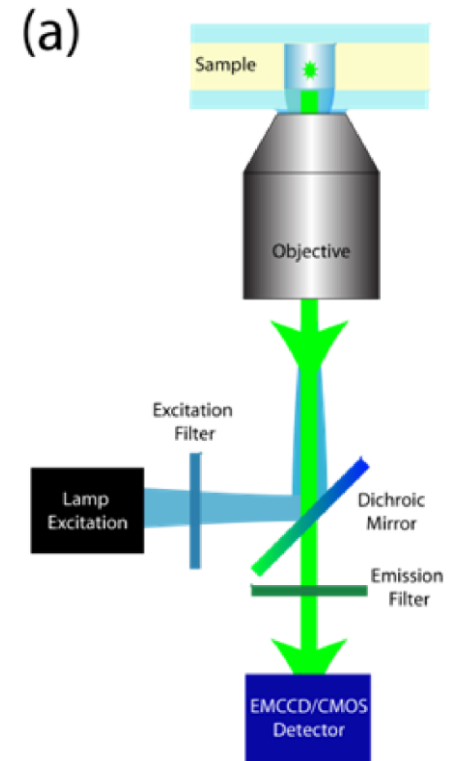
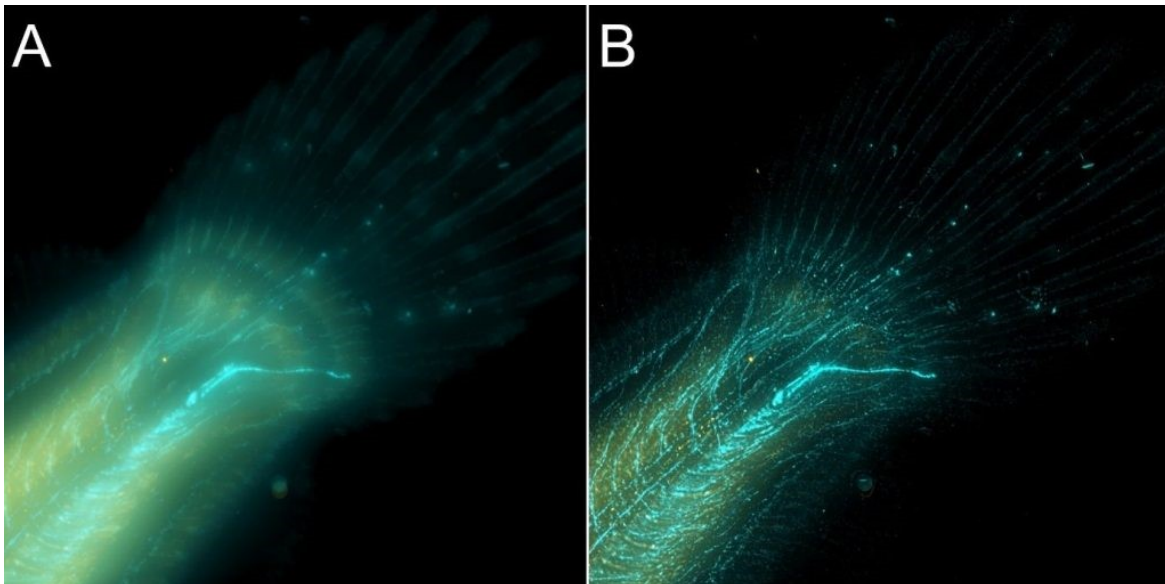
The oil has the same refractive index than the glass.



Widefield microscopy – Pros and Cons

Pros	Cons
Simple setup (compared to other techniques)	Limited contrast : background fluorescence from widefield illumination
Fast imaging	
Low photo-toxicity (no laser)	

Deconvolution can be used to improve signal-to-background :
reversing the image-formation process (i.e reversing the convolution with the PSF)
BUT complex computational method !



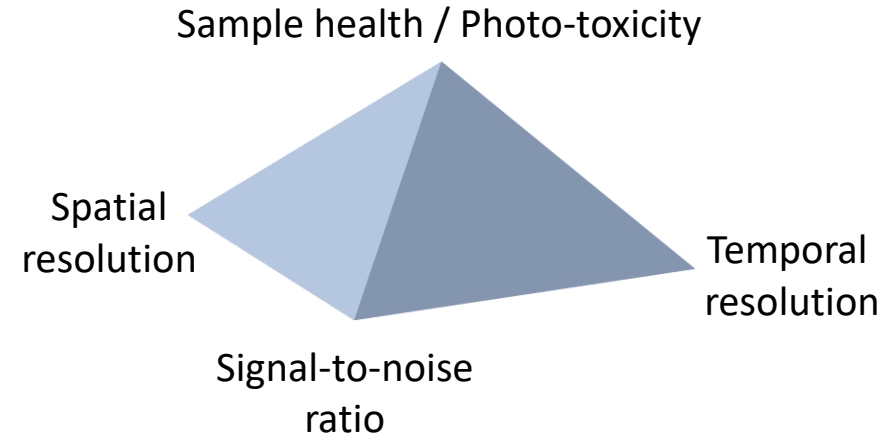
Reproduced from
Moran-Mirabal, Jose. (2013). Advanced-Microscopy Techniques for the Characterization of Cellulose Structure and Cellulose-Cellulase Interactions *. 10.5772/56584.

Other techniques

Each technique has its own pro/cons

- **Confocal** (better signal-to-background)
- **Spinning disk** (better signal-to-background + fast)
- **TIRF** (better signal to background)
- **Structured Illumination** (higher resolution)
- **Single-Plane Illumination Microscopy (SPIM) / Light-sheet** (fast and large volume)
- **Super-resolution** (STED, STED/PALM) : single-molecule resolution but slow
- Etc...

For any technique, prioritizing one summit of the pyramid... means sacrificing the others



Resources

Manufacturers' resources

- Nikon MicroscopyU <https://www.microscopyu.com/>
- [Molecular Probes School of Fluorescence | Thermo Fisher Scientific – DE](#)
- [Microscopy Resource Center | Olympus LS \(olympus-lifescience.com\)](#)
- [Scientific and educational portal for microscopy | Science Lab | Leica Microsystems \(leica-microsystems.com\)](#)

Community resources

- [Overview of Microscopy Techniques: Confocal, Widefield, Transmitted Light and Deconvolution \(biologists.com\)](#)
- [Microlist – Microscopy courses, software, meetings & jobs](#)
- Oxford Micron Course (2017) - [Index of /lectures/micron_course_2017 \(ox.ac.uk\)](#)
- Oxford Micron Course (2019 and on) - [micron-facility/micron-course-2019 \(github.com\)](#)
- iBiology YouTube channel
- [Microforum - Light microscopy forum \(microlist.org\)](#)
- ImageInLife MOOC series – Week 3 : Microscopy
https://youtube.com/playlist?list=PL7149X9aKjLF_bbOEa1osyqDIFvzyYW25

Acknowledgements

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A significant fraction of illustrations also comes from the course material of the Micron Facility in Oxford, which slides are available online (see previous Resource slide).