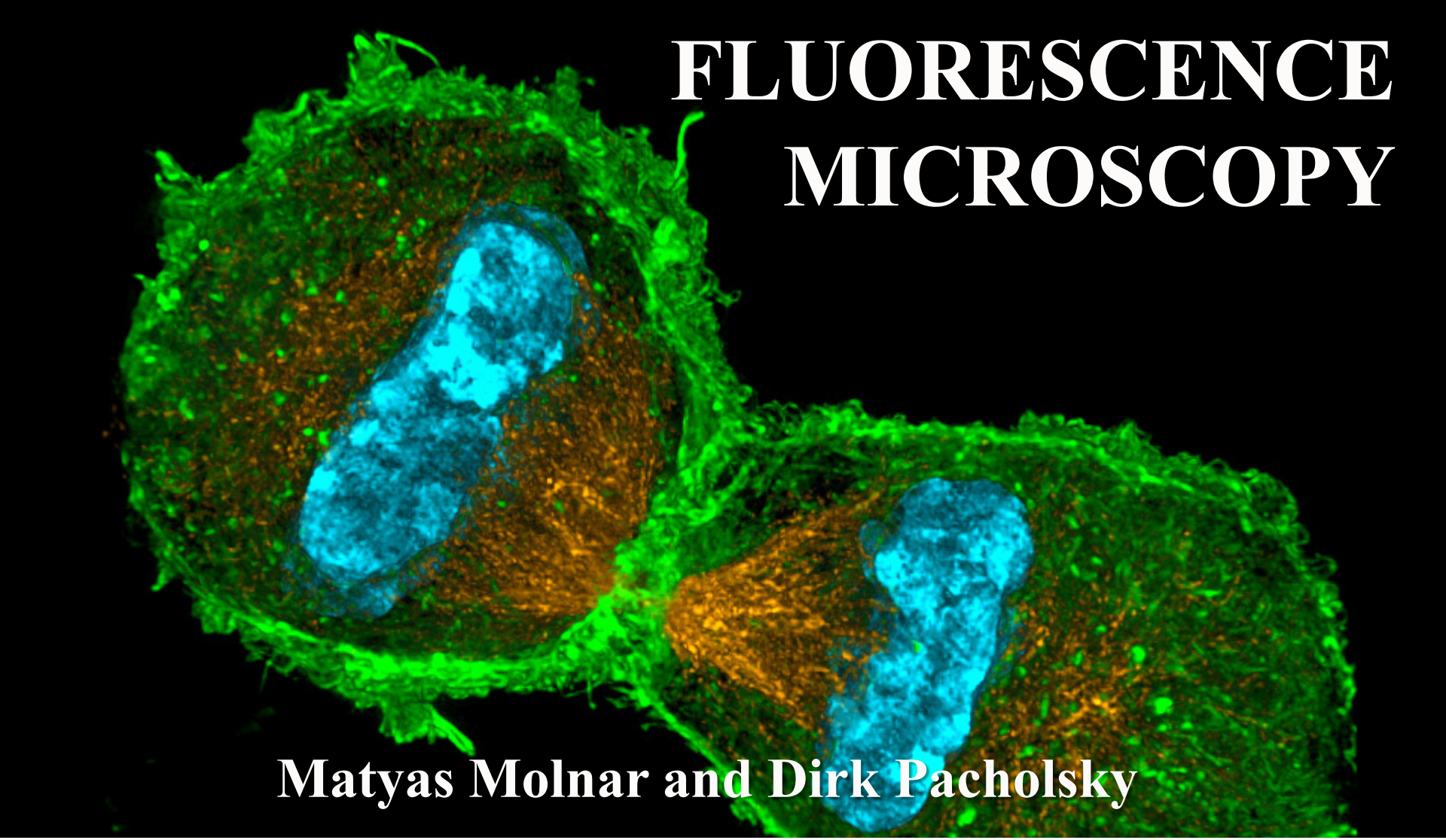
FLUORESCENCE MICROSCOPY



Matyas Molnar and Dirk Pacholsky

. Uppsala University . Platform . Biological Visualization .

BIOVIS

Light & Electron Microscopy : Flow Cytometry : Image Analysis

The human eye

- perceives app. 400-700 nm; best at around 500 nm (green)

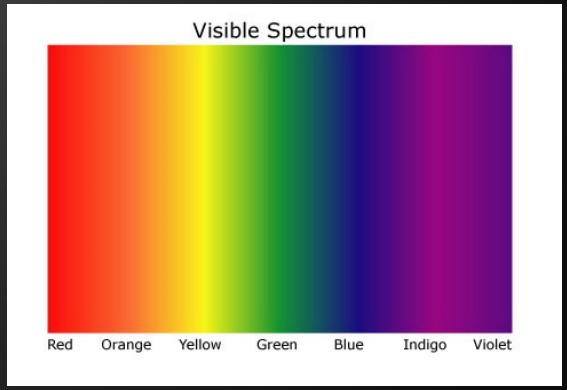
- Has a general resolution down to150-300 µm

  (human hair: 40-250 µm)

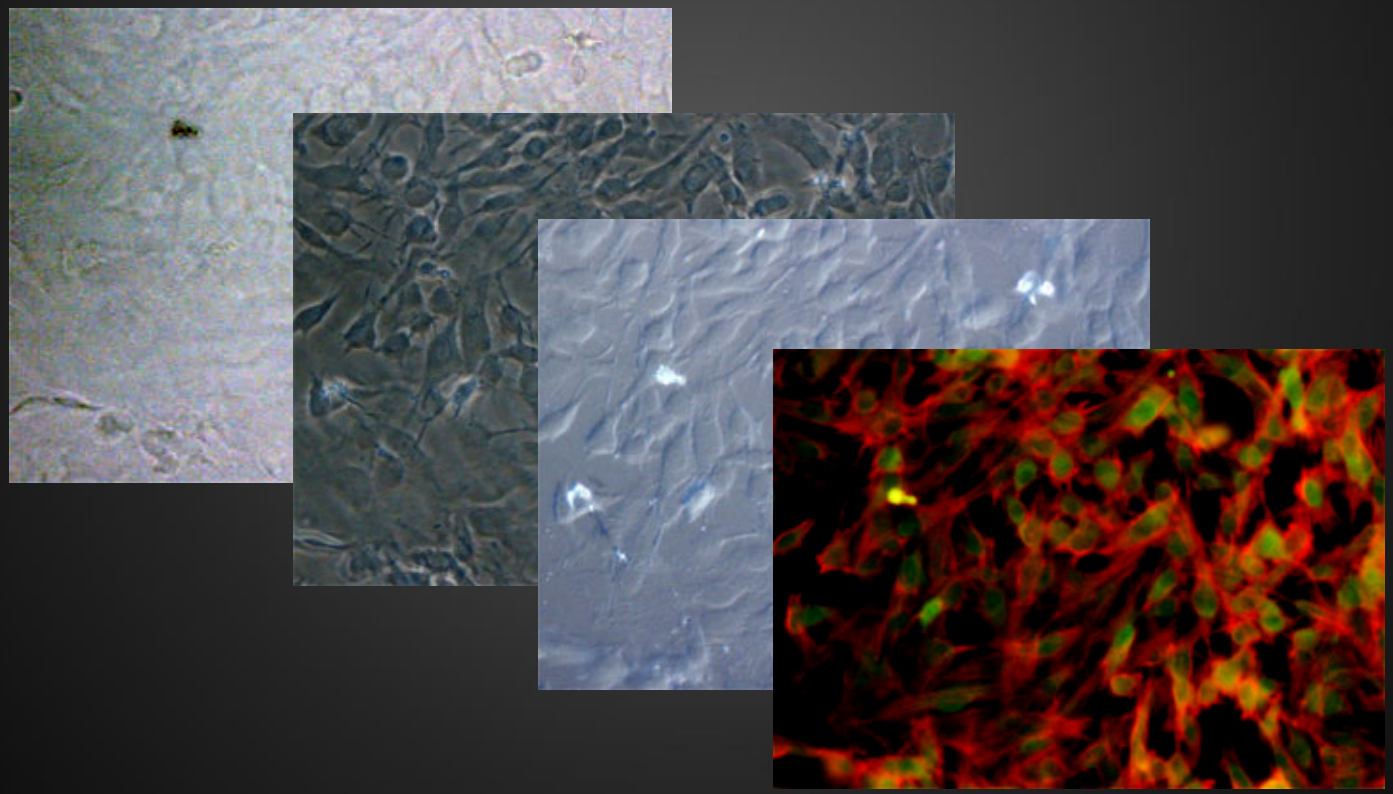
- We need a tool to see smaller things or more of the spectral range

  → Microscope (Objective/Filter)

  → Camera/Film



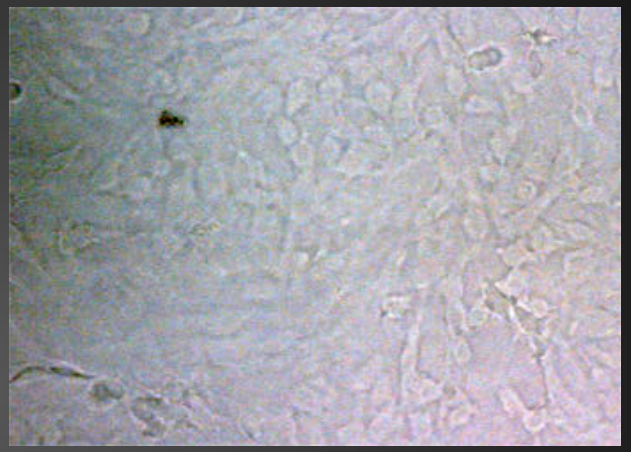
WHY FLUORESCENCE MICROSCOPY?

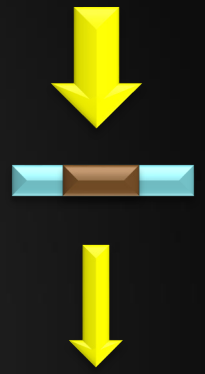


Microscopical techniques

Brightfield

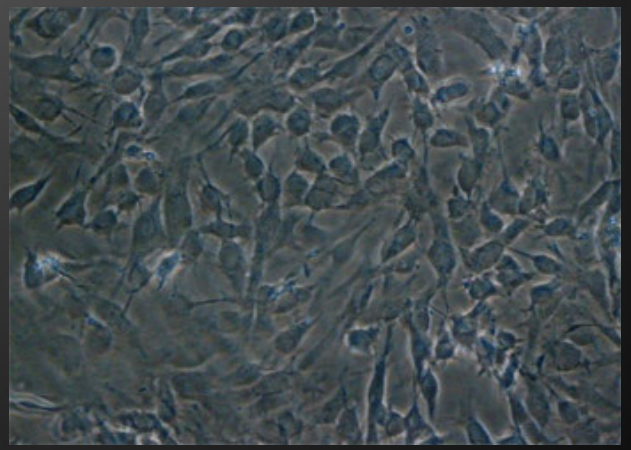
- low contrast for thin or transparent specimen
- staining to enhance contrast needed
  (histochemical staining)

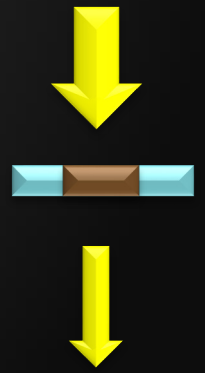




Phase contrast

- contrast via optical element
  (Phase ring)
- intracellular structures can be seen
- good for cell culture applications
- negative: halos around cell bodies
- combining with other techniques is generally poor (e.g. overlay with fluorescent image)

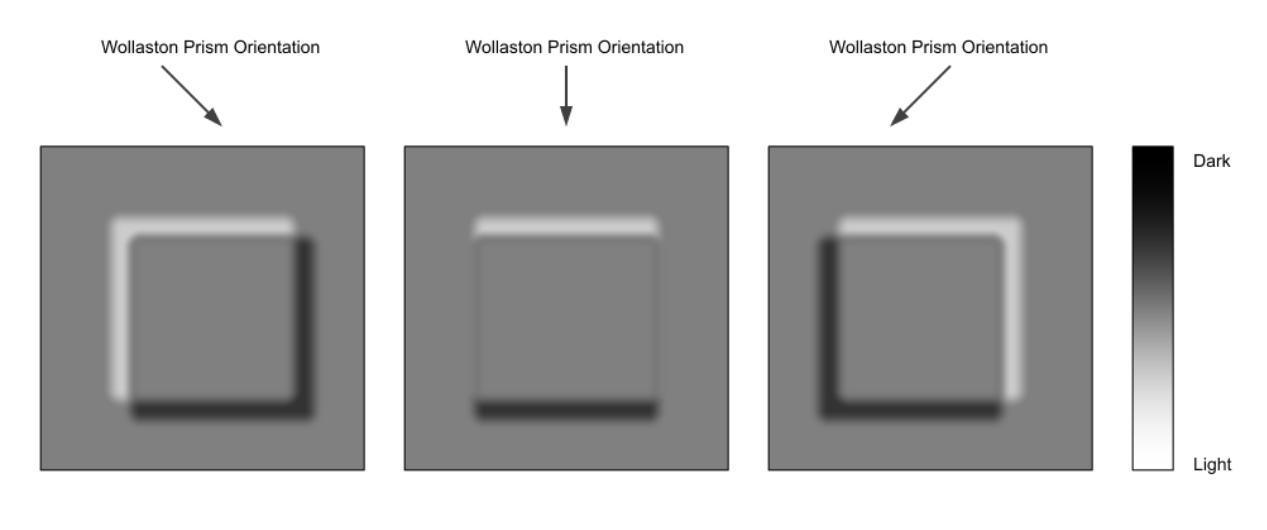




Microscopical techniques

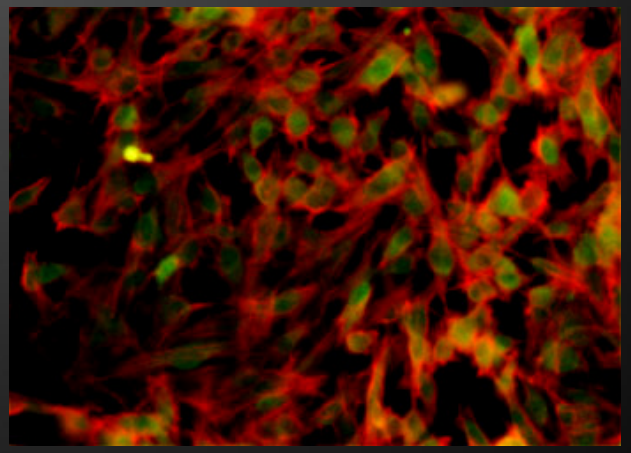
DIC/Nomarski

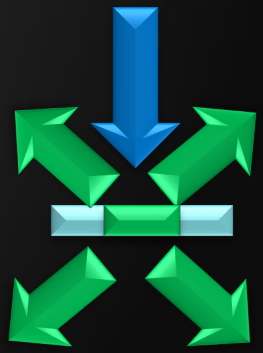
- contrast via polarized l
  element (Wollaston pri
- gives a (fake) topograp
- excellent for combinati
  fluorescence and hist
  staining



Fluorescence

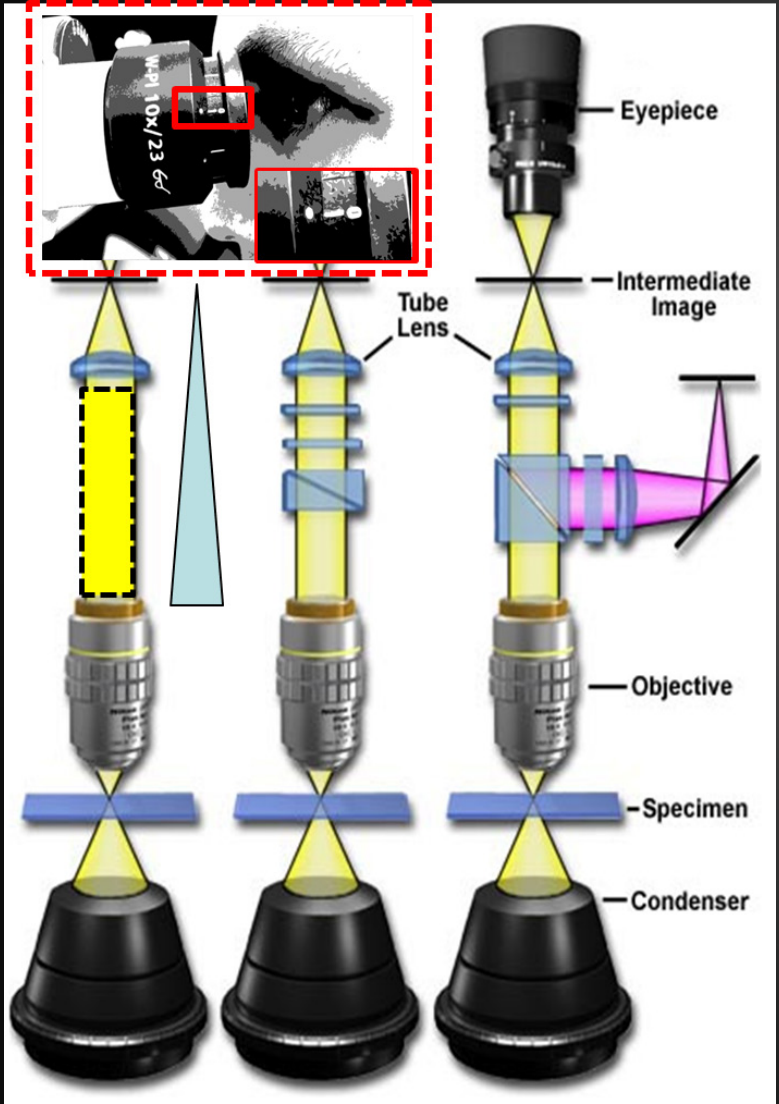
- contrast via fluorescent staining
- **You see only what you stain**
  (...in the perfect world)
- special optical elements are needed
  (filter cubes)
  high resolution, high contrast, good
  for quantification (area + intensity)
- Staining is sensitive - it can fade.





THE MICROSCOPE

Optical pathway of a microscope



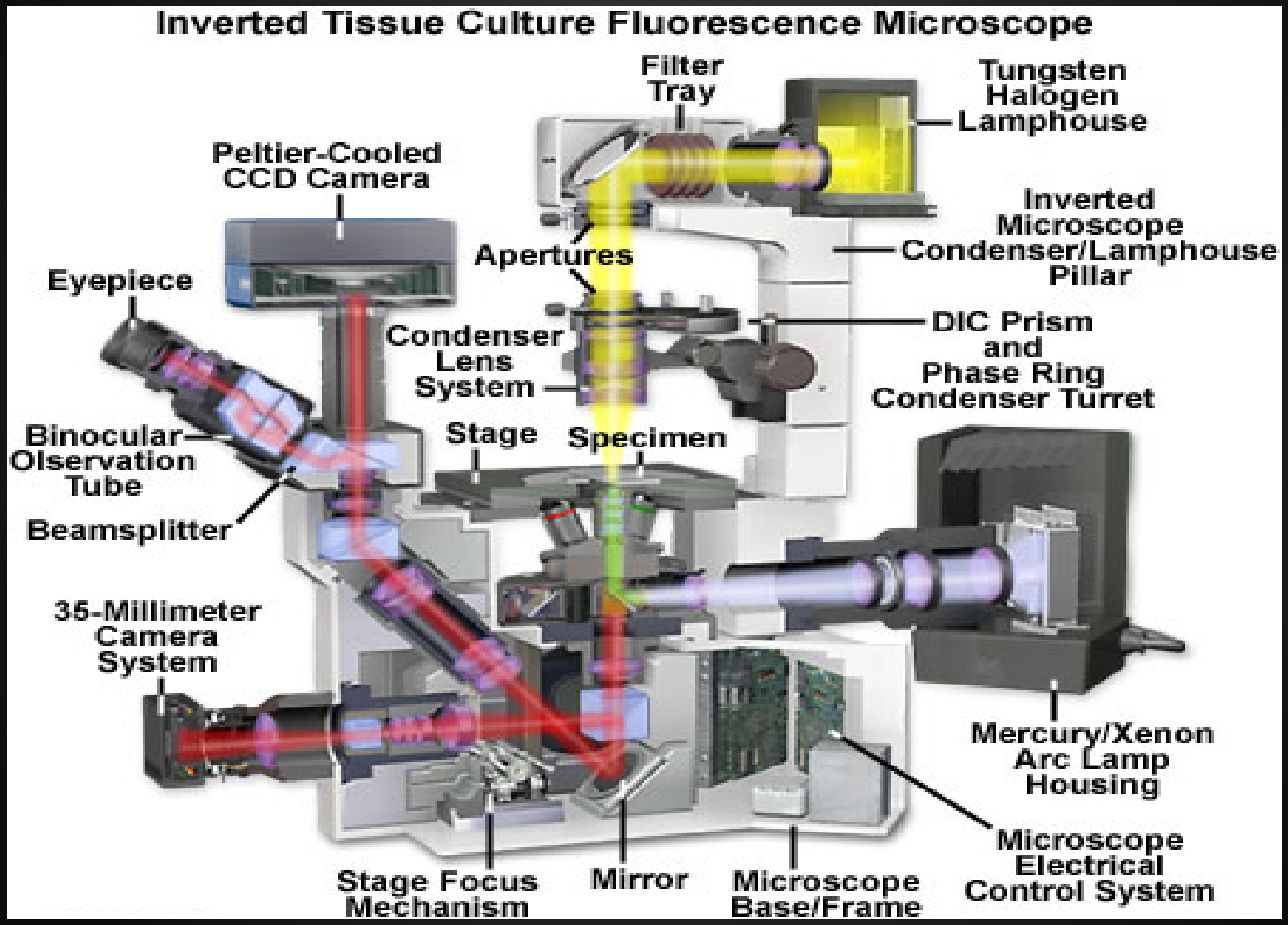
Objective projects image of specimen via Tube lens to Primary image plane
Eyepiece magnifies this image. *

* infinity corrected microscopes have parallel light beams between Objective and tube lens
→ space for different optical elements for different microscopical techiques

Old microscopes had „tube lenght“ of spec length & specific objectives which ´produce´ not convergent Light: vs
Objectives are not interchangeable

! Eyepiece/Ocular : set it to „0 – dot“ for Relaxed viewing (w/out glasses, lenses)

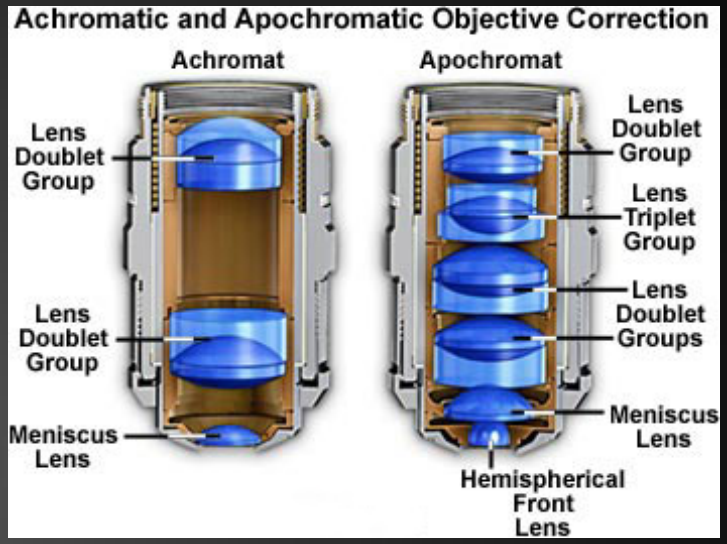
Optical pathway of a microscope

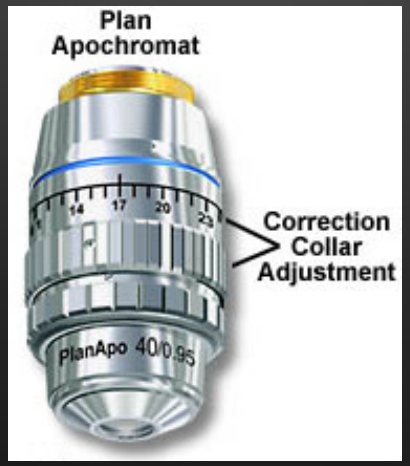


The objective or lens

The heart of a microscope,
may contain up to e.g. 12 lenses

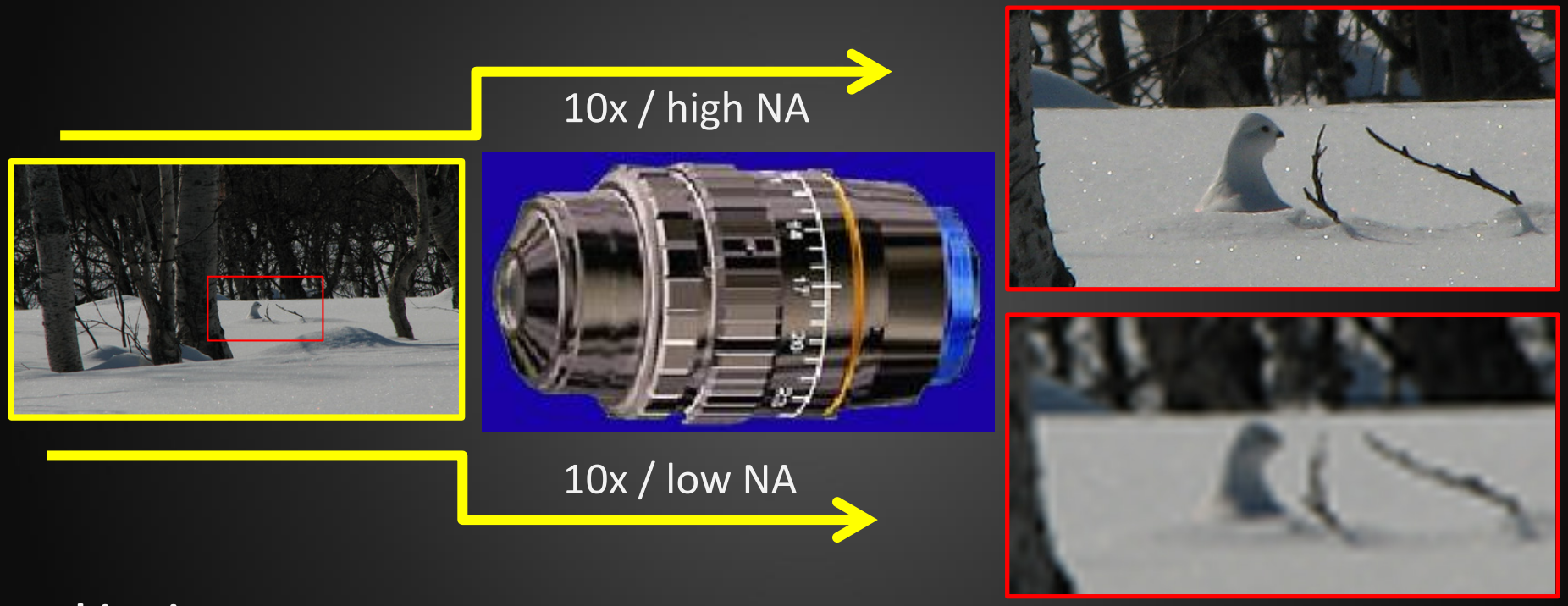
Specification and Identification
- Magnification (enlargement)
- Numerical aperture (resolution)
- Immersion medium (should fit to embedding medium)
- corrections (spherical; chromatic)
- working distance
- tube length
(infinity or 160 mm)
- coverslip thickness





Objective – magnification and resolution

A microscope magnifies a specimen with a certain resolution.



objectives

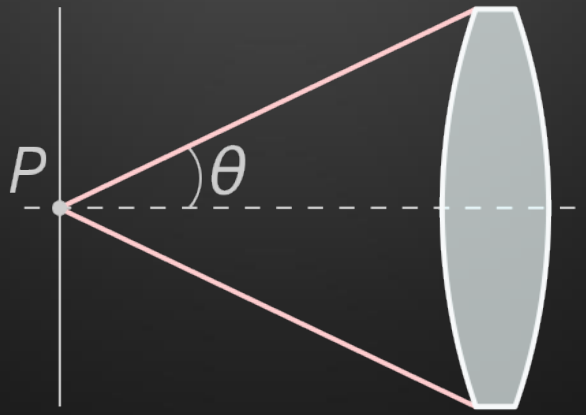
20x: N.A. 0.75 → 0.37 µm resolution
40x: N.A. 0.75 → 0.37 µm resolution
60x: N.A. 0.75 → 0.37 µm resolution

Magnification without resolution is useless : empty magnification!

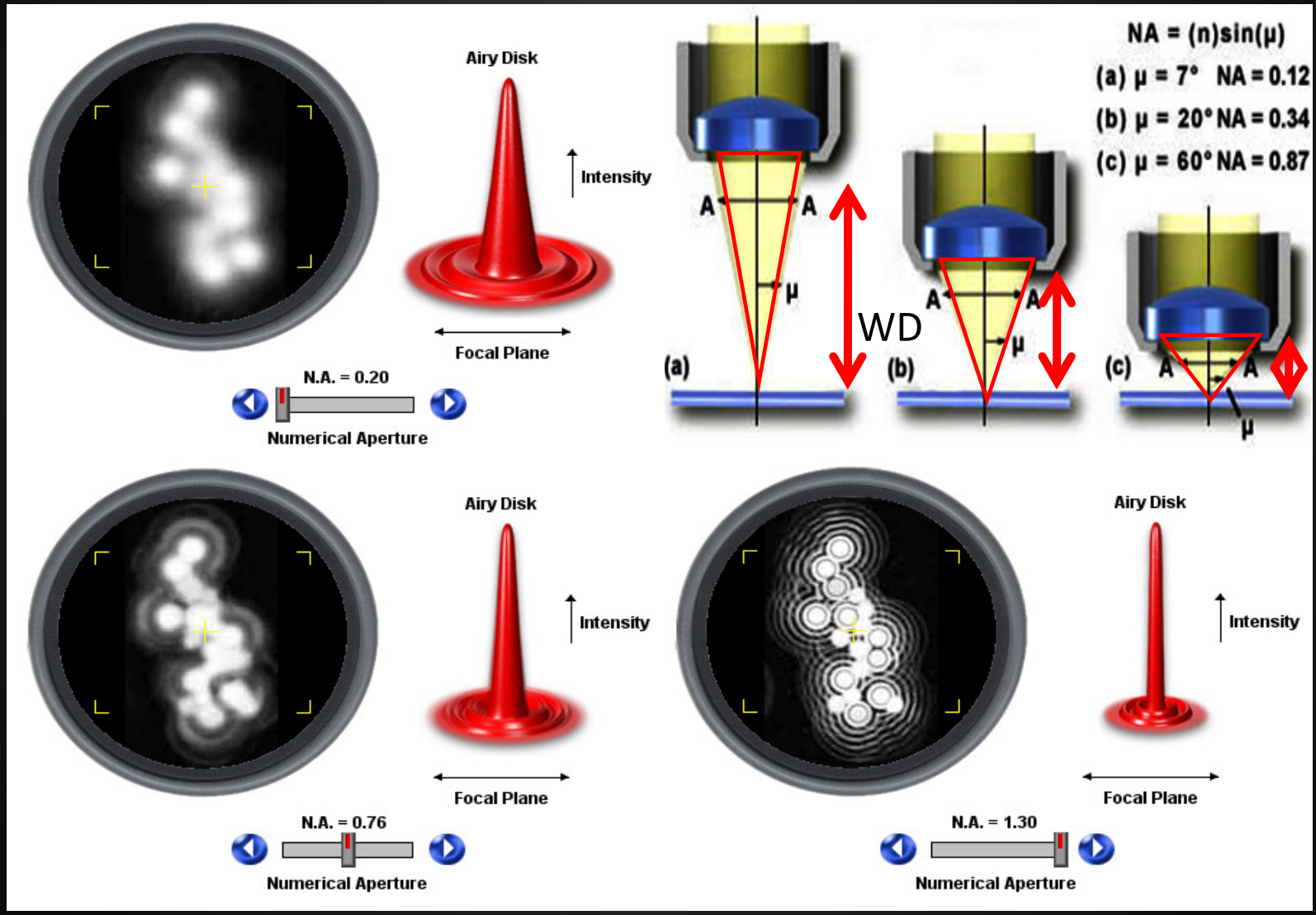
Illumination of the specimen - resolution

$$d = \frac{\lambda}{2n \sin \theta}$$

d: point resolution, the shorter the lenght is better for us [nm]
λ: wavelength of light used ([nm], visible light 400-700 nm)
n: refractive index (air: 1, water: 1.3, oil: 1.4-1.5)
ɵ: the maximum cone of light that can enter or exit the lens
nsinɵ: numerical aperture (NA)

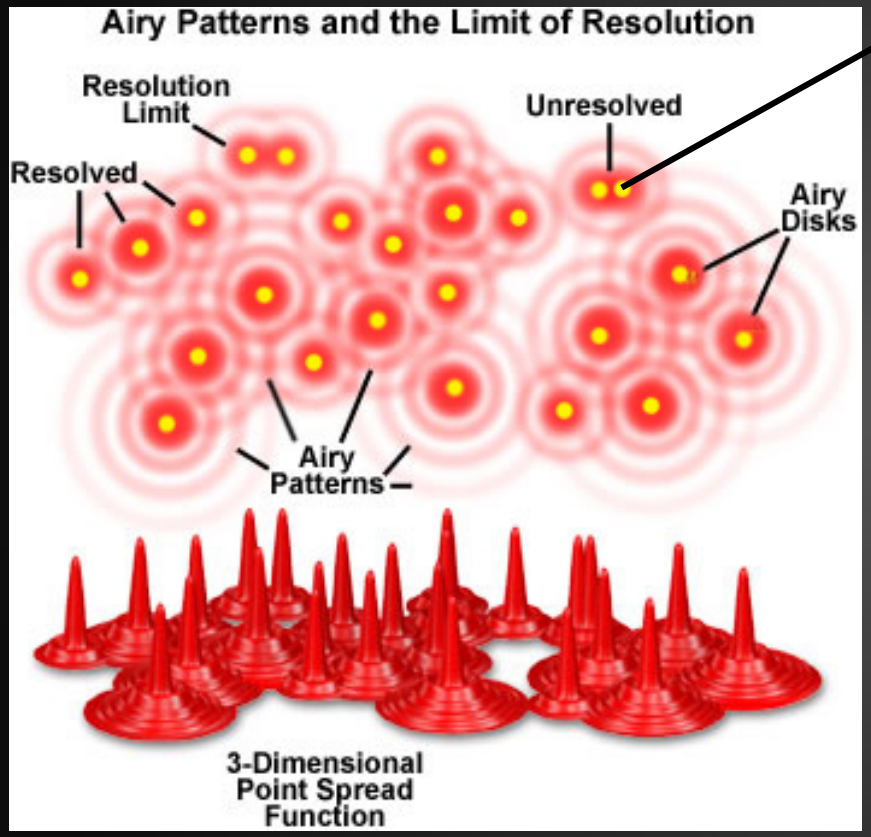


Resolution, Airy disk, NA & WD



Objective – Resolution and Airy disk

A point of light will not be a point of light



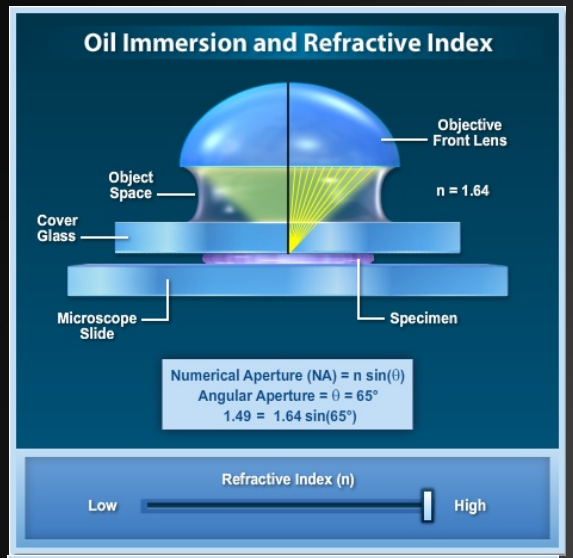
The yellow dots shall indicate infinite points, where light originally came from.

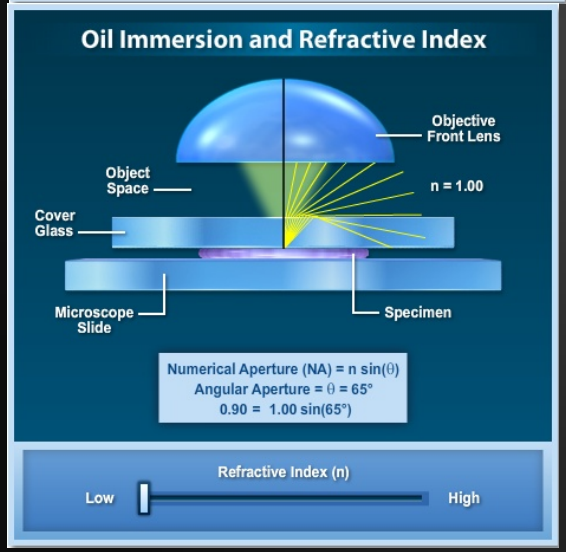
Light originally coming from a point and passing through lenses etc. will not be a point again in the image, but rather a dot (1st maxima, AiryDisk) with several side maxima separated by mininima (interference pattern).

The Spreading from Point light source to a Airy disk Image is called Point Spread Function (PSF).

PSF gets bigger with mismatch of embedding medium and objective

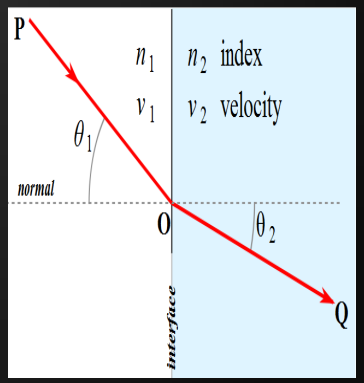
Match embedding medium to objective





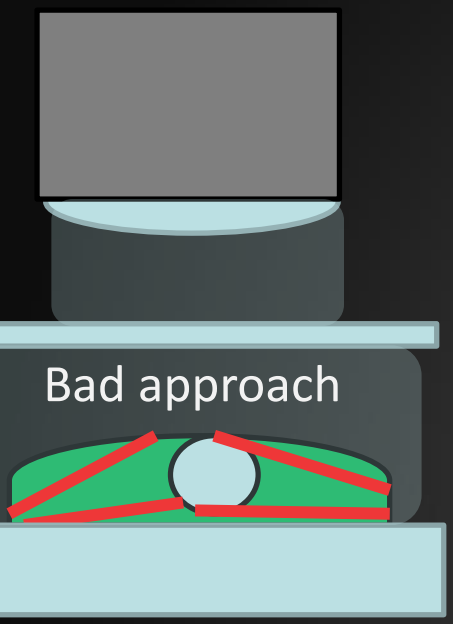
Light coming from high to low density medium (glas vs air) gets refracted away from the vertical of the incident angle, eventually misses the lense and is lost for imaging.
Appliance of immersion (oil, glycerin, water) between Coverslip and Lense with similar refractive index as Glas will reduce refraction and enhance light yield which in turn gives better Airy pattern (resolution)

Light coming from one point source will get scattered and refracted into different angles – the point gets spreaded. By applying High Numerical Aperture this effects are kept to a minimum.

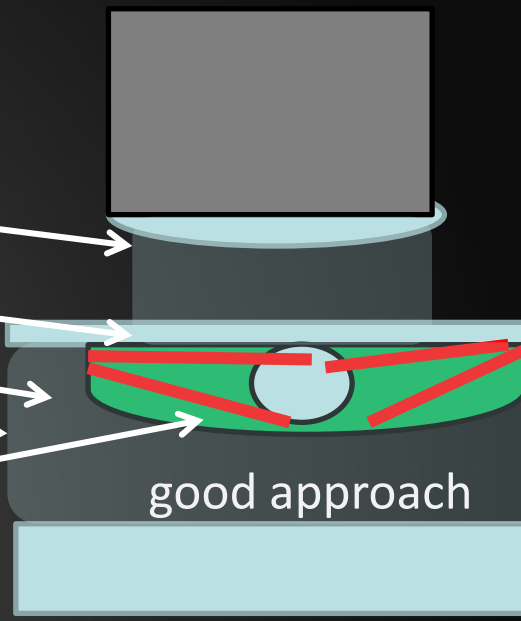


PSF gets bigger with mismatch of embedding medium and objective

Practical tips

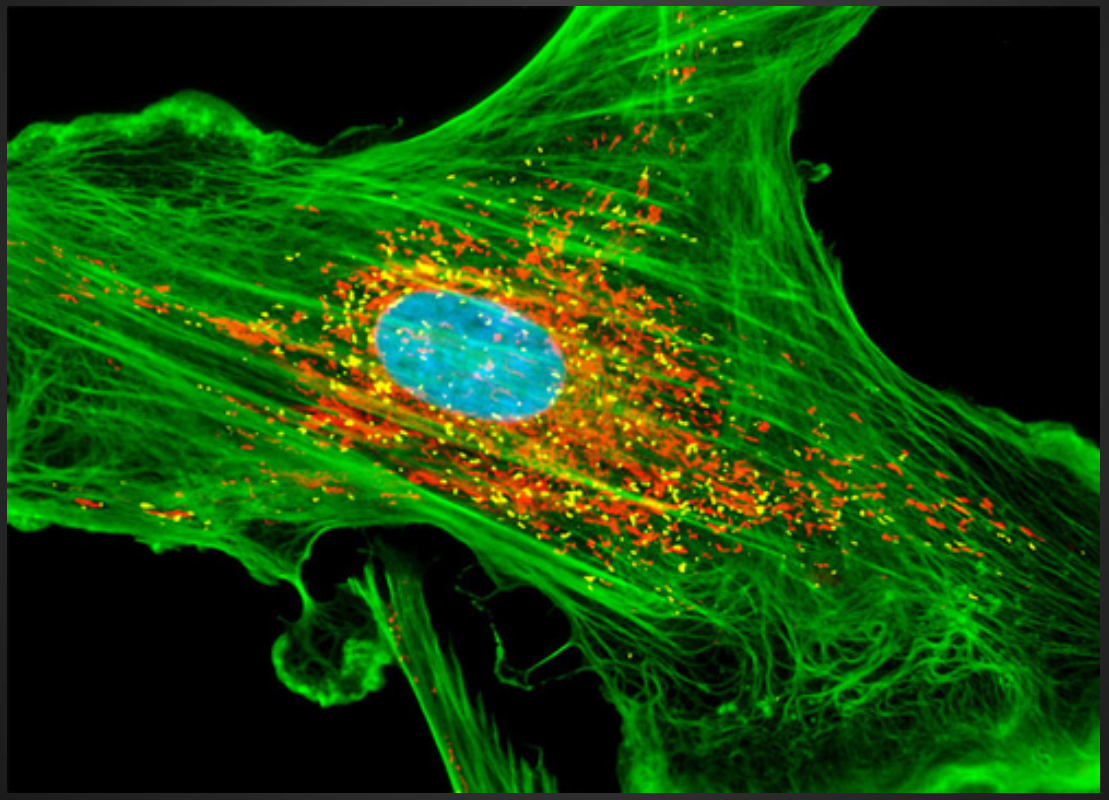


Match objective with correct
- Immersion media
- Coverslip thickness
- Embedding media
- Imaging setup
- Sample preparation



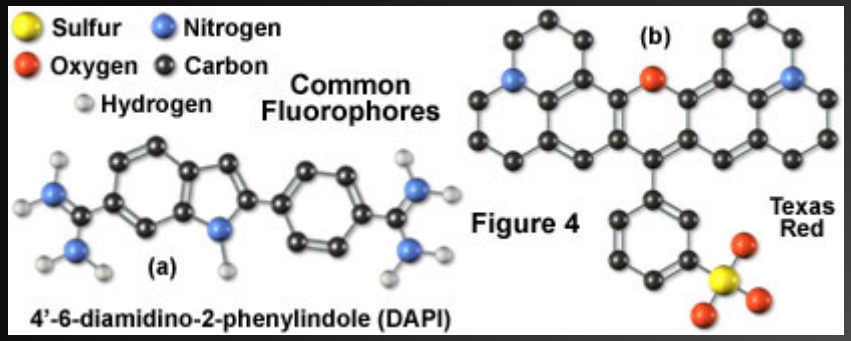
- Objectives indicate for which immersion media they are made for
- Objectives indicate for which coverslip thickness they are made for
- Embedding medium has optimally same RI like immersion media
- Place the sample as close as possible towards the objective lens
- RI of sub-cellular components considerably lower than that of immersion media, and in many cases these RI are uncertain and vary throughout the specimen. Different fixations might destroy antigen to be targeted or might quench fluorescence (of e.g. GFP)

THE FLUORESCENCE MICROSCOPE

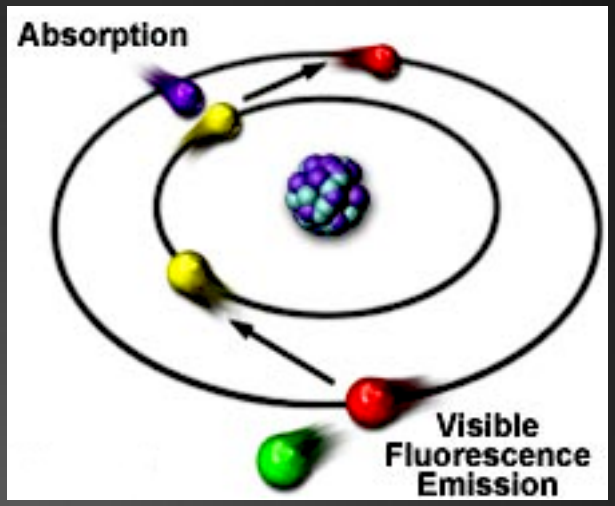


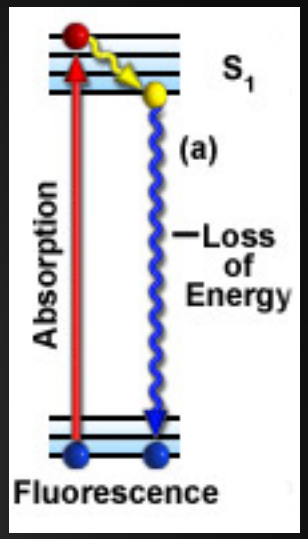
Fluorescence

Examples of fluorescent probes

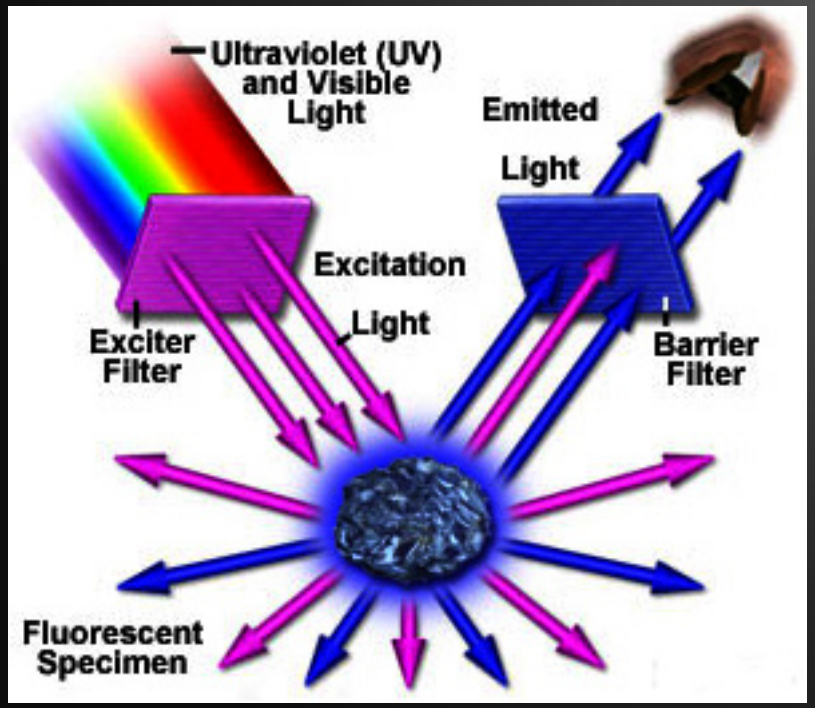


Principle of fluorescence

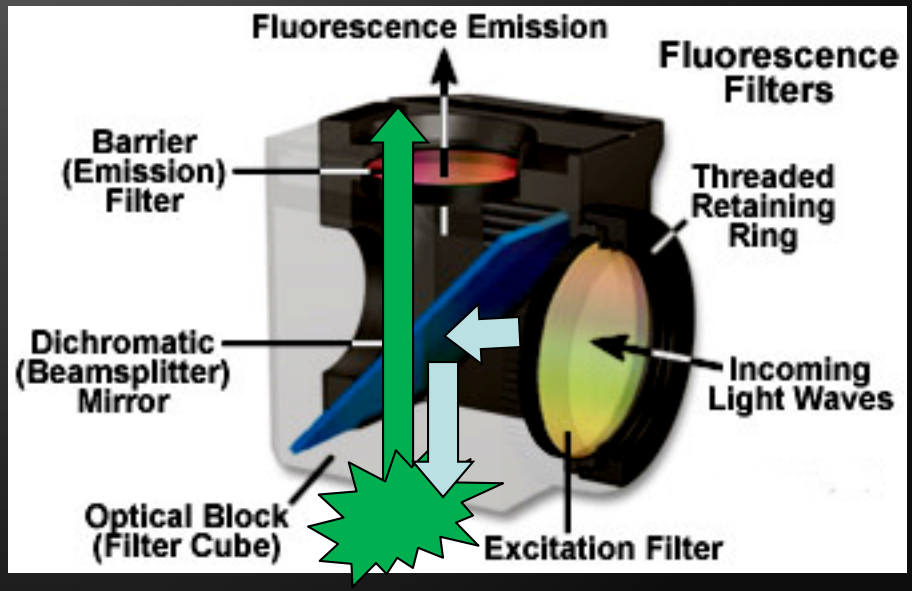




Principle of fluorescent microscope



Excitation-Emission filter cube



Combining fluorescent dyes - crosscheck

To avoid false positive images in Fluorescence microscopy check for

Seeing is Believing
BUT
Is it true?

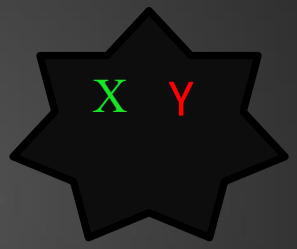
What's to be seen in pos/neg control

stained

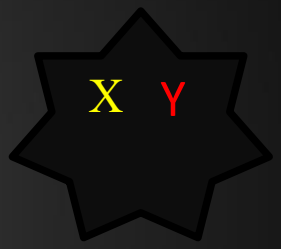
unstained

Crossreact AbX with AbY?

AbX AbY(1)



AbX AbY(2)



Unspecific binding by Ab?

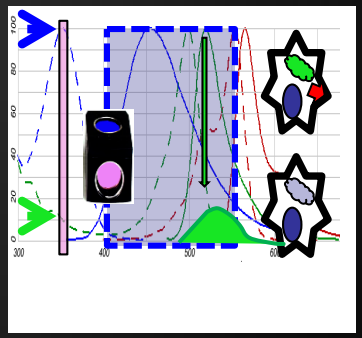
cell with - without target X

Appropr. fixation?

Fixation A

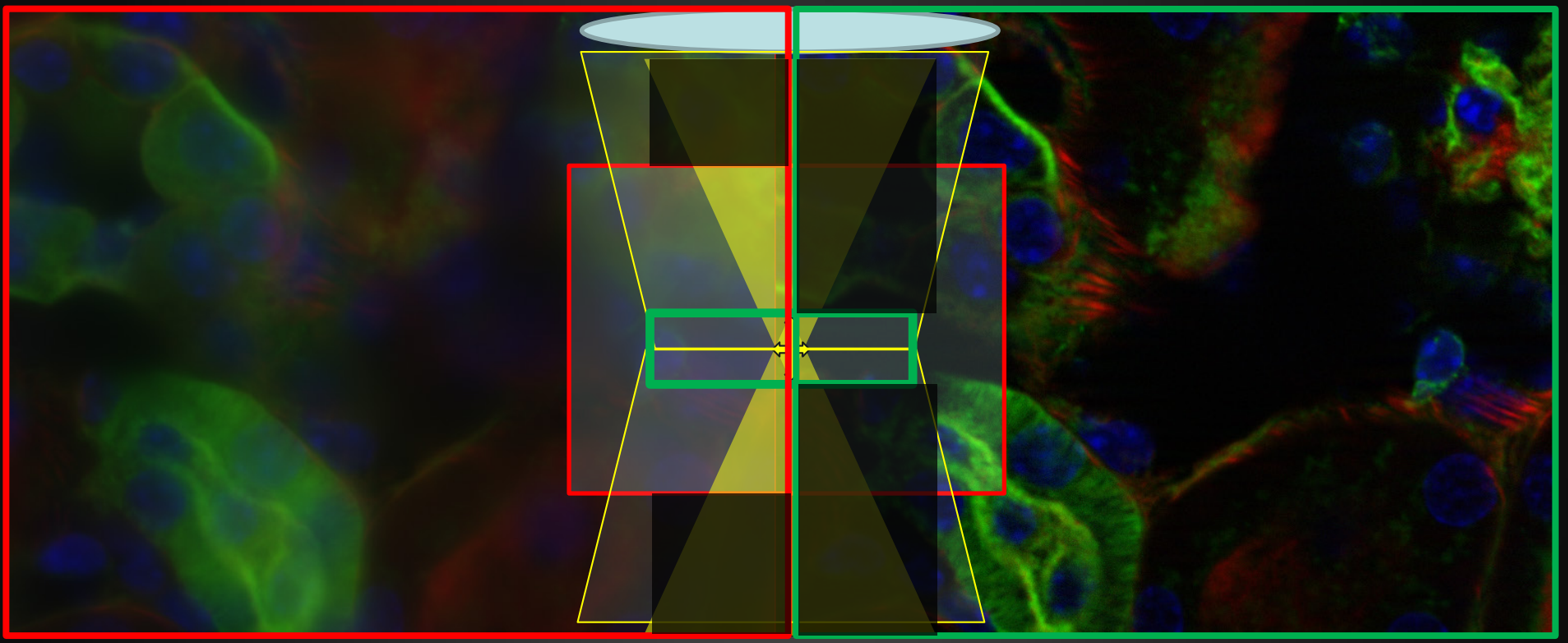
Fixation B

Crosstalk/ Bleeding through?



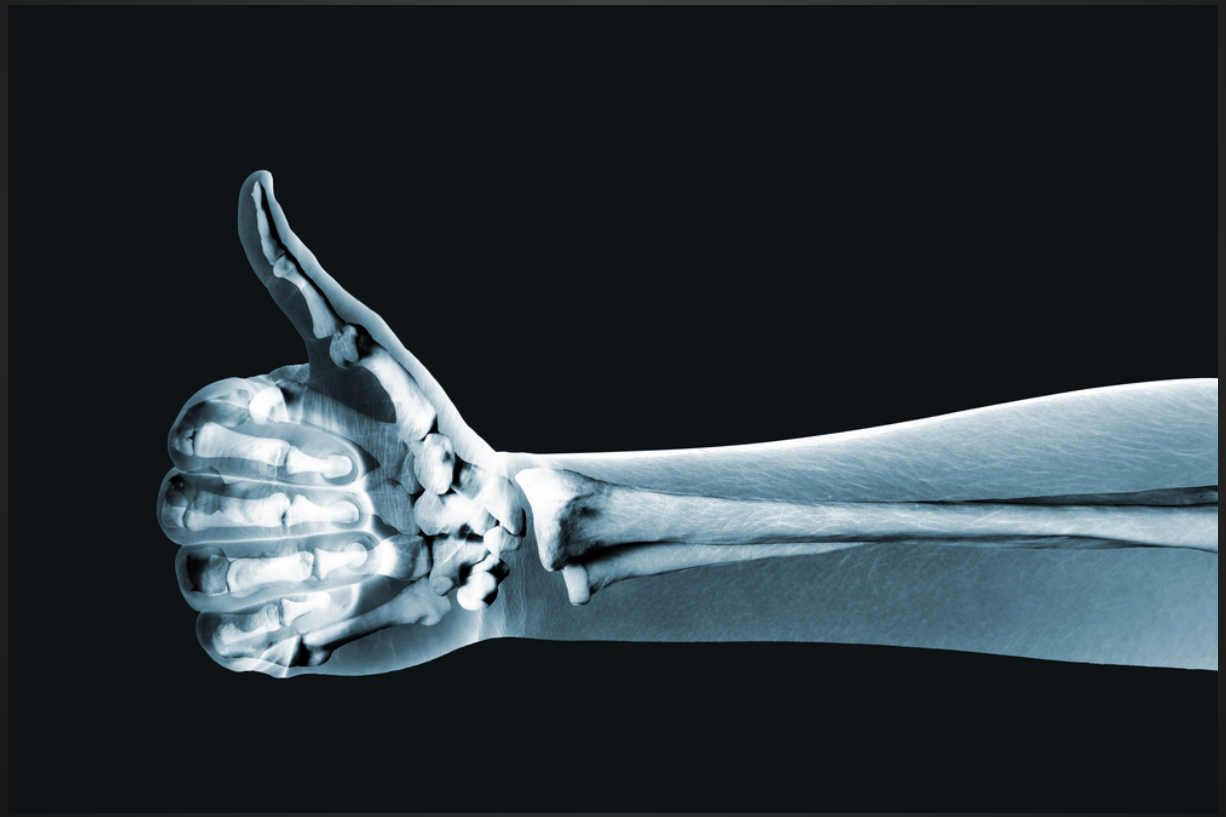
Use quality objectives, correct filter, embedding medium to avoid abberations

Widefield vs Optical section

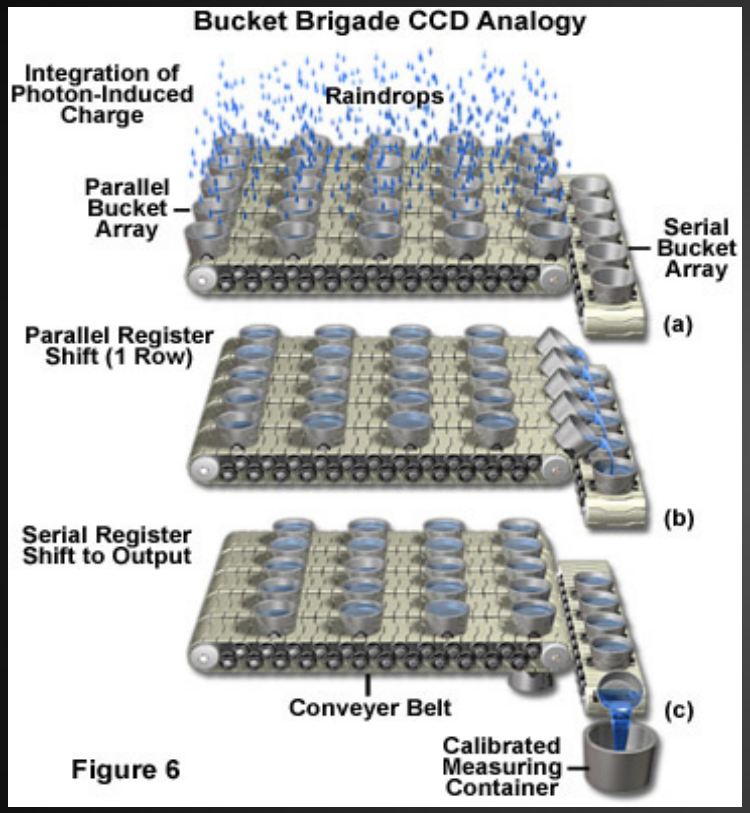


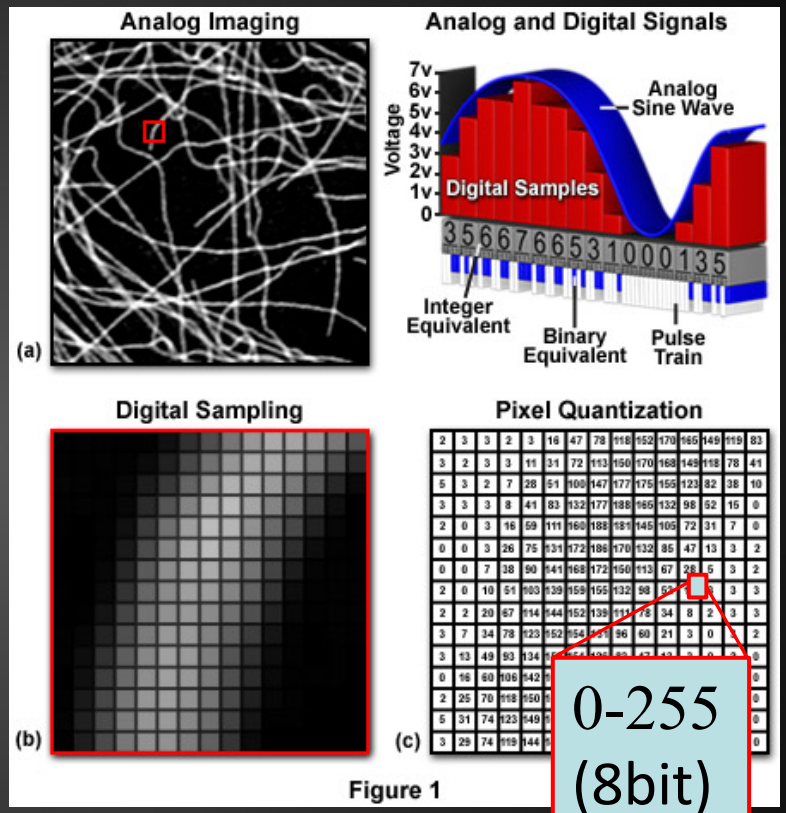
Kidney sample 10µm thick, 63x/NA 1.43,
Widefield image and optical section using Apotome technique.

IMAGING



Imaging – digital camera - pixel





- All pixels of the camera will be exposed to light at once; image is processed all pixels at once
- Black&White cameras pixel does not care about color.

For Fluorescence microscopy use B&W cameras (with appropriate filtercubes)

Imaging – Features of a digital camera

Spatial Resolution:

ability to capture fine specimen details without pixels being visible in image
(1308x1040 pixel, 6.45x6.45µm pixel on 2/3 ´´ chip)

Light-Intensity Resolution:

dynamic range or number of gray levels that are distinguishable in image.
(12 bit or 16 bit)

Time Resolution:

frame rate - the ability to follow movement or rapid kinetic processes
(38 fps)

Signal-to-Noise Ratio:

visibility and clarity of specimen signals relative to the image background

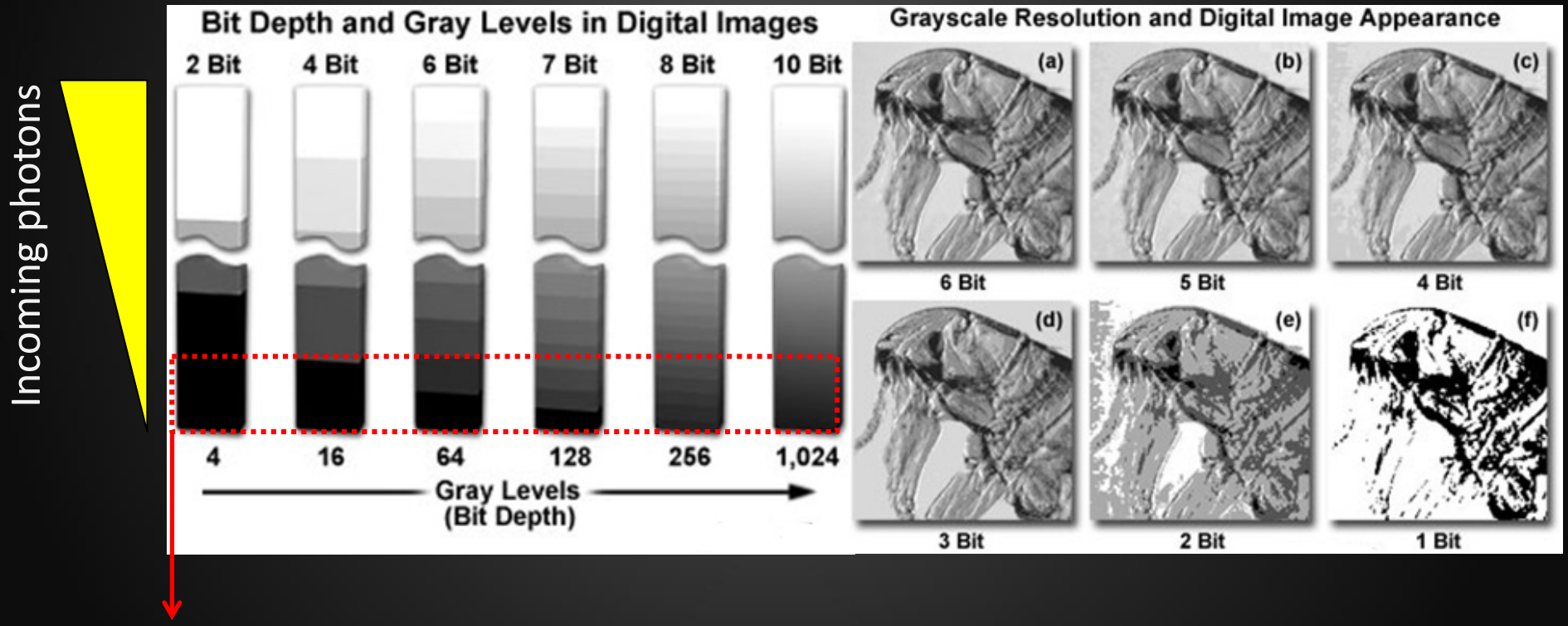
Spectral Sensitivity:

range of wavelength on which camera reacts
(350-1000 nm)

(data from Zeiss Axiocam MRm)

Image quality – dynamic range

Acquire your image with appropriate grey level (8bit at least) to represent different intensity levels .

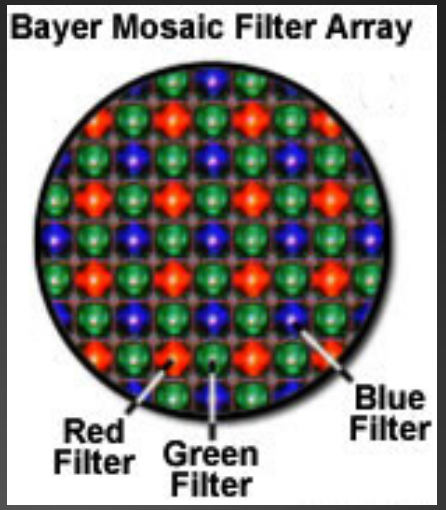


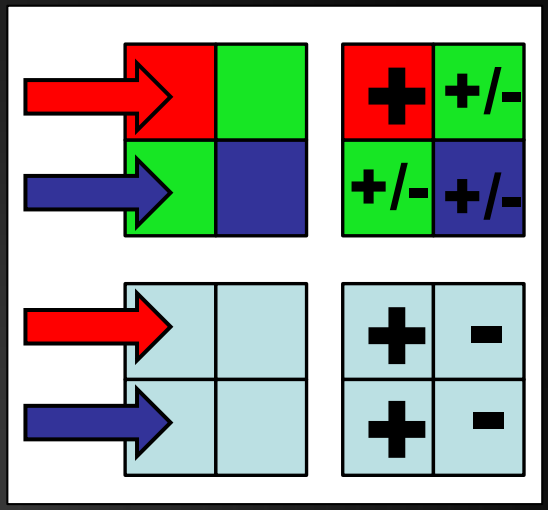
→12 or 16 (4096 – 65536 grey levels)bit images would also allow you e.g. to enhance features lying in the dark to stretch them into the light...

Imaging – color vs black&white

In Color Cameras each pixel is overlaid by color filter lense pattern <u>The Bayer mosaic</u>.

→Reduction of sensitivity and actual resolution





Color pixel ”red” only lets pass light in ”red” range (**+**) → signal. Rest of pixels are calculated in respect to surrounding pixels (**+/-**). i.e. 66% (2 of 3 colors /px). More green in the Bayer mosaic, therefore human eye is more sensitive to green.

Problem: Actual resolution is 2x2 pixel i.e. 4x less
Solution: camera with moveable chip are used → each pixel will sample light from (9) different positions. → High resolution Brightfield

Black&White cameras pixel does not care about color.
For Fluorescence microscopy use B&W cameras (with appropriate filtercubes)

Publishing photos

- Use the highest bit depth if needed, and take care about over/underexposure
- Use the highest quality/resolution as possible
- Use TIF files
- Crop image if neccessary
- Use measure bar to show scale
- Do not use total magnification, e. g. objective magnification - 60X (which is of course not 60X magnification but 600X at least)

Magnification
”1000x”

VS

Measure bar
(20.000 light years)

http://apod.nasa.gov/apod/ap090917.html

THANKS FOR YOUR ATTENTION!