Imperial College London

Facility for Imaging by Light Microscopy

FILM

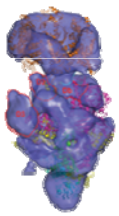
Observing Life As It Happens

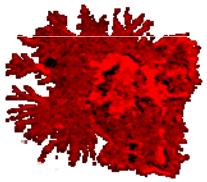
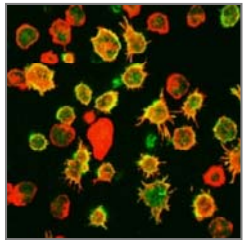
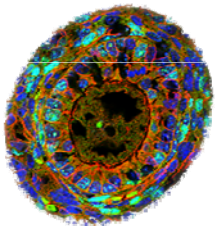
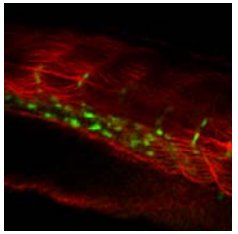
MICROSCOPY DAY 2011:

Basics of Microscopy

Martin Spitaler

- Properties of light:
  - Magnification and defraction limit
  - Contrasting techniques in transmitted light microscopy
- Fluorescence microscopy techniques:
  - Epifluorescence technique
  - Widefield microscopes
  - Confocal microscopes
- Light as a tool:
  - photo-bleaching, activation and switching
  - phototoxins
  - laser tweezers
- Light detectors:
  - noise
  - resolution and sampling rate



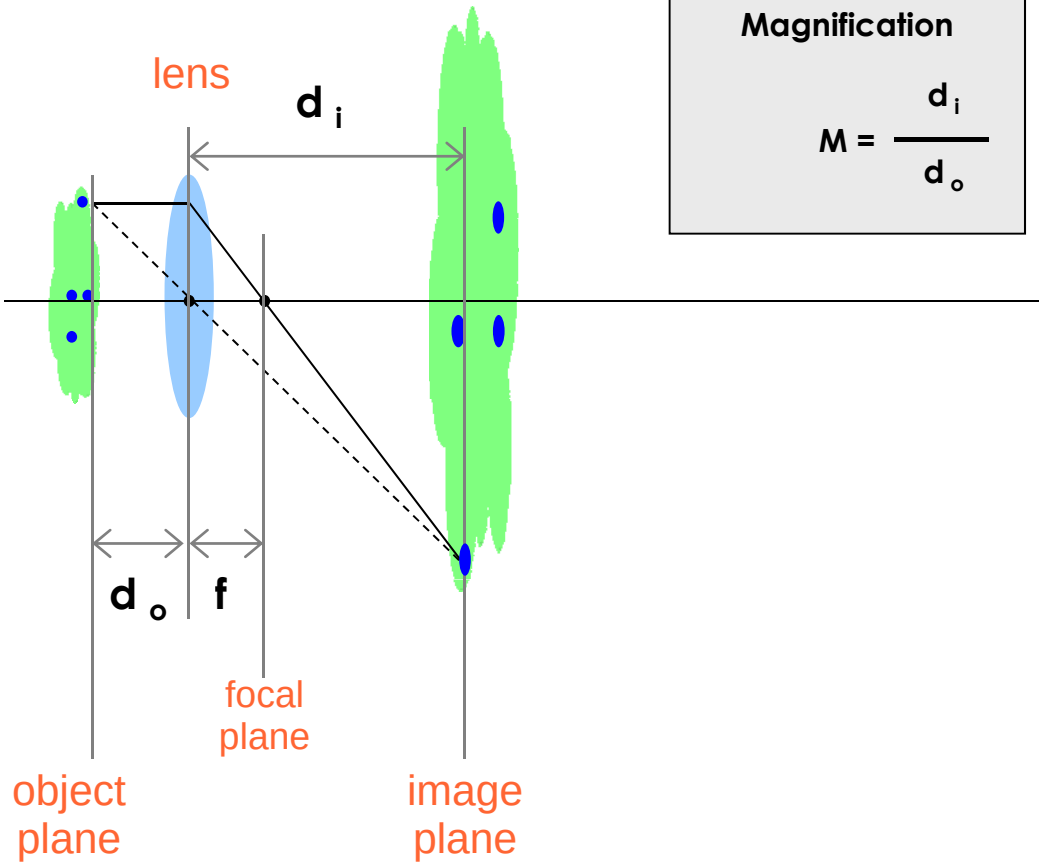






Microscopy tools in FILM

- **Conventional (widefield) microscopes** (WF1, WF2)
  - low-light live imaging
  - automated multi-position XYZT acquisition
  - ratiometric imaging
  - high-speed acquisition
- **Confocal microscopes** (CF1, CF2, CF5)
  - fixed and live 3D imaging
  - high-speed imaging
  - photobleaching, photoswitching, laser tweezers
  - spectral imaging
  - automated multi-position XYZT acquisition
- **Multiphoton / Flim microscopes** (CF3, CF4)
  - in vivo imaging
  - fluorescence lifetime, FRET
- **Superresolution microscope** (WF3)
  - TIRF
  - PALM
  - STORM

Transmitted light microscopy: basic design

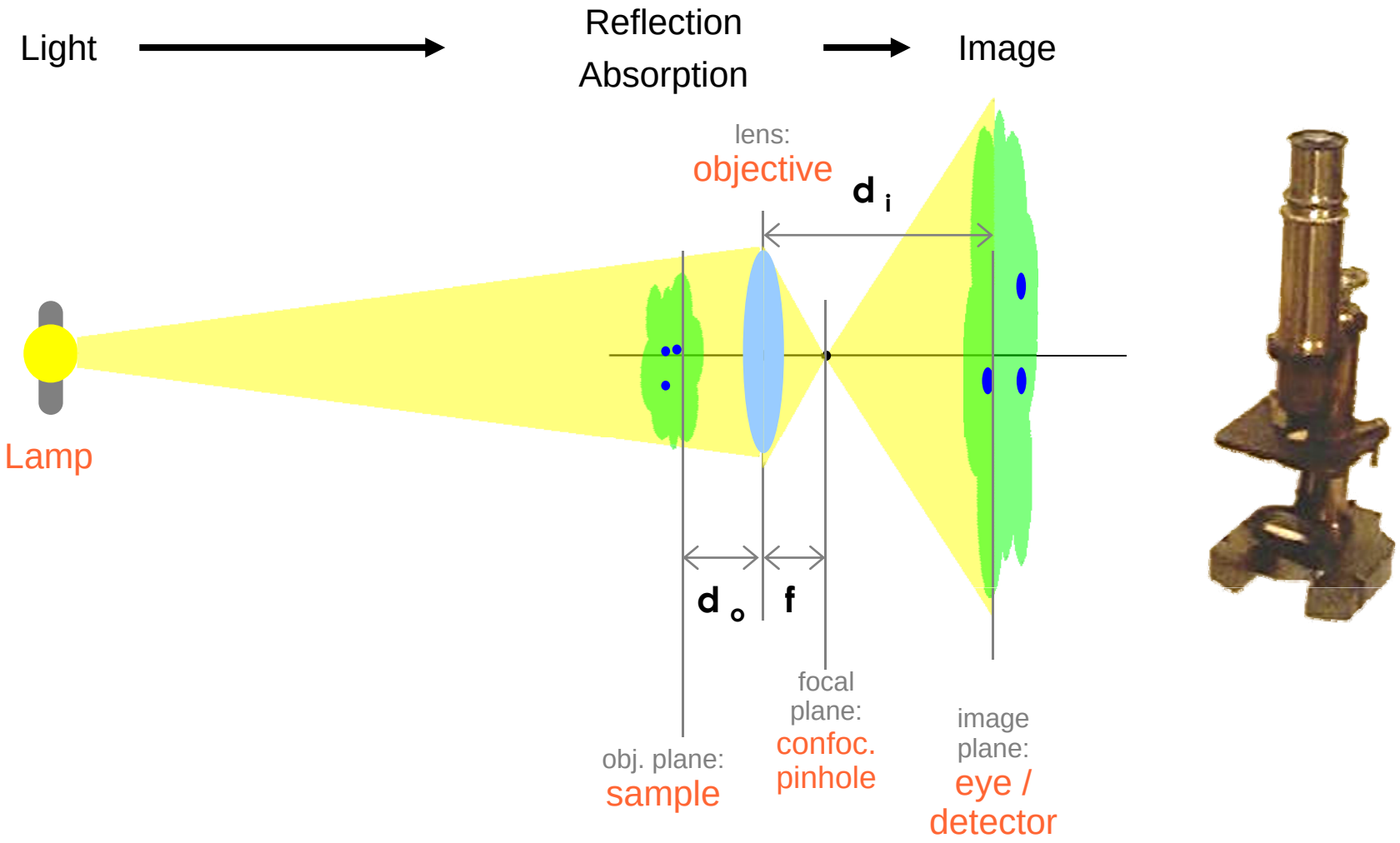
Louis Pasteur's microscope
(ca. 1850)



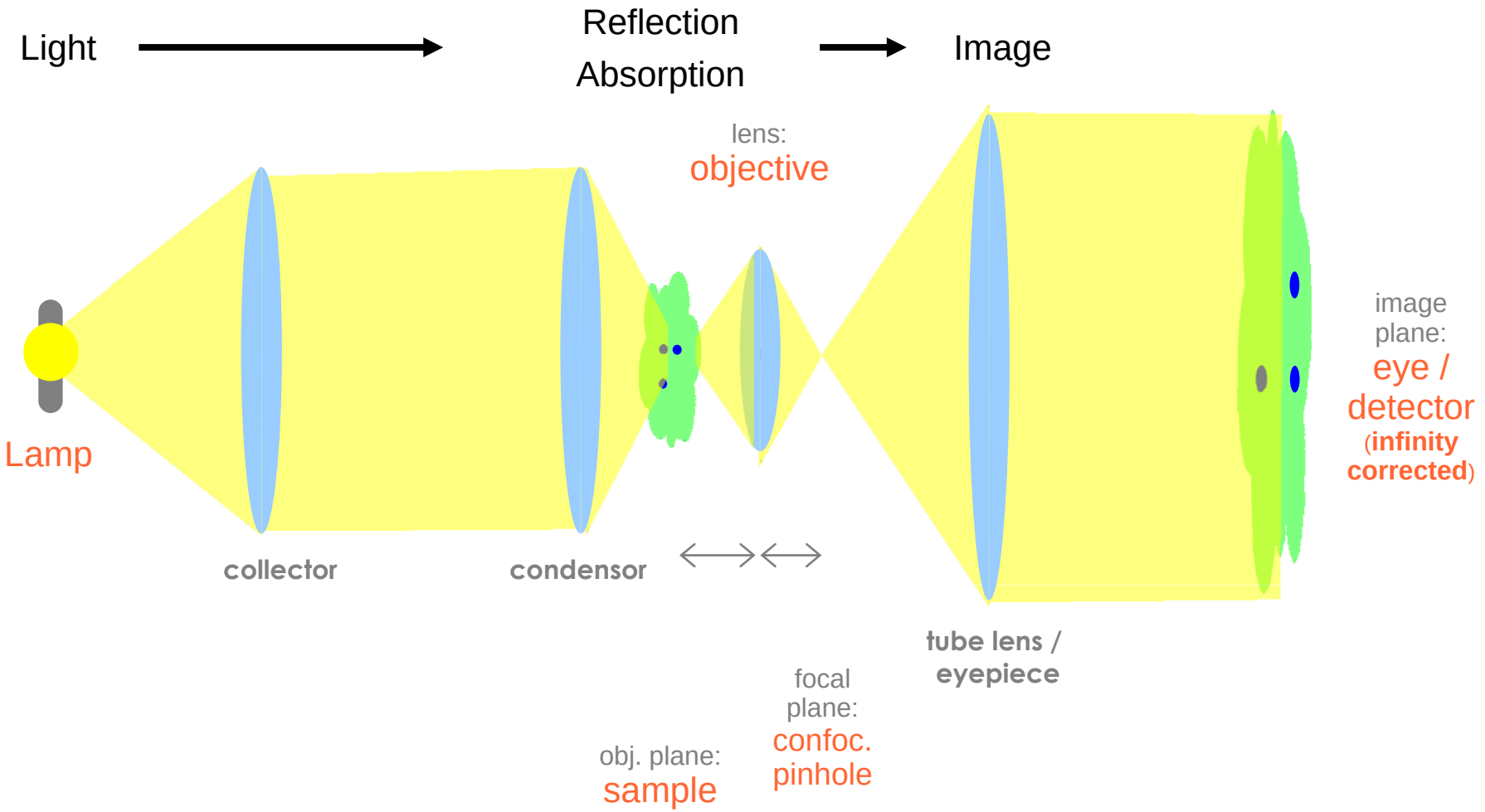
Magnification

$$M = \frac{d_i}{d_o}$$

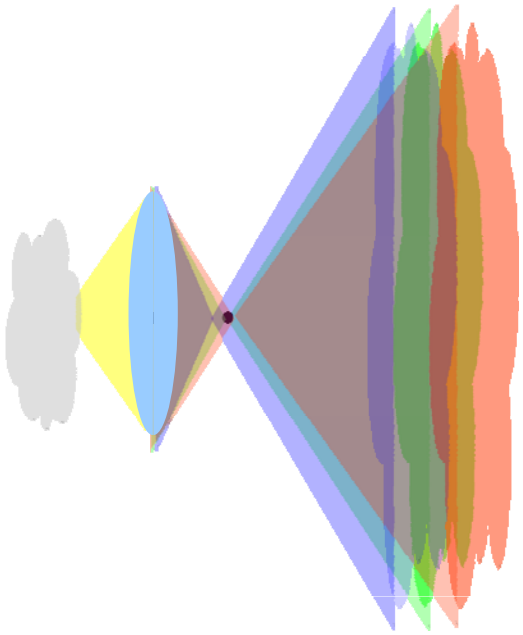
Transmitted light microscopy: basic design



Transmitted light microscopy: basic design

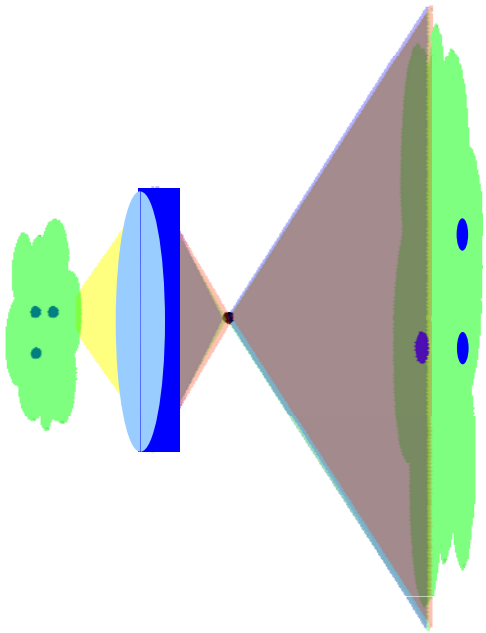


Transmitted light microscopy : basic design



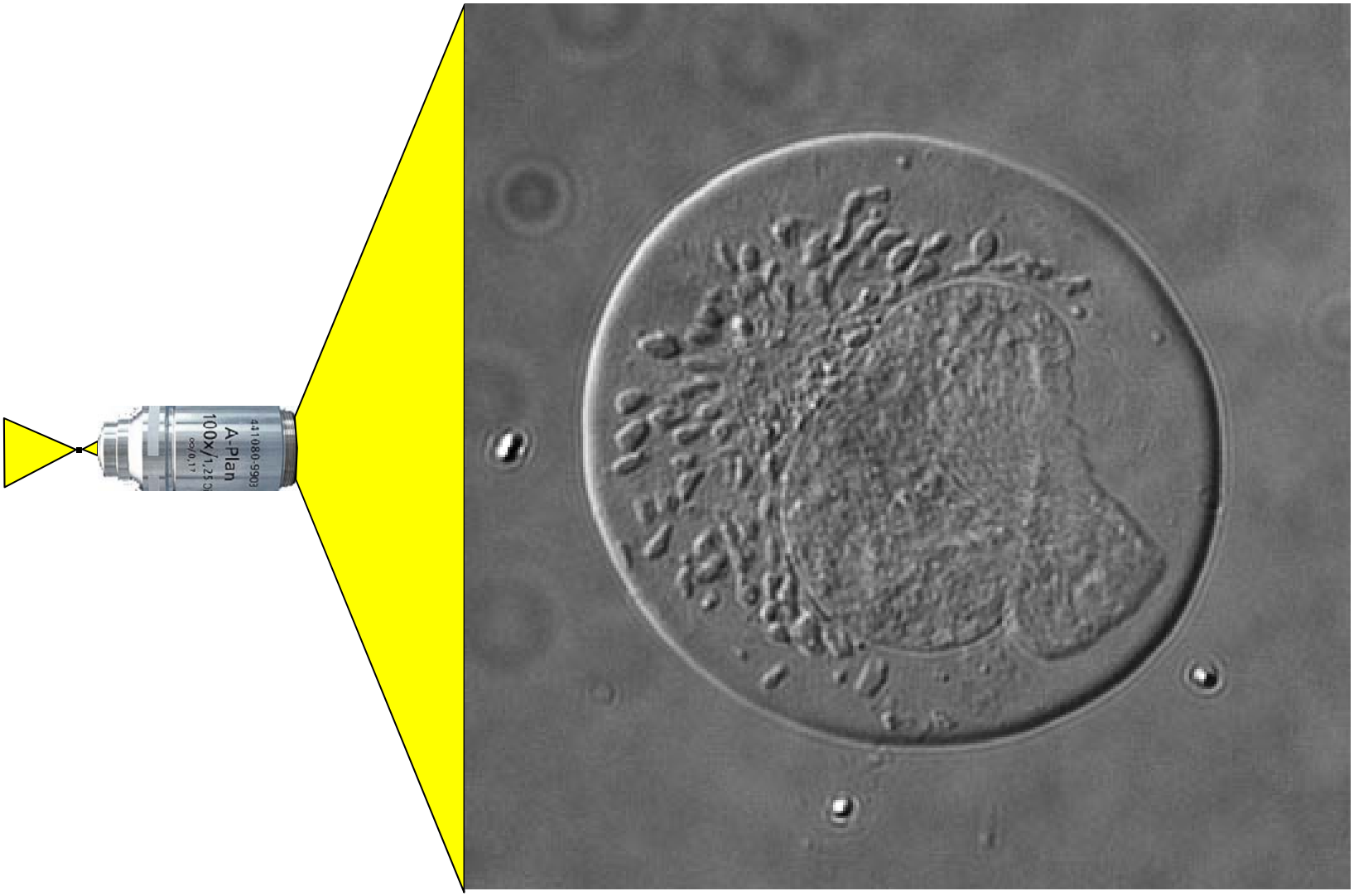
chromatic aberration

Transmitted light microscopy : basic design



chromatic correction
(Achromat, Apochromat, ...)

Transmitted light microscopy



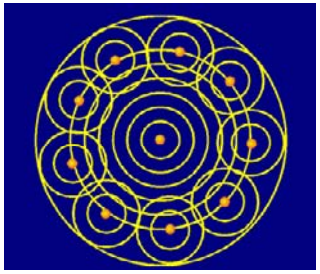
Transmitted light microscopy

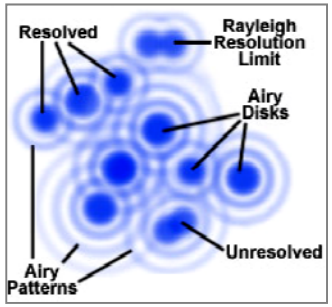
Zebrafish (*Brachydanio rero*)

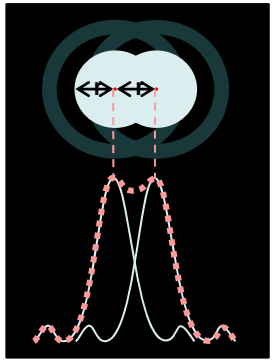
long time courses (4 days)

Mariya Moosaje: **Zebra fish embryo development**

Transmitted light microscopy: diffraction limit







$$d_{xy} = 0.61 * \frac{\lambda}{n*\sin(\mu)}$$

Abbe's Law

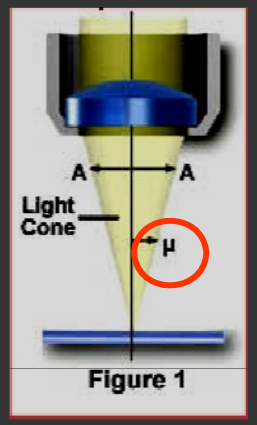
λ = wavelength

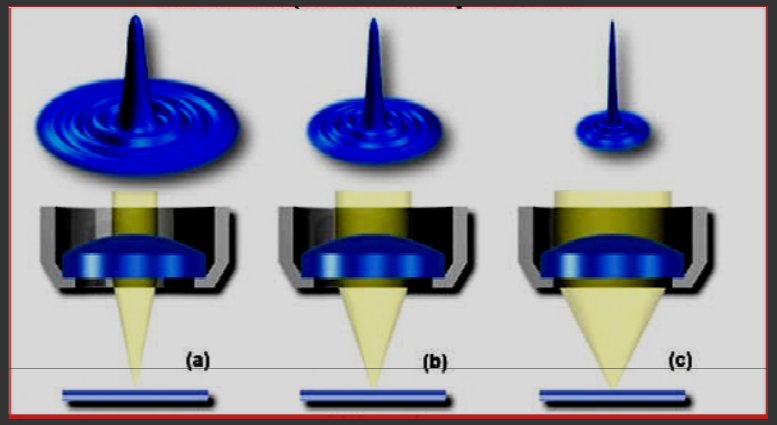
n*sin(μ) = numerical aperture (NA)

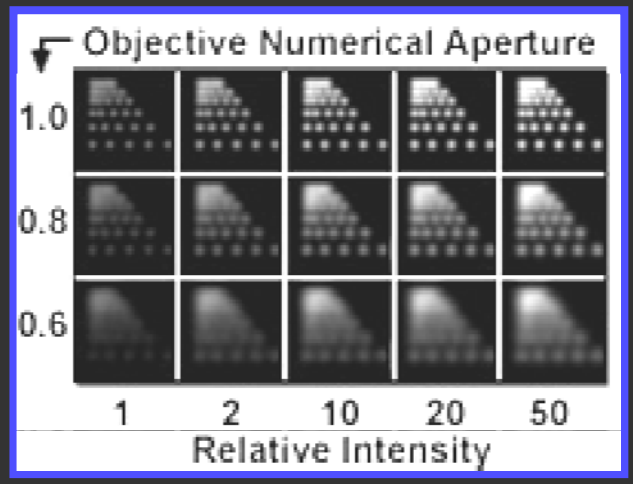
Transmitted light microscopy: diffraction limit

$$d_{xy} = 0.61 * \frac{\lambda}{n * \sin(\mu)}$$

Abbe's Law





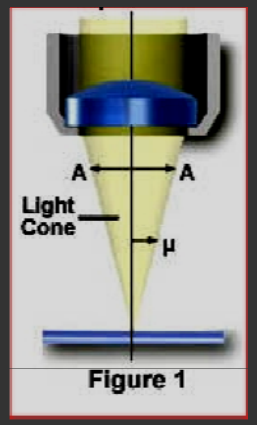


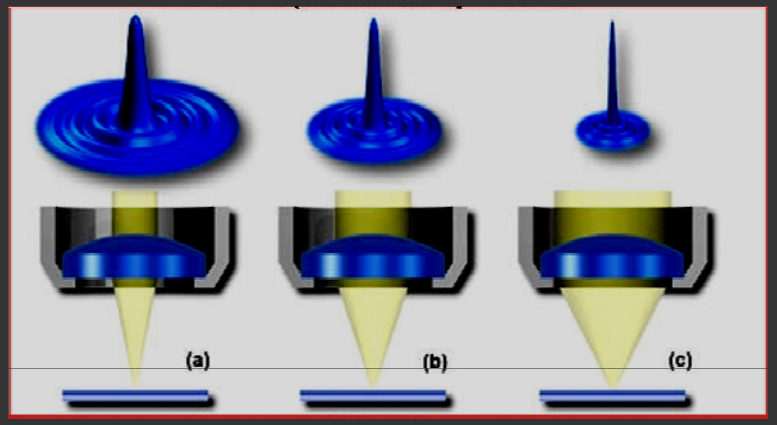
Transmitted light microscopy: diffraction limit

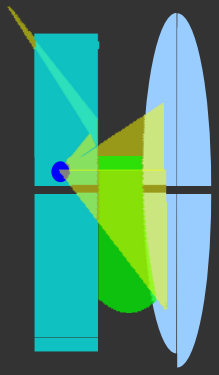
Refractive Index n = speed of light in vacuum / speed of light in medium

$$d_{xy} = 0.61 * \frac{\lambda}{n * \sin(\mu)}$$

Abbe's Law



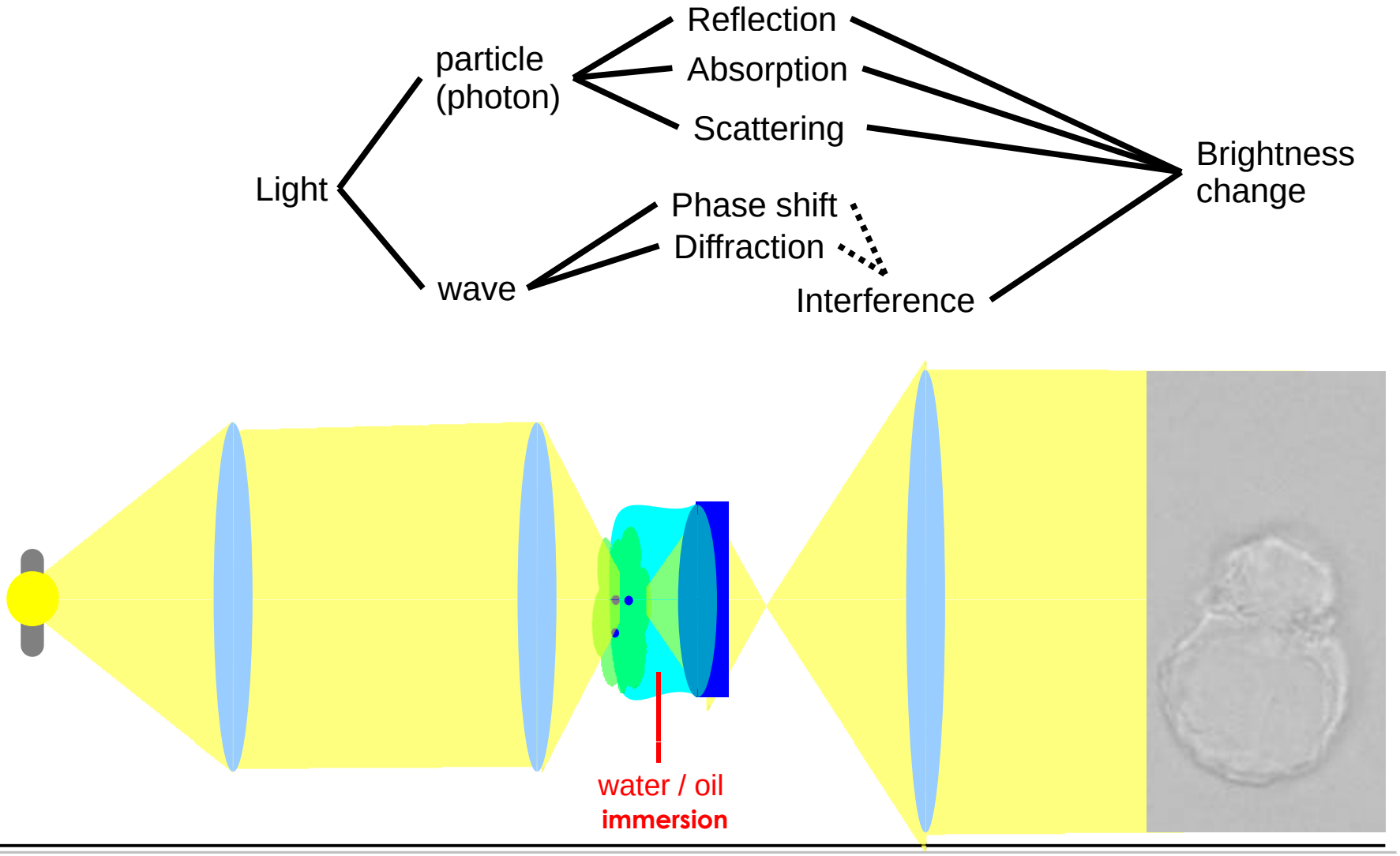




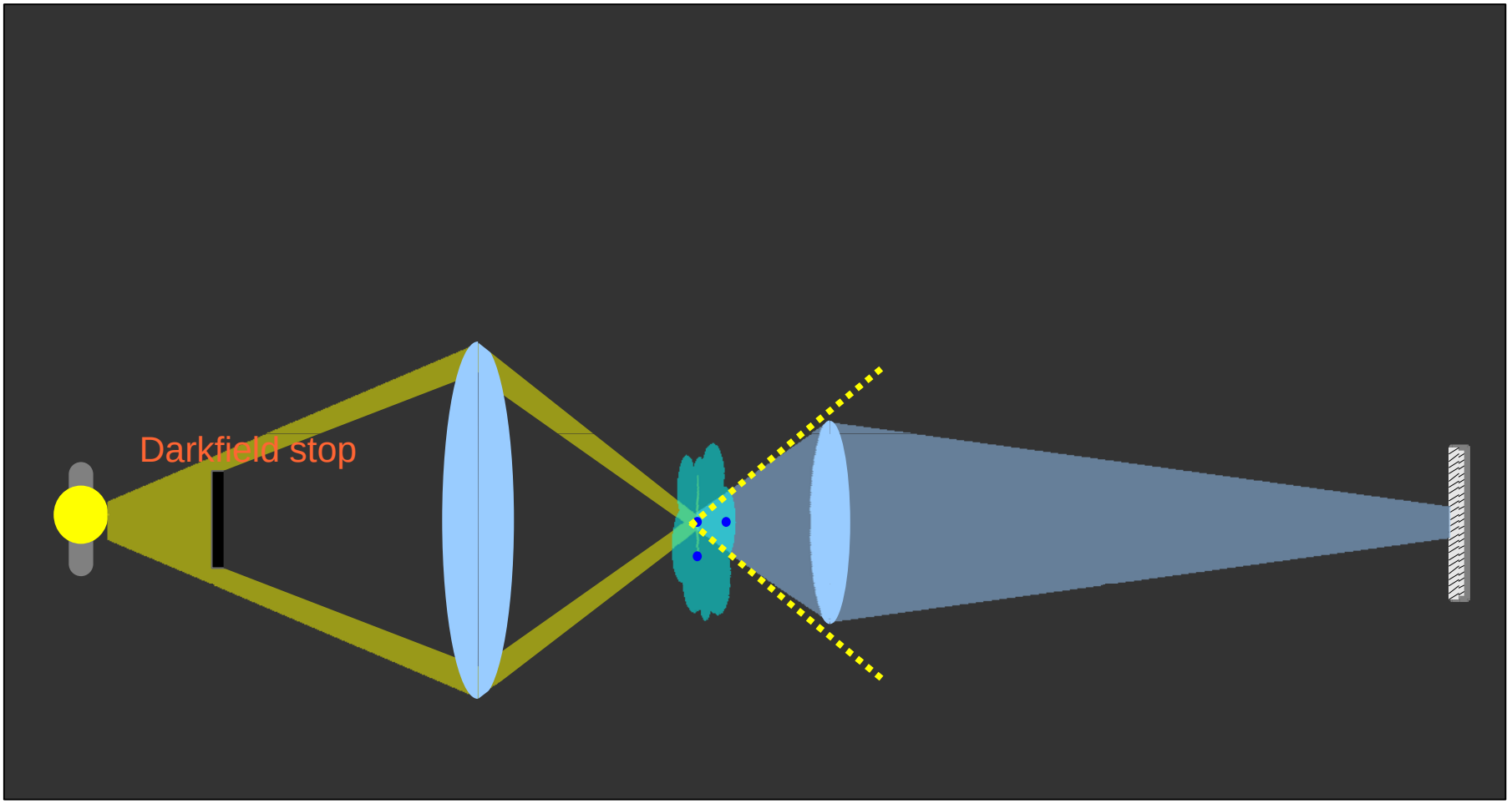
Immersion:

Air	(n=1)
Water	(n=1.33)
Glycerin	(n=1.47)
Oil	(n=1.51)

Transmitted light microscopy: increasing contrast

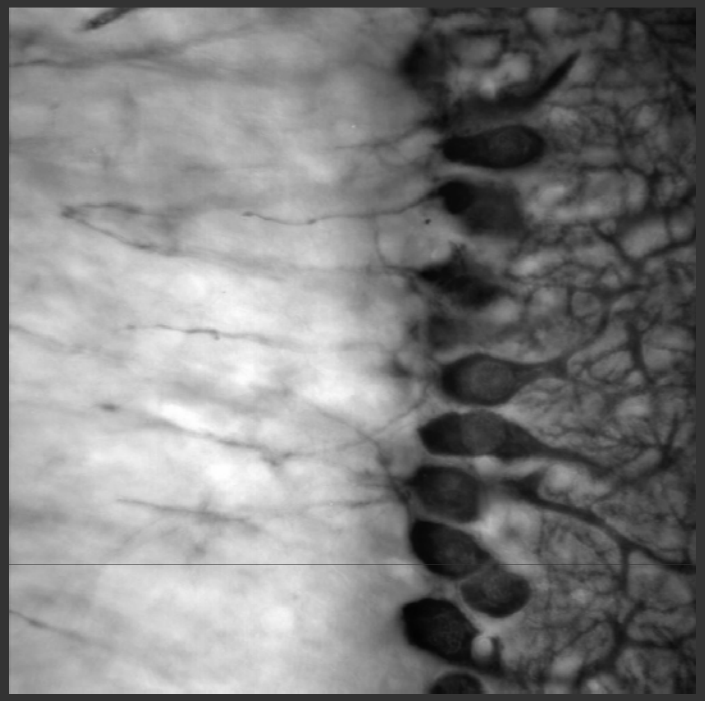


Darkfield illumination

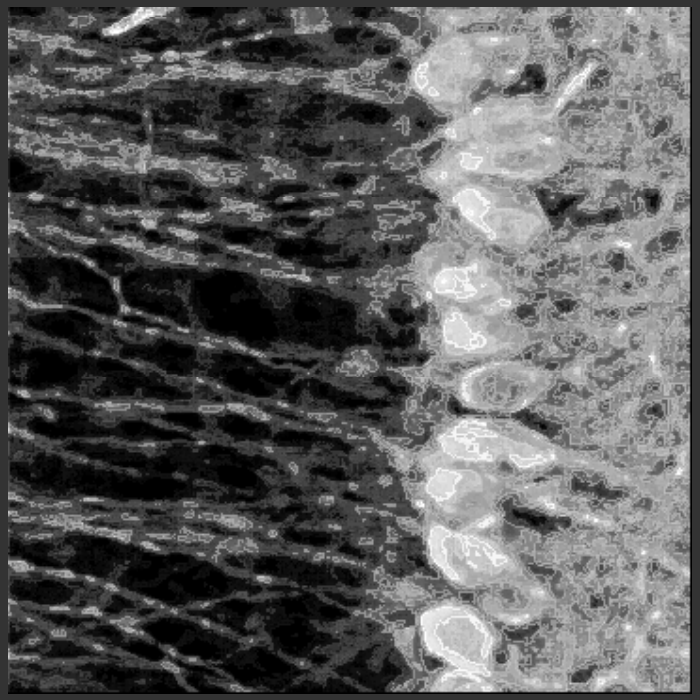


Transmitted light microscopy: increasing contrast

Darkfield illumination



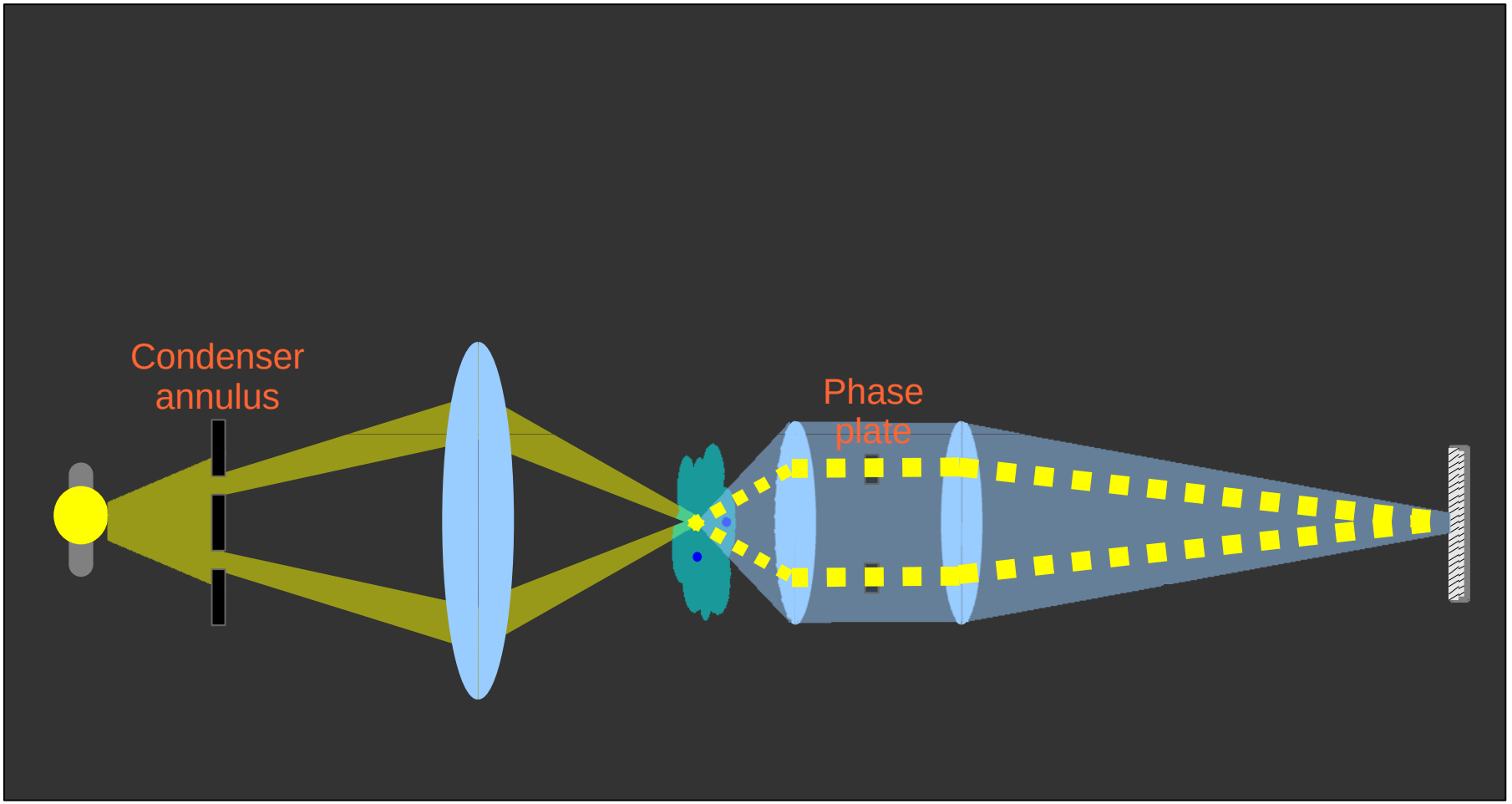
Brightfield



Darkfield

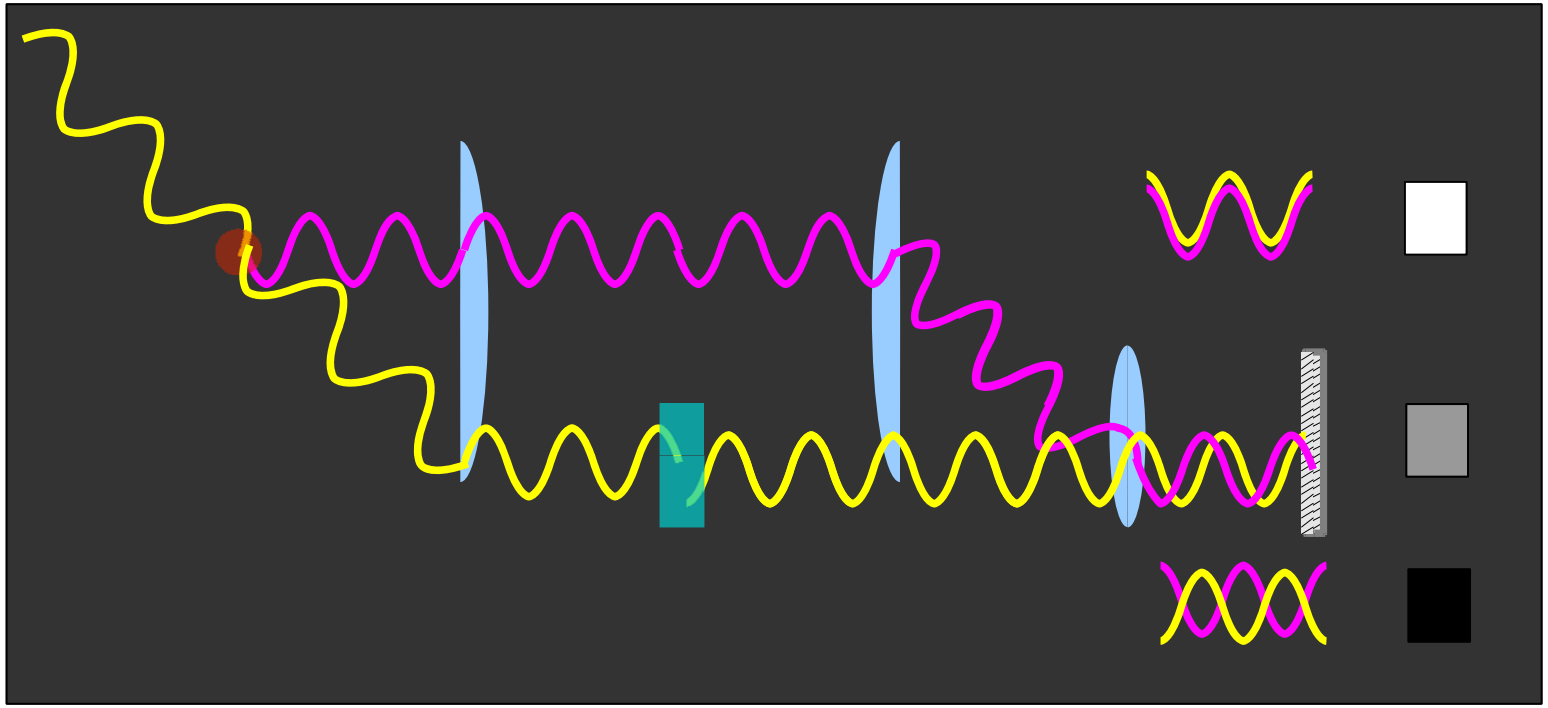
Transmitted light microscopy: increasing contrast

Phase contrast



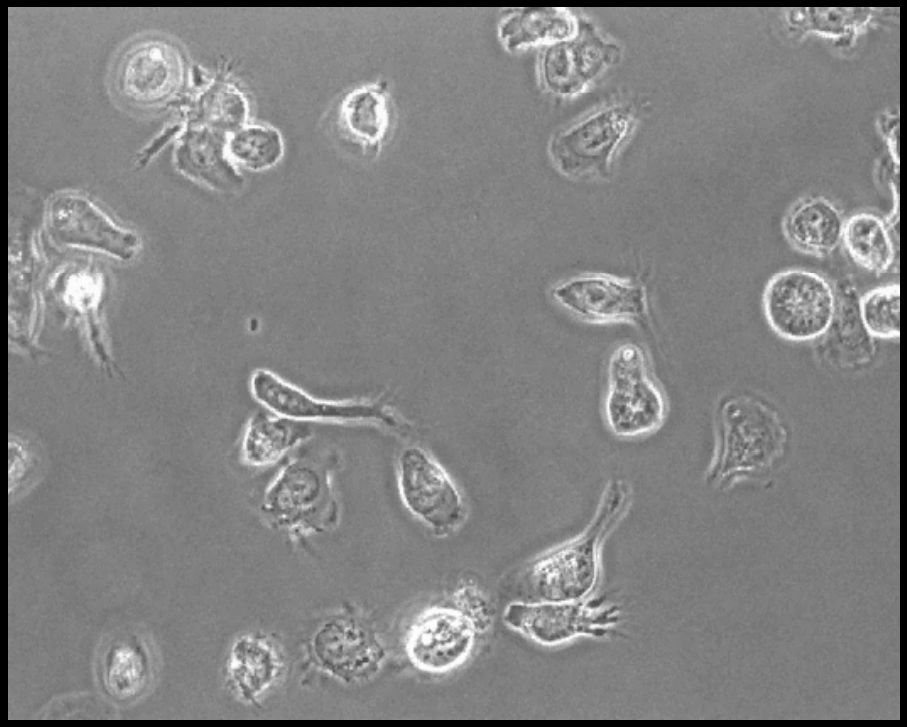
Transmitted light microscopy: increasing contrast

Phase contrast



Transmitted light microscopy: Increasing contrast

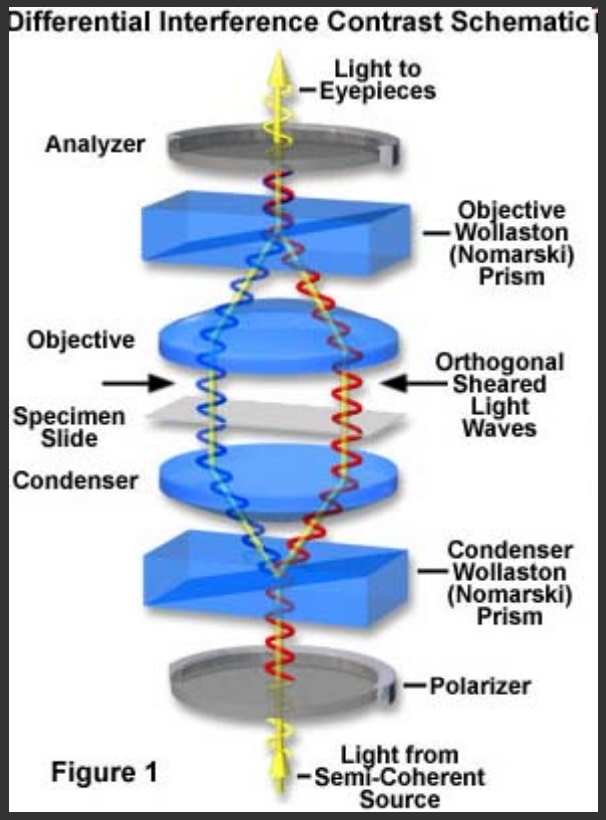
Phase contrast

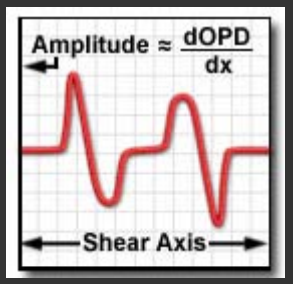


Georgina Cornish: **Migrating T cells**

Transmitted light microscopy: increasing contrast

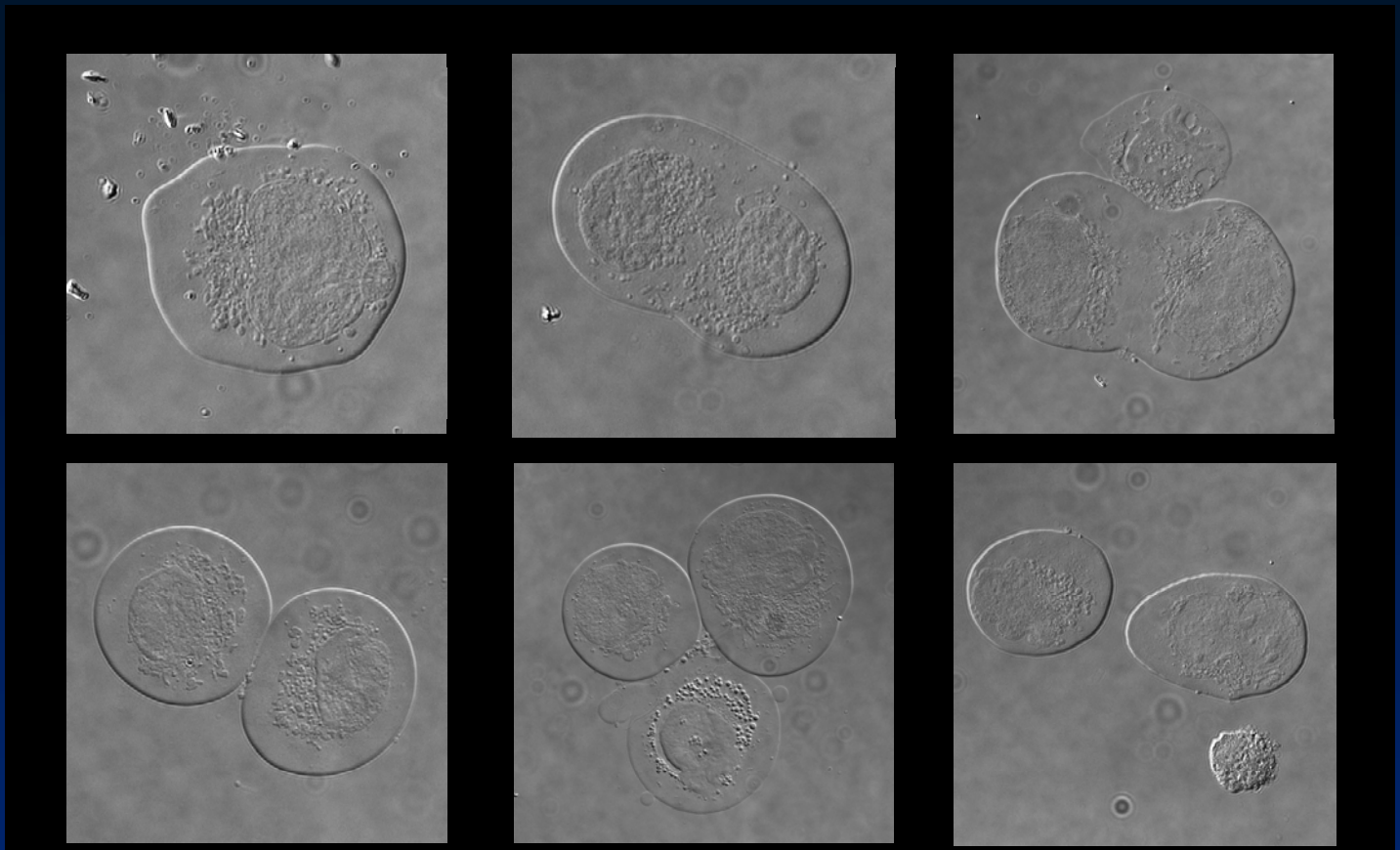
Differential Interference Contrast (DIC)





from 'microscopy primer' (http://micro.magnet.fsu.edu)

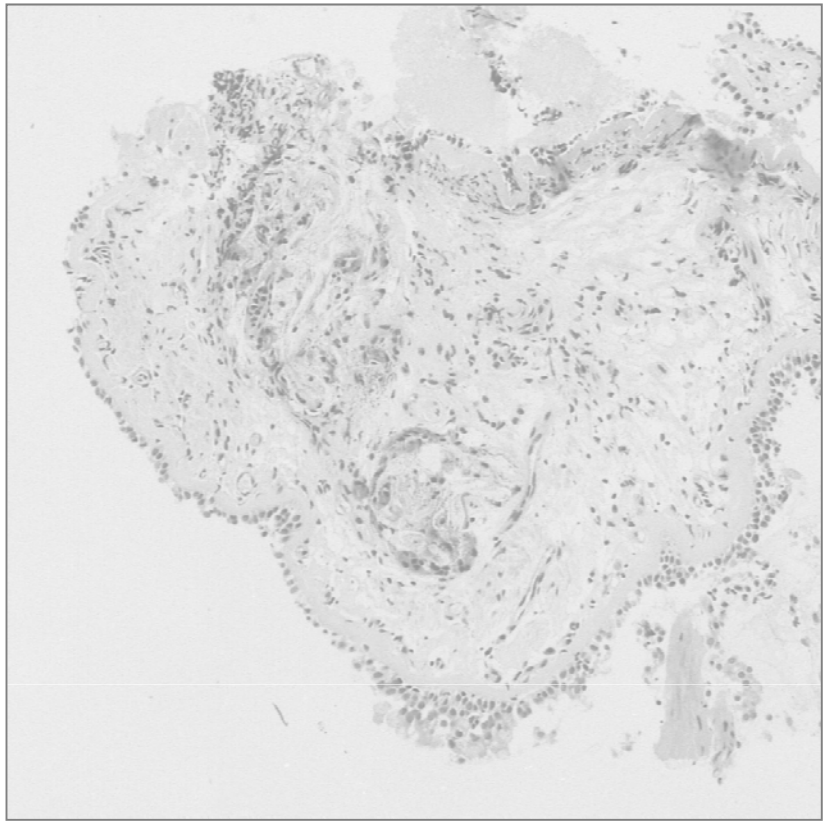
Transmitted light microscopy : Increasing contrast

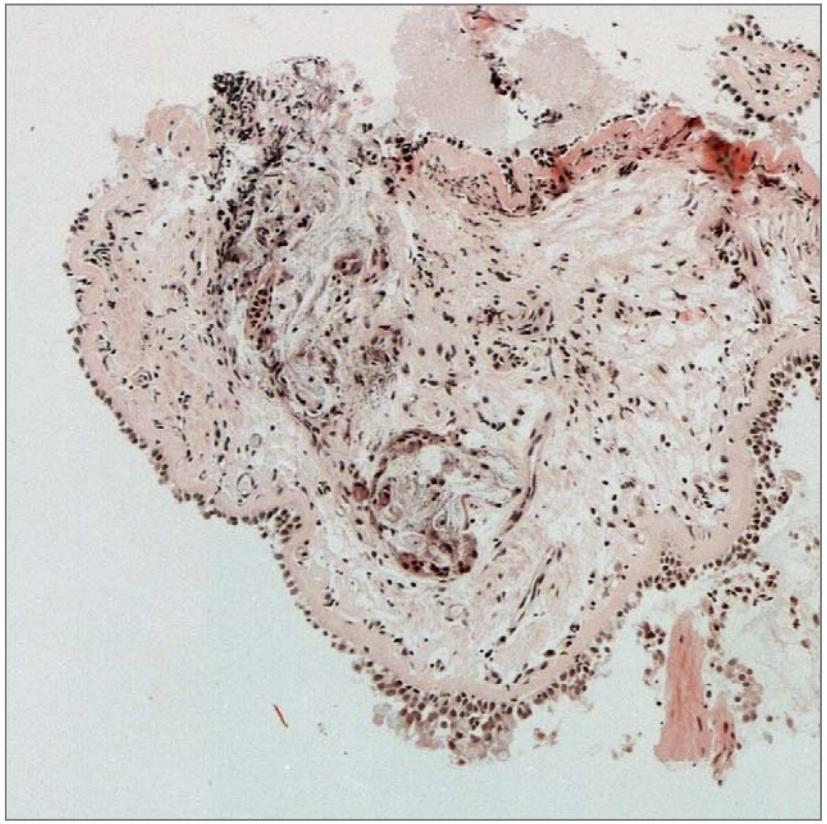


Cell growth, division and death

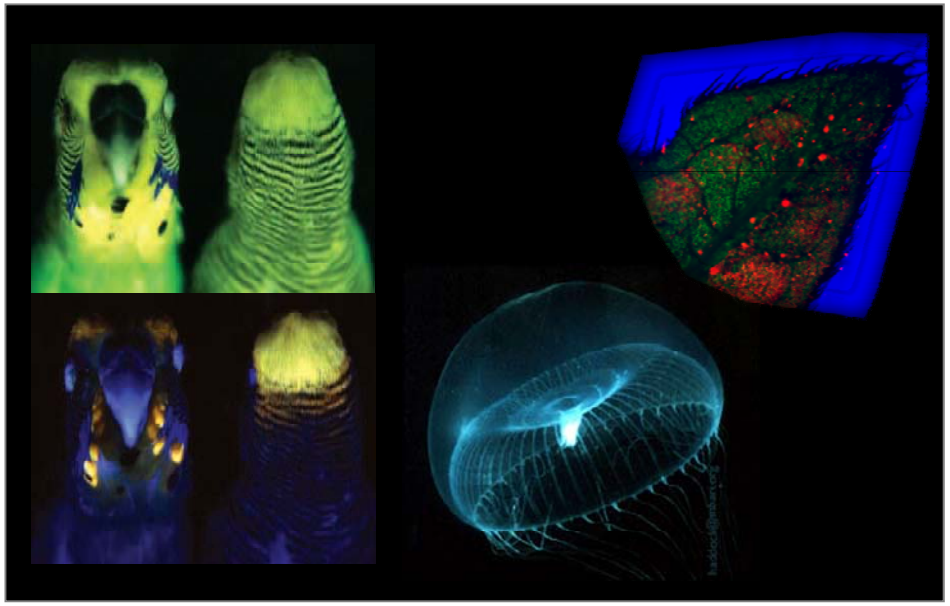
Transmitted light microscopy: Increasing contrast

Sample staining

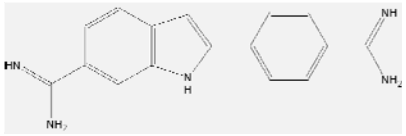




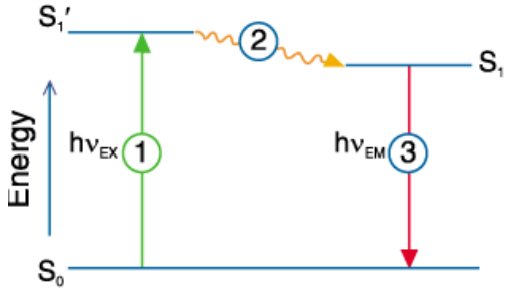
Fluorescence microscopy

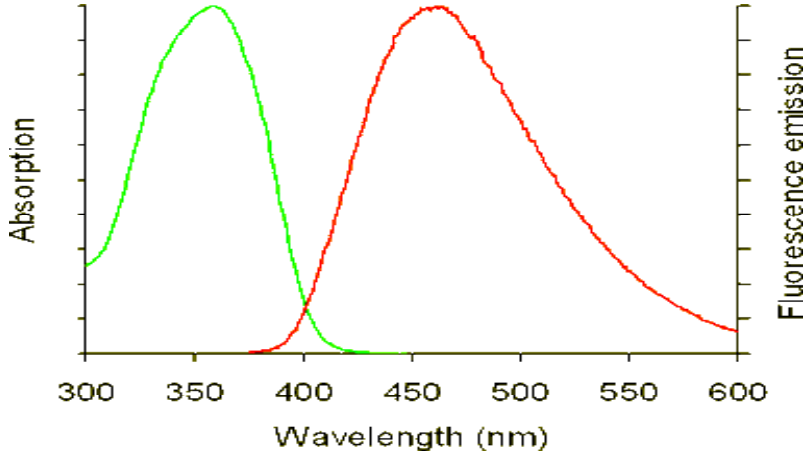


Fluorescence microscopy: Basics

exciting light →  → emitted light

fluorophor

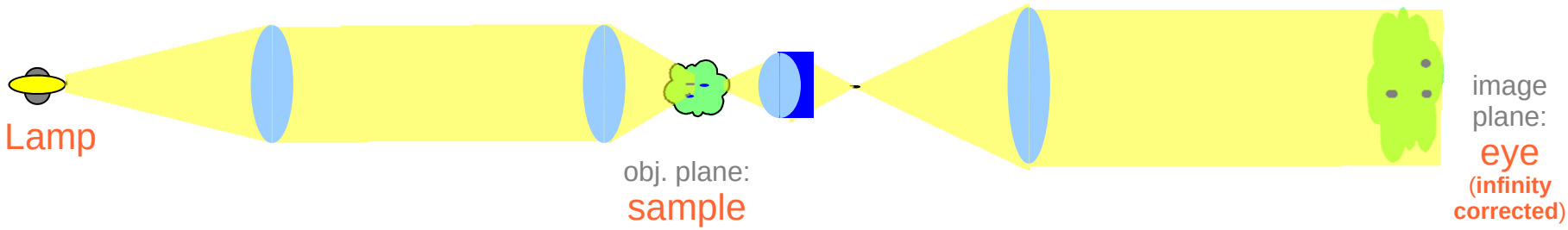




Jablonsky diagram

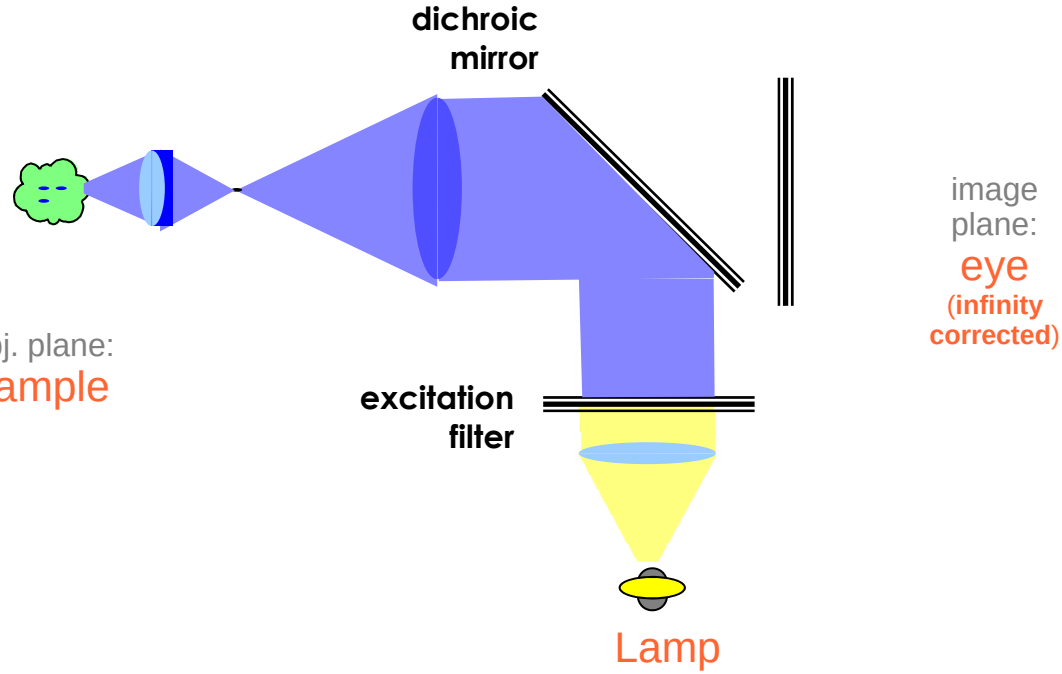
Fluorescence microscopy: Epifluorescence

Transmitted light path



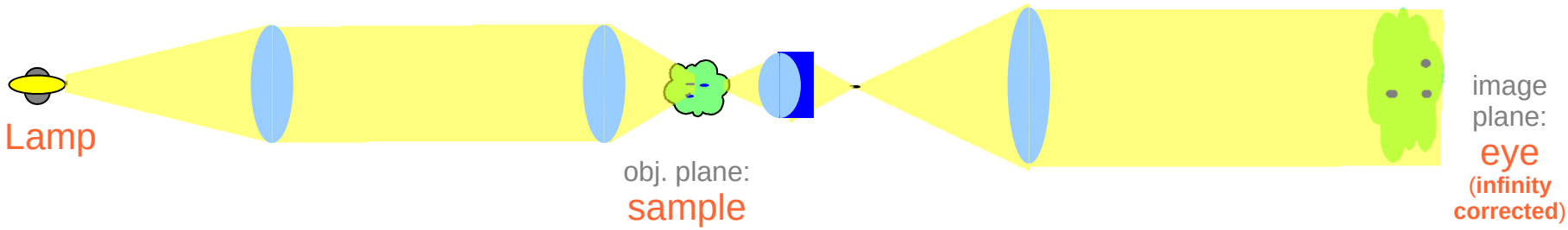
Epifluorescence light path

(Invented 1965 by Johan Ploem)



Fluorescence microscopy: Epifluorescence

Transmitted light path



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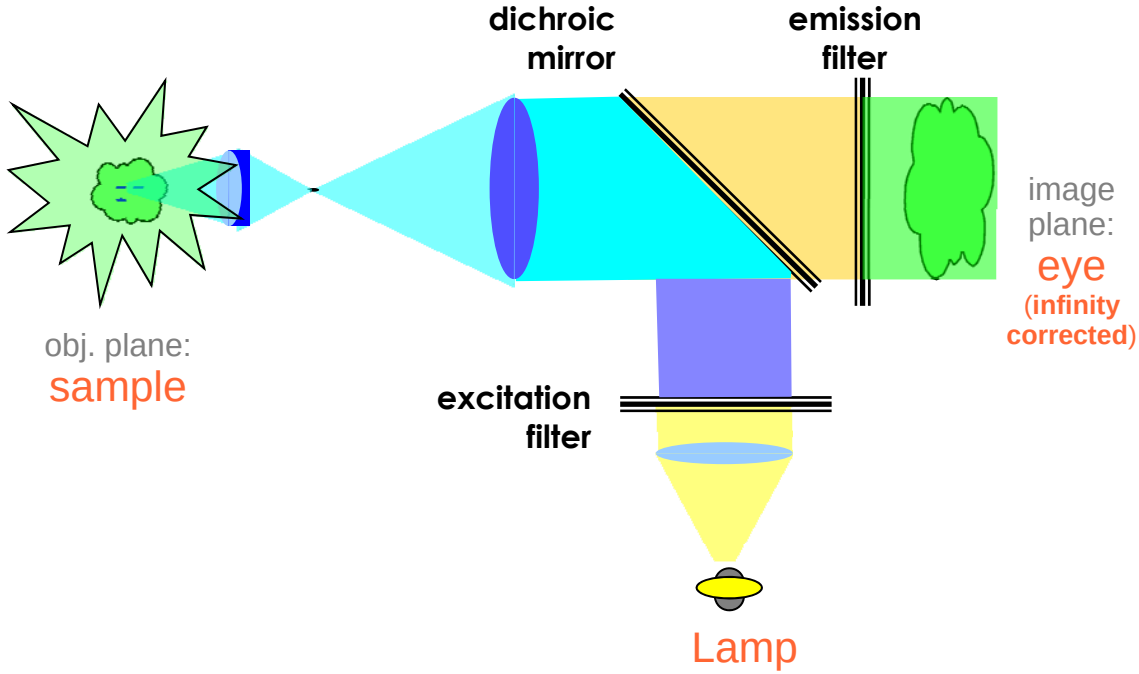
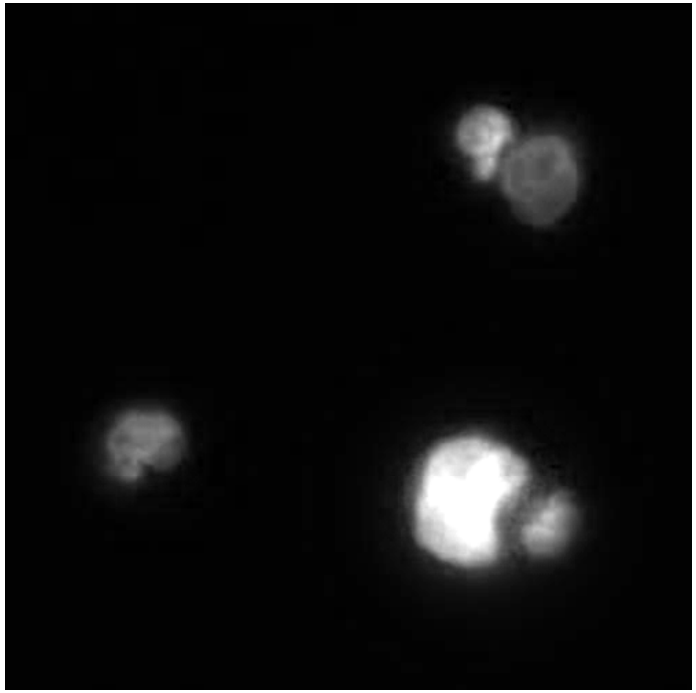
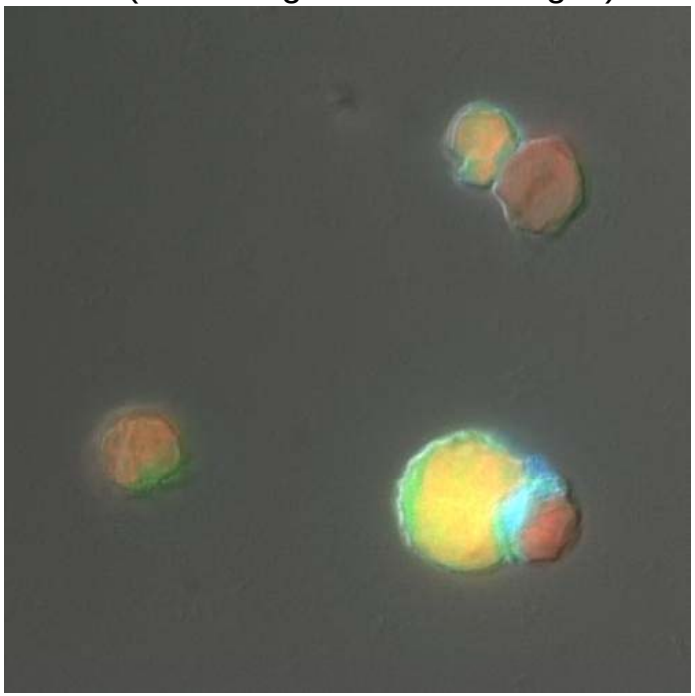


Fluorescence microscopy: Epifluorescence

single channel

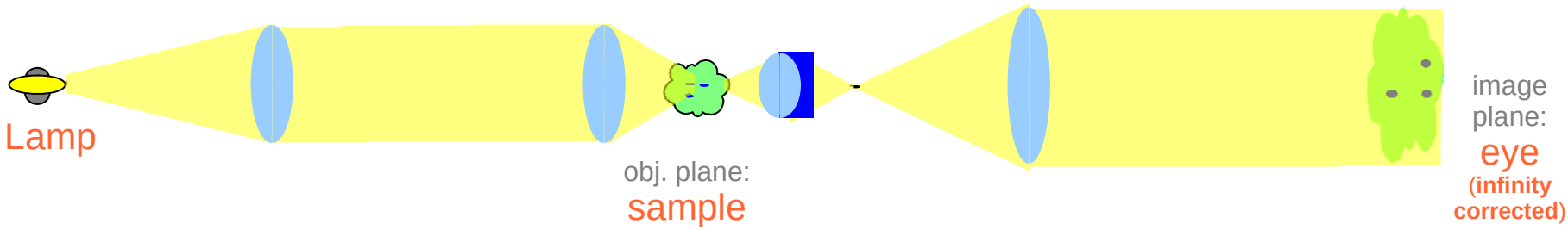


channel overrlay
(including transmitted light)



Fluorescence microscopy: Epifluorescence

Transmitted light path



Epifluorescence light path

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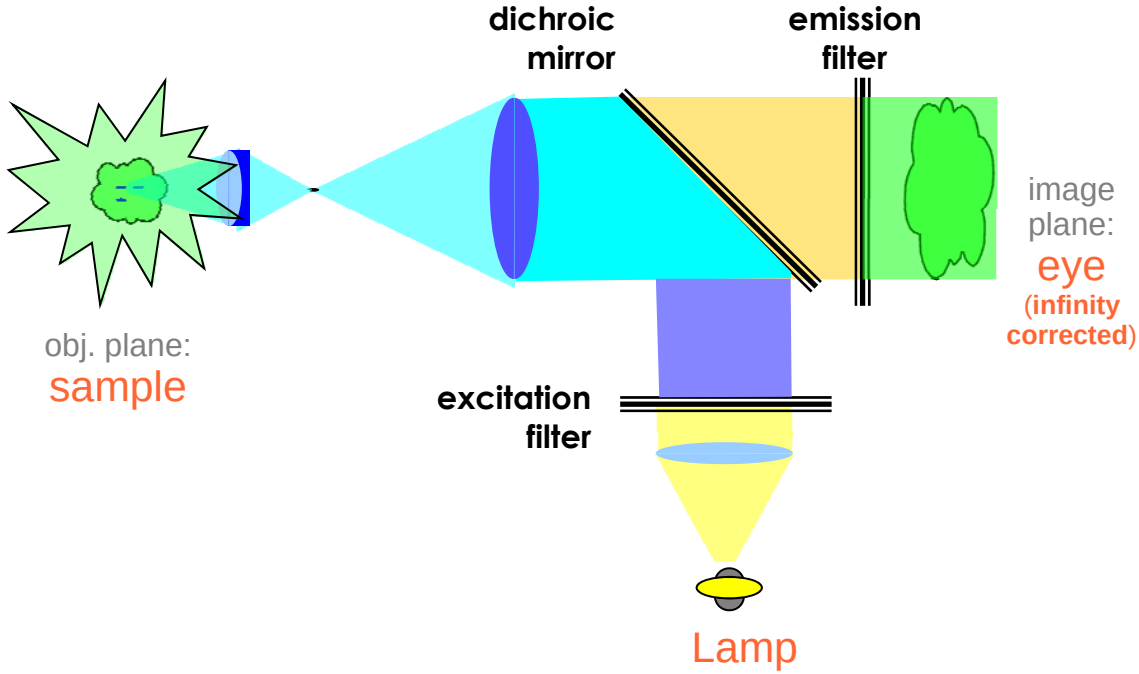
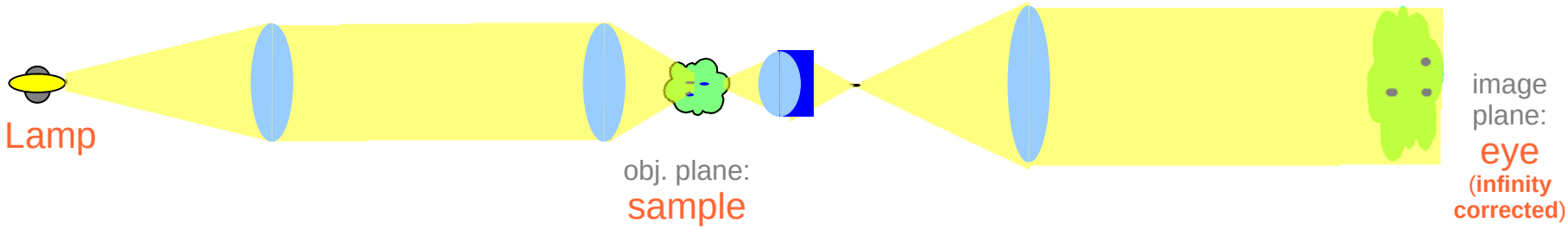


Fluorescence microscopy: Epifluorescence

Transmitted light path



Epifluorescence light path

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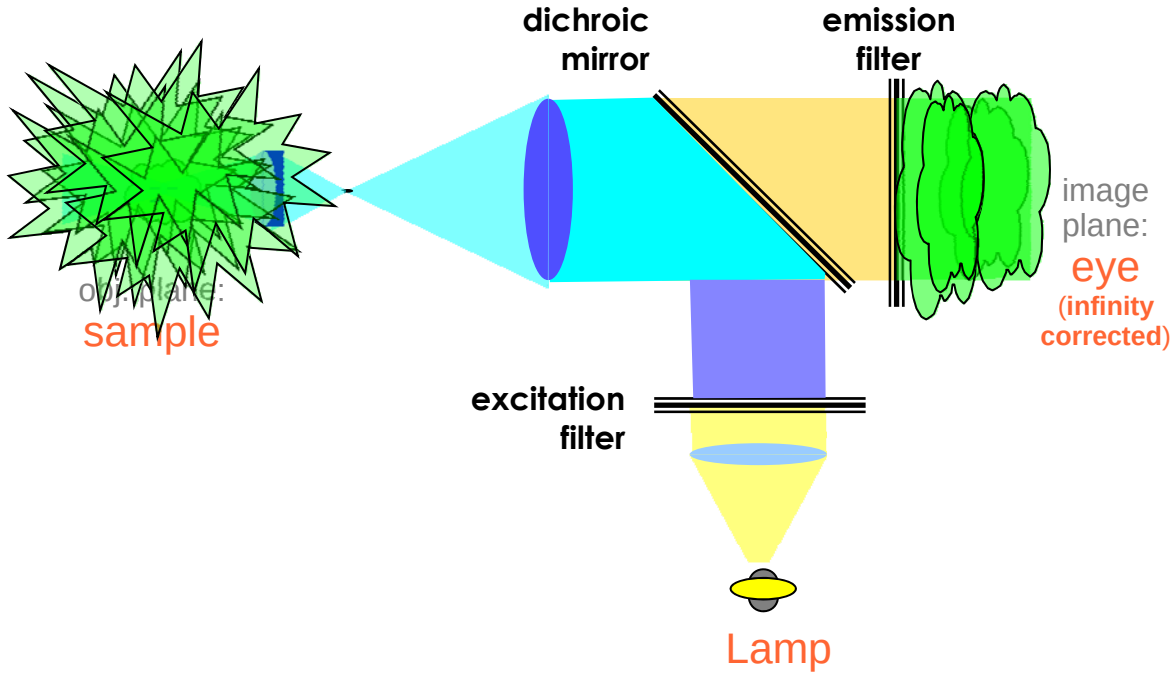
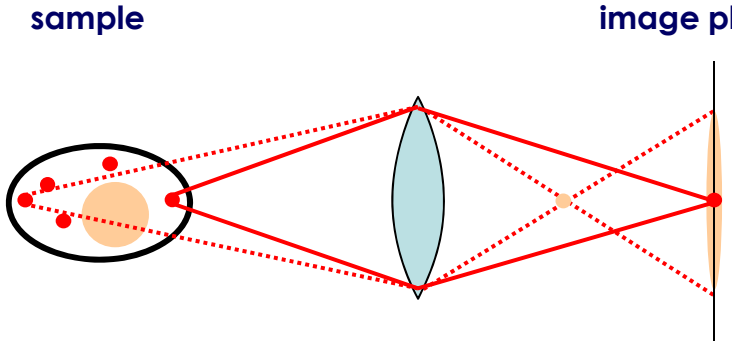
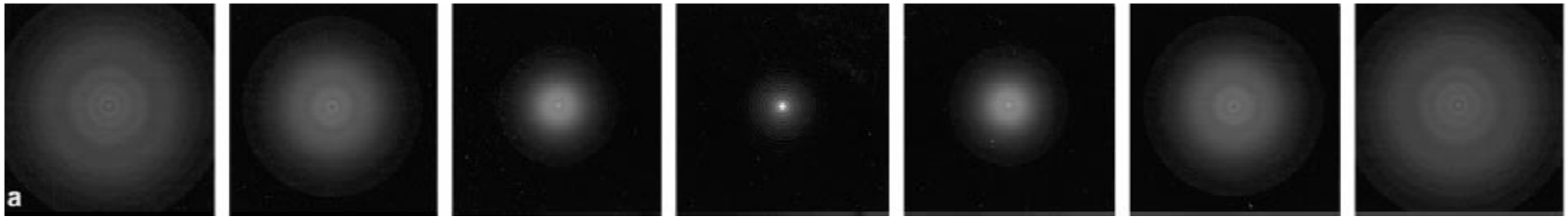


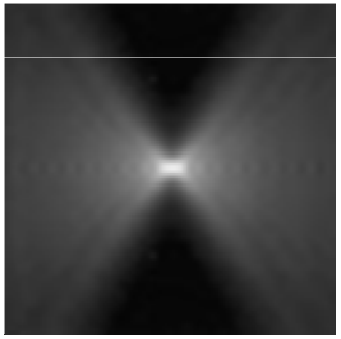
Fluorescence microscopy: Deconvolution



Stack of images along z axis

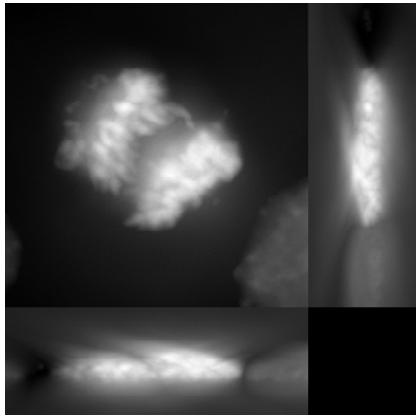


-3 -2 -1 0 1 2 3µm

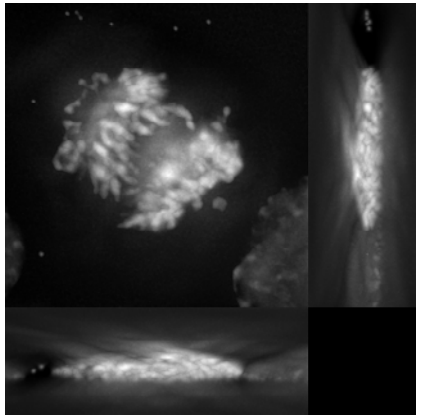


Point spread function (**PSF**)

Fluorescence microscopy: Deconvolution



original



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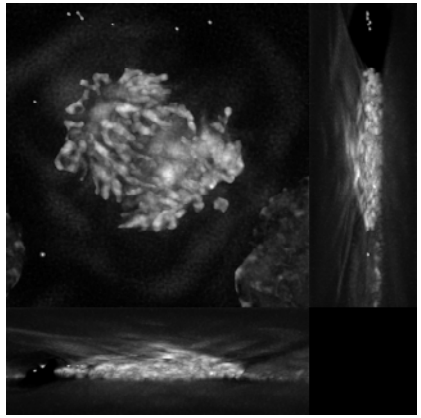

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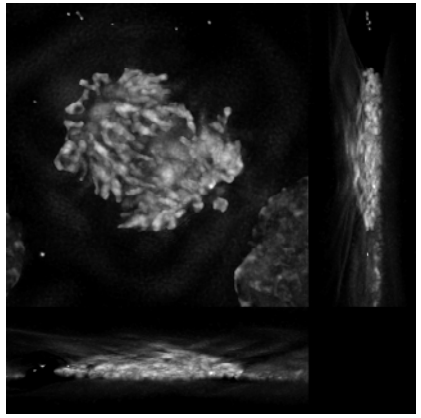
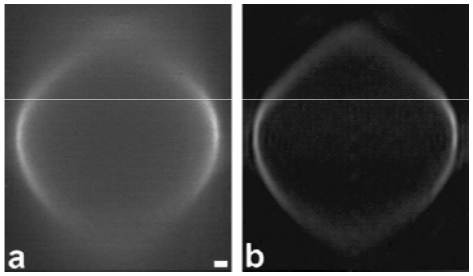


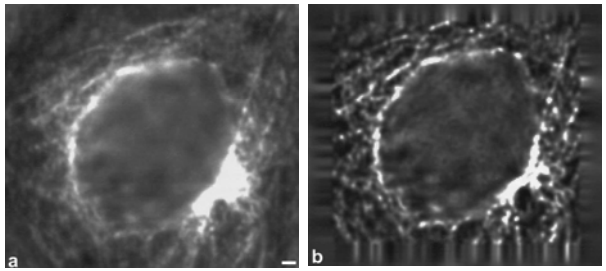
1000 iterations

Potential artefacts

Stretching

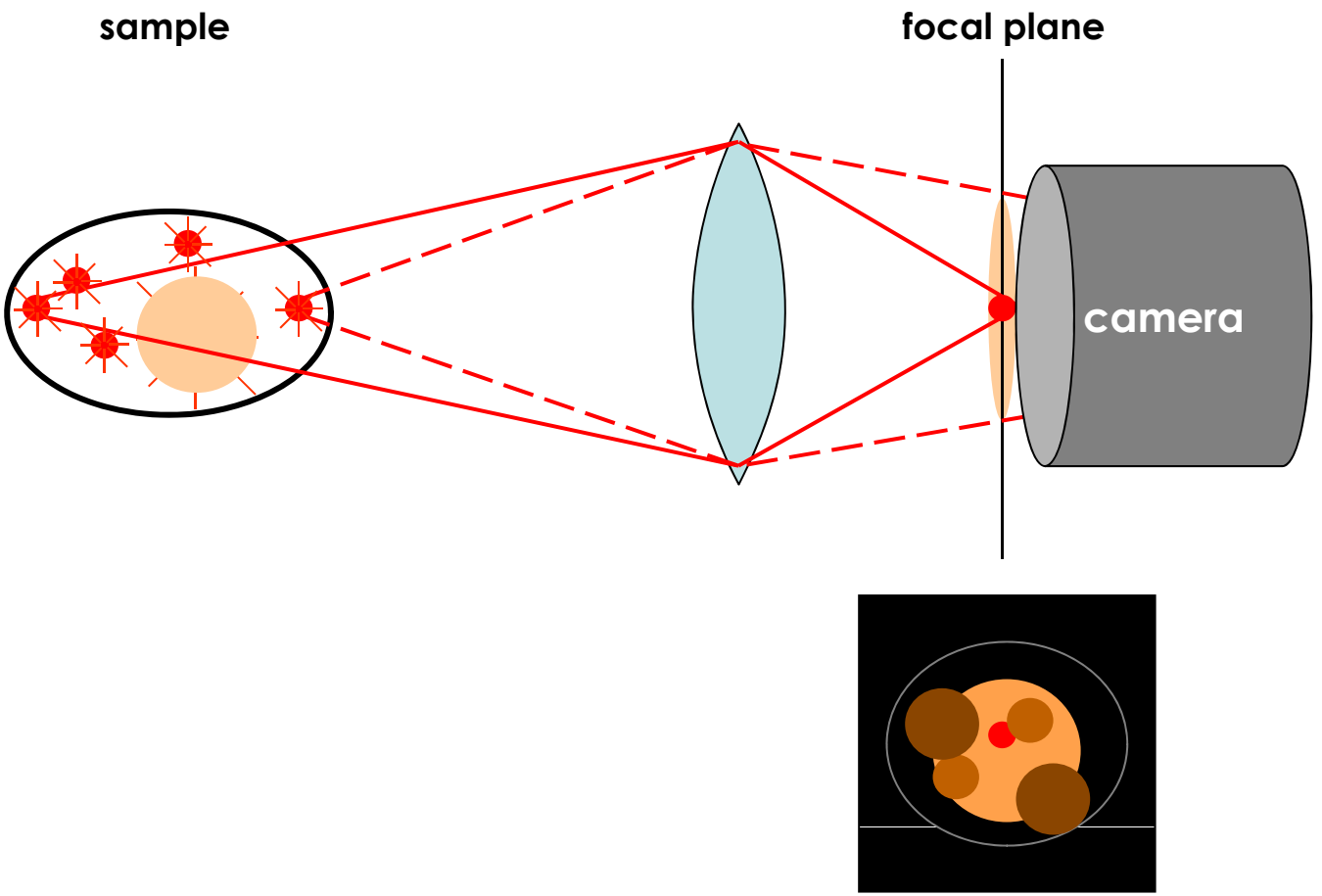


Edge artefacts

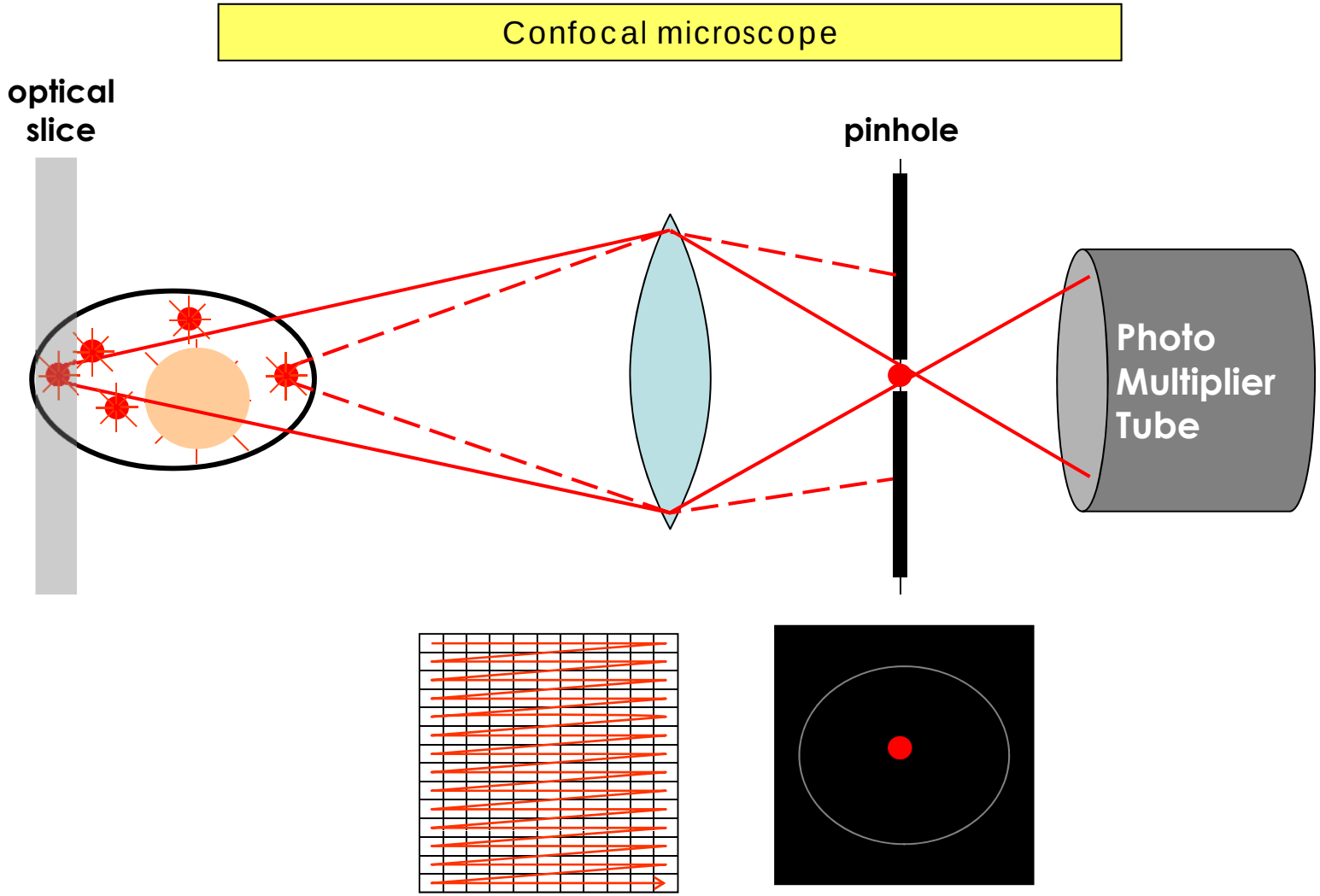


Fluorescence microscopy: Confocal

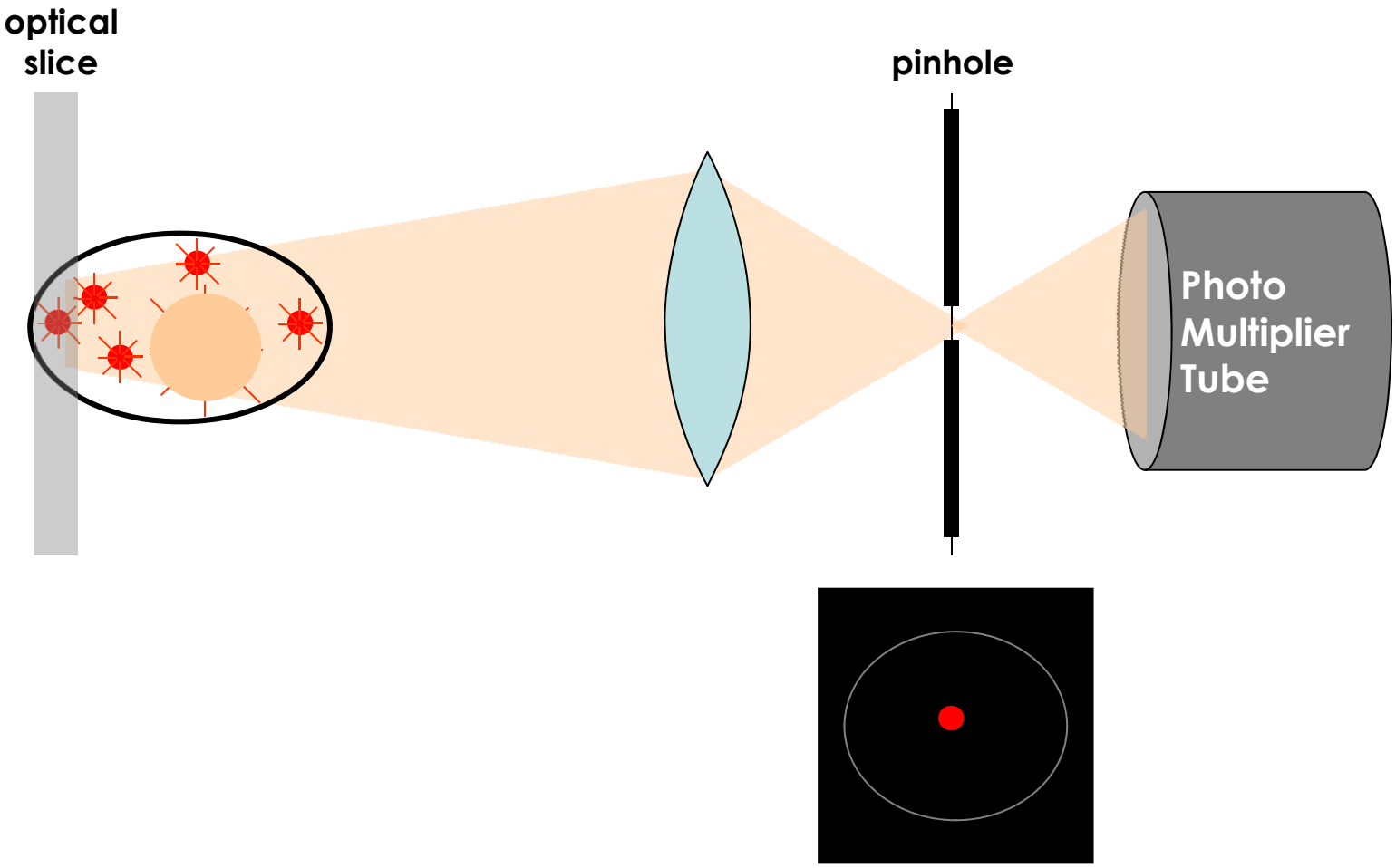
Standard (widefield) microscope



Fluorescence microscopy: Confocal

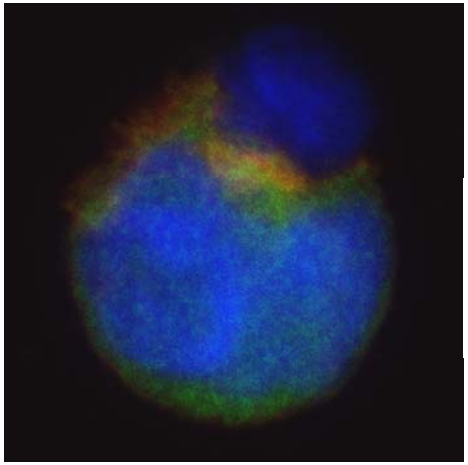


Fluorescence microscopy: Confocal

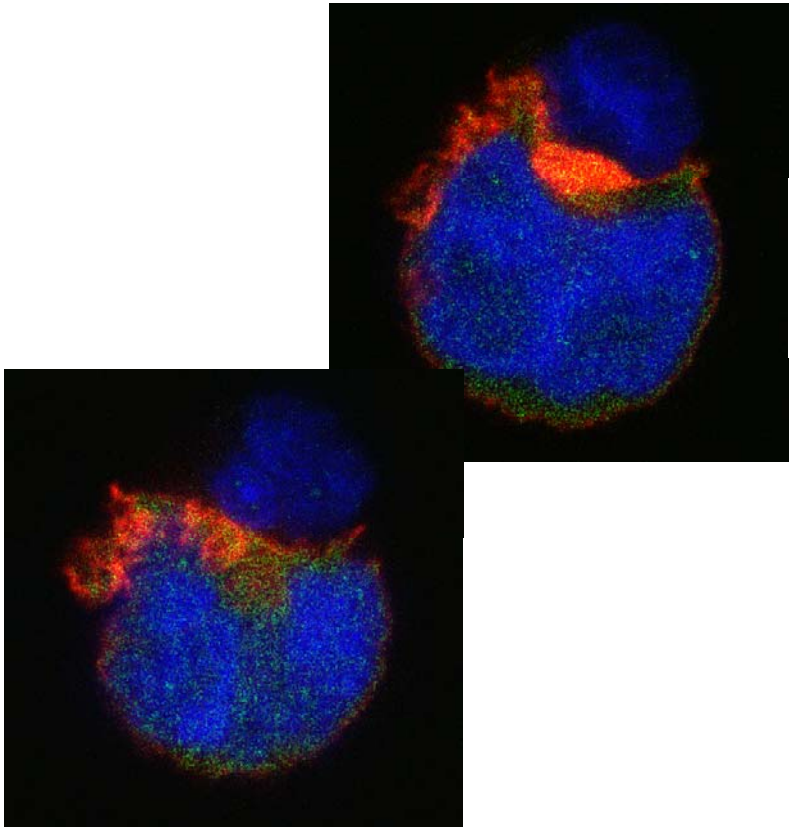


Fluorescence microscopy: Confocal

Widefield



Confocal



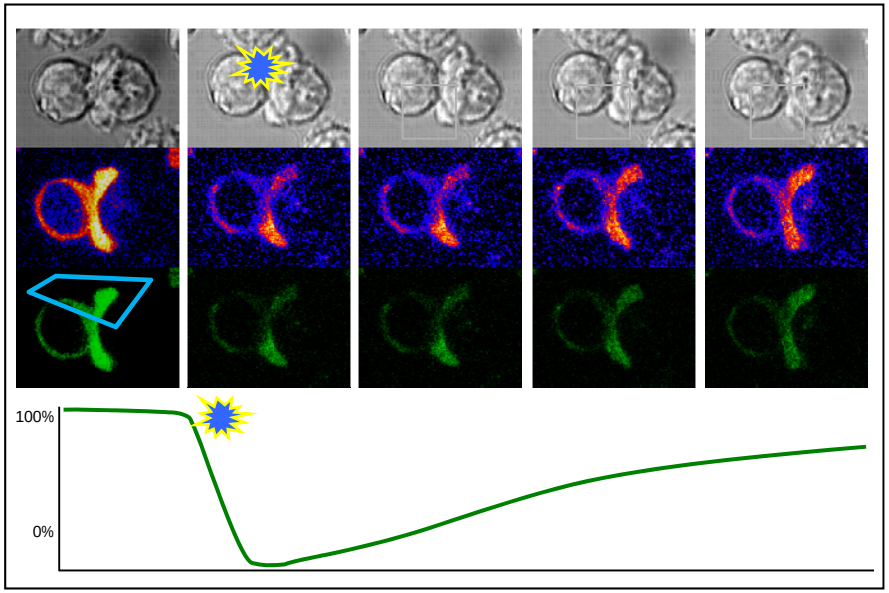
Light as a tool

Other uses of lasers in microscopes:

- pulsed lasers (2P, SHG, Flim, Flim-Fret) → Mark Scott: ***Special techniques I***
- switching / uncaging → Steve Rothery: ***Special techniques II***
- bleaching (FRAP, FLIP)
- phototoxins
- laser tweezers

Photobleaching techniques:

FRAP (Fluorescence Recovery After Photobleaching) / **FLIP** (Fluorescence Loss In Photobleaching)



0 1min

Principle:

- An Region Of Interest (ROI) is bleached
- Movement (diffusion, transport) of the visible fluorescence into the ROI (FRAP) or loss of fluorescence outside the ROI is measured over time

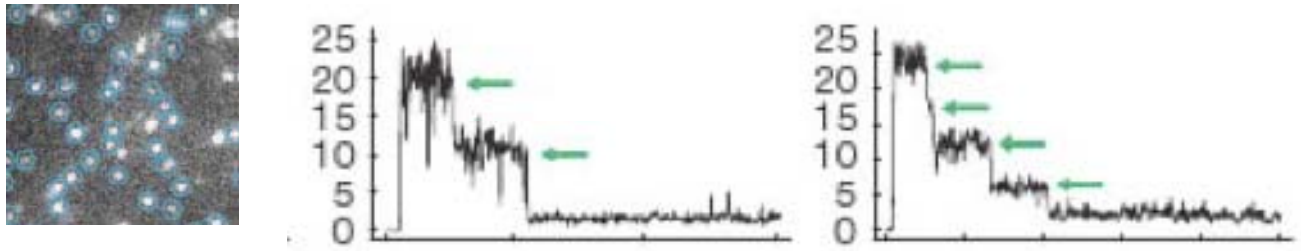
Problems:

- incomplete bleaching
- slow (sample movement)
- high phototoxicity

$$\frac{F(t)}{F_p} = \alpha \sum_{n=0}^{\infty} \left[\left(\frac{(-K)^n}{n!} \right) \left(\frac{1}{1 + n(1 + 2t/\tau_D)} \right) \right] + (1 - \alpha) \frac{F_o}{F_p}$$

Photobleaching techniques:
FRAP (Fluorescence Recovery After Photobleaching) / **FLIP** (Fluorescence Loss In Photobleaching)

Single-molecule analysis with photobleaching



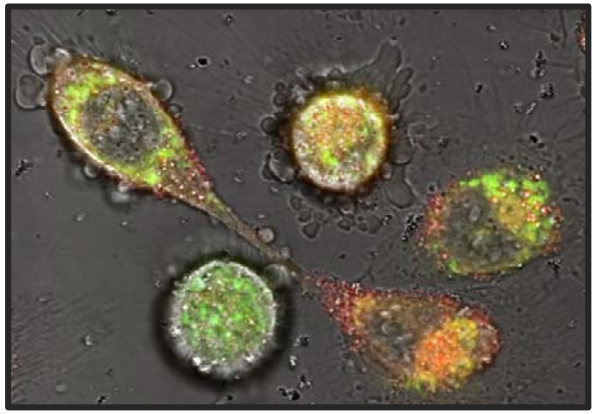
source: Kevin Teng, Univ. Illinois, USA

Light as a tool

Light-switches: Phototoxins, uncaging

Light pulses are used to:

- activate phototoxins
- uncage drugs
- activate photo-sensitive ion channels (opsins)
  - Optogenetics

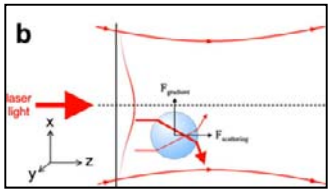


Ioanna Stamati (Mahendra Deonarain lab):
Apoptosis (cell death) induced by photoactivation of a phototoxin

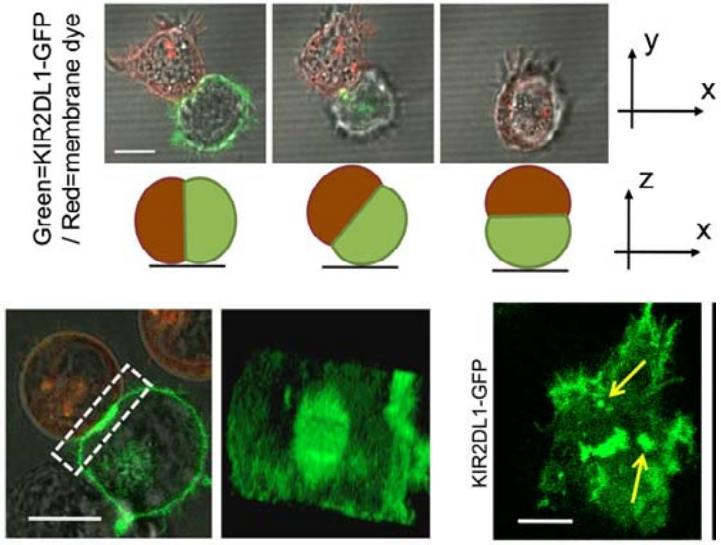
Light as a tool

Laser tweezers / laser dissection

Intensive infrared laser light is used to cut (laser dissation) or move objects (e.g. whole cells)

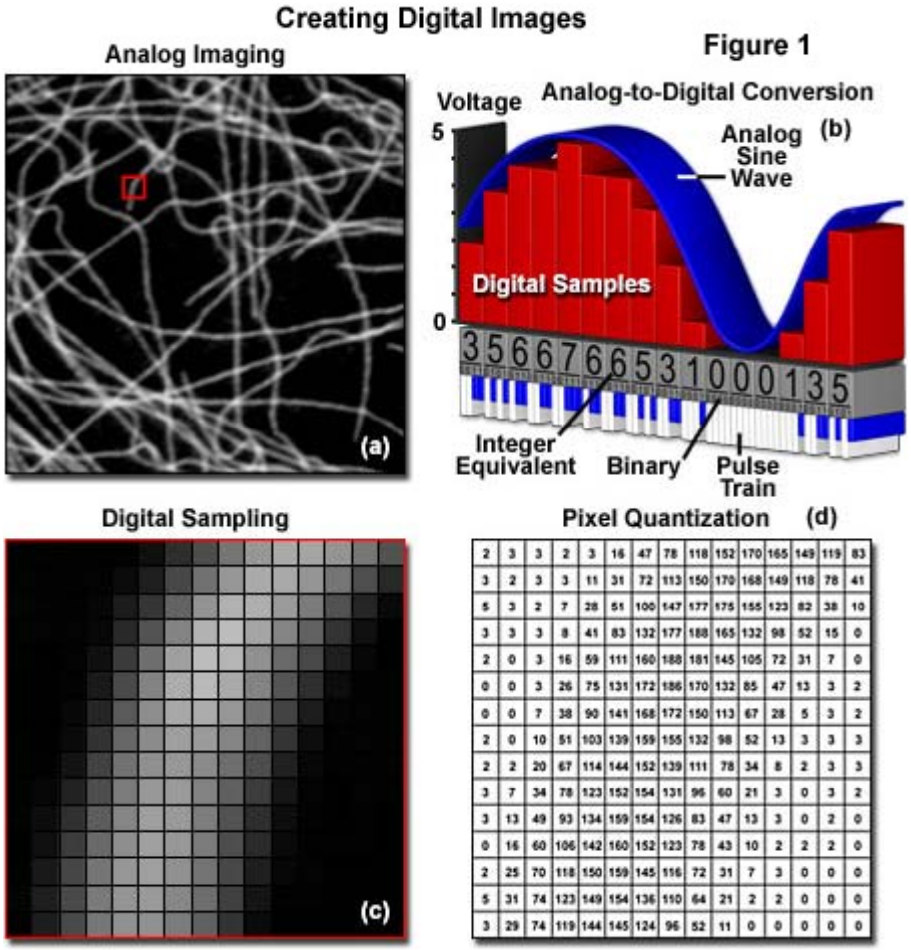


http://www.stanford.edu/group/blocklab/OpticalTweezersIntroduction.htm



Stefane Oddos (French / Davis labs):
Signalling clusters in the immunological synapse

Light detectors



source: zeisscampus online

Light detectors

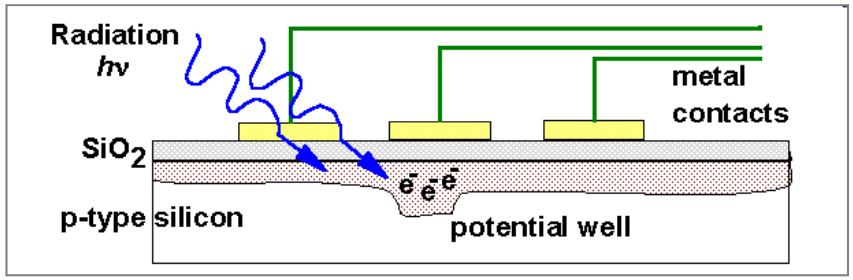
Types of detectors in light microscopes:

- **cameras:**
  - CCD
  - EM-CCD
  - back-illuminated EM-CCD
  - CMOS
- **photomultiplier tubes (PMT)**

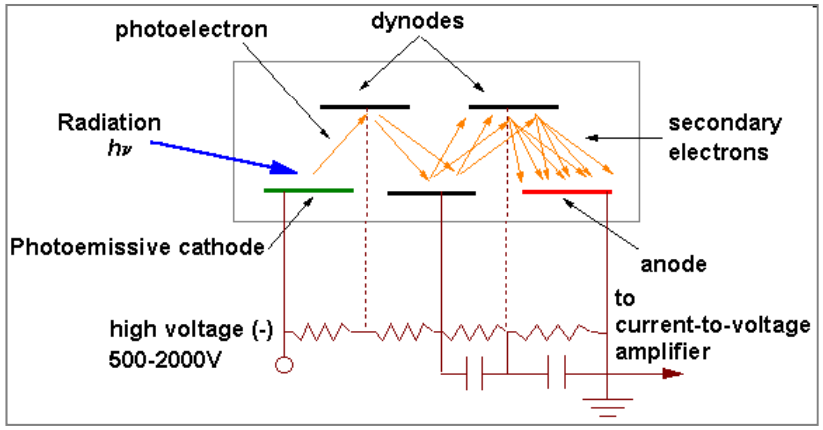
Essential considerations:

- sensitivity (signal-to-noise ratio)
- dynamic range
- linearity
- sampling rate ('pixel size)

CCD

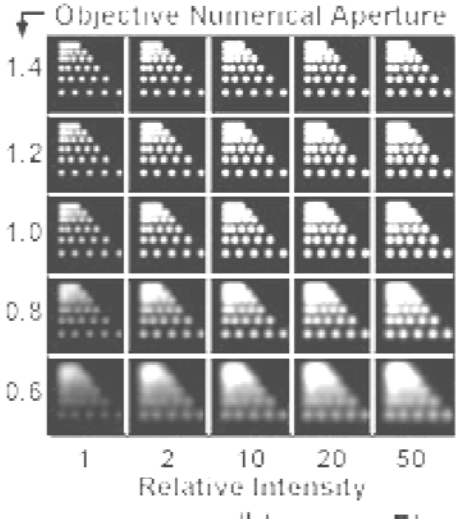


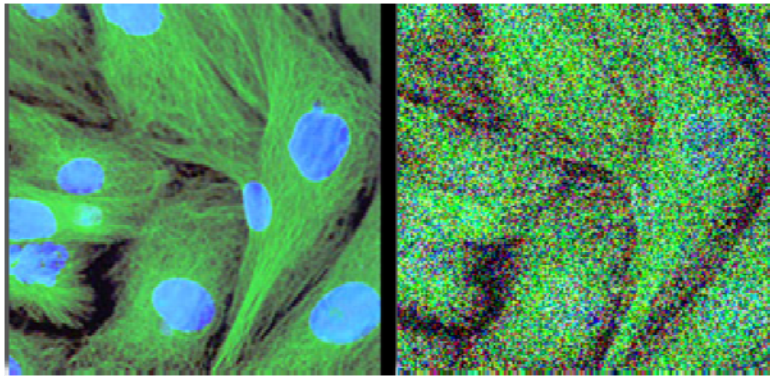
PMT

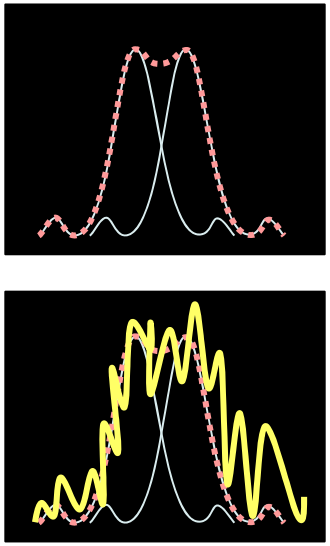


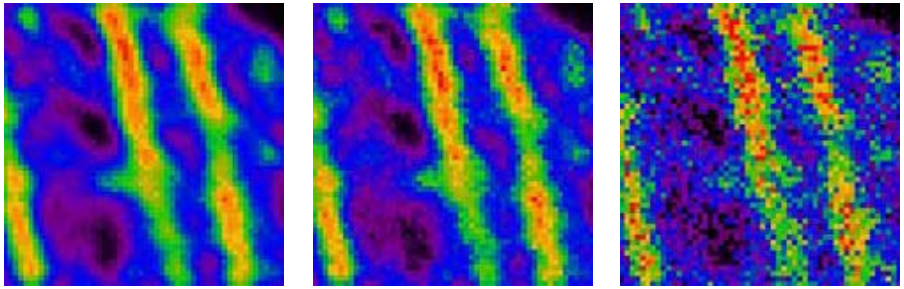
source: http://elchem.kaist.ac.kr/vt/chem-ed/optics/detector/detector.htm

Light detectors: noise









Signal-To-Noise ratio (SNR): **30** **15** **5**

SNR: commonly measured as

$$\frac{\text{intensity (objects)}}{\text{STDEV (background)}}$$

Imperial College London
FILM

Light detectors: noise

Types of noise:

1) Photon noise:
- inherent statistical variation in the arrival rate of photons (Poisson statistical distribution)
- equivalent to the square-root of the signal.

2) Dark noise:
- electrons thermally generated within the silicon structure of the CCD
- independent of photon-induced signal
- cooling the CCD reduces the dark current dramatically

3) Read-out noise:
- inherent to the process of converting CCD charge carriers into a voltage and the subsequent processing and analog-to-digital conversion
- Usually added uniformly to every image pixel (except CMOS)

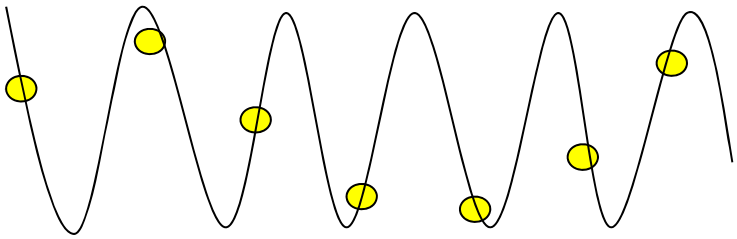
Reducing noise:

- brighter labelling
- higher-sensitivity detector
- longer integration time / lower scan speed
- averaging multiple exposures
- image processing: median / Gauss filter

Light detectors: sampling rate

$$d_{xy} = 0.61 * \frac{\lambda}{n*\sin(\mu)}$$

Abbe's Law



To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency

Nyquist theorem

Miroscopy:
sampling distance
=< 0.5 smallest structure or diffraction limit

Light detectors: sampling rate

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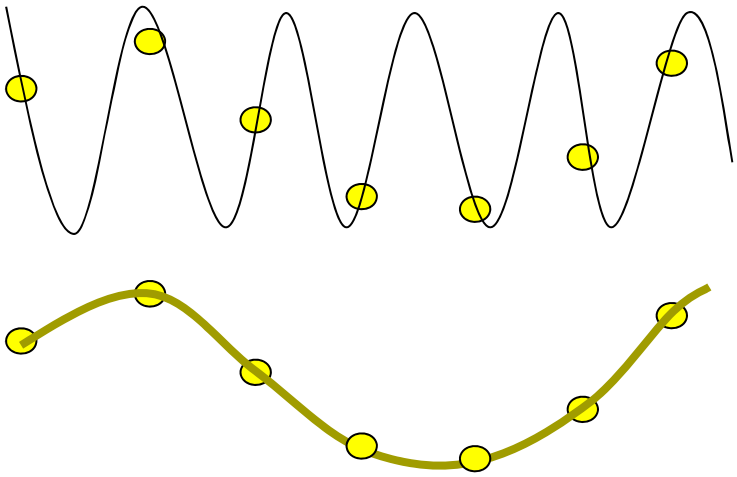
Abbe's Law



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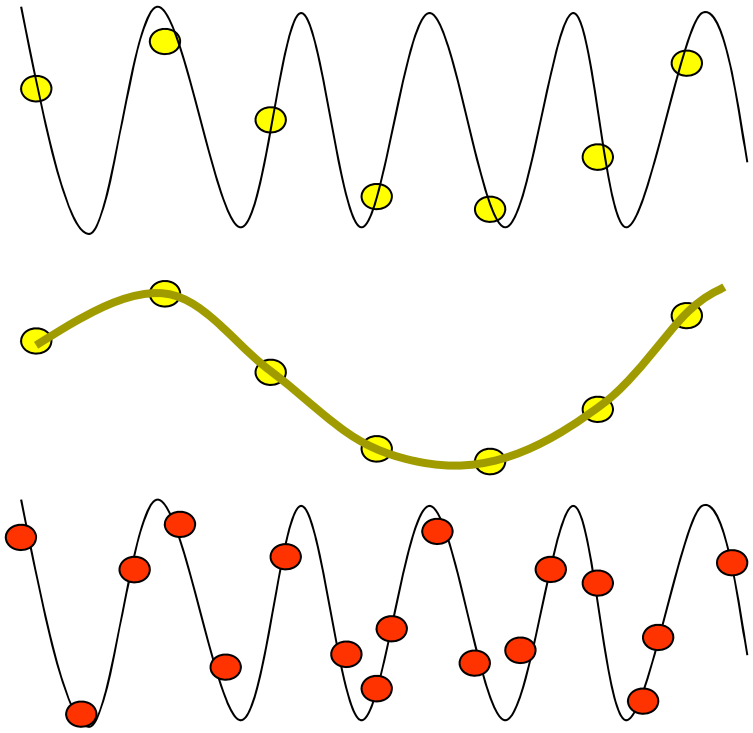

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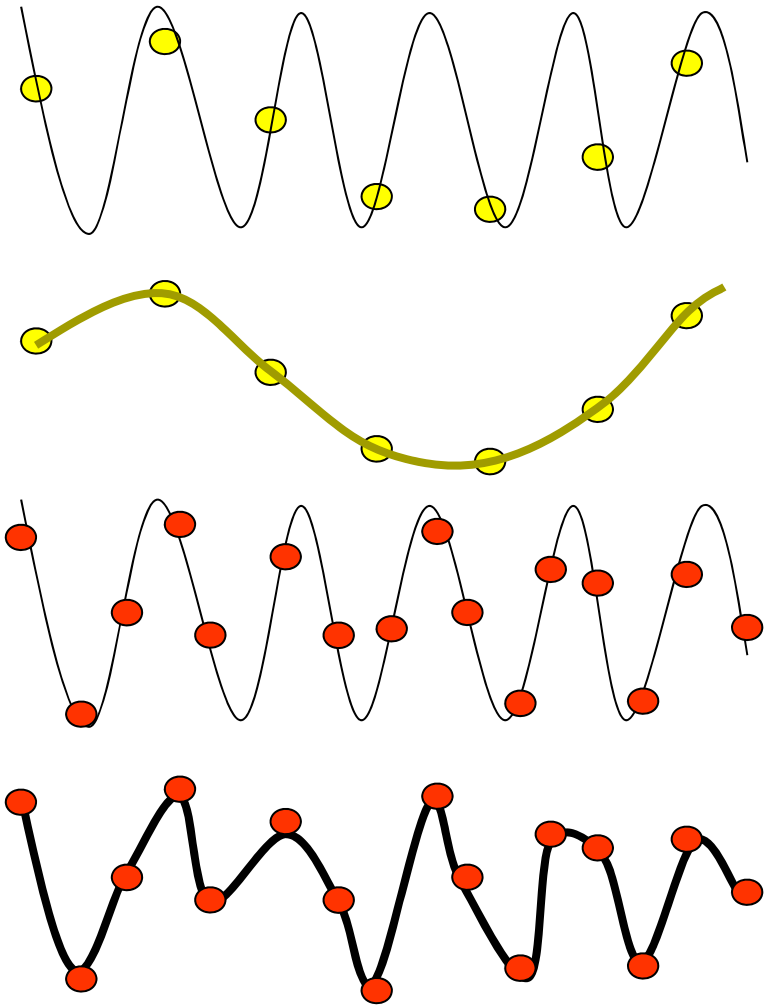

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How to achieve the 'Nyquist Rate':

- Widefield:
  - combination objective / pixel size
  - distance of Z slices
- Confocal:
  - zoom
  - distance of Z slices