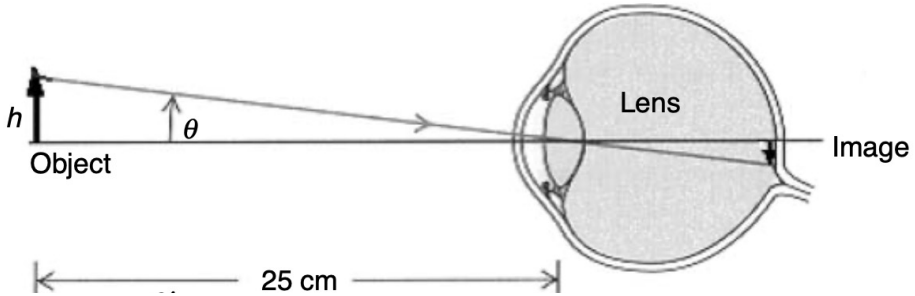
Georg Fantner

Introduction to Microscopy and imaging systems

Why do we need microscopes?

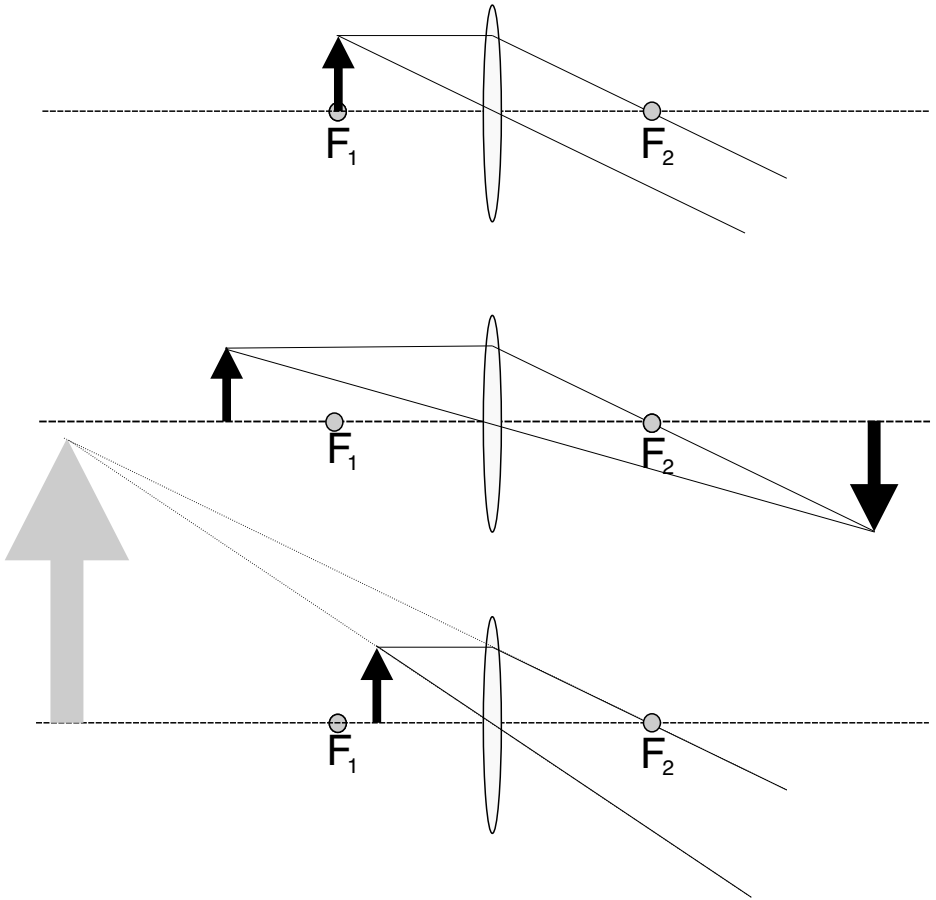
- Magnify objects so we can see them with our “eyes”.
  - Our eye is an “imaging system” that projects an object on our retina using one lens.
  - The strength of this lens is adjustable between totally relaxed (projects infinity onto the retina) to maximum curvature (projects objects that are at the *near point* of the eye on the retina). The near point is the minimum distance an object can be away from the eye to still be focussed on the retina.
  - The closer an object is to the eye, the larger its viewing angle θ, the larger its projection on the retina will be.



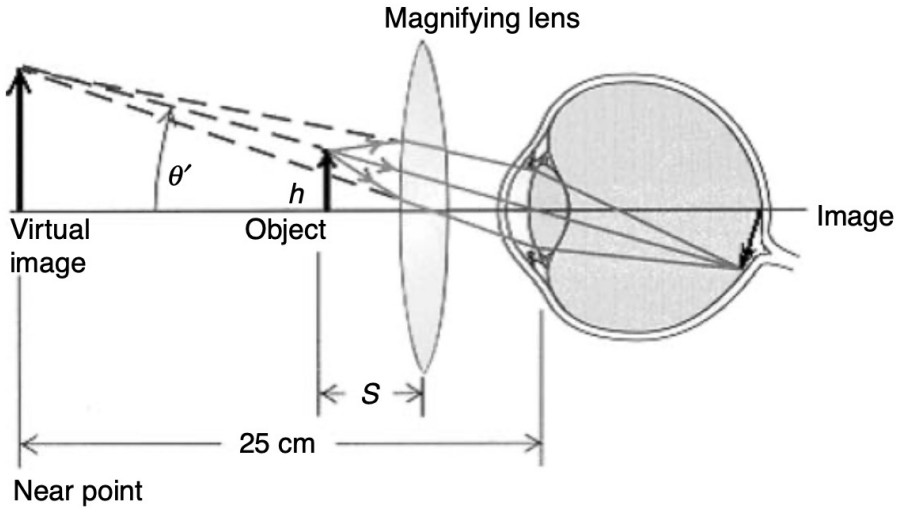
- If we go closer than the near point, the object can not be focussed on the retina anymore →*there is a maximum resolving power of our eyes*
- To increase the resolving power of our eyes, we can use lenses to increase the object on the retina. The simplest is the *magnifier glass.*

Short geometrical optics refresher:

- Convex lenses (lenses with positive curvature) bend light towards the optical axis.
- If an object is at the focal distance of the lens, the image is projected into infinity.
- If an object is further away than the focal distance, the lens will create a *real image* on the other side of the lens, opposite where the object is. The image is inverted.
- If an object is closer to the lens than the focal distance, the lens will create a *virtual image* on the same side of the lens where the object is



- With a magnifying glass, we create a virtual image farther away from the eye, so that when it is focussed on the retina by the eye lens, it is larger than the original object.



- The ratio of the viewing angle with the magnifying lens over the viewing angle without the magnifying lens is the *angular magnification*

$$M_a = \frac{\theta'}{\theta}$$

- For comfortable viewing, the object is placed slightly closer than the focal distance f of the lens. The virtual image then appears at infinity. The angular magnification in that case reduces to:

$$M_a = \frac{25}{f}$$

- So while theoretically we could increase the magnification by decreasing the focal length of the lens. But in practice the maximum magnification we can get with a magnifying glass is 3x-4x

- That is why compound microscopes were invented. They use two lenses to create much larger magnifications.

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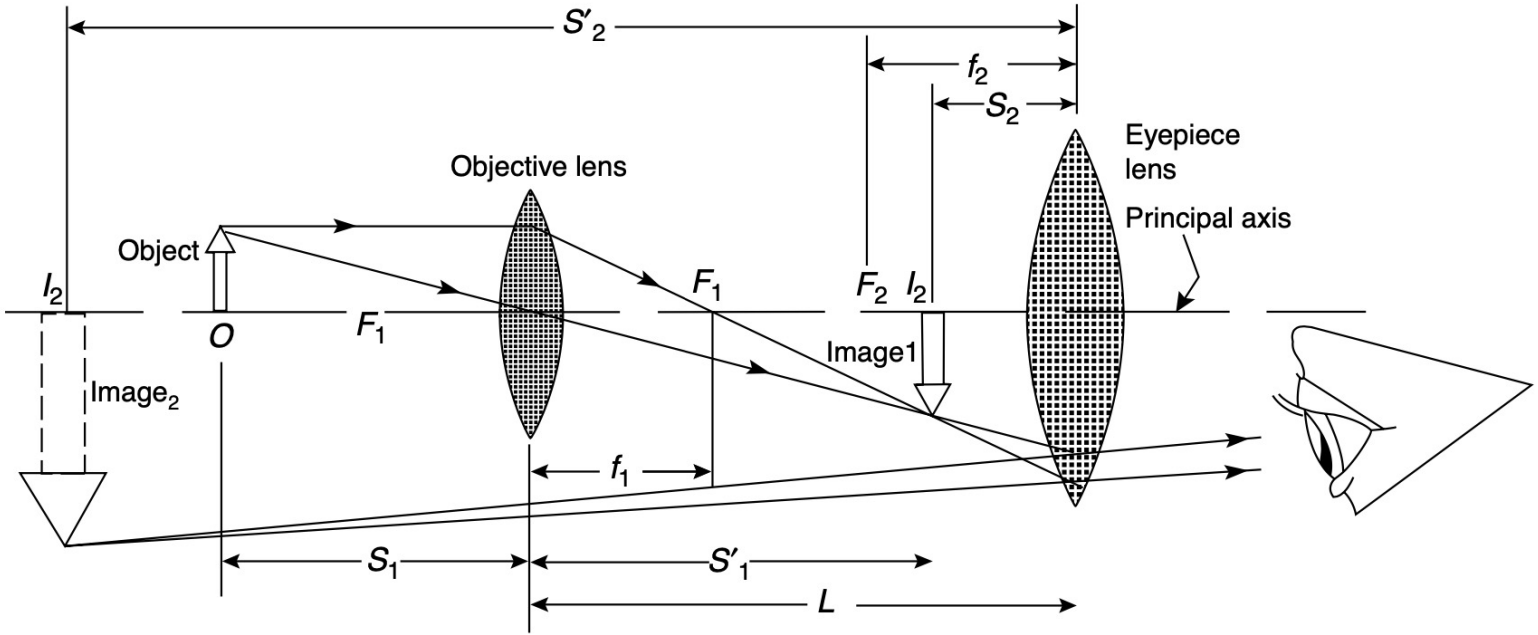
The compound microscope



- The first lens (the objective lens) generates a real image of the object that is already magnified.
- The second lens (the eye piece lens) is placed so that the image that the $Image_1$ is formed just inside of the focal distance of the eyepiece. The eye, piece then acts like a magnifier and makes a virtual image ($Image_2$) that the eye can project on the retina.
- The magnification of such a compound microscope is the product of the magnification of the first lens M_1 with the angular magnification of the second lens M_2.

$$M_1 = -\frac{S_1'}{S_1}$$

- Since in most microscopes the object is placed very close to the focal point of the objective lens, $S_1 \approx f_1$

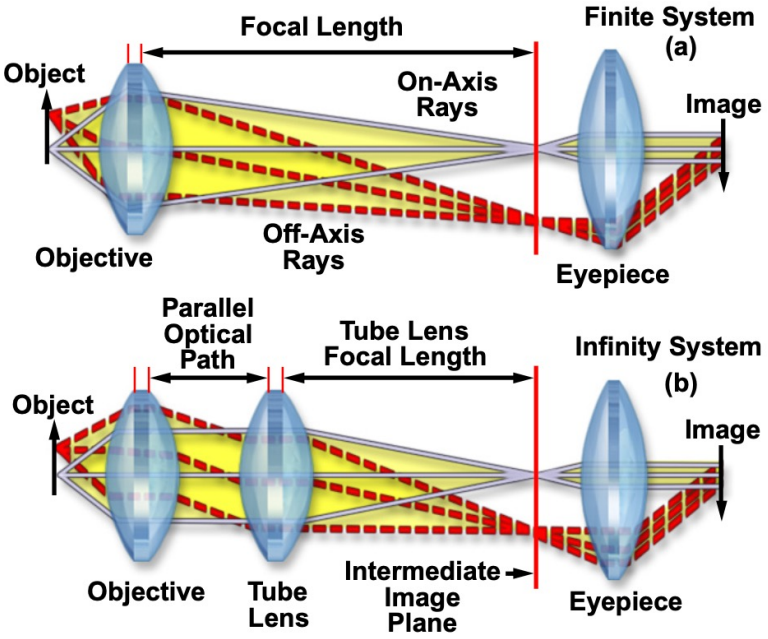
$$M = M_1 * M_2 = -\frac{25\ S_1'}{f_1 f_2}$$

(Distances are is cm. The minus is because the second image is inverted)

- Using compound microscopes it is possible to generate microscopes with up to 200x magnification.
- One problem with this kind of compound microscope is that they have a specified distance from the nosepiece opening, where the objective barrel is secured, to the ocular seat in the eyepiece tubes. This distance is the *tube length*. Any deviation from this length causes a change in the behaviour of the microscope. This is particularly a problem if additional optical components have to be placed in the beam path for advanced microscopy techniques such as fluorescence microscopy, phase contrast, or DIC.
- To overcome this, *infinity corrected microscopes* have been invented in 1930 and have become standard since the 1980's

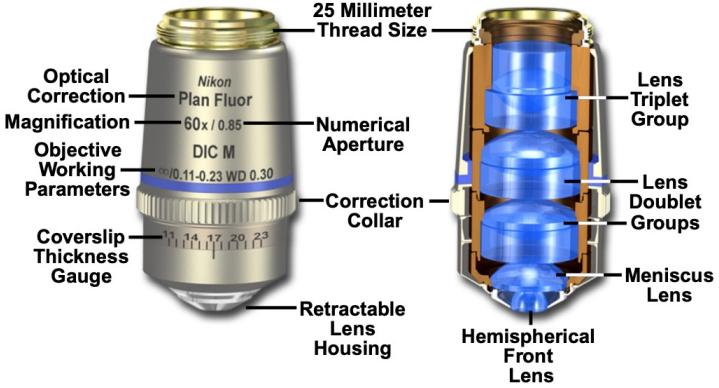
Infinity microscopes

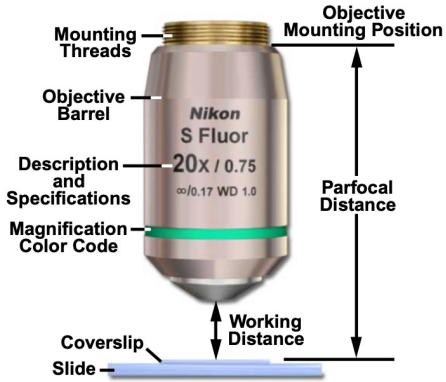
- Infinity microscopes have an additional lens (Tube lens)
- The object is placed in the focal point of the objective lens, so that the rays after the objective lens are parallel.
- The tube lens focusses the rays to form the intermediate $Image_1$
- The distance between the objective lens and the tube lens is called the infinity space. This space can be varied without changing the magnification of the microscope.
- This space can be used to introduce beam splitters, filters, dichroic mirrors, etc.



Microscope objectives

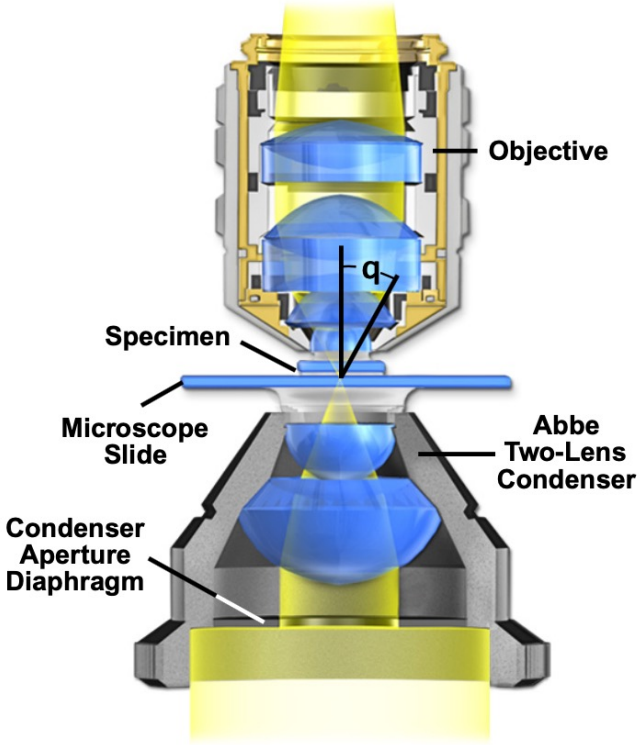
- The quality of the objective is paramount for the image quality. Objectives can be very complex and can cost 10s of thousands of CHF!
- They consist of a combination of many different lenses to correct for lens errors such as spherical or chromatic aberration.
- Some important parameters for objectives are:
  - Magnification
  - Numerical aperture
  - Working distance
  - Oil/air/water immersion
  - Parfocal length
  - Thread size





Illumination system

- Unless an object emits light on its own, we need to illuminate it to see it.
- Illumination can be grouped in different categories
  - Through illumination (for microscopy techniques that measure how ”transparent” samples interact with light)
  - Top illumination (for microscopy techniques that measure how ”reflective” samples interact with light)
- For illumination it is important that we Illuminate the sample with enough light and that the light is uniform.
- The optical principles behind illumination are the same as those for the image formation system.



- The preferred type of illumination is Köhler illumination:

- Köhler illumination ensures uniform illumination and can be used to regulate the amount of light that illuminates the sample using apertures.
- An image of the lamp is projected using the collector lens onto the front focal plane of the condenser. The condenser transforms this image into a parallel ray of light illuminating the sample.

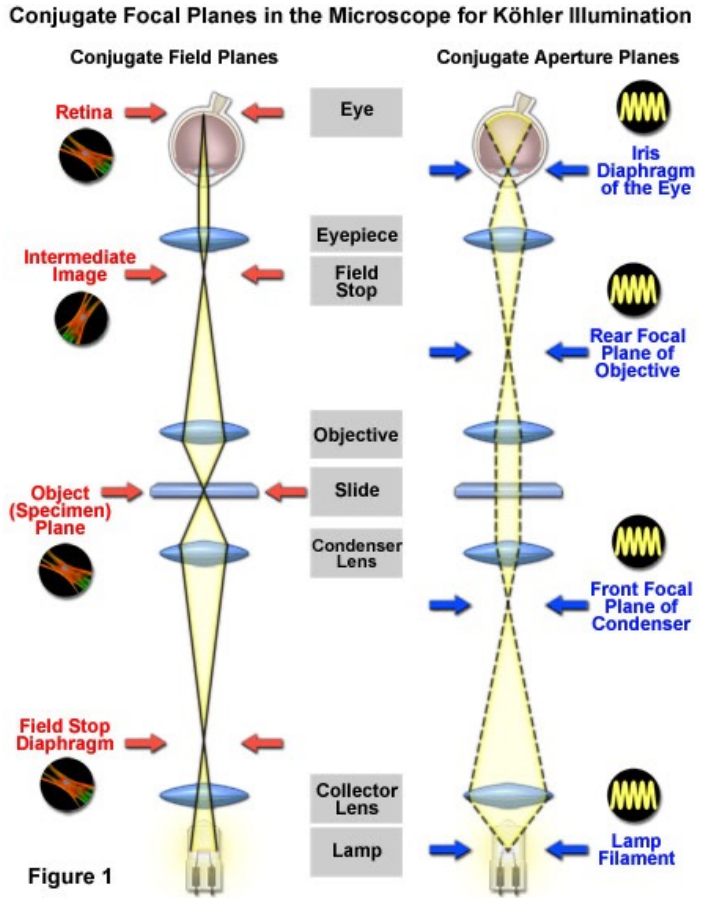


Figure 1

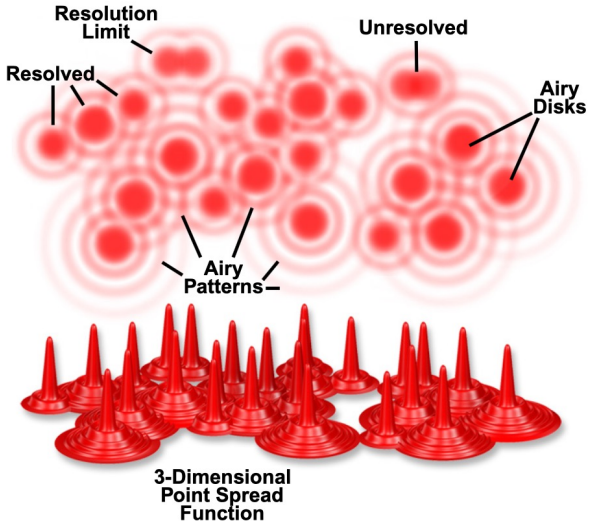
Resolution

- Resolution for a diffraction-limited optical microscope: the minimum detectable distance between two closely spaced specimen points.
- Three characteristics of an objective determine the ultimate resolution limit of the microscope:
  - The wavelength of the light used to illuminate the sample: λ
  - The angular aperture of light captured by the objective: $\sin(\theta)$
  - The refractive index of the environment between the objective and the object: n

$$R = \lambda / 2n(\sin(\theta))$$

This formula is empirical.

- *Magnification does not influence specimen resolution!*



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- $n(\sin(\theta))$ is called the Numerical Aperture (NA). NA values range from 0.1 to 1.6. https://www.microscopyu.com/tutorials/imageformation-airyna

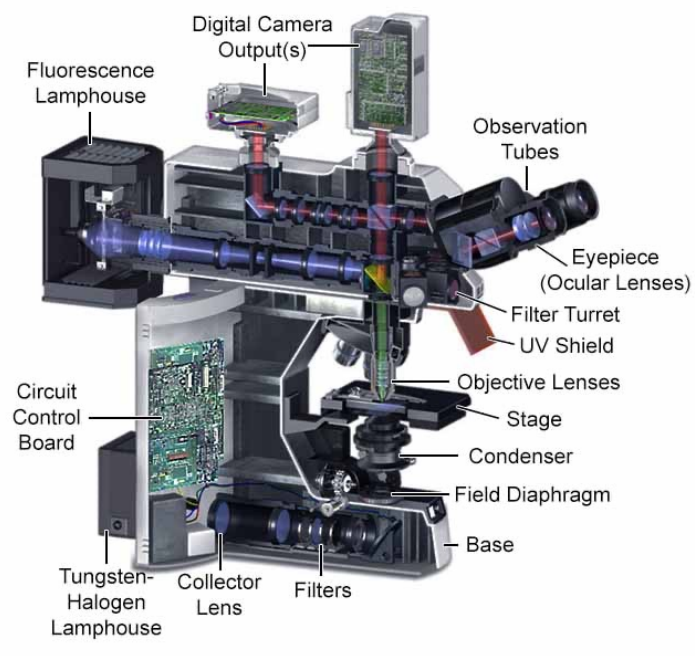
- Because n directly affects the resolution, high magnification objectives are used with immersion oil (n =1.5) between the objective and the cover slip.

- The wavelength also is important. Blue light can be better resolved than red light.

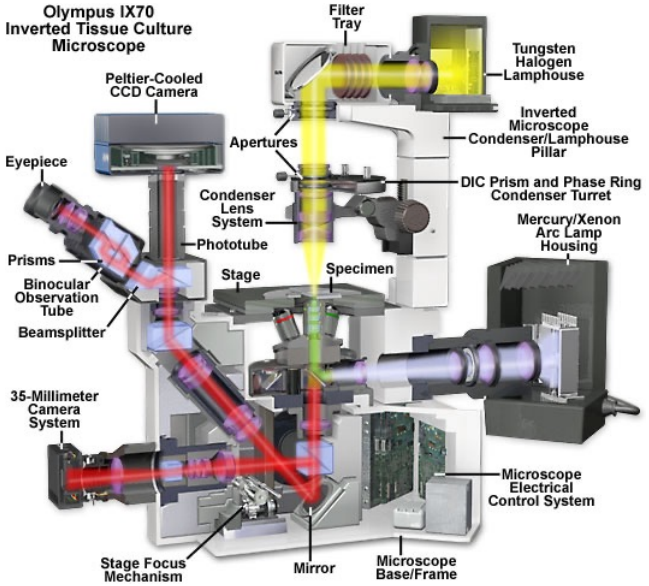
Wavelength (Nanometers)	Resolution (Micrometers)
360	.19
400	.21
450	.24
500	.26
550	.29
600	.32
650	.34
700	.37

Different microscope setups

Upright microscope



Inverted microscope



Many different optical microscope modes exist

- Bright-field
- Dark field
- Phase contrast
- Differential interference contrast (DIC)
- Fluorescence
- …

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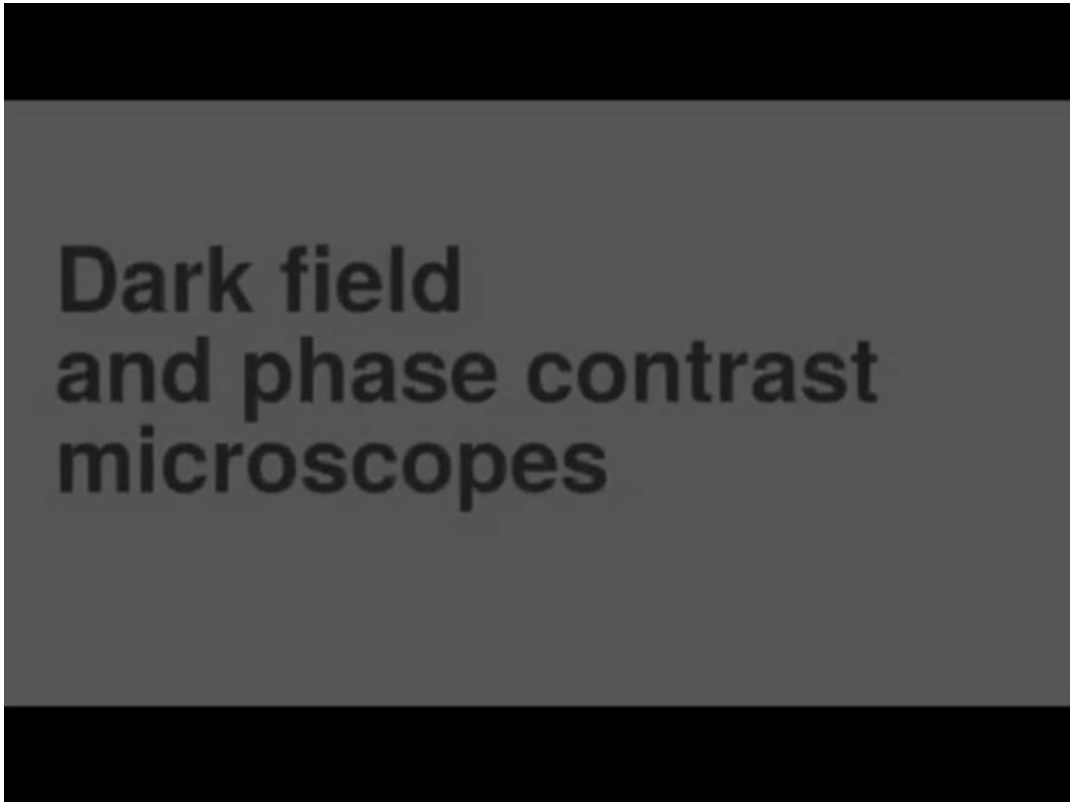
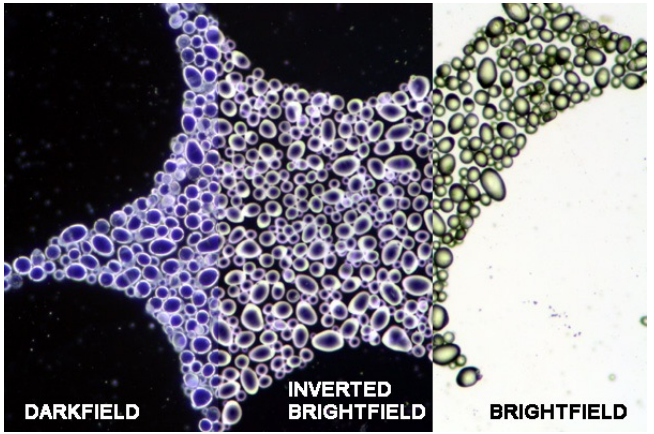
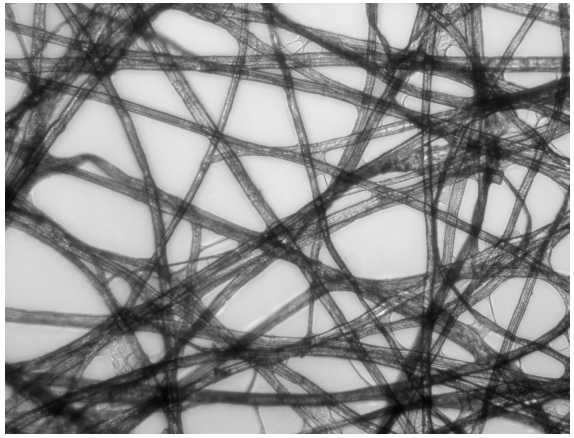
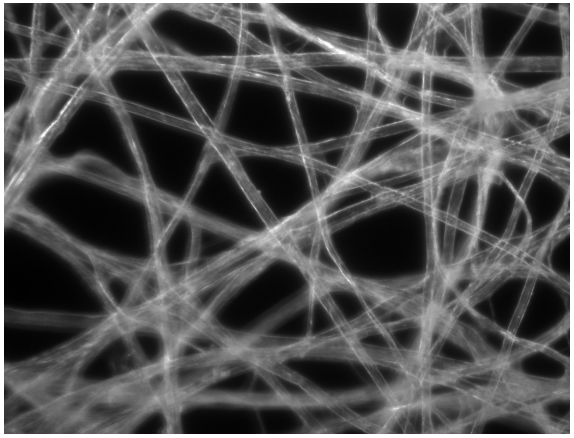
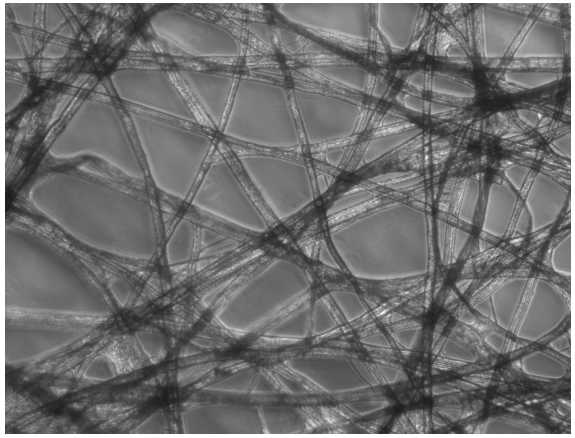
Darkfield and phase contrast





Brightfield | Dark field | Phase contrast

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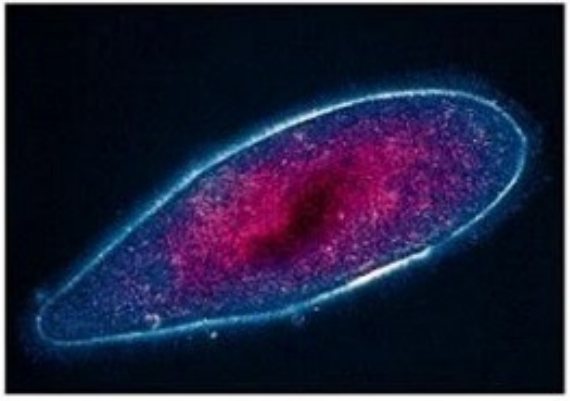
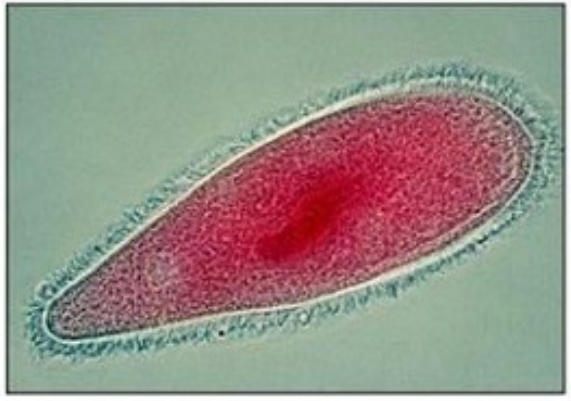



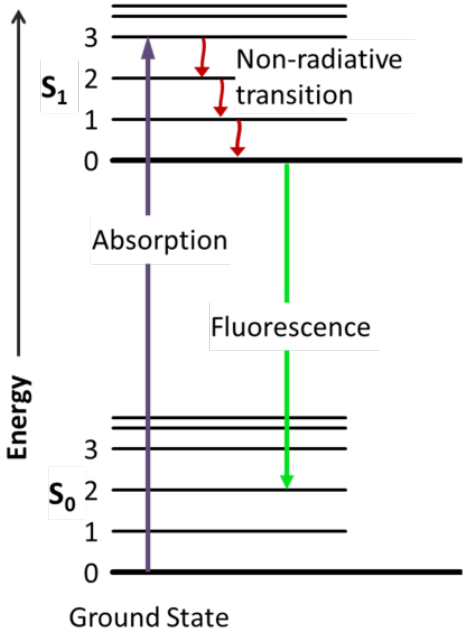
Brightfield | Darkfield | Phase-contrast

Fluorescence microscopy

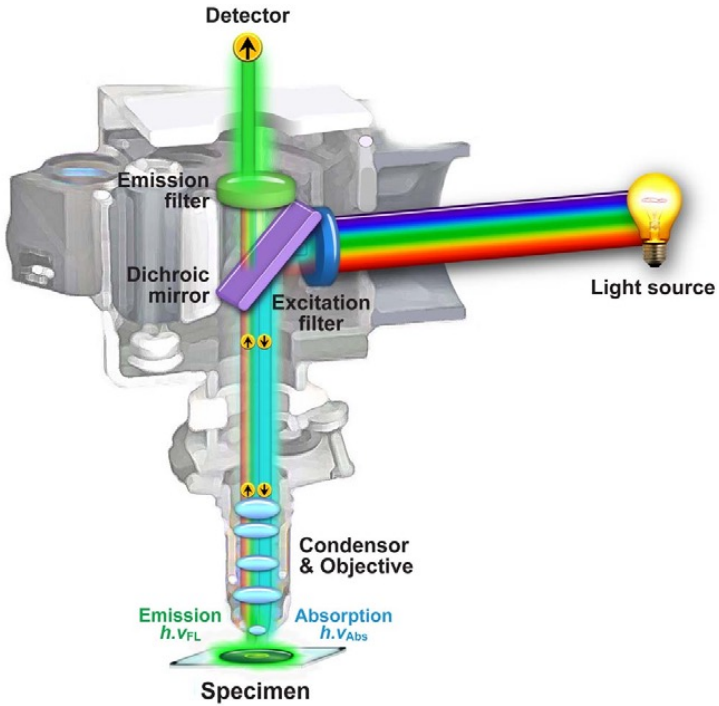
Fluorescence microscopy

- In fluorescence microscopy we image the light **emitted by the sample itself**.
- In order to make the sample emit light, it has to be excited with a wavelength having more energy.
- When a photon is absorbed, molecules can be excited from the ground state to an excited state.
- When the molecules relax from the excited state into the ground state they emit a photon.
- The difference in wavelength between excitation and emission is called the *Stokes shift.*

Jablonski diagram



- The sample is excited by one wavelength (excitation wavelength)
- The image is formed by collecting the emission wavelength
- The excitation wavelength and the emission wavelength are separated by filter cubes. A filter cube exists of an excitation filter, a dichroic mirror and an emission filter



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Fluorescent filters

- You need the right filter set for each fluorophore
- The dichroic mirror reflects the excitation wavelength but passes the emission wavelength, thereby separating the incoming and outgoing light.
- Some filter cubes exist for dual colour viewing

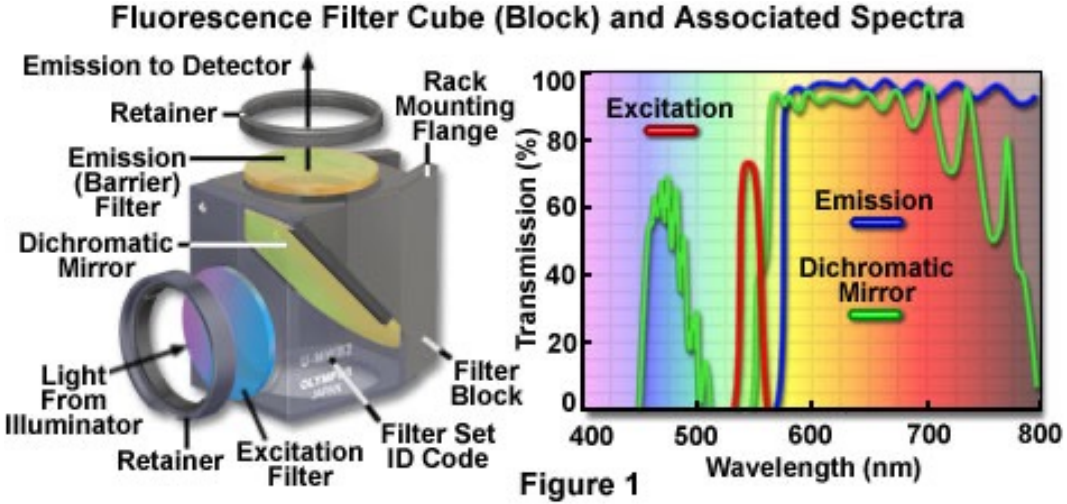
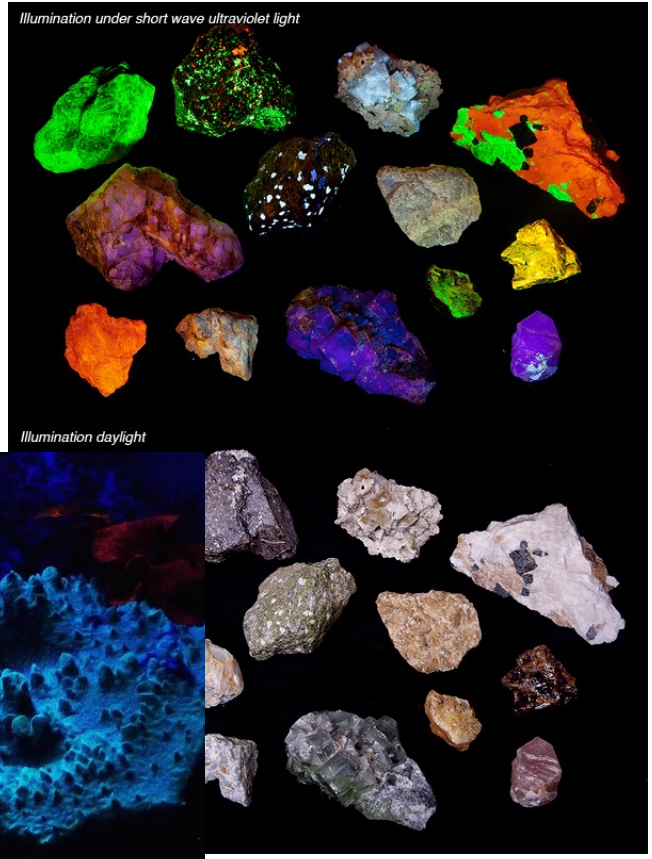
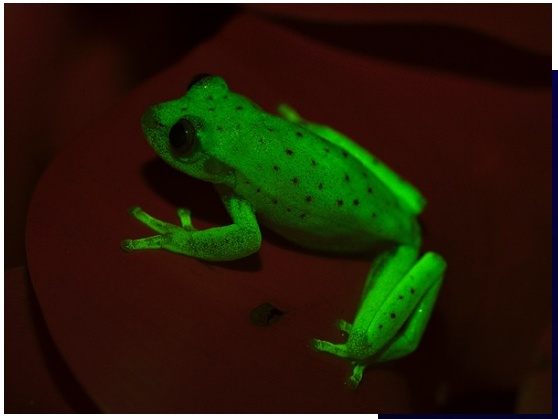
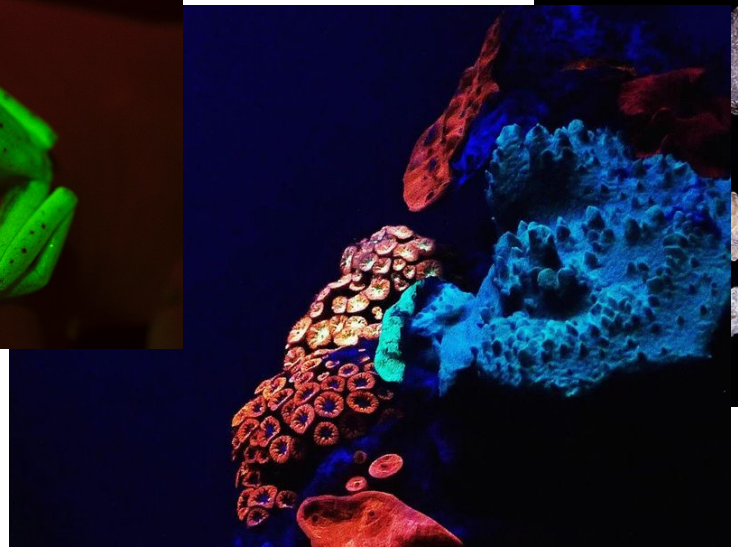


Figure 1

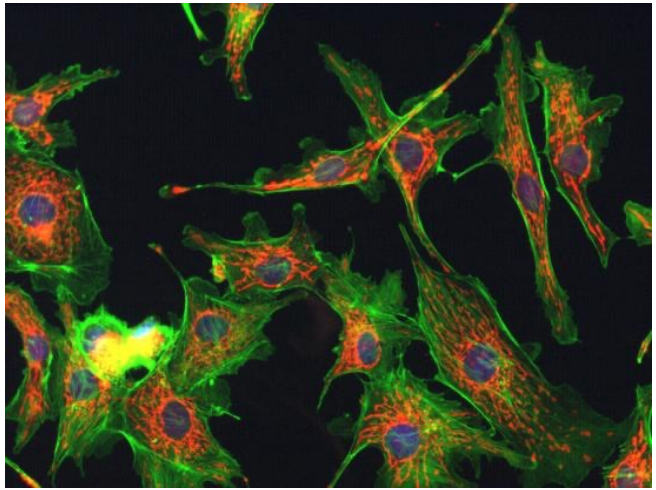
- Samples can either be naturally fluorescent or can be labelled.
- Naturally occurring fluorescence happens in minerals, coral, animals,…



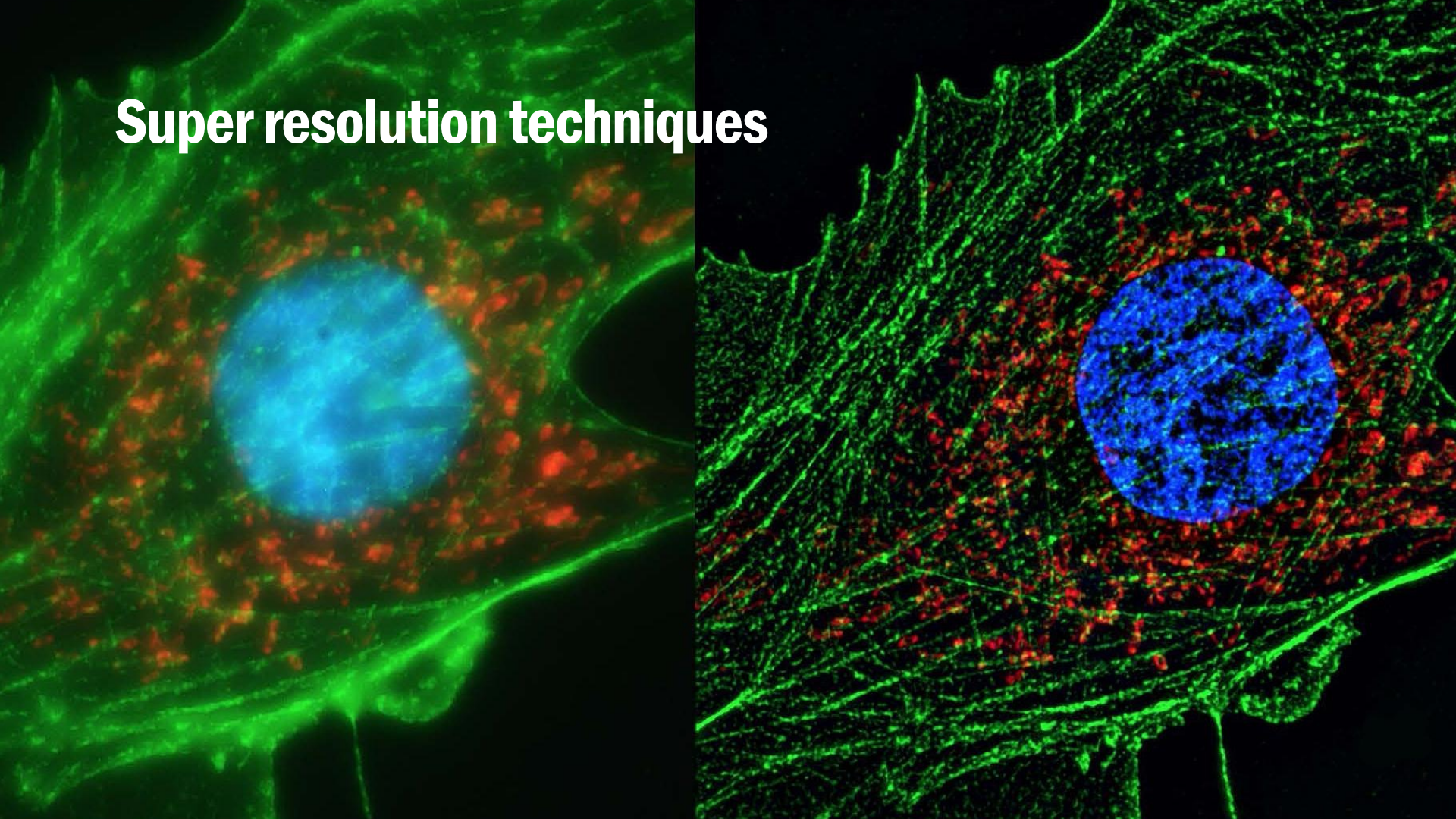




- To make non fluorescent specimens fluorescent, they can be labelled with fluorescent molecules (fluorophores).
- Some molecules are fluorescent due to their electron-structure. Examples are *fluorescine, rhodamine, DAPI,…*
- Some proteins are fluorescent: green fluorescent protein (GFP), red fluorescent protein (RFP), etc. These proteins can be cloned into organisms to make specific proteins in cells light up in the microscope. This is widely used in biology.

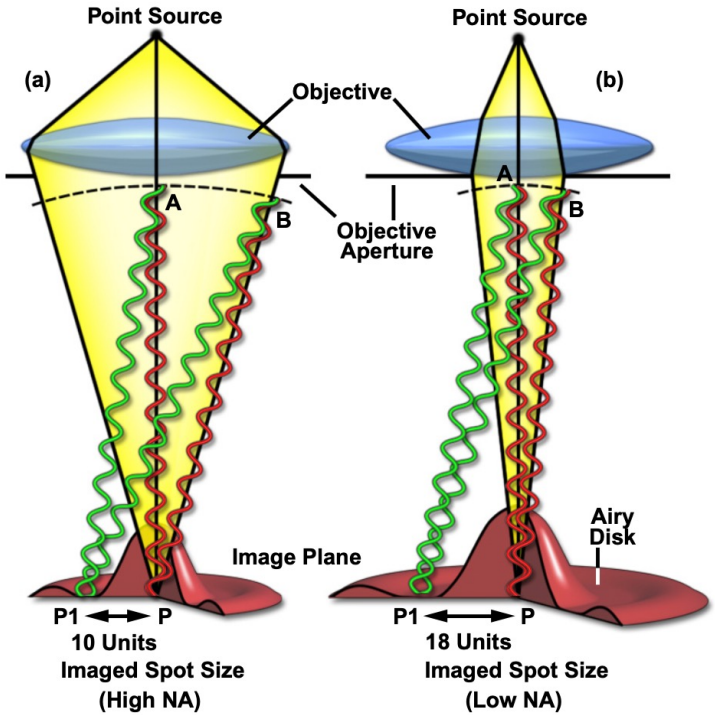


Super resolution techniques



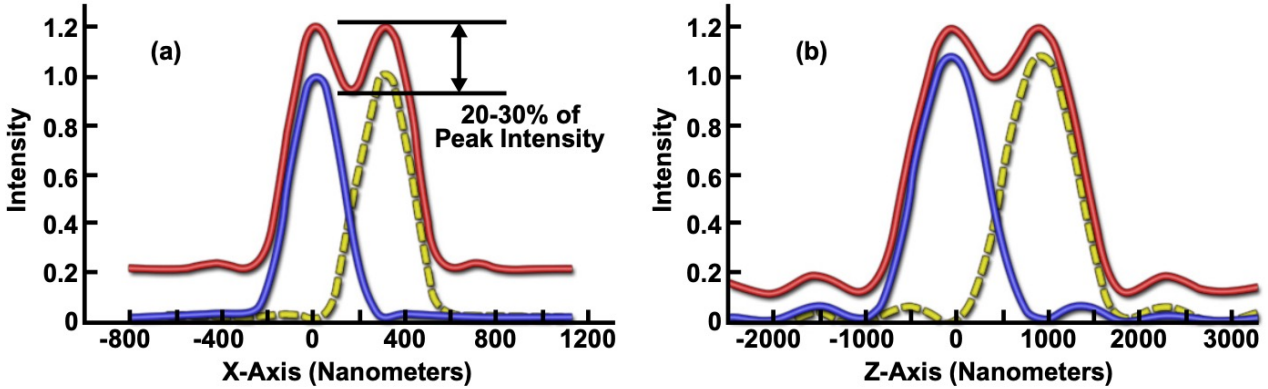
Wavelength nature of light reduces resolution

- Point sources are transformed by the microscope into an image that has a finite diameter.
- The smaller the NA of the microscope is, the larger the spot is
- The transformation of a point source into a finite spot is called the point spread function.



- The point spread function has dimensions in X,Y, and Z
- It determines how close point sources can be together to still be able to resolve them as two separate identities
- The resolution can be approximated by:

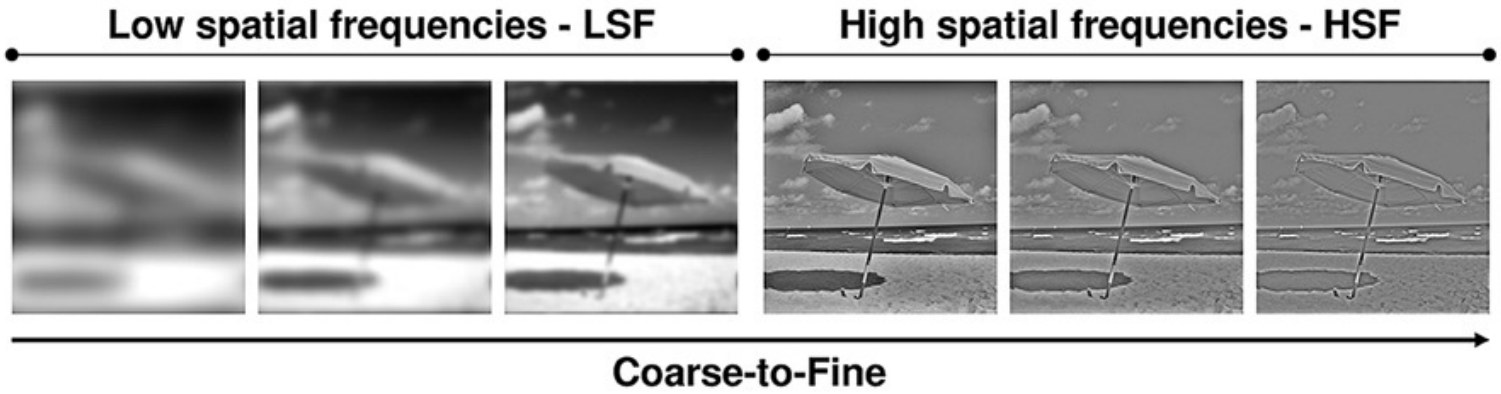
$$Abbe\ Resolution_{XY} = \lambda/2NA$$
$$Abbe\ Resolution_{Z} = 2\lambda/NA^2$$



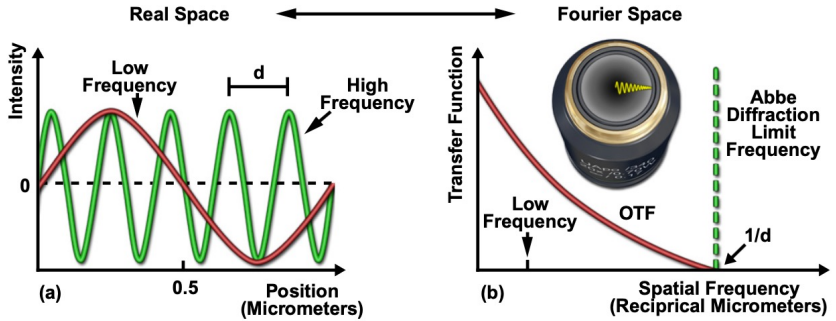
- For a perfectly sharp object viewed through a microscope it means that the resulting image is not infinitely sharp.

The microscope as a filter

- The resolution limit can also be described in terms of signal processing.
- *Remember:* any temporal signal (waveform) can be described as a sum of Fourier components in time.
- Images can be similarly decomposed into spatial frequencies.

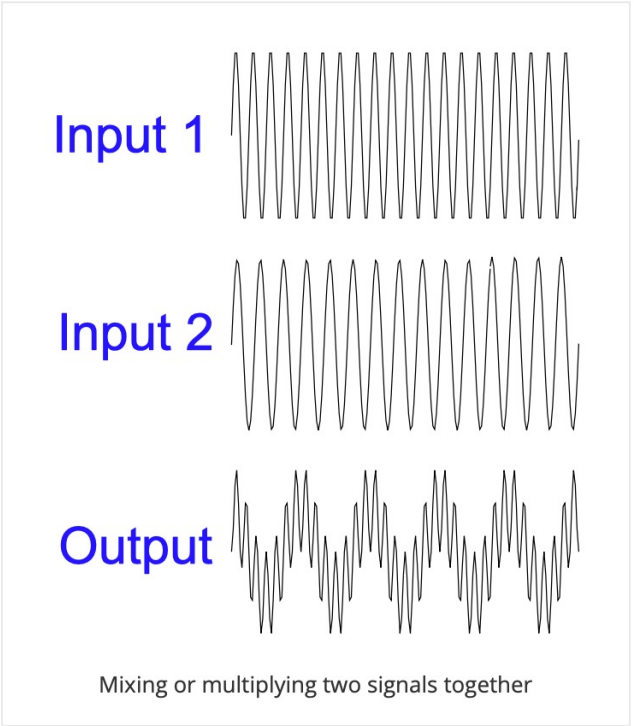


- Just like any other measurement instrument, a microscope has am maximum bandwidth. This bandwidth is given by the abbe diffraction limit.



- In 2D the bandwidth can be plotted in Fourier space as a circle. The larger the circle, the higher the achievable special resolution.

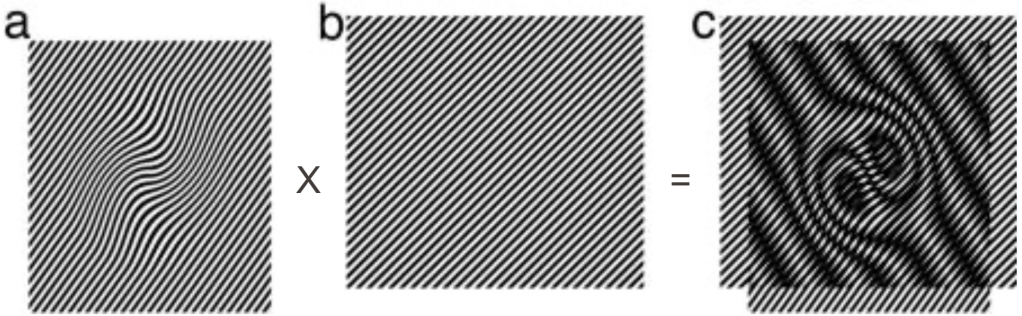
- In signal processing, frequency modulation techniques have been developed to measure the amplitude of very high frequencies with low frequency electronics (for example in radios).
- Signals of two slightly different frequencies are mixed.
- Mixed signal = $\sin(\omega 1t)*\sin(\omega 2t)$
- = $\frac{1}{2} \cos(\omega 1 - \omega 2)t - \frac{1}{2} \cos(\omega 1 + \omega 2)t$
- $(\omega 1 + \omega 2)$ is above the cut-off frequency of the electronics
- Only $\cos(\omega 1 - \omega 2)t$ remains.
- If you know the frequency and amplitude of the mixing frequency $\omega 2$, you can back-calculate the amplitude and frequency of $\omega 1$.
- We can use a similar «trick» in microscopy



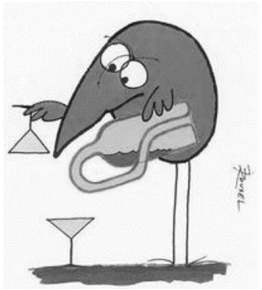
Mixing or multiplying two signals together

Structured illumination microscopy (SIM)

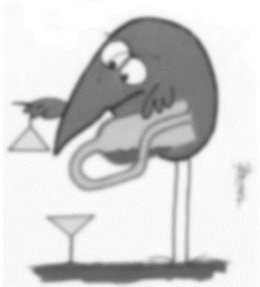
- Frequency mixing is done by illuminating the sample not with a uniform illumination, but with a striped pattern of known frequency.

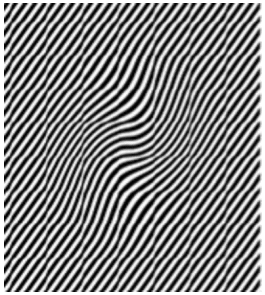


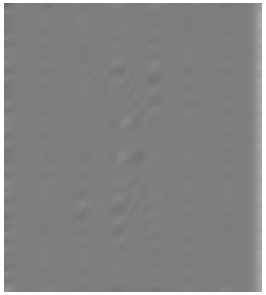
$$A_\omega \cos(\omega_{sample} x) \times \cos(\omega_{ill} x)$$
$$= \frac{A_\omega}{2} \cos\left((\omega_{sample} - \omega_{ill})x\right) + \frac{A_\omega}{2} \cos\left((\omega_{sample} + \omega_{ill})x\right)$$



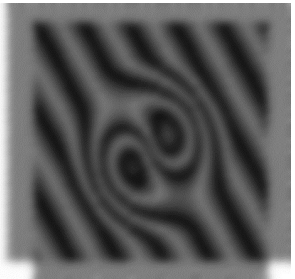
Microscope removes high frequencies



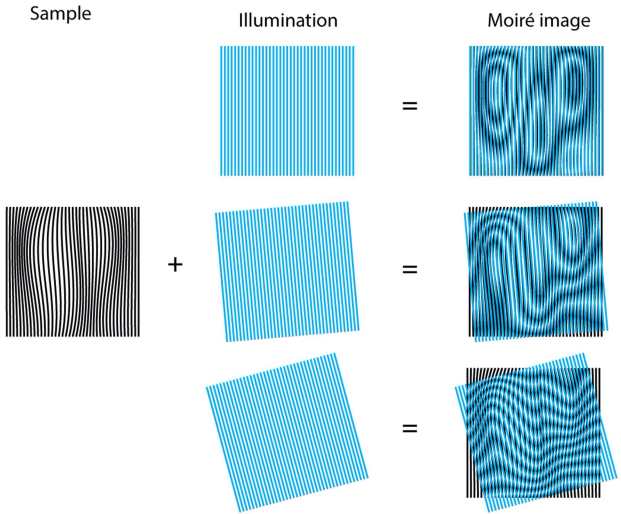


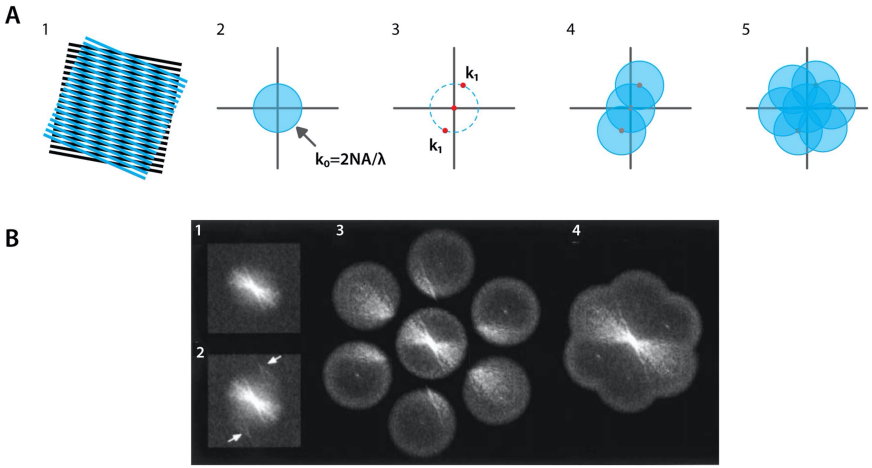


$$A_\omega \cos(\omega_{sample} x) \times \cos(\omega_{ill} x)$$
$$= \frac{A_\omega}{2} \cos\left((\omega_{sample} - \omega_{ill})x\right) + \frac{A_\omega}{2} \cos\left((\omega_{sample} + \omega_{ill})x\right)$$

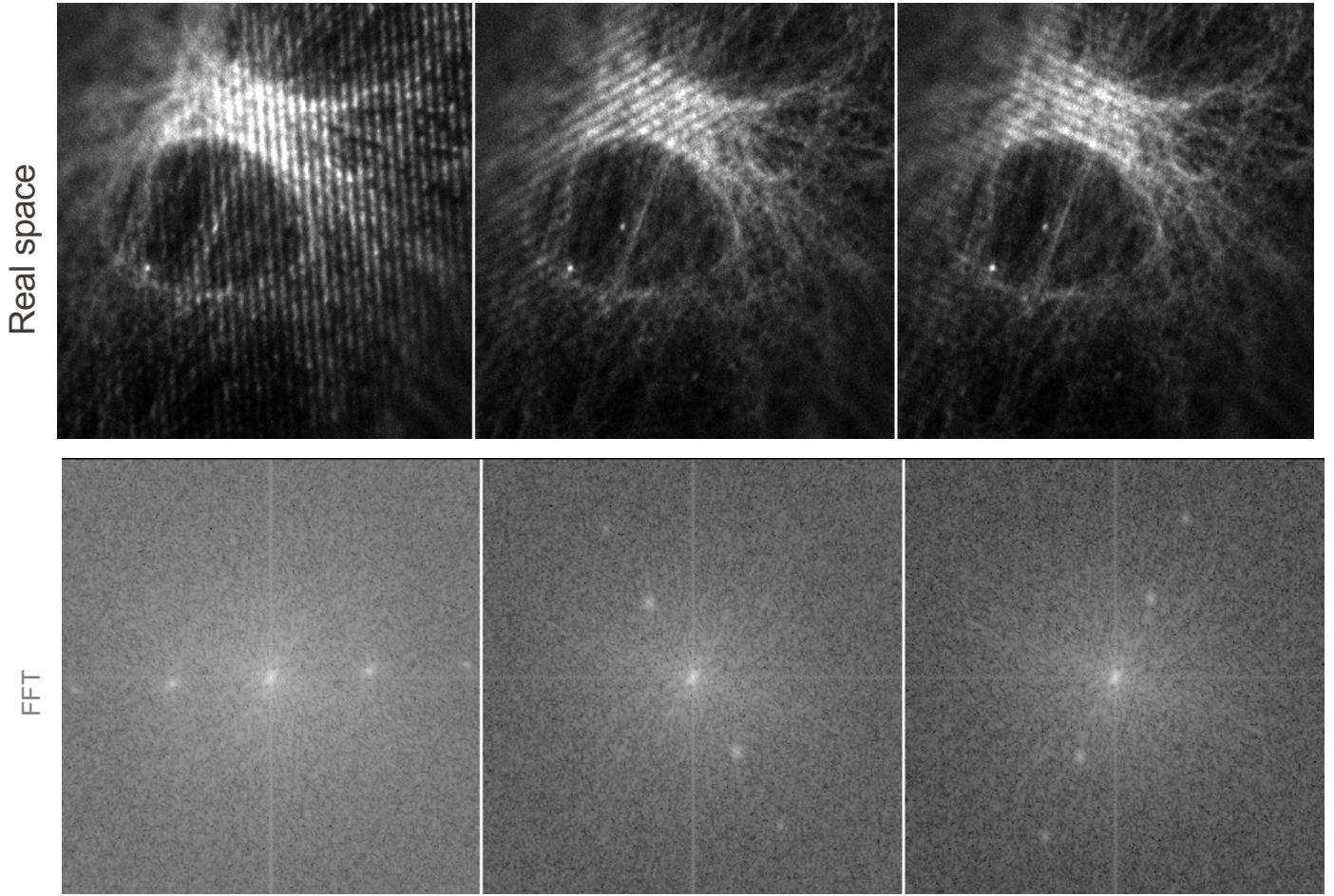


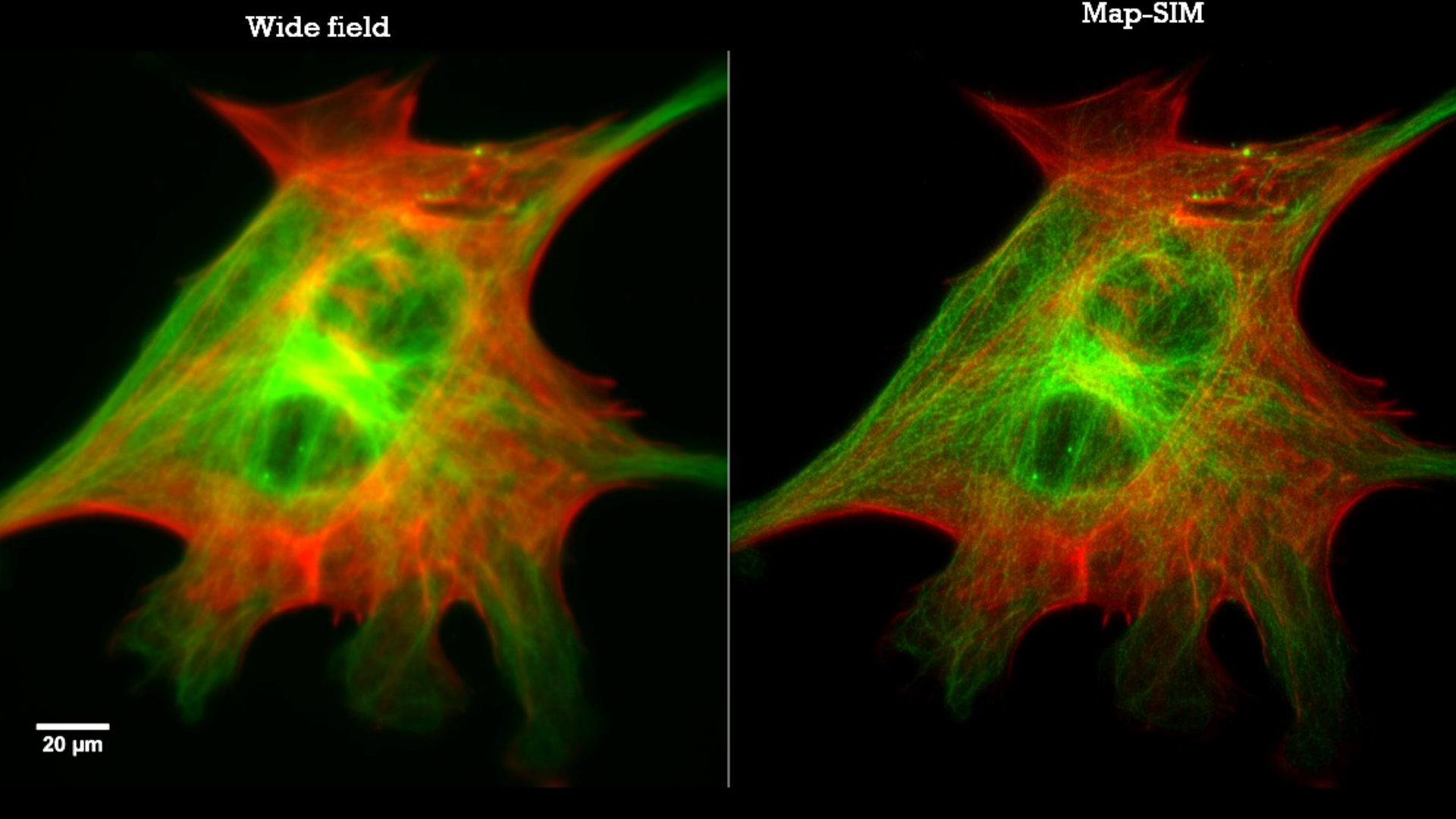
- We can do the structured illumination with different displacements and rotations.





- Each image with striped illumination expands the imaged Fourier space in a different direction. Reassembling the Fourier transforms of the individual images results in information with twice the frequency content.

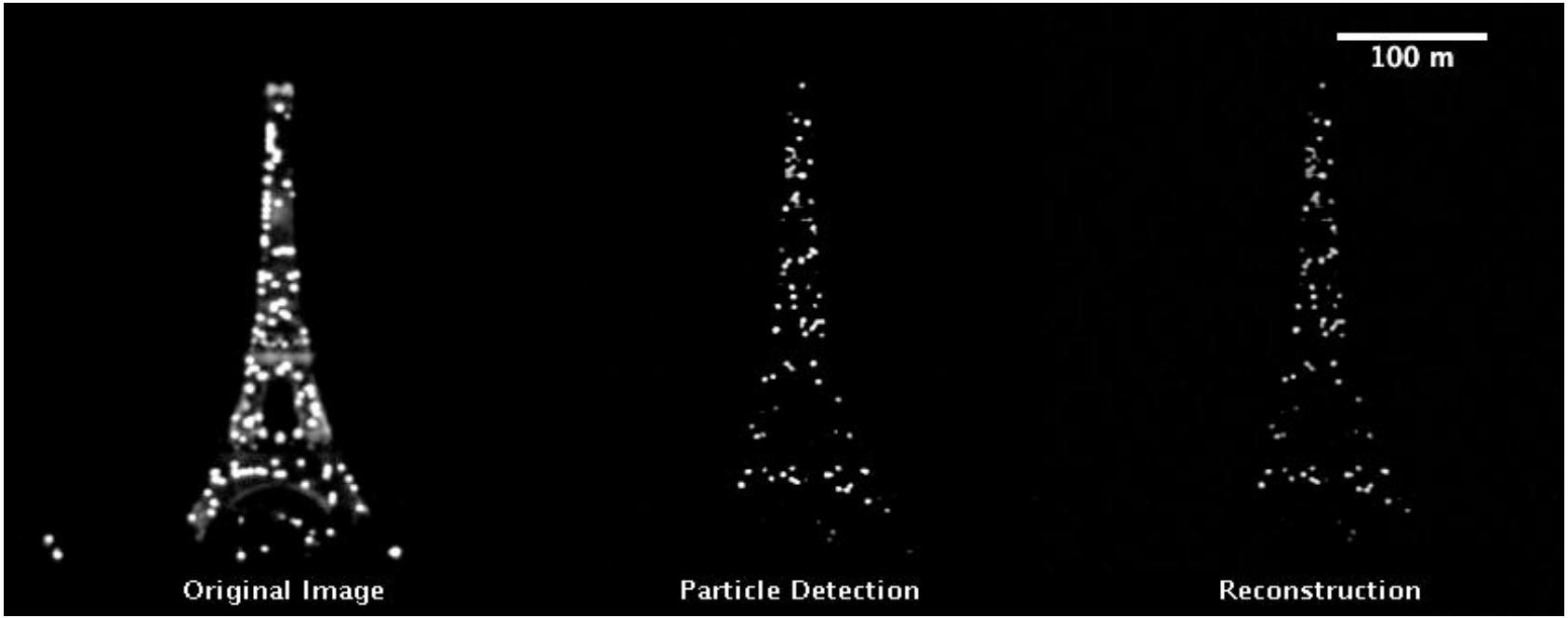

Real space
FFT

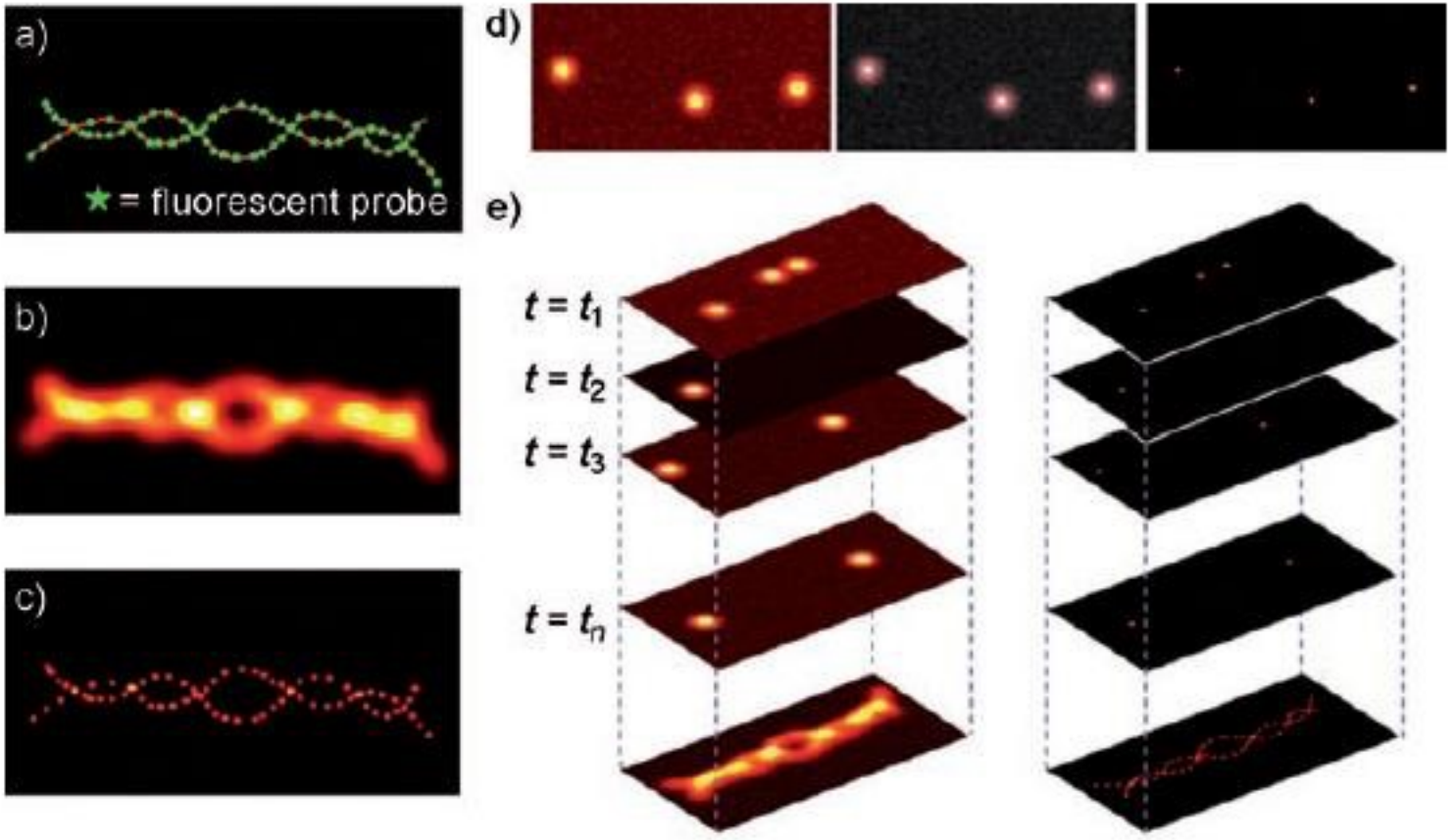

Wide field
Map-SIM
20 µm

Single molecule localization microscopy

- Basic principle: Separate spatially overlapping light sources in time

- If you plot the centre of each light source as a dot, and sum them all up, you can reconstruct a super resolved image.

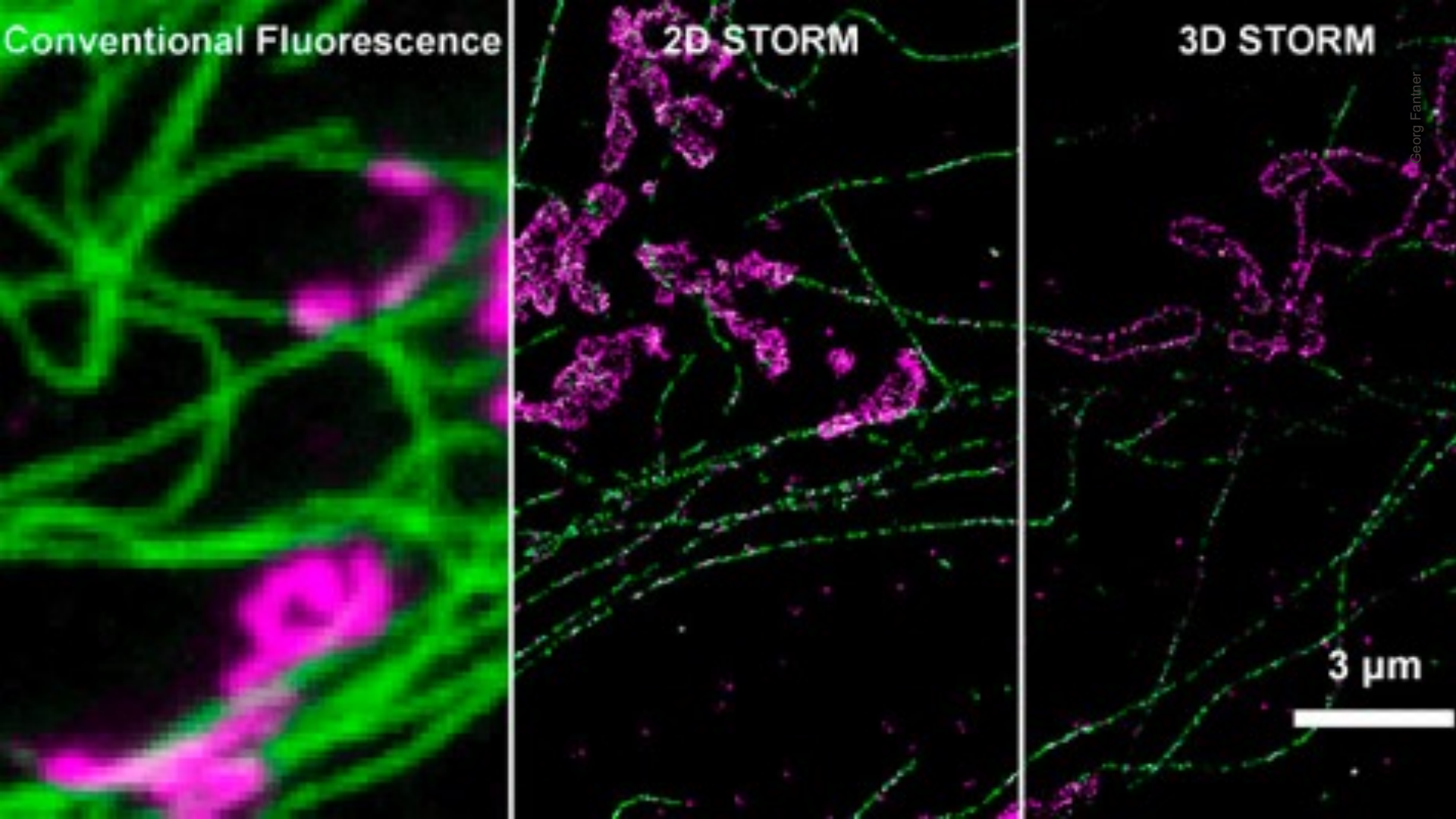




http://bme240.eng.uci.edu/students/08s/mkotlarc/images/PALM2.JPG

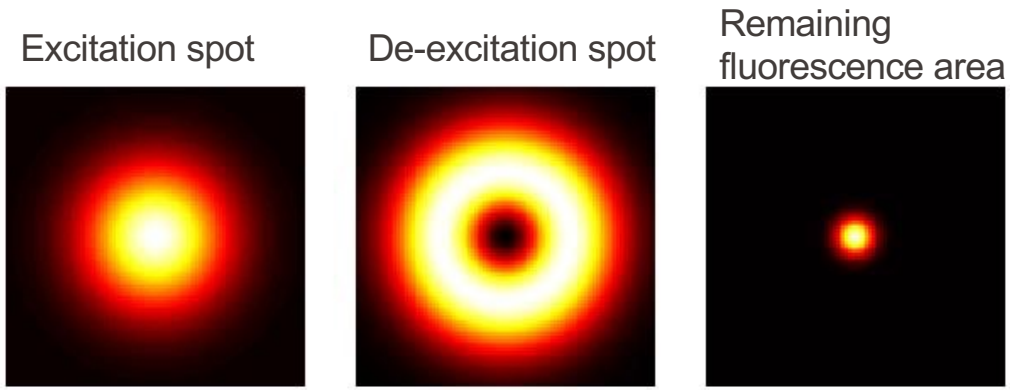
How to make fluorophores blink

- Fluorophores can be made to occupy a non-emitting “dark” state. From this state, a small number of molecules are returned to the emitting state at any given time, where thy can be excited and emit photons that can be detected.
- Stochastic Optical Reconstruction Microscopy (STORM) uses stochastic photosetting of organic dyes. For STORM one only needs one wavelength.
- Photoactivation Localization Microscopy (PALM) uses photoactivatable fluorescent proteins (PS-FPs) such as photoactivatable GFP. The emission intensity can be tuned using light of particular wavelengths. These PA-FPs are initially in a dark state. Using a flash of light of appropriate wave length, a small subset of PA-FPs is moved into the dark state where it can be excited with an excitation wavelength after which it emits at the emission wavelength.

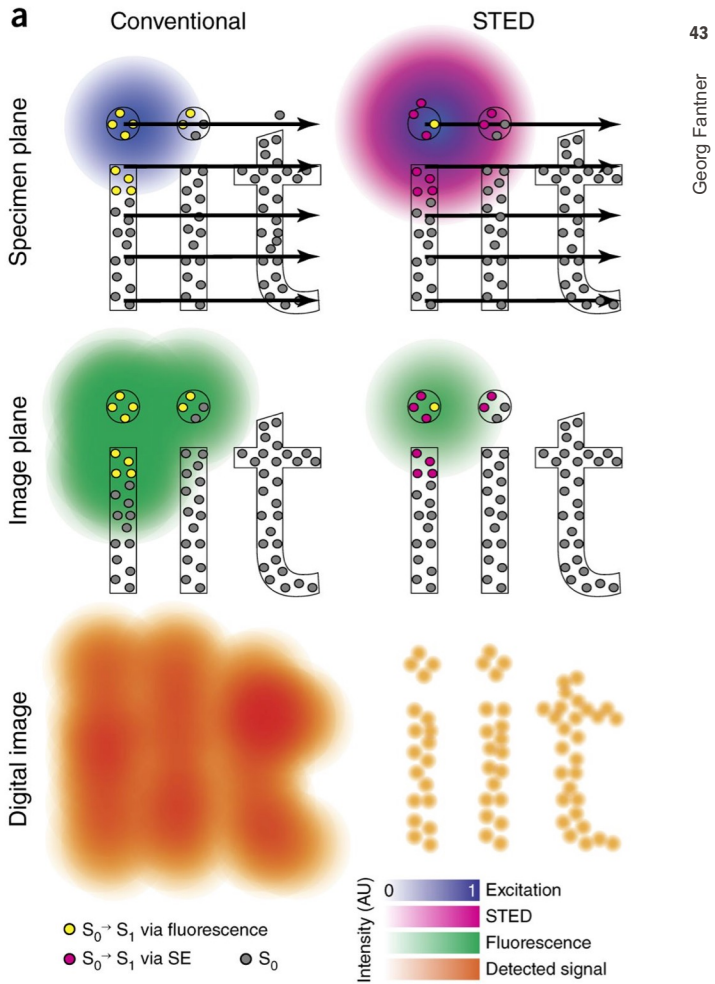

Conventional Fluorescence
2D STORM
3D STORM
Georg Fantner
3 µm

Stimmulated Depletion Emission microscopy (STED)

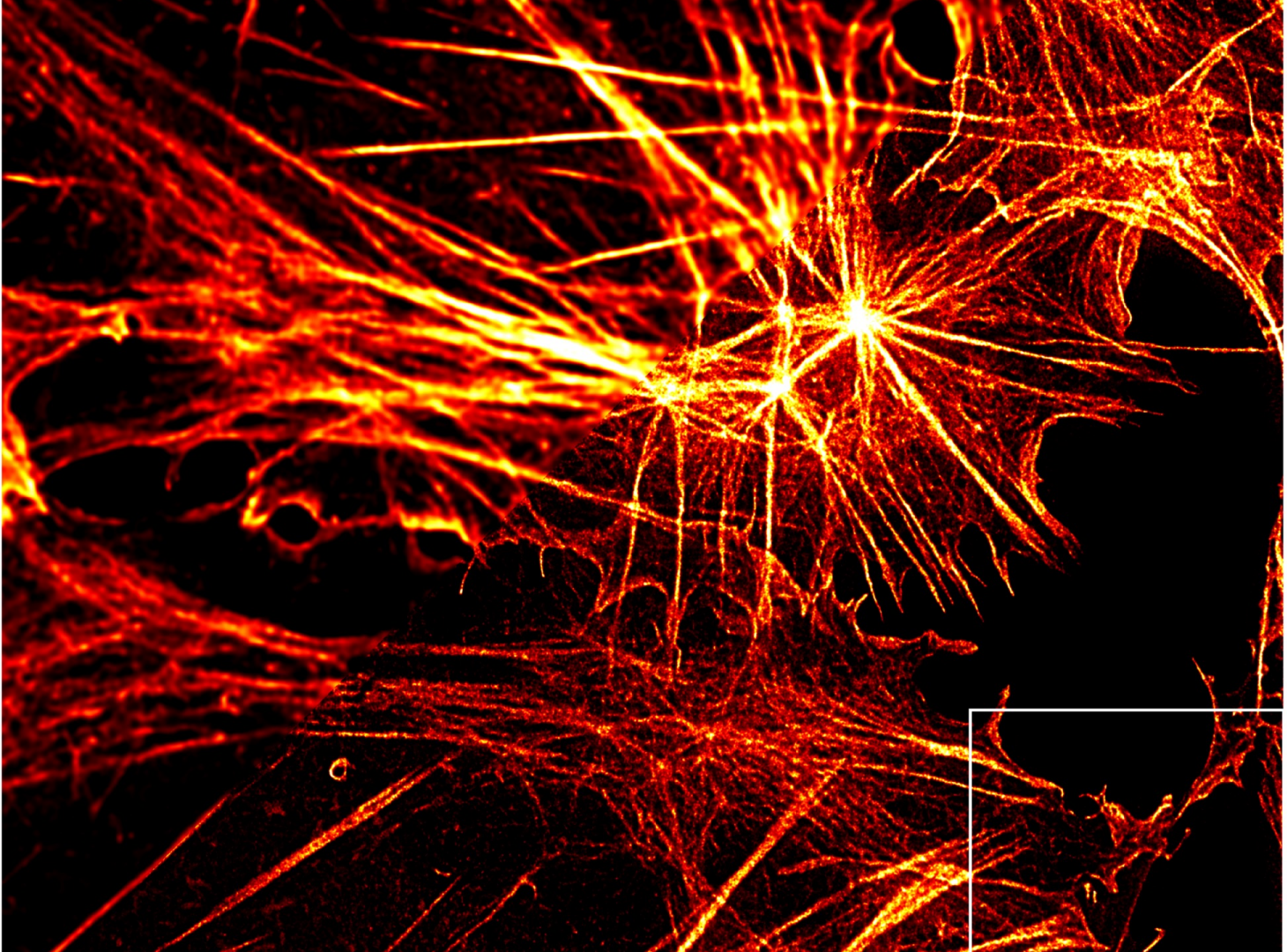
- Confocal microscopy: In scanning confocal, only one pixel at a time is illuminated. The beam is raster scanned over the sample and the image is reconstructed digitally. The resolution is limited by the size of the illumination spot.
- STED is a scanning confocal microscopy technique, where the size of the emitting area of the spot is reduced by depleting fluorescence in specific regions of the sample while leaving a centre focal spot active to emit fluorescence.

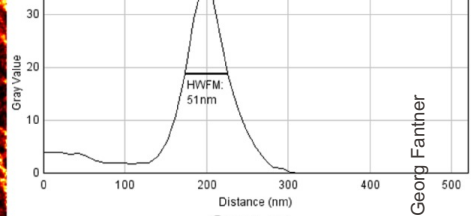


- When scanning the STED beam, only the fluorophores inside the excitation spot are excited.
- Those fluorophores that also are inside the STED doughnut are stimulated to emit. This emission occurs at a wavelength red-shifted from the normal fluorescence. These red shifted photons can be filtered out from the detected signal.
- Only the fluorophores that fall inside the excitation beam, but NOT in the STED doughnut are used for image reconstruction.

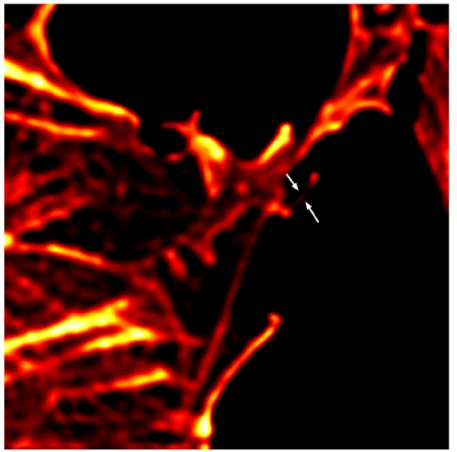


Vicidomini et al. Nature Methods volume 15, pages173–182(2018)

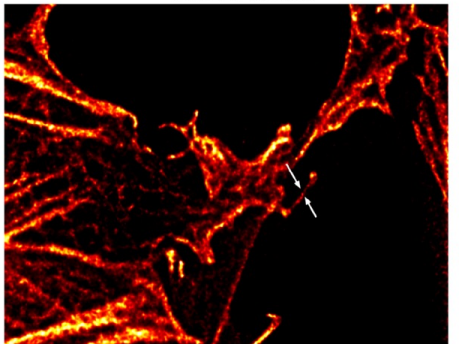




STED



Confocal



Summary

- Microscopes are imaging systems based on lenses, filters, apertures. Dichroic beam splitters and phase elements. Many different microscopy methods are possible depending how these elements are combined.
- In traditional imaging systems, the maximum obtainable resolution is determined by the diffraction limit. It depends on the wavelength, the numerical aperture, and the medium that the object is imaged in.
- Super resolution microscopy techniques are advanced microscopy techniques that overcome the diffraction barrier. The microscopes however do no longer create a “real image”. The image is reconstructed in a computer.

Useful resources

- MicroscopyU: https://www.microscopyu.com
- iBiologyEducation: https://www.youtube.com/user/iBioEducation/playlists?view=1&shelf_id=2&sort=dd
- **“An introduction to optical super-resolution microscopy for the adventurous biologist”** J Vangindertael *et al* 2018 *Methods Appl. Fluoresc.* **6** 022003, https://doi.org/10.1088/2050-6120/aaae0c