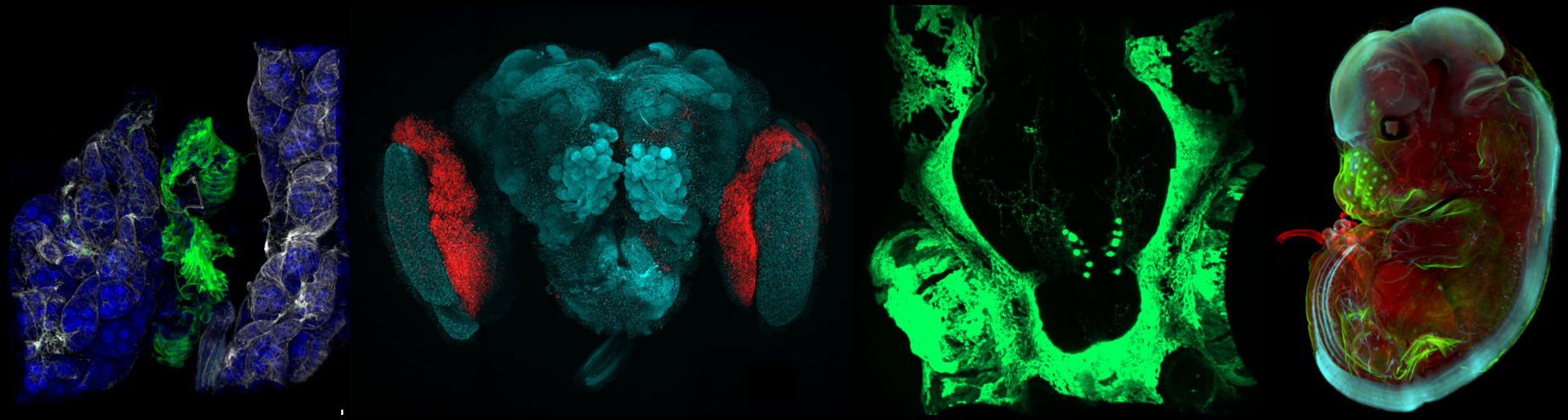
Microscopy in Biomedical Sciences

“Through the Looking Glass”
A Graduate Introduction to Biological Microscopy
Across History, Scales and Techniques



Christina Pyrgaki, PhD
Head of the Advanced light microcopy lab (ALMIL), MSKCC
09/04/2025

ALMIL

Advanced Light Microscopy and Innovation Lab

Mission of the lab:

- **To provide access to** cutting-edge imaging tools and expertise as needed for transformative discoveries
- **To advance light microscopy techniques** through innovation, collaboration, and education.
- To serve as a **center of excellence** for the design, optimization, and application of advanced optical imaging solutions within MSK and the Tri-I community.

Available Technologies at the ALMIL

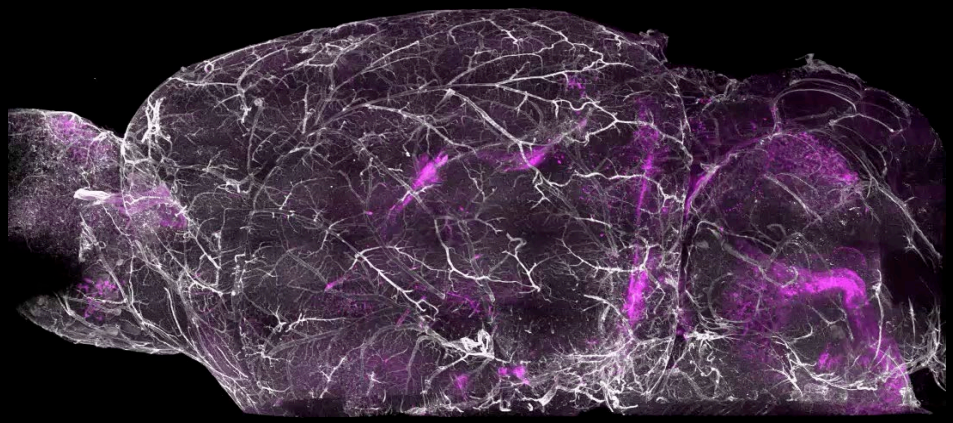
Light Sheet Microscopy (RL1024)

Super Resolution (SoRa RL1139)
(STED/MINFLUX RL561)

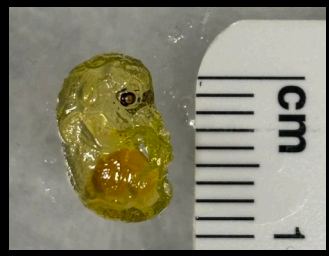
Multiphoton Imaging (RL651)

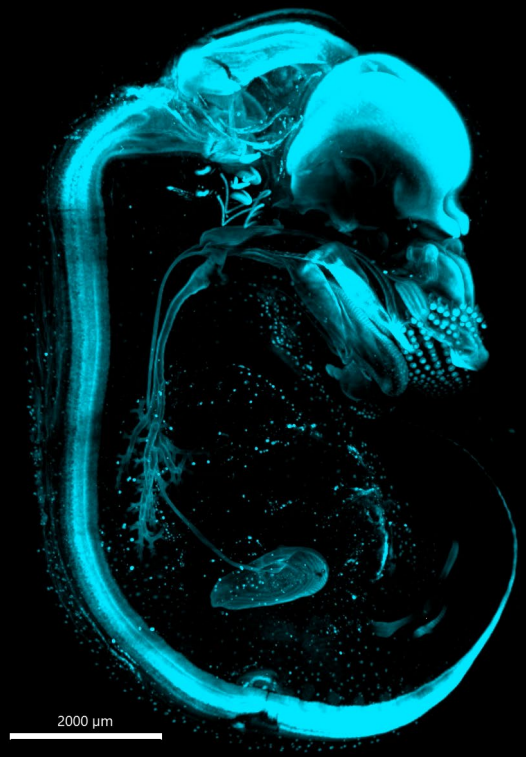
LCS Light Sheet From Luxendo

Optically Cleared brain



E12.5 Mouse Embryo

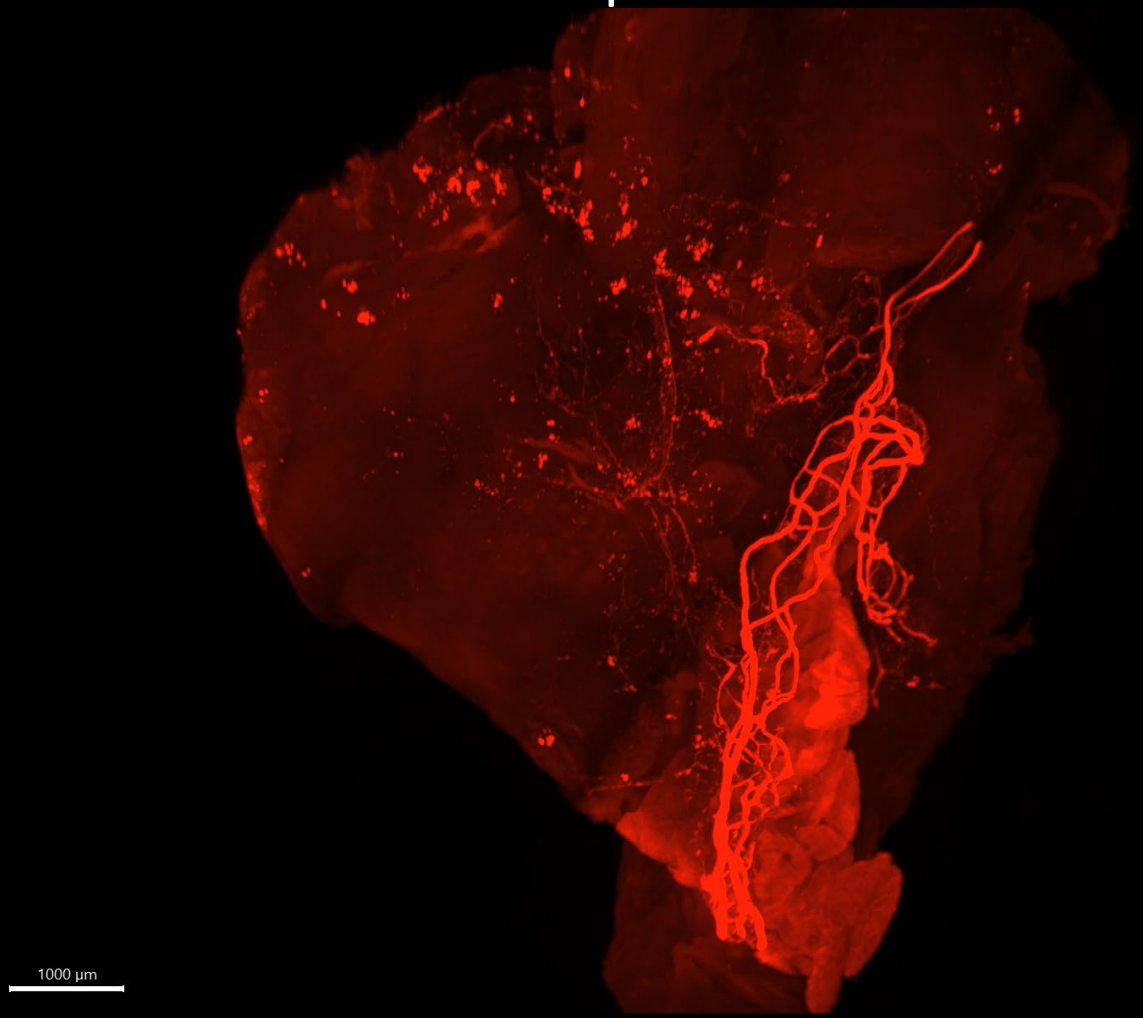




Sample provide by MO Gaite

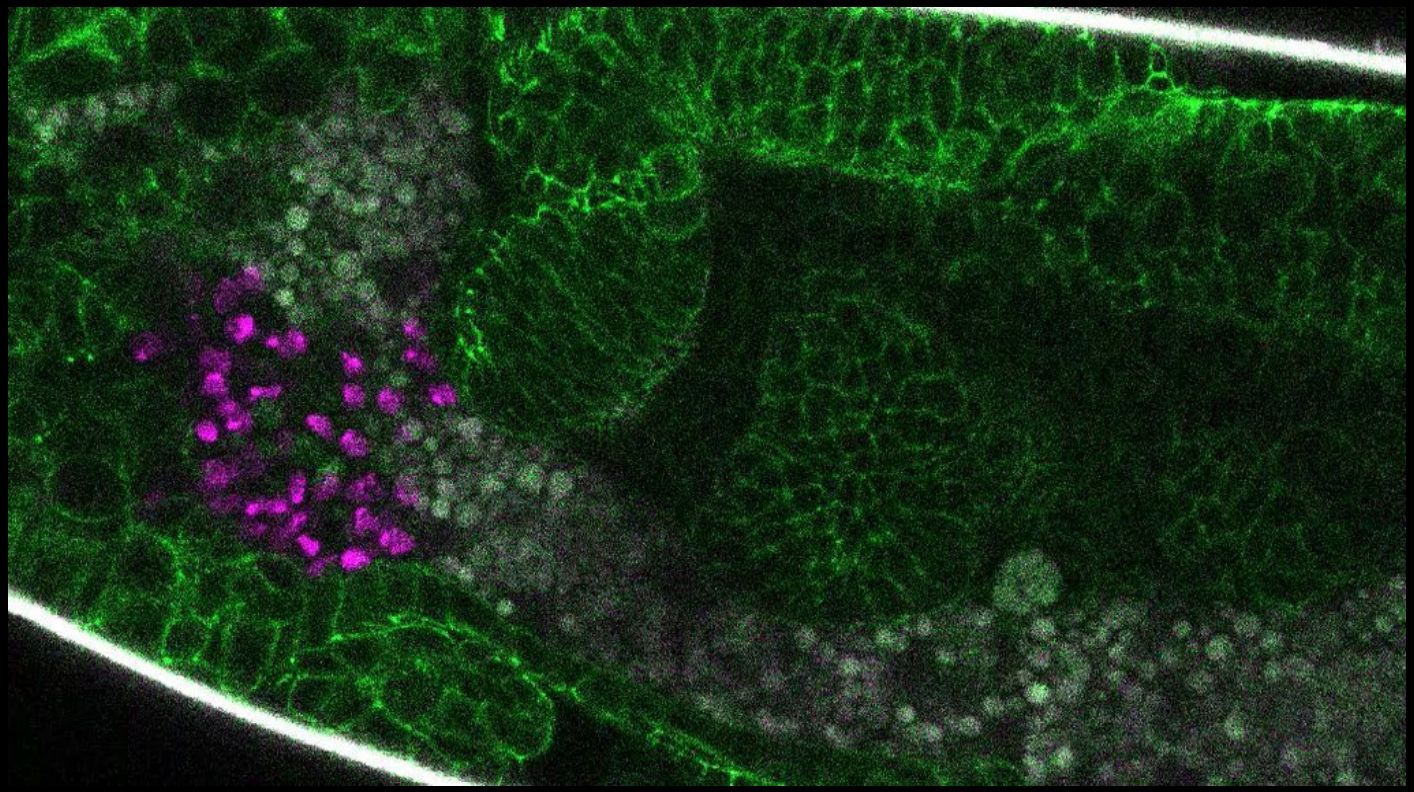
LCS Light sheet

Whole mouse pancreas Innervation



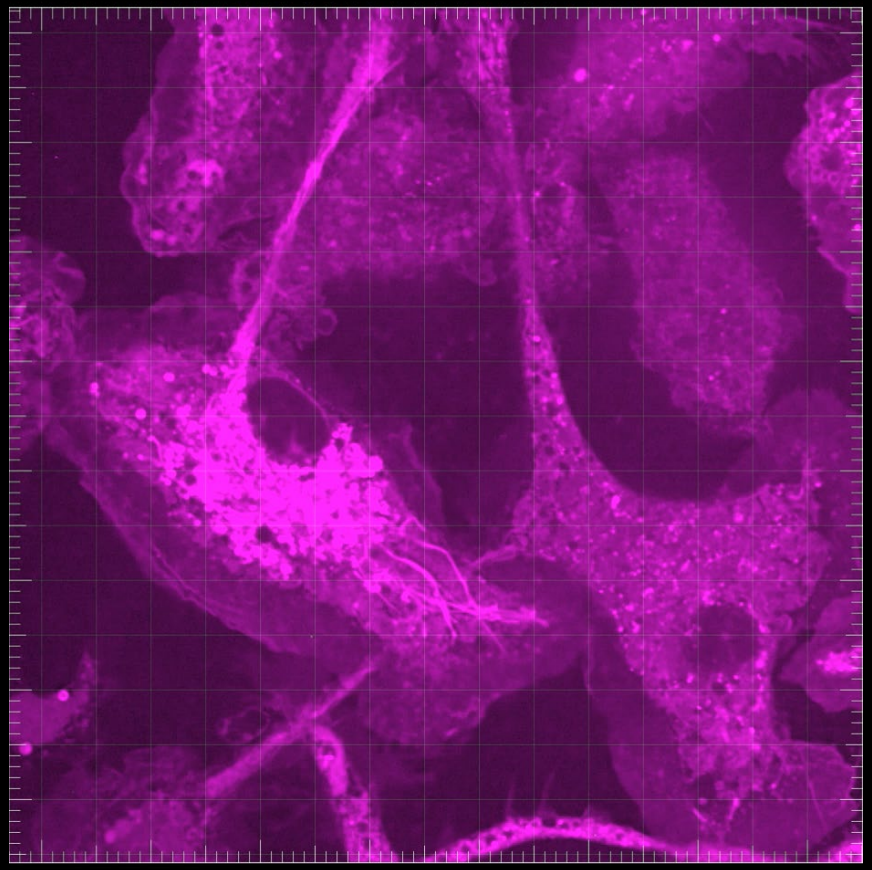
Sample provided by Zainab Hussain

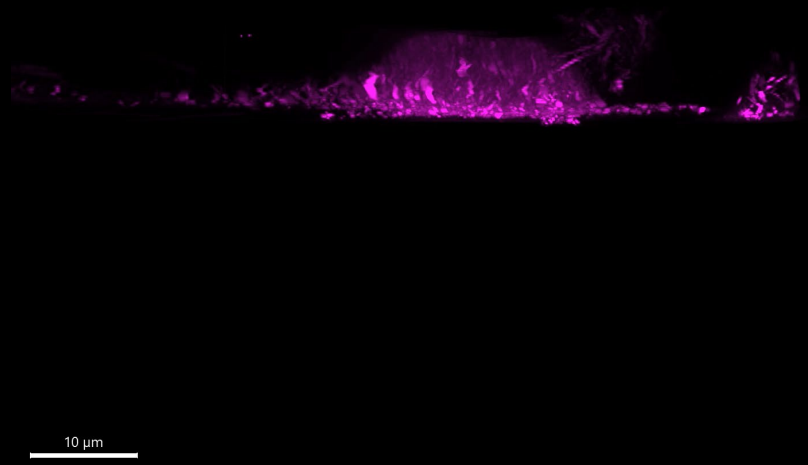
Leica Stellaris Dive Multiphoton



Drosophila Embryo E-caherin GFP and H2A-mCherry expressed in macrophage nuclei. 40x lens
Image provided by Masha Akhmanova.

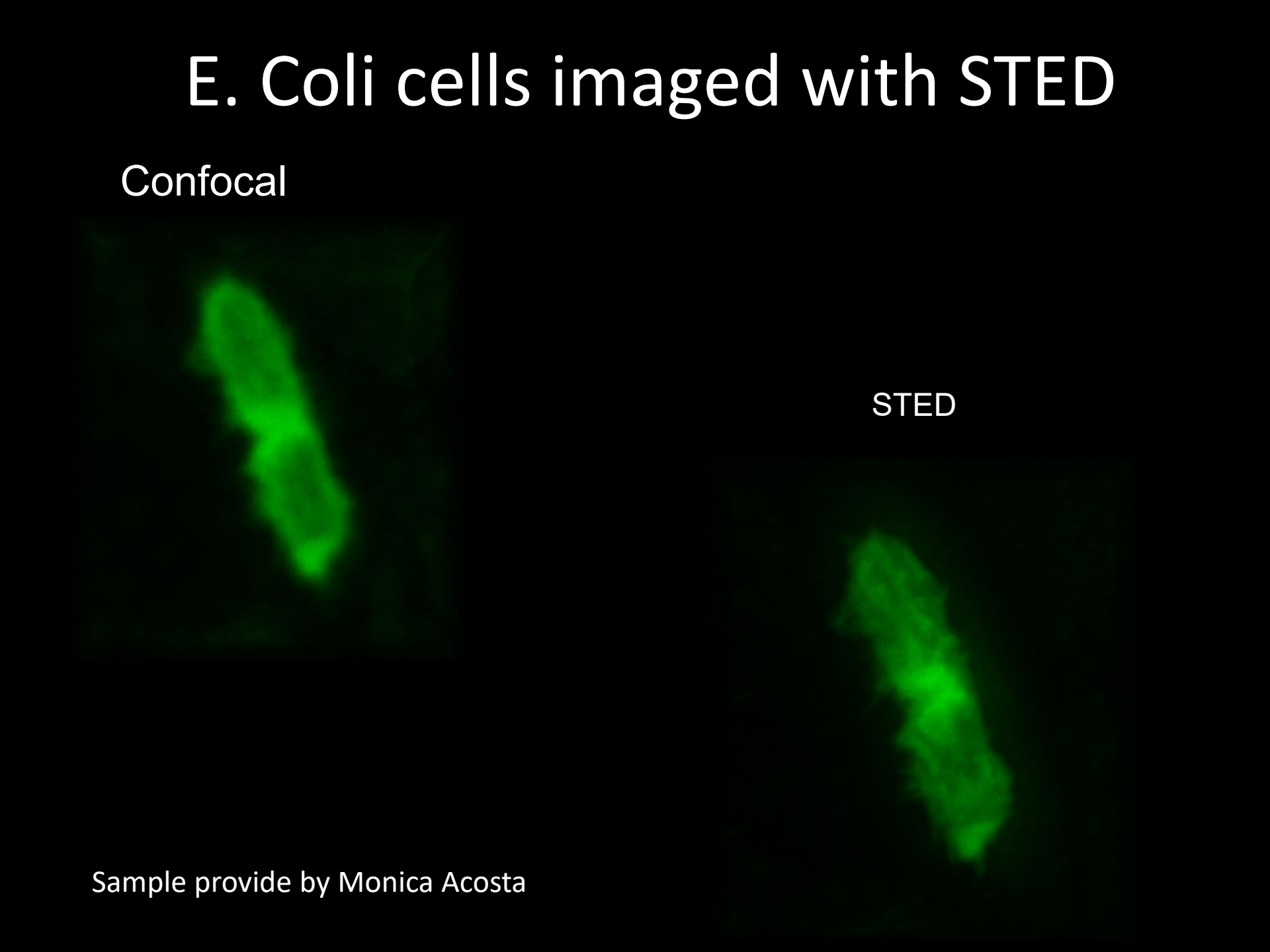
SoRa Spinning Disk





Live imaging of lysosomes
Sample provide by Monica Acosta

E. Coli cells imaged with STED



Sample provide by Monica Acosta

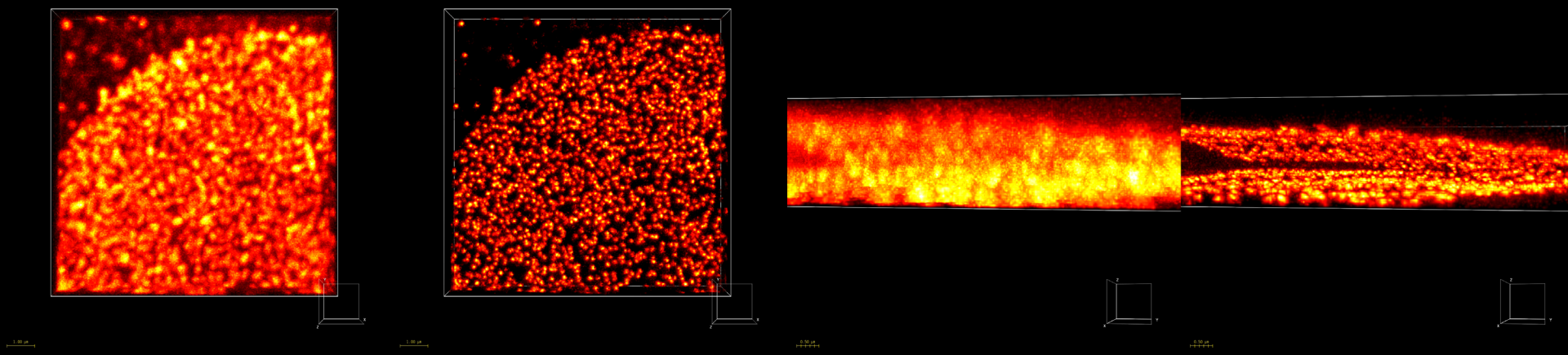
Isotropic 3D STED super-resolution imaging

Confocal

90% 3D STED

Confocal

90% 3D STED



Nuclear pore complexes were labelled with STAR RED / Pan-FG repeat antibody that detects the central spoke (~80 nm in diameter) of the nuclear pore complex.

Sample provided by Abberior Instruments

Why microscopy?

Microscopy and Biology: More than meets the eye

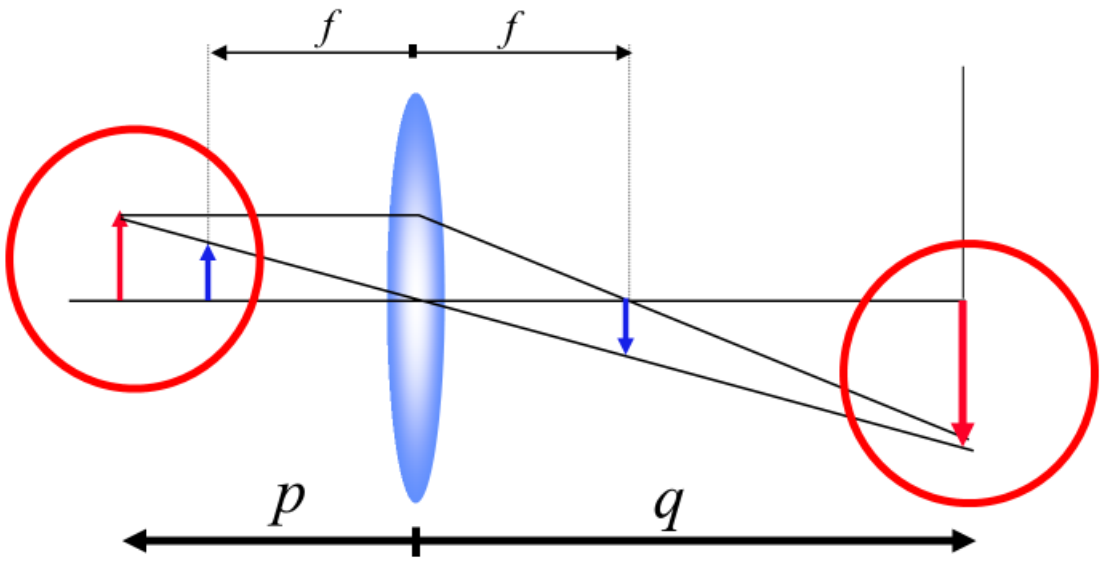
Microscopy provide information on:

- The **structural organisation** of the biological specimen;
- The **location** of a molecule (e.g. protein or DNA sequence) in relation to specific cellular structures;
- The **spatial relationship** between different molecules and structures
- **Connectivity** and **interactions** between different organelles or cellular domains
- Protein trafficking and **kinetics** of movement;
- C**hanges** in localisation or abundance of molecules during development, disease or drug treatment.

Convex Lenses and Image Formation

Thin lenses and magnification

Properties of thin Lenses



$$\frac{1}{p} + \frac{1}{q} = \frac{1}{f}$$

$$\text{Resolution } (R) = 0.61 \times \frac{\lambda}{NA}$$

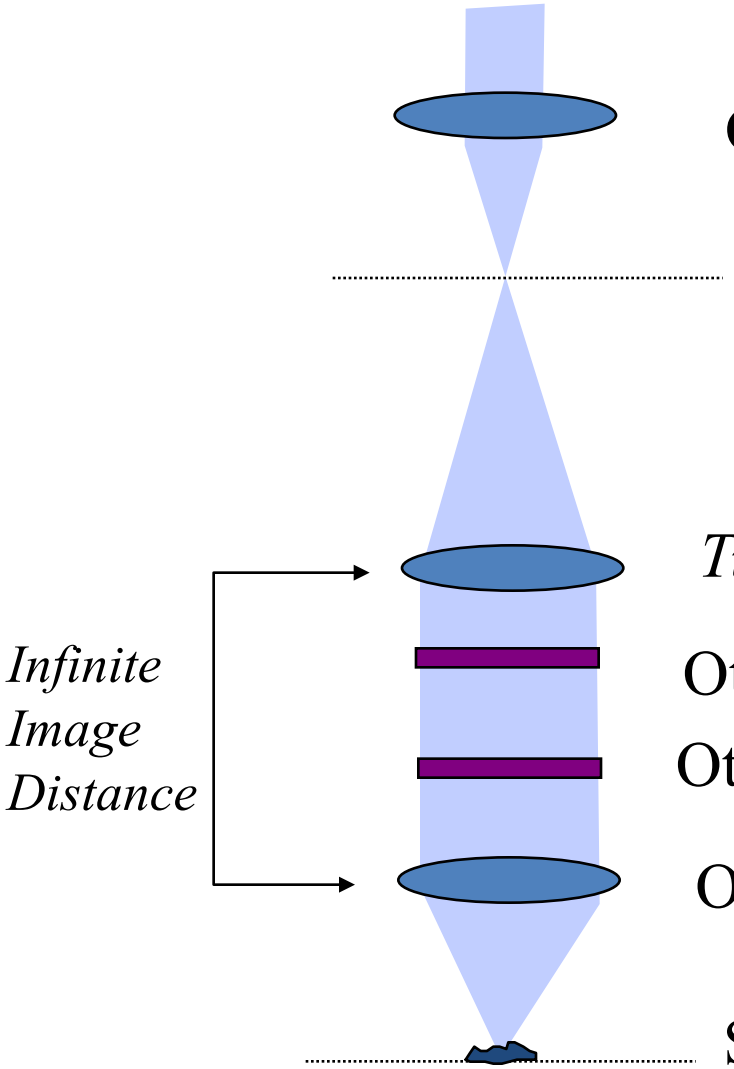
(lateral)

(Rayleigh criterion)

$$\text{Magnification} = \frac{q}{p}$$

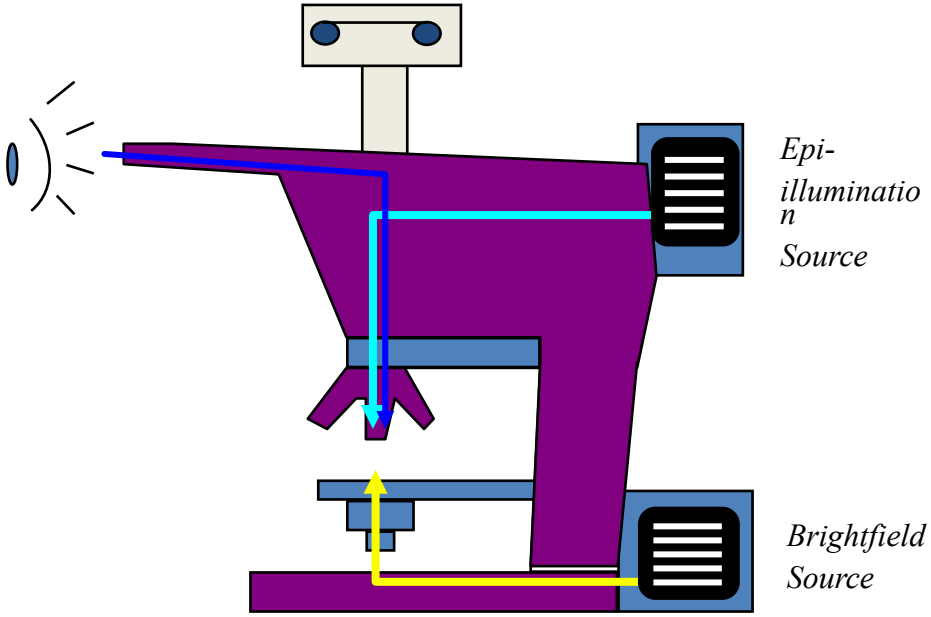
Compound Microscope Design

Infinity Optics

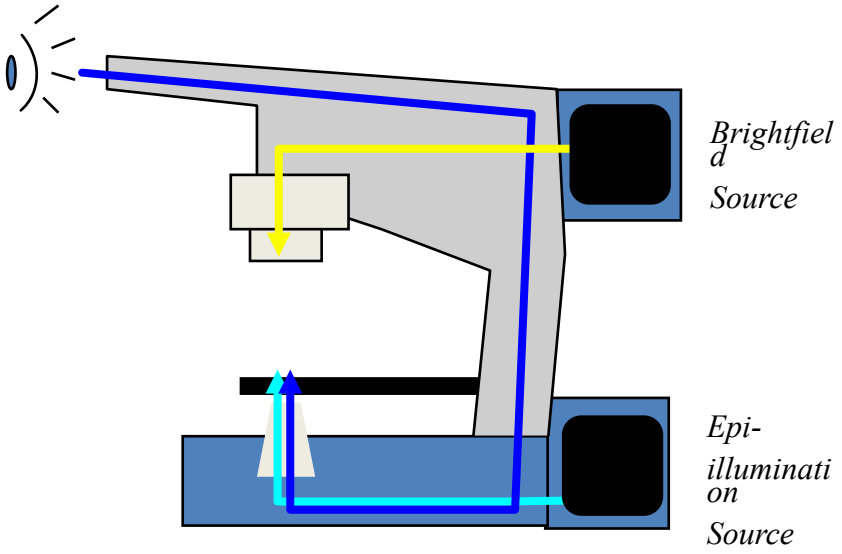


The main advantage of infinity corrected lens systems is the relative insensitivity to additional optics within the tube length. Secondly one can focus by moving the objective and not the specimen (stage)

Upright Microscope



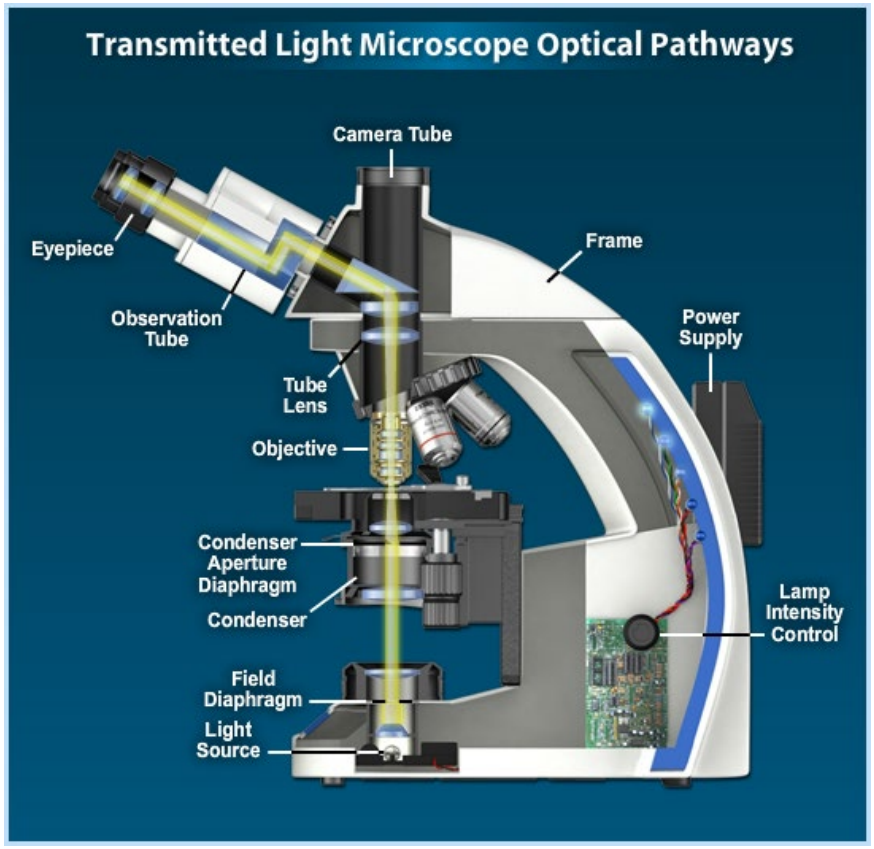
Inverted Microscope



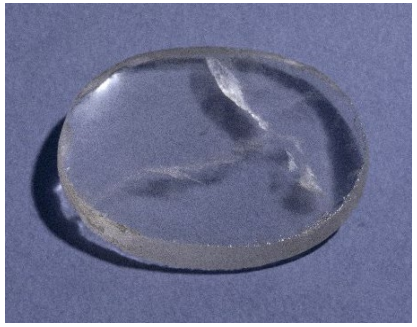
Different configurations serve different applications. Take that into account when you design your experiments

Know your light path. Troubleshooting bad imaging can become a painful experience if you don't know your microscope

The transmitted light train



The evolution of lens:
The ultimate star of the microscope



“Half” of a Zeiss Microscope objective

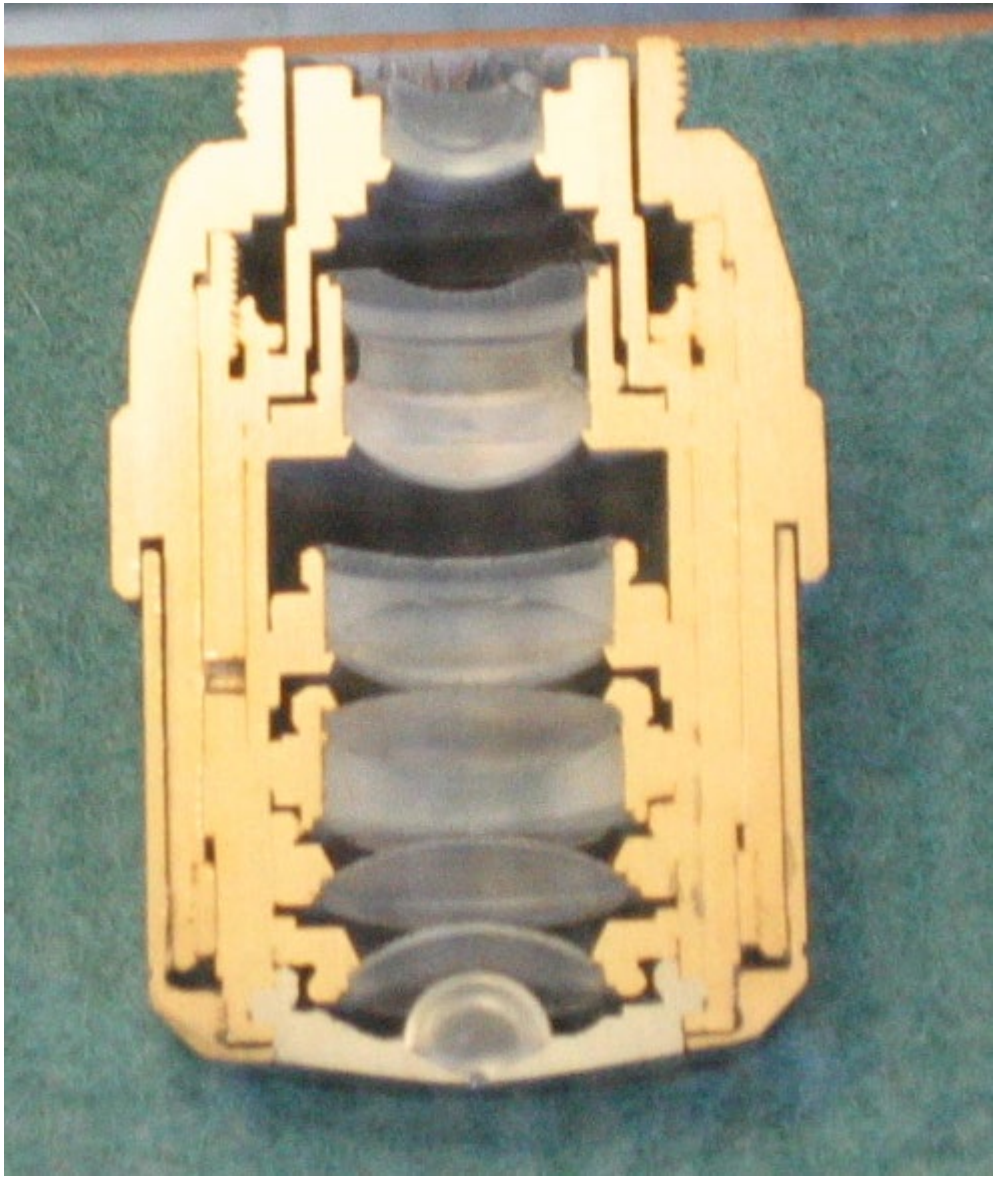
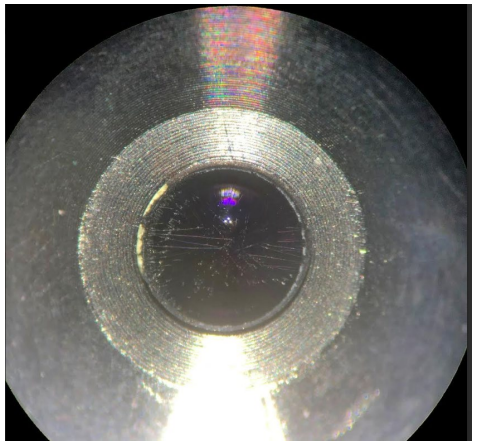
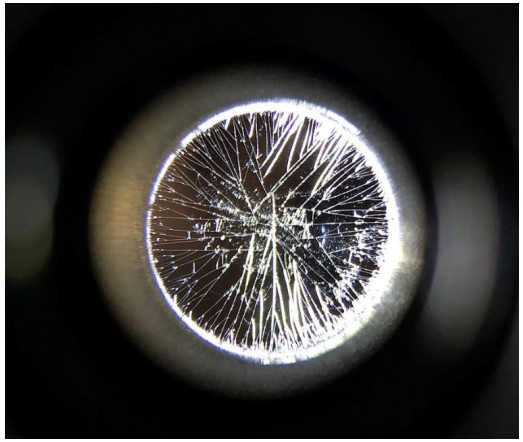


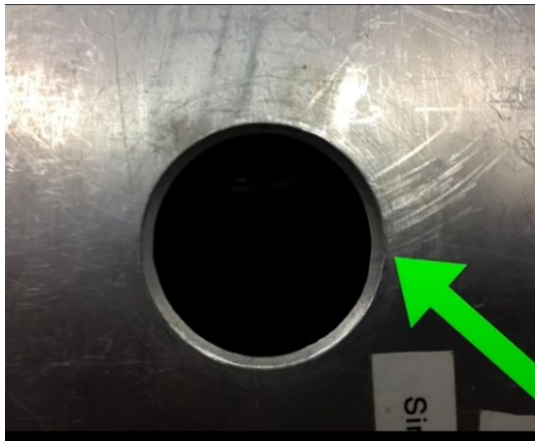
Photo from
Zeiss Museum

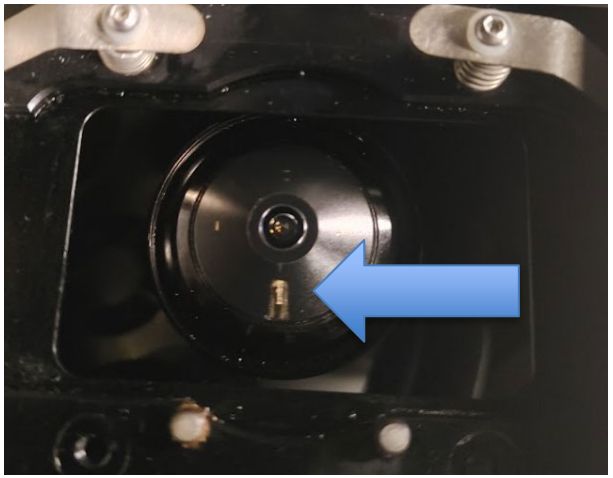
Be extremely careful when you are handling objective lenses

A lens can be damaged due to a variety of reasons: collisions, immersion media, lasers, etc
Repairs even if they are possible, they are costly and time consuming.









Also be extremely careful when selecting an objective…

- ✓ Magnification
- ✓ Resolution
- ✓ (NA)
- ✓ Working Distance
- ✓ Image Quality (flatfield, corrections for various aberrations)
- ✓ Spectral Transmission
- ✓ Immersion choices
- ✓ Correction collars
- ✓ Contrasting Technique (Phase, Pol, DIC)
- ✓ Mechanical, Thermal, Chemical Stability
- ✓ Minimized auto-fluorescence

Plan Apo 100x/1.30 oil

Lens resources

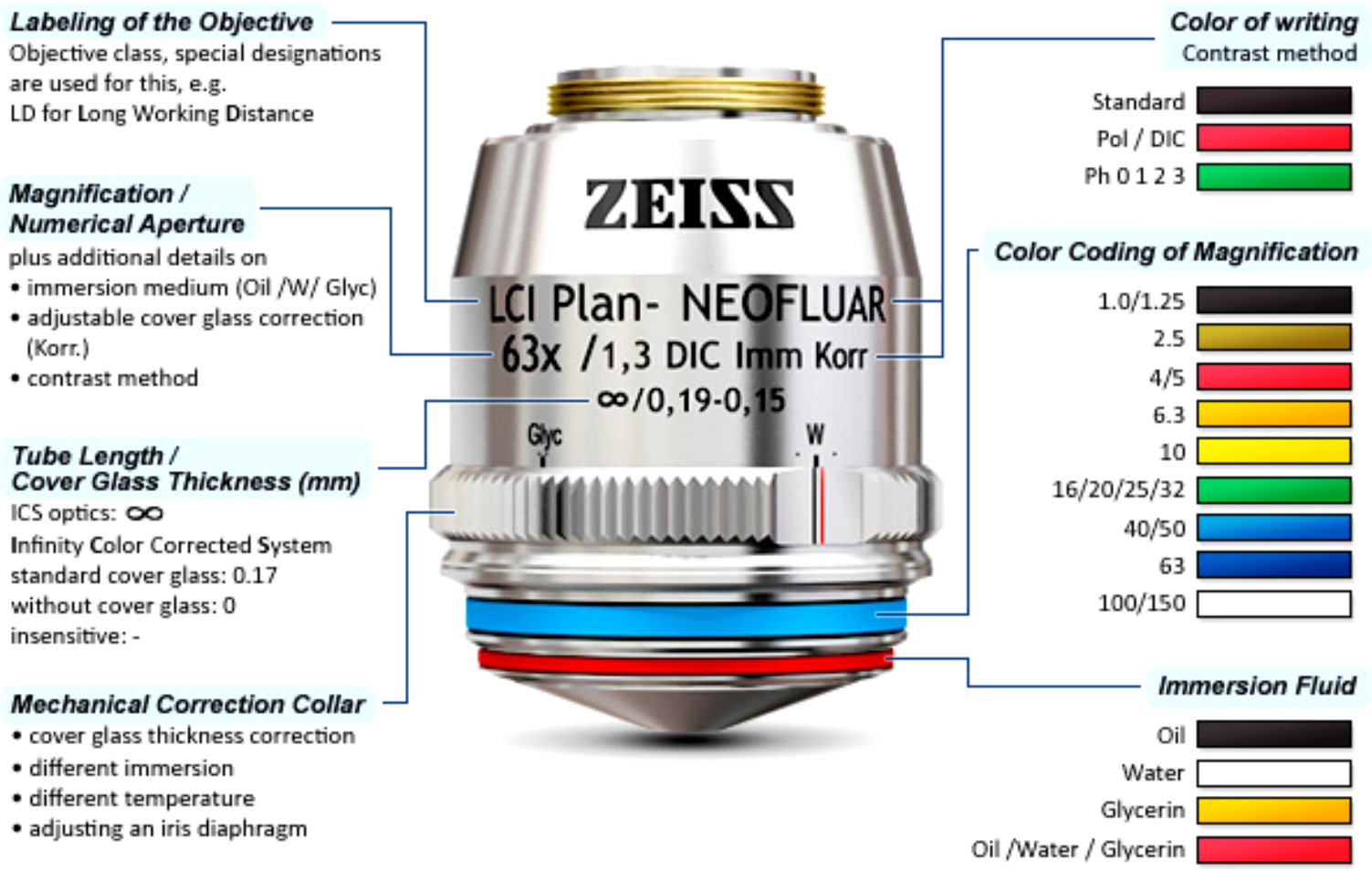
https://www.micro-shop.zeiss.com/en/us/shop/objectives

https://microscopecentral.com/collections/olympus

https://www.microscope.healthcare.nikon.com/products/optics/selector

https://www.leica-microsystems.com/products/microscope-accessories/microscope-objective-lens/

Objective labelling

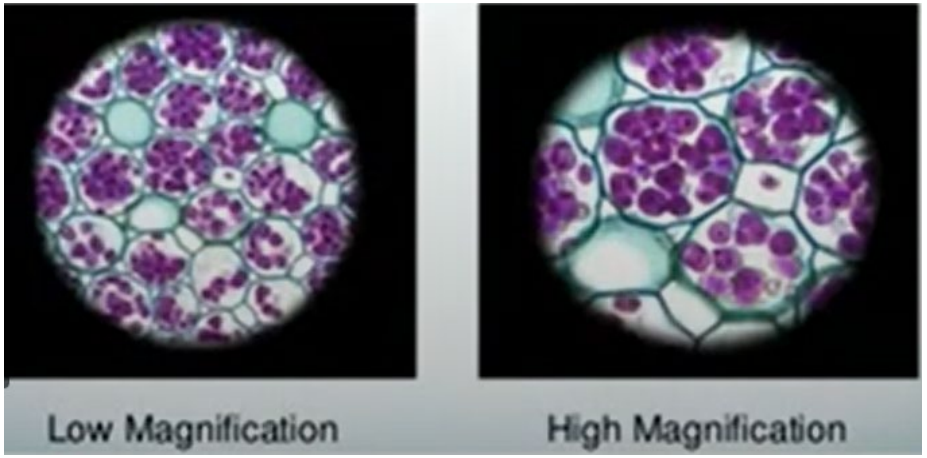


Microscope objectives follow a color code that allows immediate recognition of important objective parameters (e.g. magnification, N. A.).
The standard colour code of objectives was introduced to microscopy in 1953 by Dr. Kurt Michel at CARL ZEISS

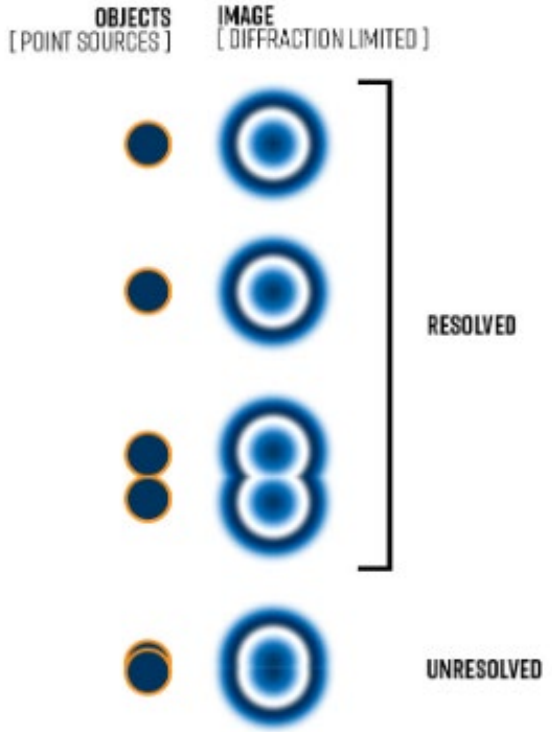
Magnification vs Resolution

- Total **magnification** =

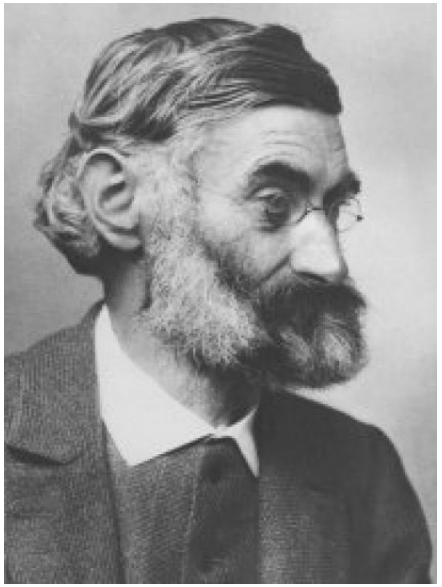
eyepiece lens magnification x objective lens magnification



- **Resolution** is the ability of a microscope to distinguish two separate point emitters on a sample as separate objects

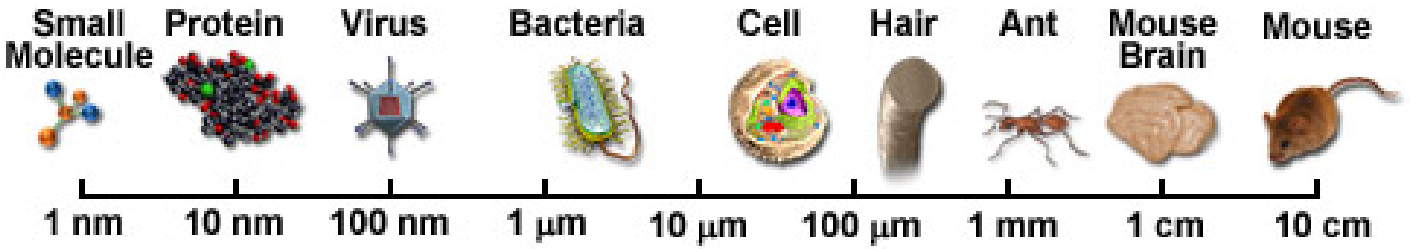


Diffraction Limited Optics
The Abbe equation

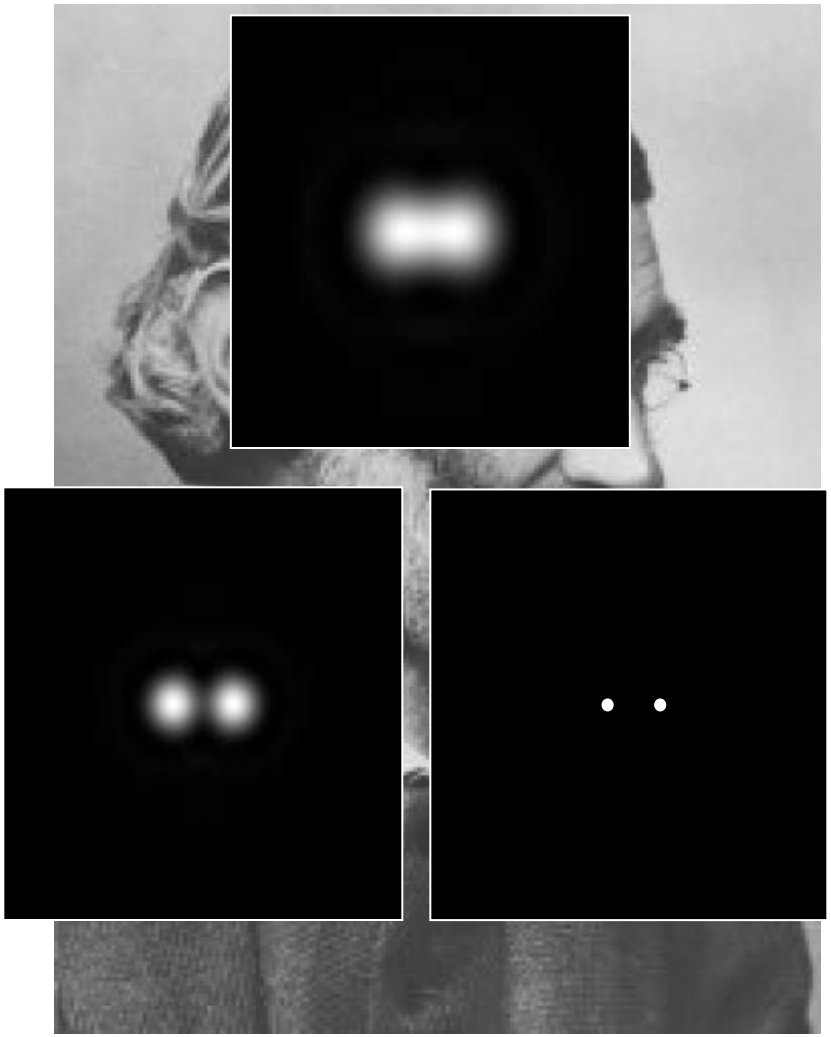


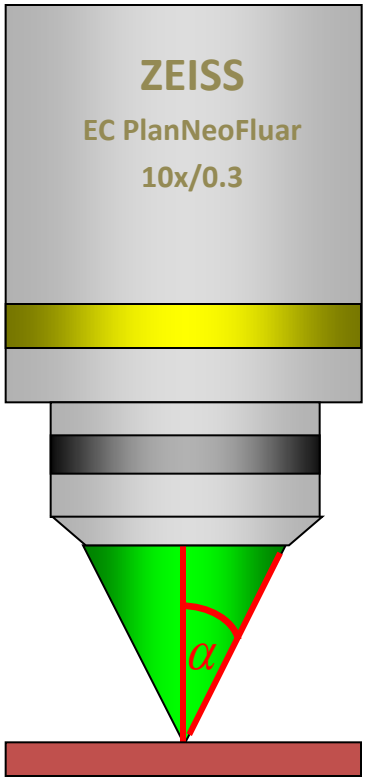
$$d = \frac{\lambda}{2n\sin\alpha}$$

$$\frac{400\text{nm}}{2\,(1)\,(\sin 90)} = 200\text{nm}$$



Diffraction Limited Optics
The Abbe equation





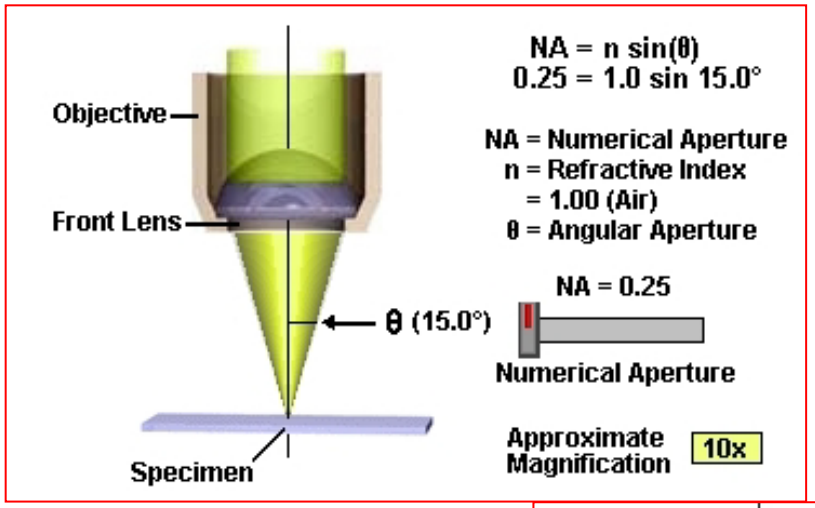
$$d = \frac{\lambda}{2n\sin\alpha}$$

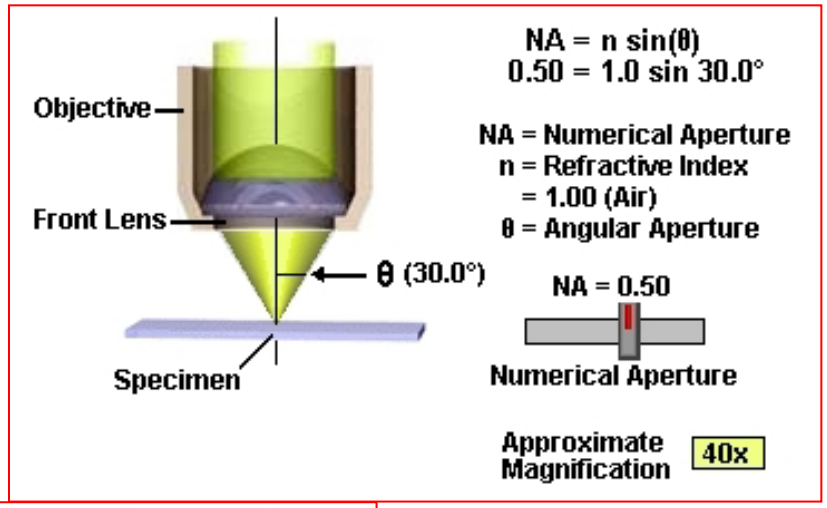
$$NA = n \cdot \sin\alpha$$

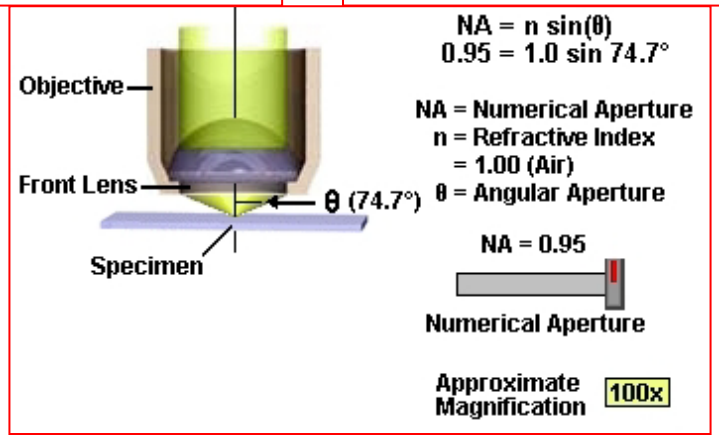
d	resolution
λ	wavelength
NA	numerical aperture
α	opening angle
n	refractive index

Numerical Aperture

- The wider the angle the lens is capable of receiving light at, the greater its resolving power
- The higher the NA, the shorter the working distance







Images reproduced from: http://micro.magnet.fsu.edu/ Go to the site and check out the tutorials. You will not be disappointed. Excellent learning tools.

How immersion medium affects the true N.A., and consequently, resolution

No immersion (dry)

- Max. Value for α = 90° (sin = 1)
- Attainable: sinα = 0.95 (α = 72°)

- Actual angle a1:

$$\alpha_1 = \text{arcsine}\frac{NA}{n} = \text{arcsine}\frac{0.95}{1.52} \approx 39^o$$

With immersion oil (3) n=1.518

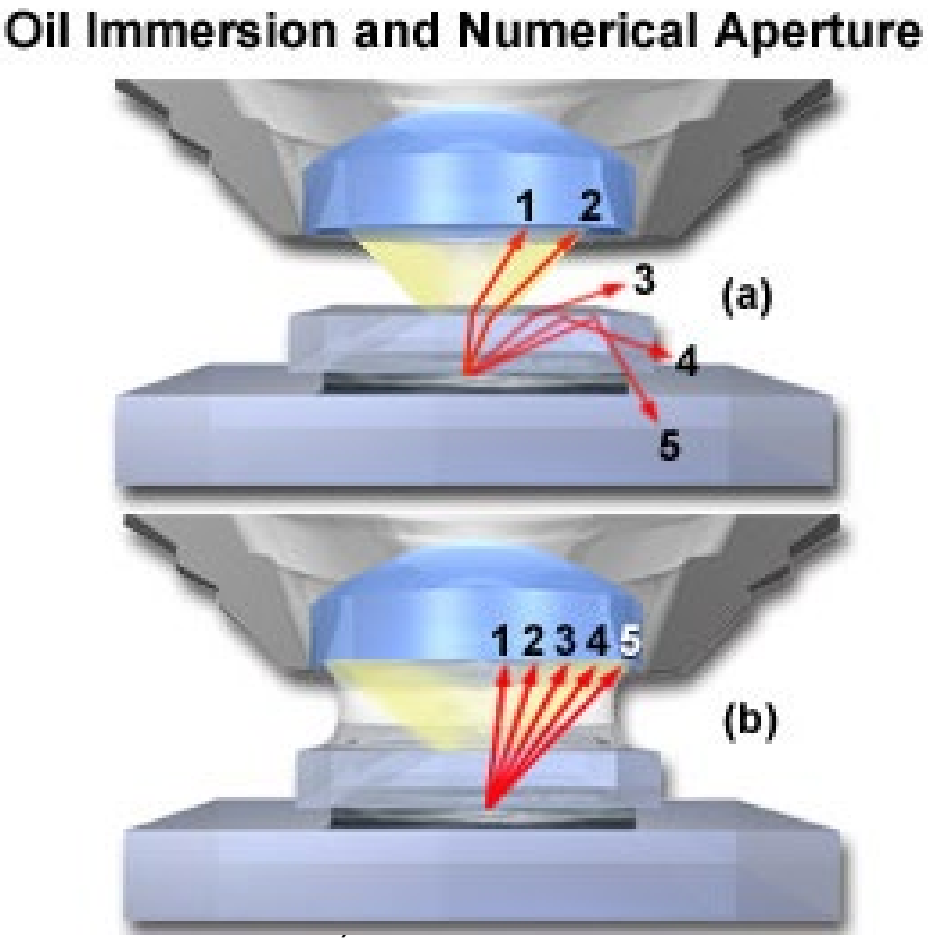
- No Total Reflection
- Objective aperture fully usable

- N.A.max = 1.45 > Actual angle a2 :

$$\alpha_2 = \text{arcsine}\frac{NA}{n} = \text{arcsine}\frac{1.45}{1.518} \approx 73^o$$

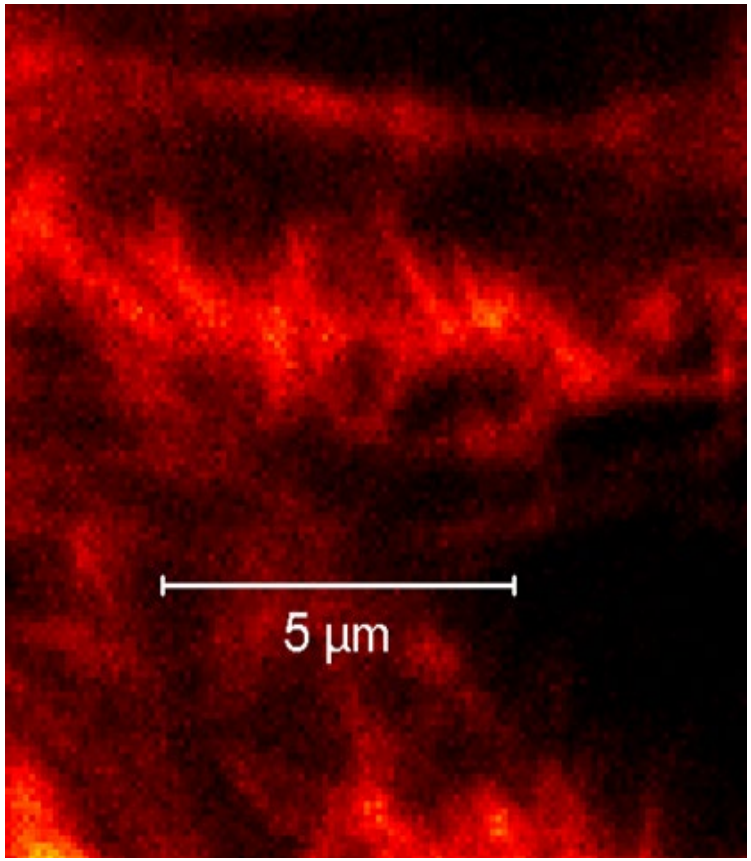
Snell's Law:

$n_1 \sin \beta_1 = n_2 \sin \beta_2$

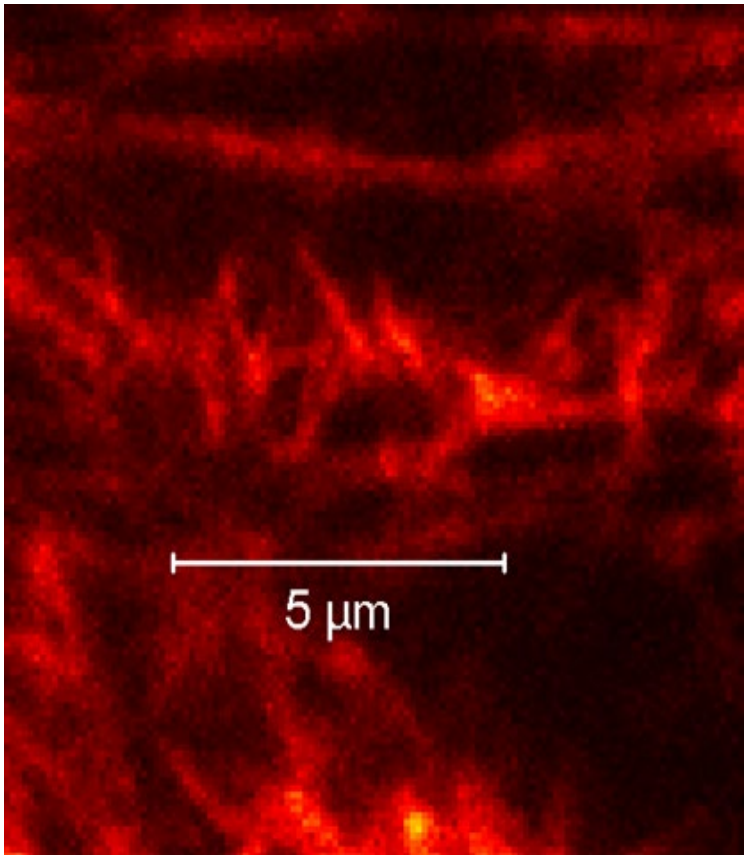


The first oil- immersion objective was introduced in 1879 by CARL ZEISS

The effect of Numerical aperture in the final image



Plan Apo 63x/1.4



Alpha Fluar 100x/1.45

Things to keep in mind

- The intensity of light collected **decreases** as the square of the magnification
- The intensity of light **increases** as the square of the numerical aperture
- The higher the NA the lower the working distance
- Thus when possible, use **low magnification**

or **high NA** objectives, while keeping in mind the working distance

Aberrations and Corrections

Optical Aberrations in Microscopy

- **Spherical Aberration**

Rays at lens edges focus differently

- **Chromatic Aberration**

Different wavelengths focus at different points

- **Coma**

Off-axis point sources appear comet-shaped

- **Astigmatism**

Point objects appear stretched in one direction

- **Field Curvature**

Flat specimen cannot be in focus across field

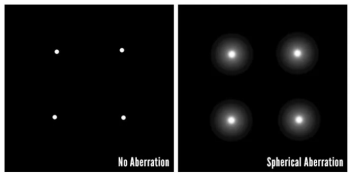
- **Distortion**

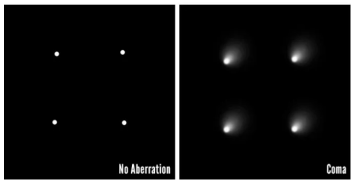
Straight lines appear curved (barrel/pincushion)

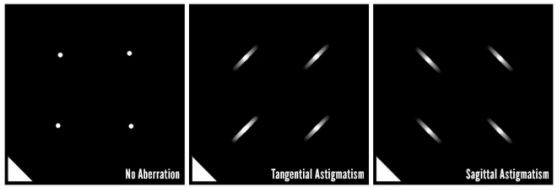
- **Photobleaching & Phototoxicity**

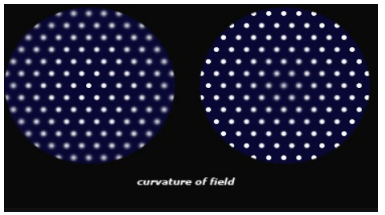
Practical fluorescence limitations

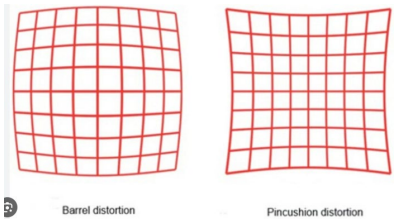
https://www.handprint.com/ASTRO/ae4.html#distortion









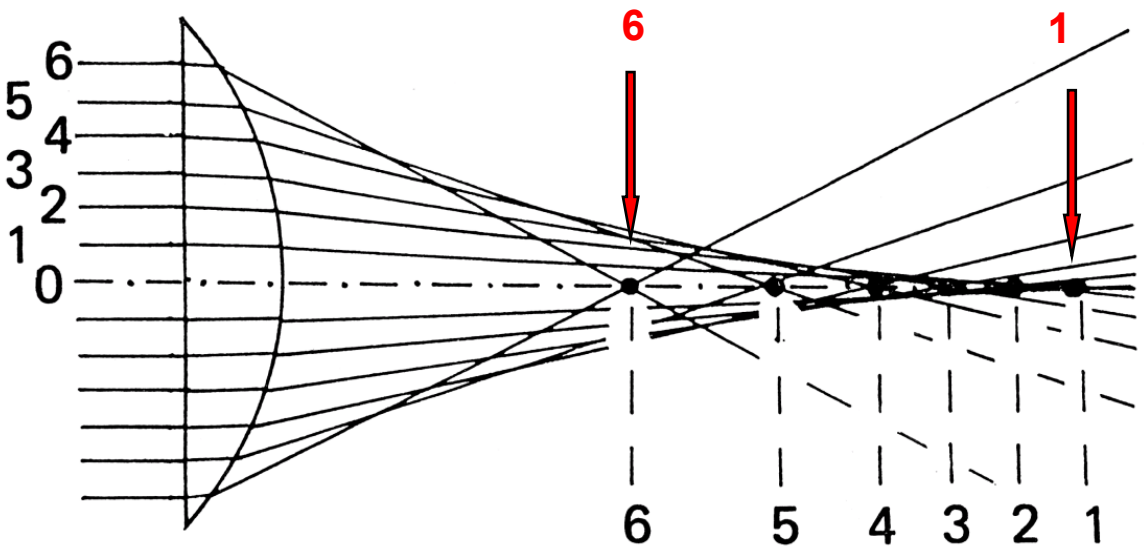


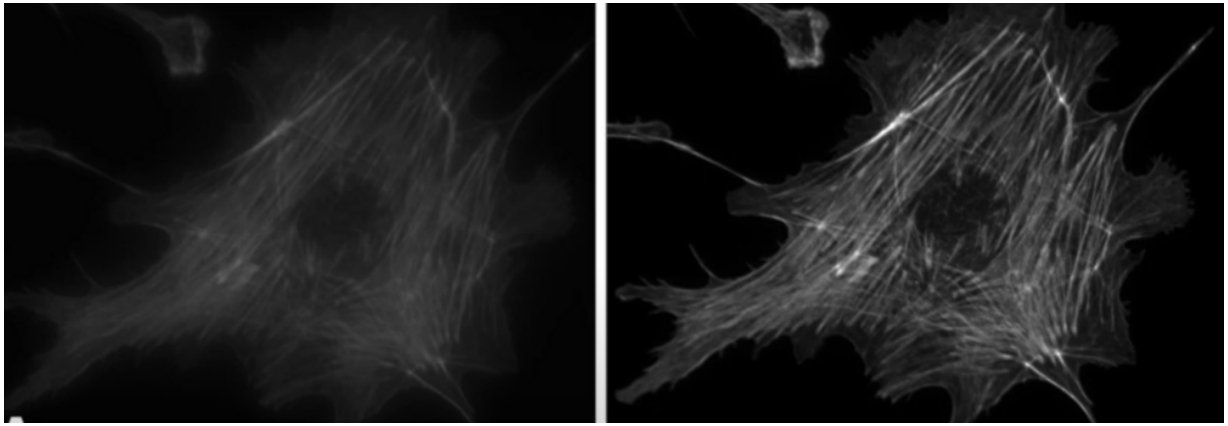
Spherical aberration inherent to the lens

A simple spherical lens has a different focal length for light rays passing the outer zones (4-6) compared to the central rays (1-3)

Spherical aberration introduces a haze into the image
It is prominent with all (dry) objective magnifications 40:1 and higher

Spherical aberration can be compensated albit not fully by the objective lens design





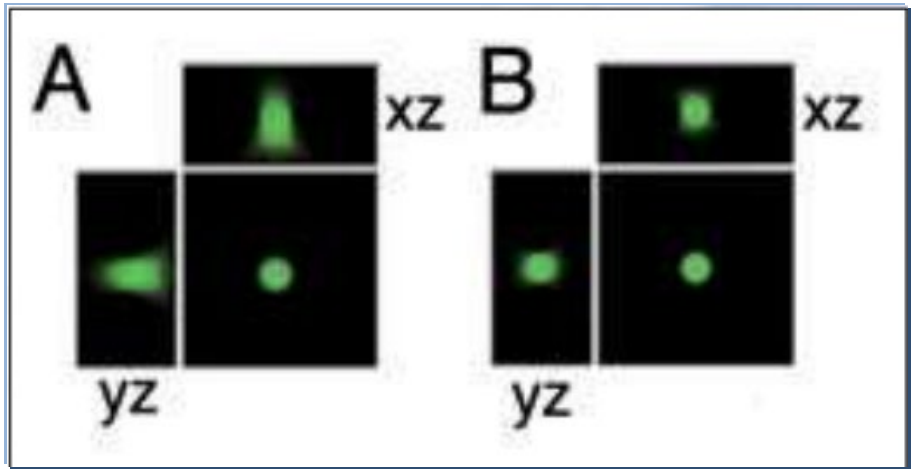
Other causes for spherical aberrations

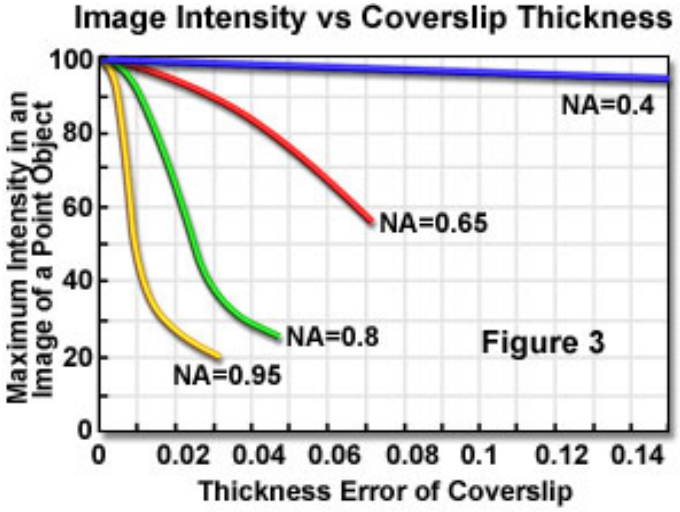
- Wrong coverslip thickness
- Sample away from the coverslip
- Refractive index mismatch between immersion media and embedding media
- Thick samples

What can you do?

1)Use the appropriately corrected lens

2) Use the right coverslips (No 1.5 or No 1.5H)





2) Sample as close to the coverslip as possible

3) Use the appropriate refractive index and embedding medium for your application

4) Utilize the correction collar of your objective

Correction collars

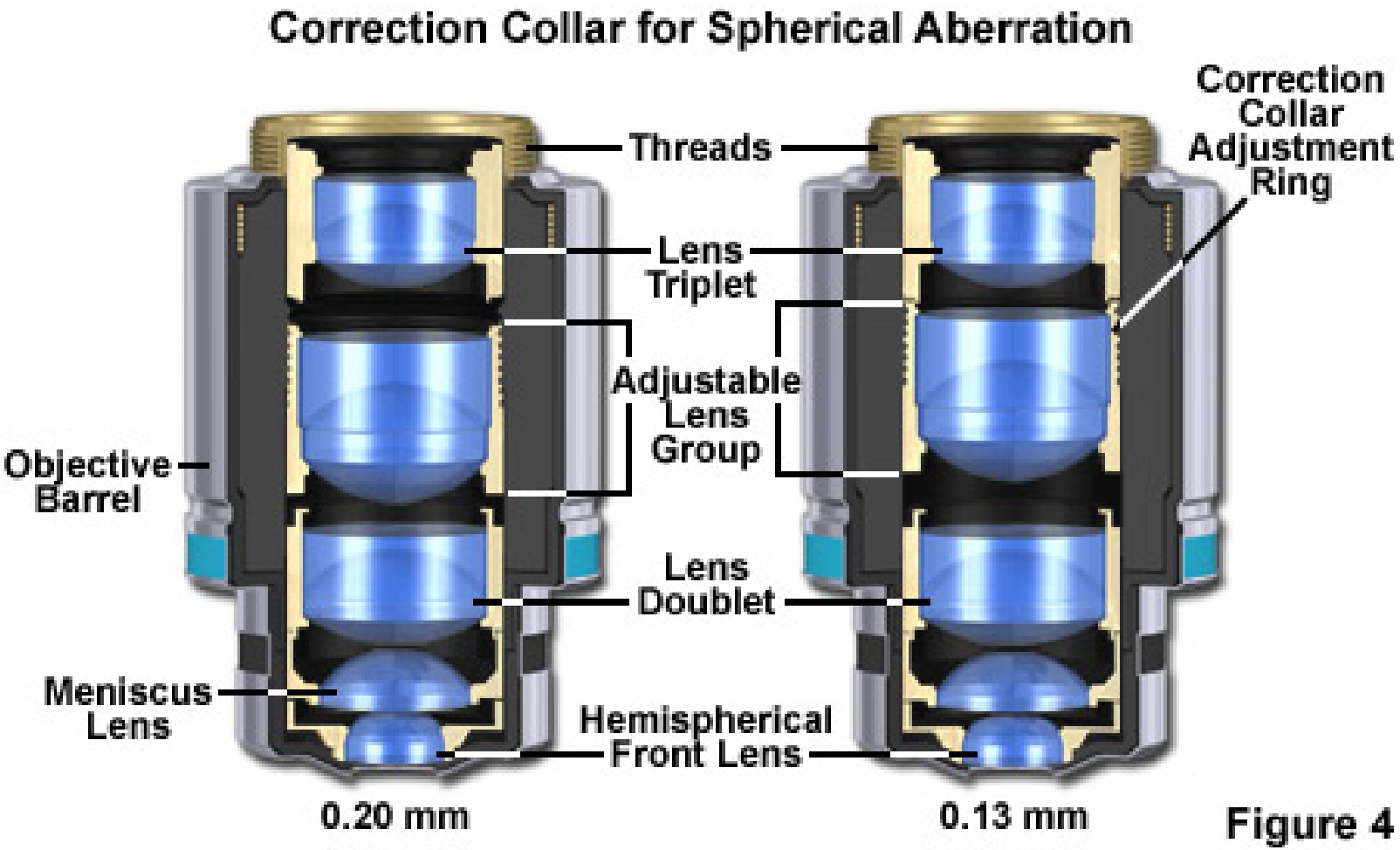
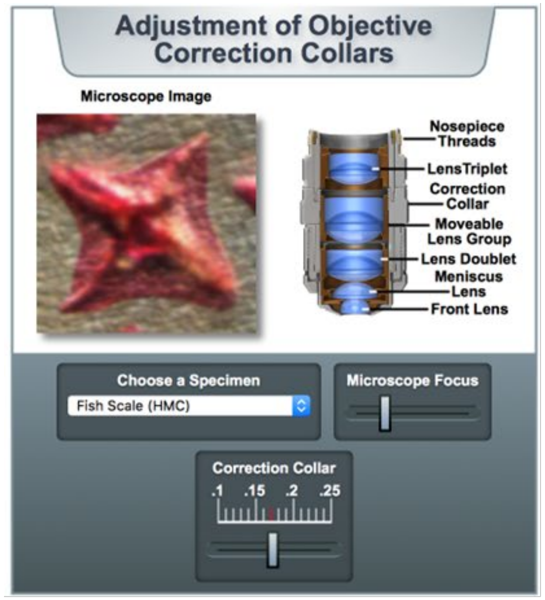
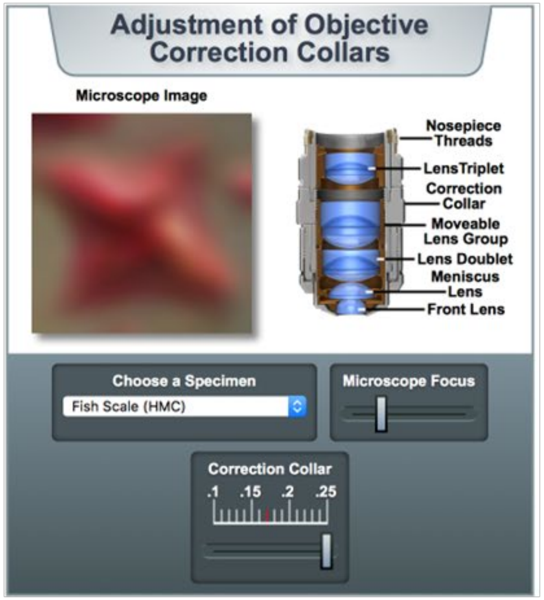
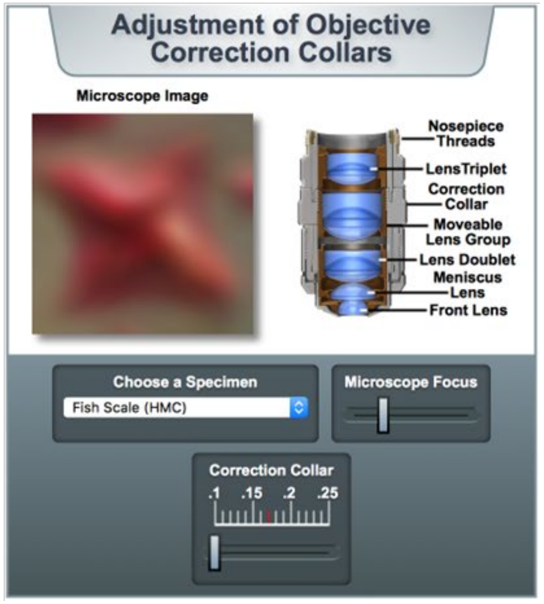


Figure 4

Lenses with Correction Collar

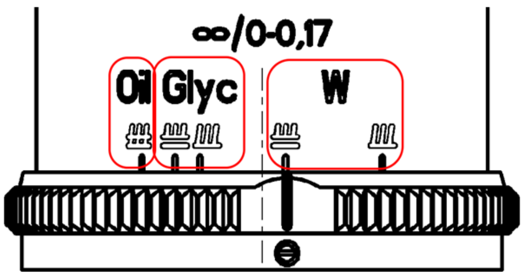






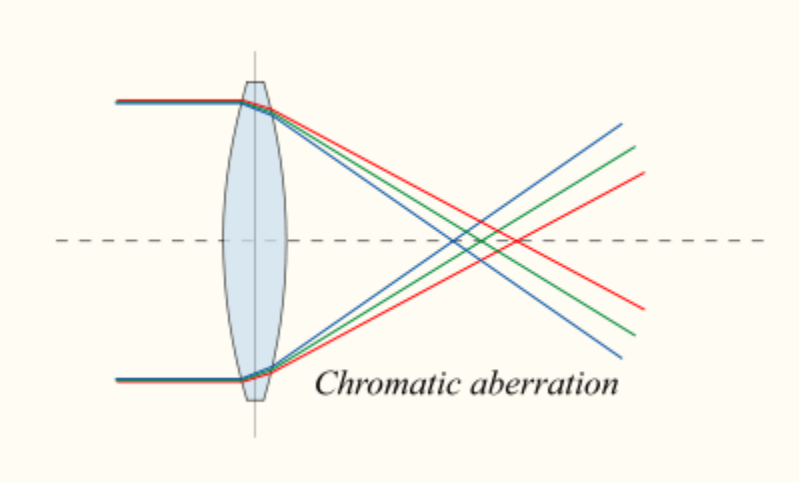
The correction collar is turned until the contrast/ signal to background noise ratio is best.

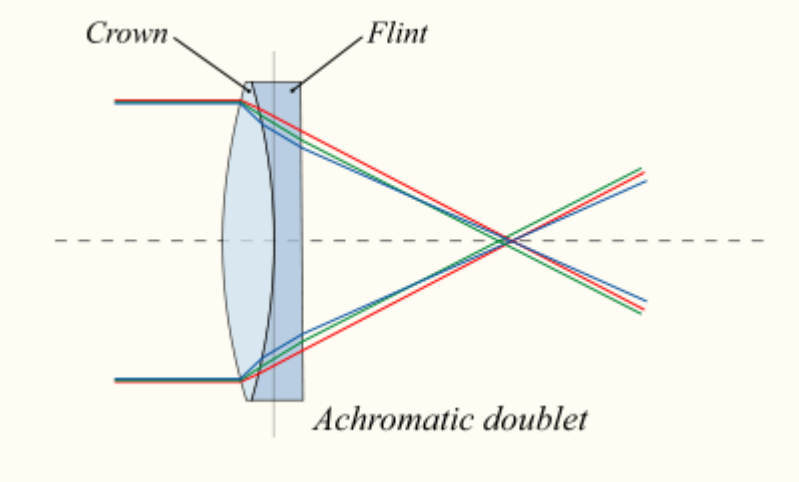
Similarly Multi- Immersion objectives can be used when working with different immersion media (oil, glycerol, water)



Chromatic aberration

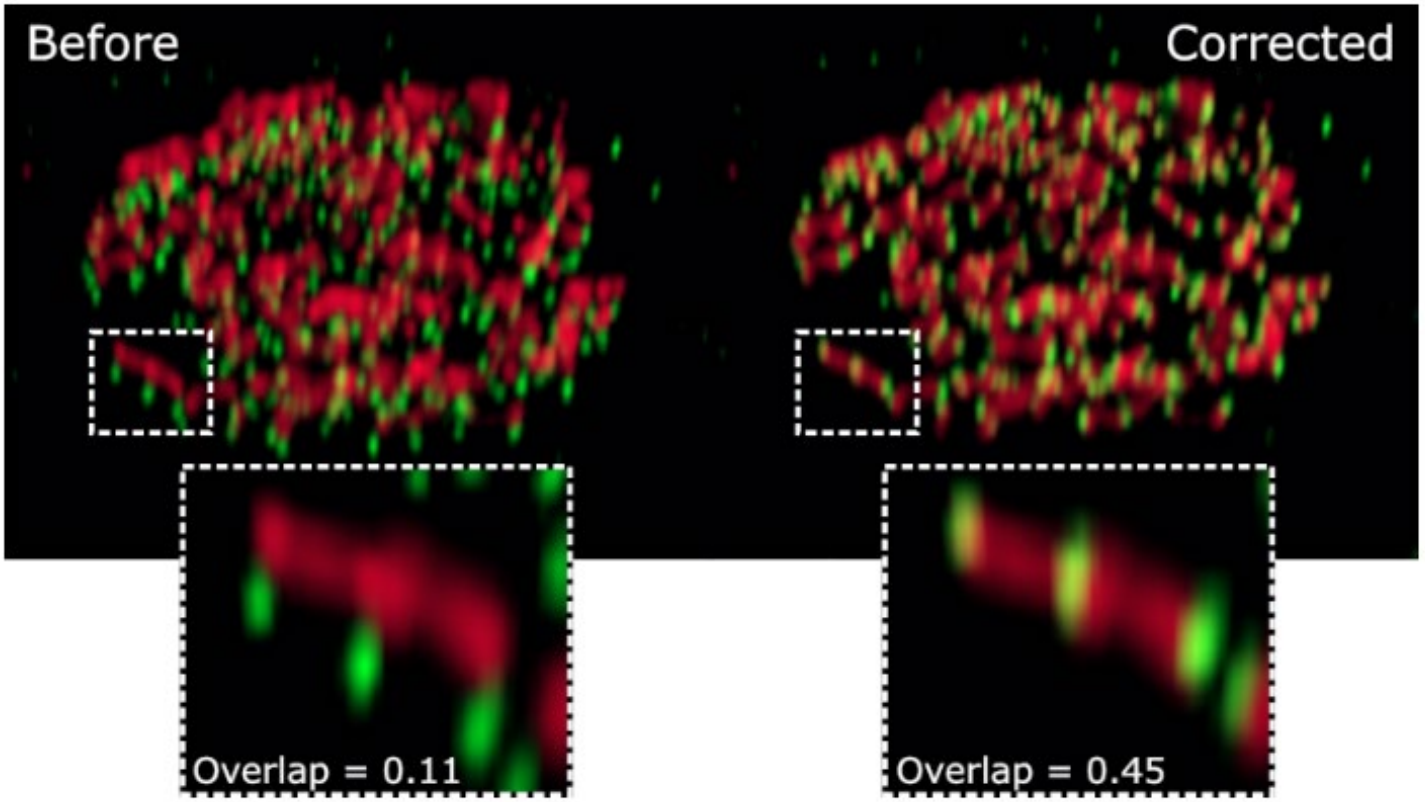
Blue light is refracted to the greatest extent followed by green and red light, a phenomenon commonly referred to as dispersion. different wavelengths will be focused on different positions in the focal plane. Chromatic aberration is seen as fringes of colour around the image.





It can be minimised by using an achromatic doublet (= achromat) in which two materials with differing dispersion are bonded together to form a single lens.

Chromatic Aberration



What can you do?

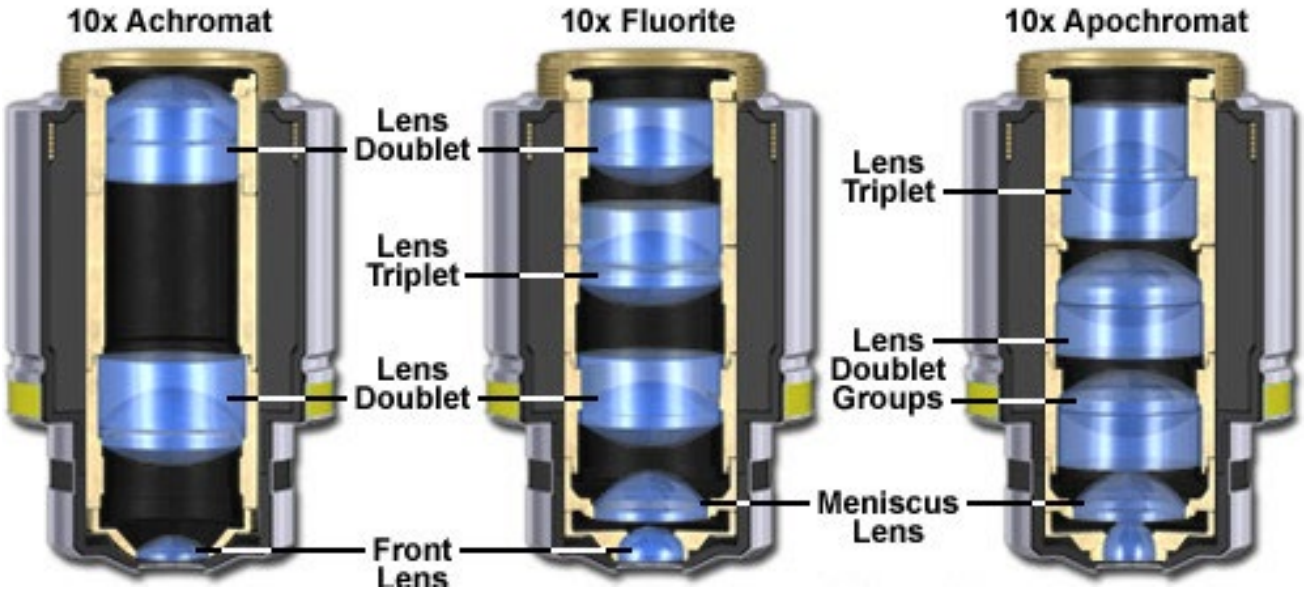
Use the appropriately corrected lens for your application.

Use appropriate controls to correct during post processing (Fiji, Huygens, etc)

https://svi.nl/Chromatic-Aberration-Corrector-Software

Choosing the right objective

Microscope Objective Optical Correction Factors



Microscope Objective Correction for Optical Aberration

Objective Specification	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

In conclusion

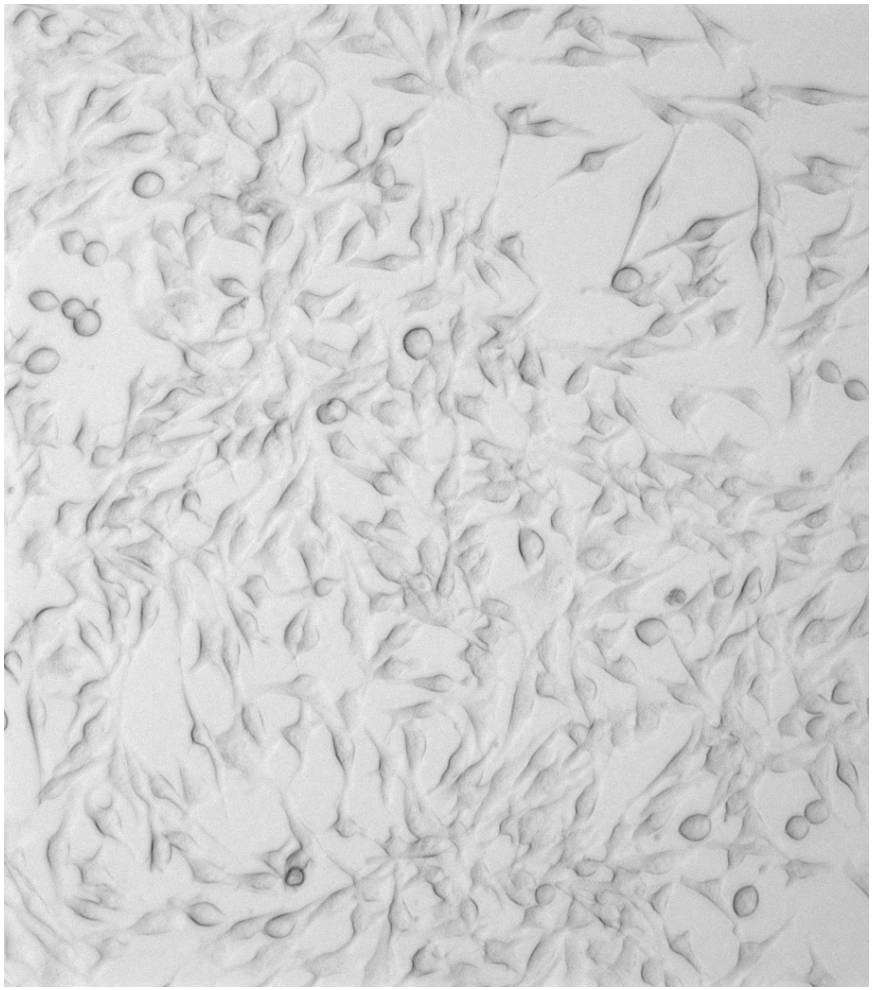
- Pick the lens with the most corrections that your budget allows
- Be meticulous in your sample prep and reagent selection
- Assume that aberrations are never completely eliminated
- Use controls that will allow you to run post processing corrections if needed

Building Contrast

Brightfield Microscopy

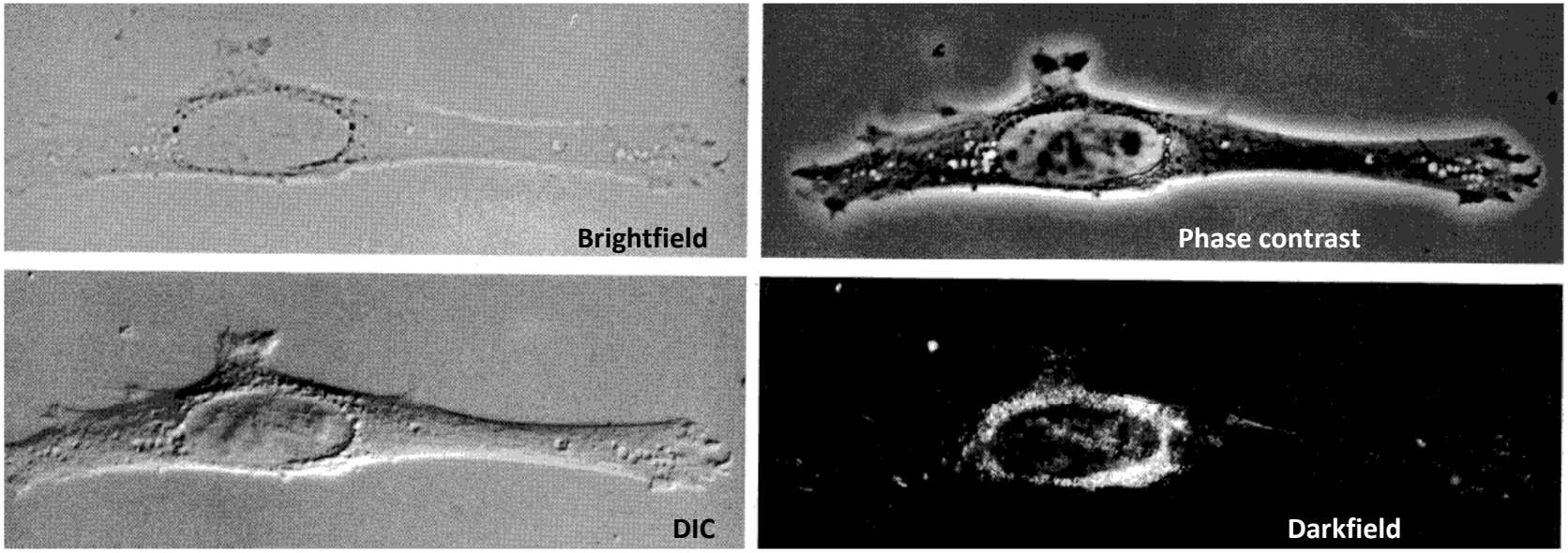
- Bright field illumination does not reveal differences in brightness between structural details - i.e. no contrast
- Structural details emerge via phase differences and by staining of components
- The edge effects (diffraction, refraction, reflection) produce contrast and detail

Properties of light used to create contrast



- Absorption
- Scattering
- Refraction
- Phase
- Polarization

Contrasting techniques

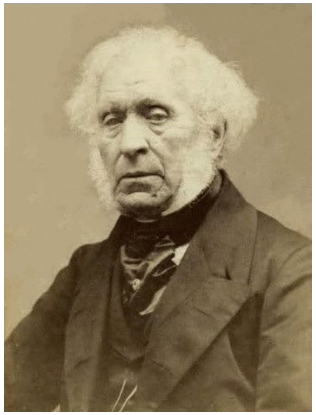


- Brightfield
- Darkfield
- Phase Contrast
- Polarization Contrast
- Differential Interference Contrast (DIC)
- Fluorescence Contrast

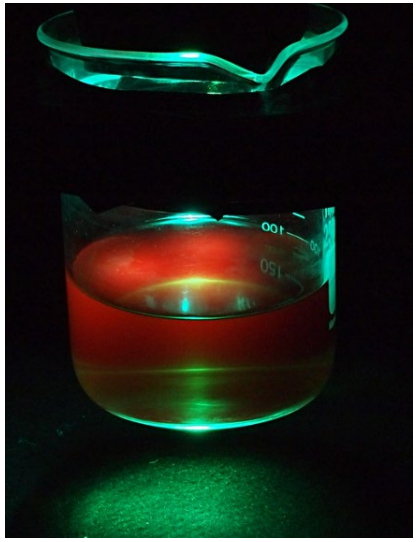
Fluorescence Microscopy

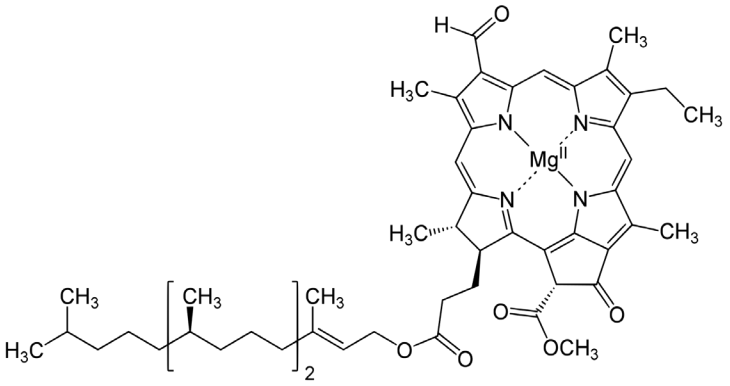
- Principles of Fluorescence
- Evolution of fluorescence as a microscopy tool
- Why use florescence anyway
- Fluorescent probes
- Fluorescent techniques
- The future or fluorescence in biological imaging

Fluorescence

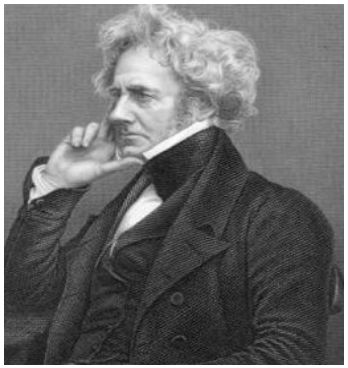


Sir David Brewster
1781-1868

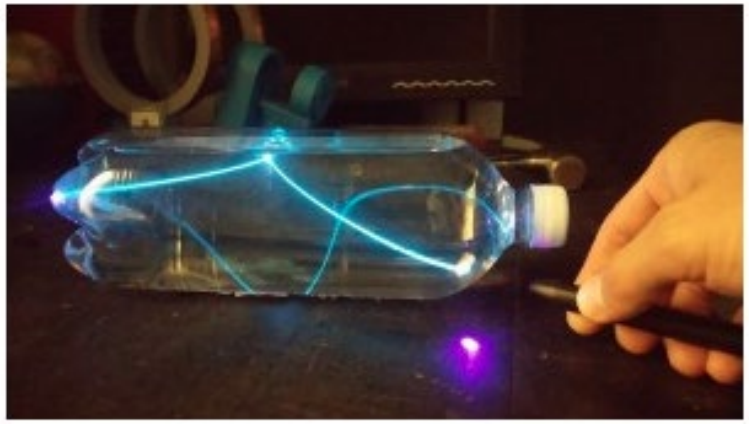


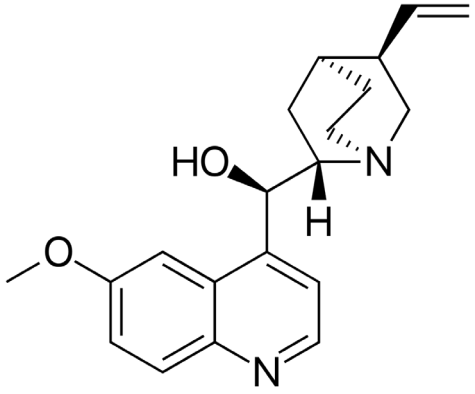


Chlorophyl



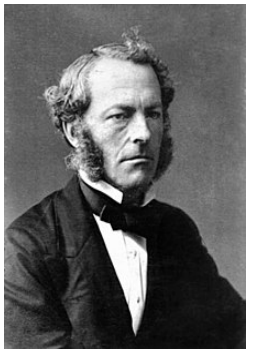
Sir John Frederick William Herschel
(1792-1871)



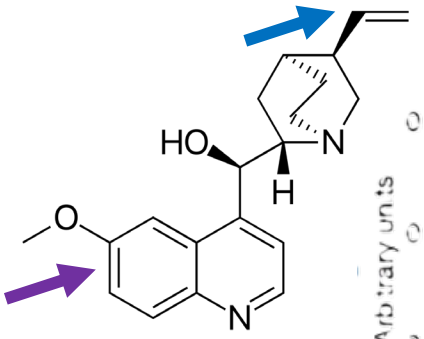


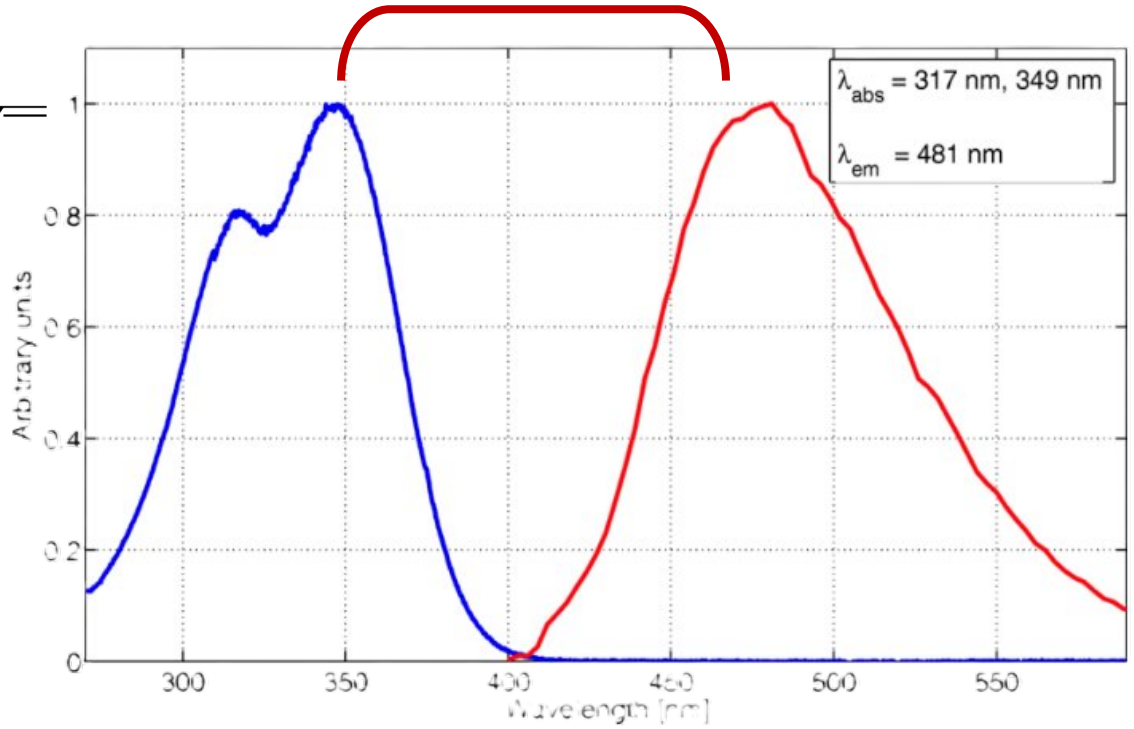
Quinine

Stoke's shift



George Gabriel Stokes
(1819 -1903)



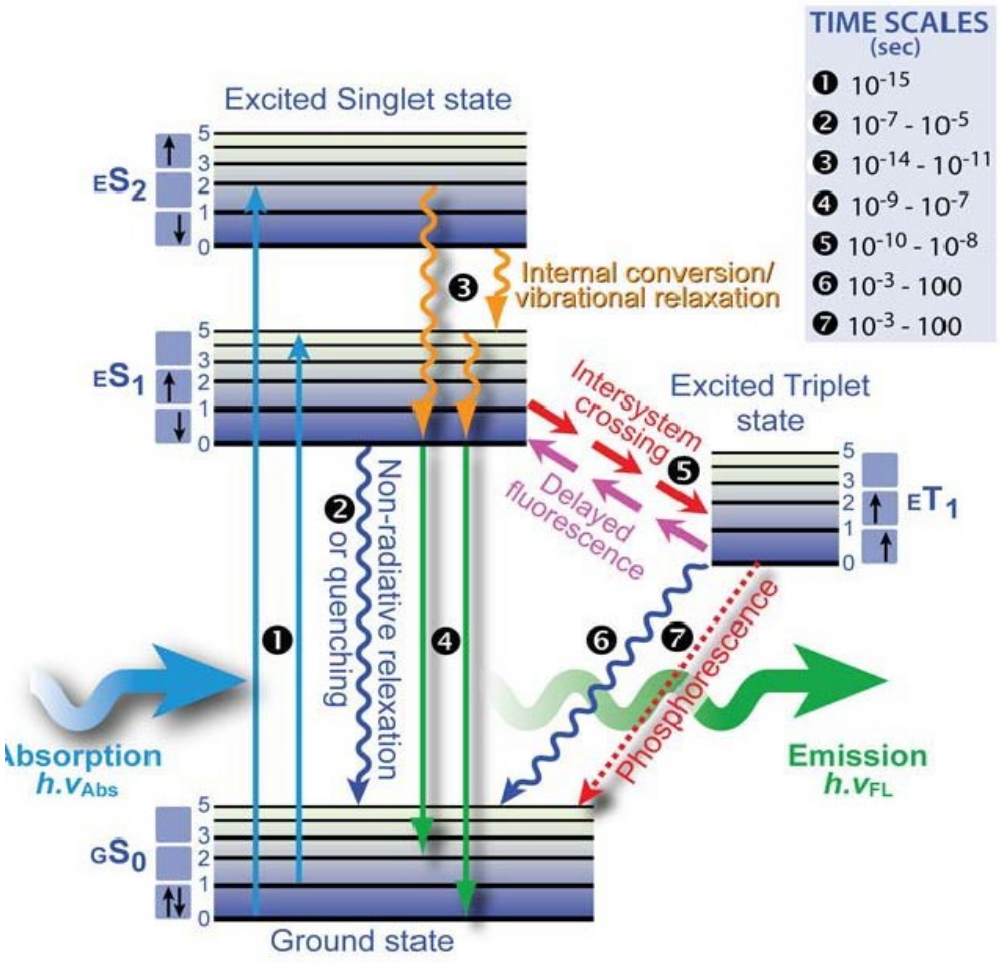


Quinine sulfate in sulfuric acid

Note: Emission of lower energy is possible (anti-Stokes shift) for example in 2-photon microscopy

Journal of Fluorescence (2018) 28:1065–1073

Jablonski Diagram

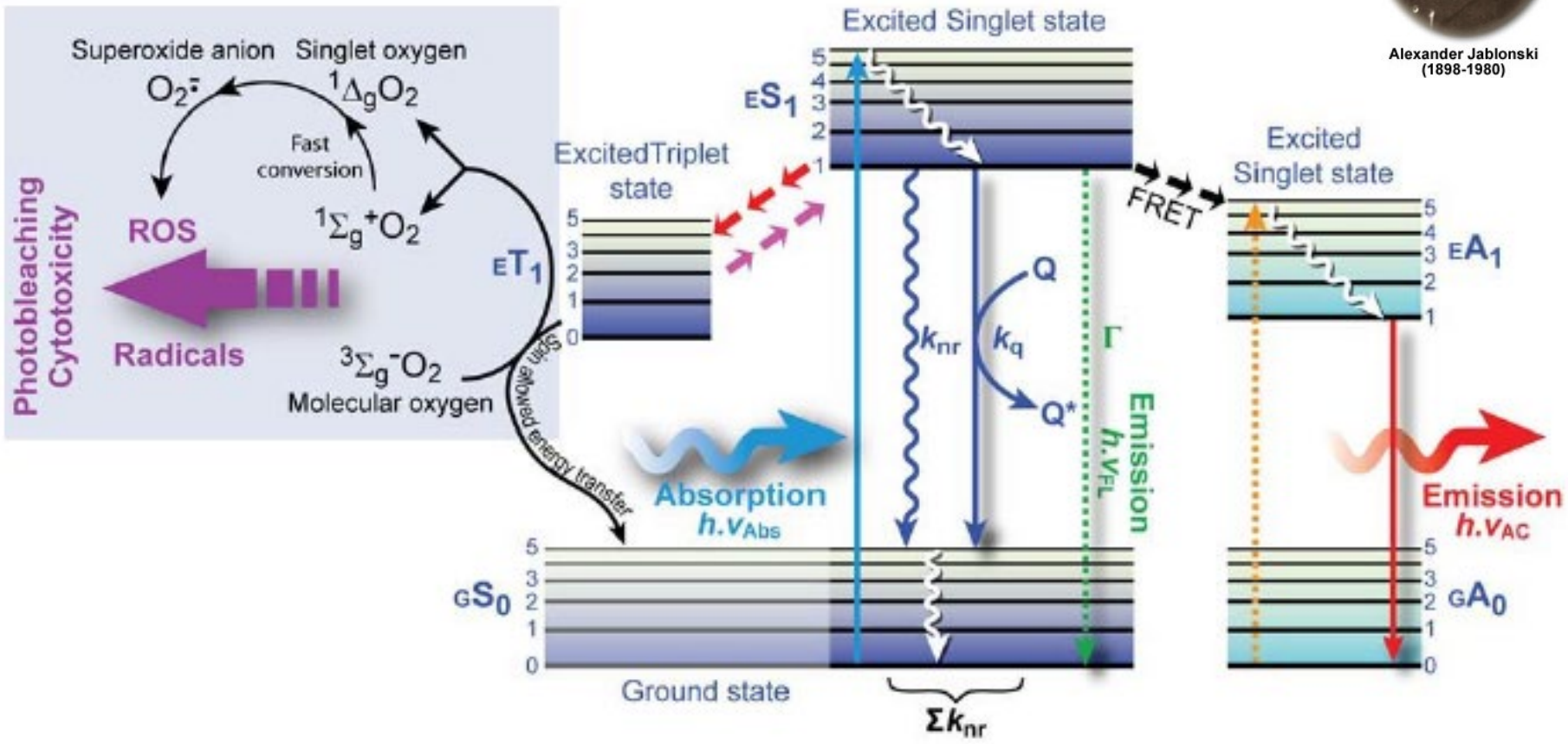


Alexander Jablonski
(1898-1980)

Molecules 2012, 17, 4047-4132

Jablonski Diagram

Alexander Jablonski
(1898-1980)



Molecules 2012, 17, 4047-4132

Florescent molecules characteristics

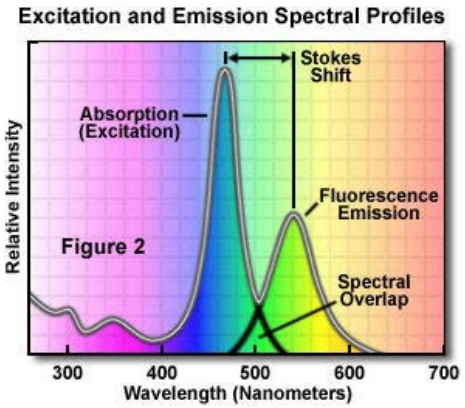
Stokes shift:
Difference in nm between Ex and Em maxima;

Fluorescent lifetime: Average time that the fluorescent molecule spend in the excited state

Quantum yield:
A maximum value of 1.0, a ratio of photons fluoresced to photons absorbed

Extinction coefficient: (e units: M−1 cm−1), a measure of the capture of excitation light by the fluorochrome

Molecular Brightness is the product of the quantum yield and extinction coefficient



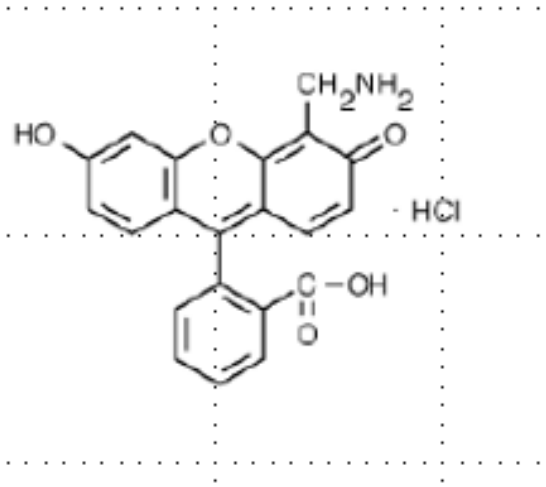
Useful fluorochromes typically have:

- High extinction coefficient
- High Quantum yield
- High Stokes shift.

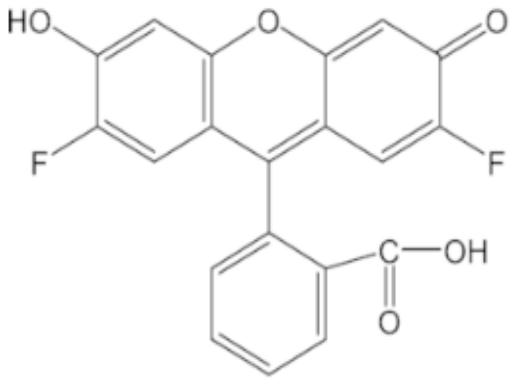
The continuous evolution of Fluorochromes

- **1942**–Coons –Fluorescein and Rhodamine dyes;
- 1993 –cyanine dyes Cy2, Cy3, Cy5, also AMCA and Texas Red;
- **1999**–AlexaFluor and DyLightdyes;
- 2003 –Quantum dots (Q-dots);
- More recently… SiRdyes; “JaneliaFluor” dyes, Abberior long stoke shift dyes, StayGold etc.

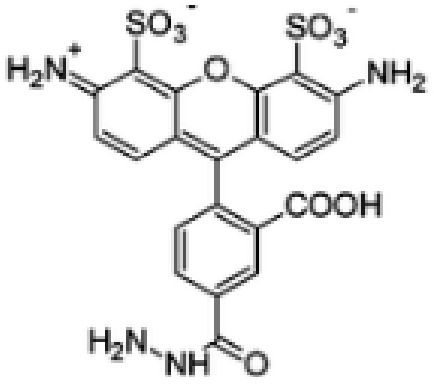
Fluorescein



Oregon Green

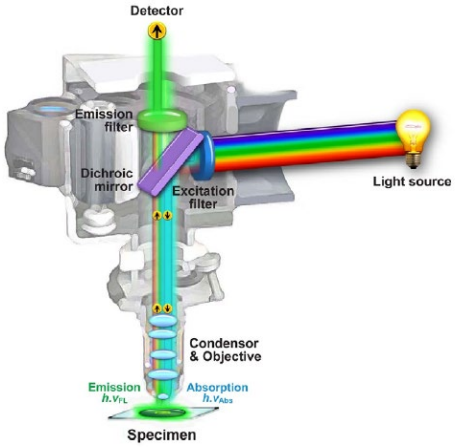


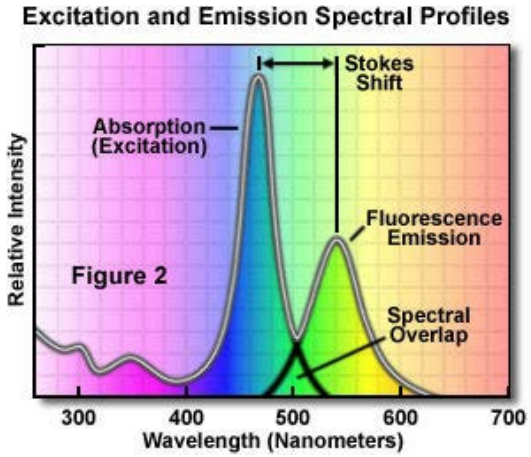
Alexa 488



Fluorescence in Microscopy

- According to Helmholtz (1874) was to be expected that an image can be better differentiated, and fine structures are more easily discernible if the object itself emits light. (Ellinger, 1940, Renz, 2013)
- First attempt to a fluorescent microscope by Kohle and Siendentopf for demonstration in a summer course in Vienna (1908)
- First fluorescent microscopy was developed independently by Carl Zeiss and C.Reichert in 1911
- Early fluorescent imaging relied on autofluorescence in animals (While Stubel, 1911) and in plants (Klemm, 1917)
- Philip Ellinger and August Hirt were also one of the very first to try and apply fluorescent stain on biological samples and the first implement the intravital epifluorescent microscope (1929)



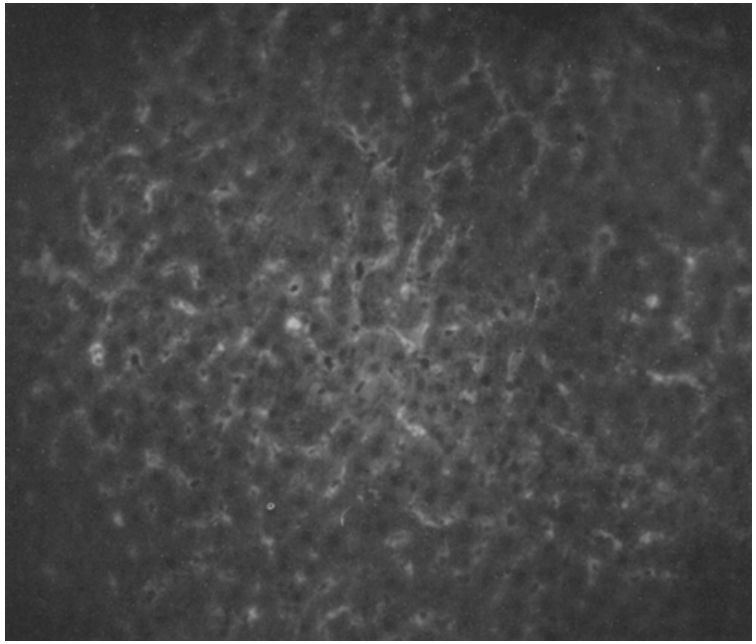


Anatomy of the epifluorescent microscope

Ishikawa-Ankelrod HC., 2012

From autofluorescence to non-specific staining to labelled antibodies

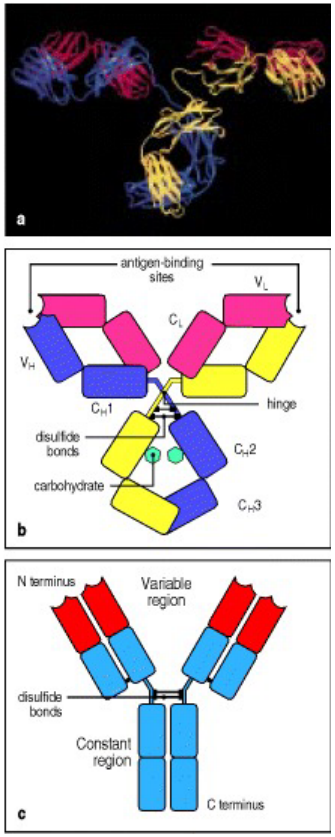
- Klemm (1917) tried to stain non fluorescent objects with fluorescent materials and the efforts continued staining bacteria
- In the early 1930 researchers started conjugating antibodies with dyes (not fluorescence), but imaging turned out to be challenging due to low intensity. (Marrack, 1934)
- Stepping on the Marrack study and after a short break to fight a world war Dr. Coon came back to Harvard and with his colleagues conjugate antibodies with fluorescein-4-isocyanate (Coons et al,1942)



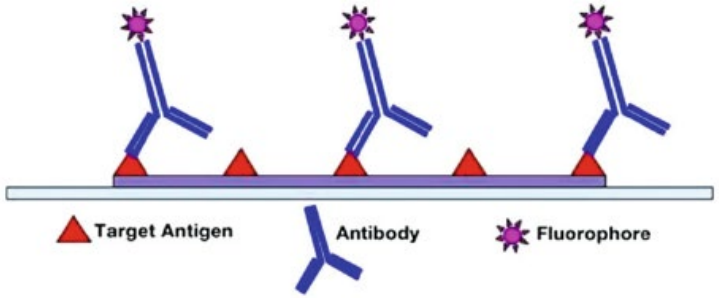
▶ FIG. 1. PHOTOMICROGRAPH THROUGH FLUORESCENCE-MIcROSCOI~E OF THE LIVER OF A MOUSE ~IORIBUND WITH PNEUMGCGCCAL 3 INFECTION

The antigen in the liver stained with fluorescein-carbamido-antipneumococcal 3 rabbit serum. Black areas are blood vessels; grey areas are the blue-grey fluorescence of formalinfixed hepatic cells; white areas and fine white lines represent the green fluorescence of fluoresccin-antibody and indicate the location of pneumococcal 3 antigenic material. 20 X apochromatic Zeiss objective, 10 X ocular; in the ocular Wratten filters //2A and %8 (K2.).* Leitz 1 × microphotographic apparatus. Eastman film Super Ortho Press. (Coons et. Al 1942) Exposure: 20 minutes.

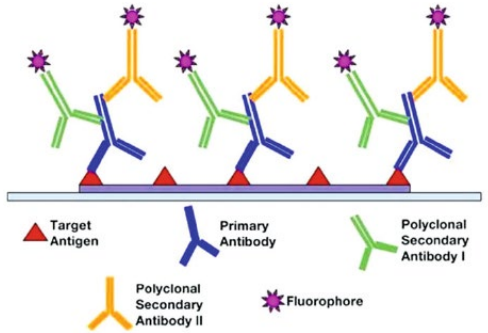
Fluorochrome labelled Antibodies



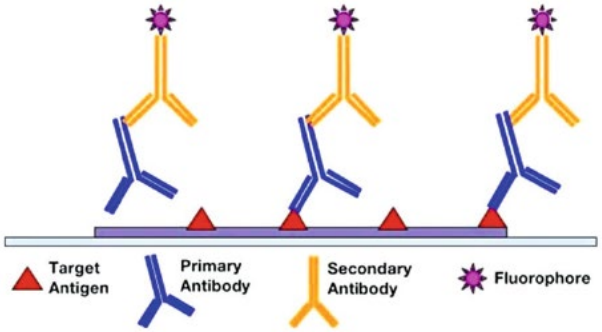
Janeway CA Jr, Travers P, Walport M, et al.
New York: Garland Science; 2001.



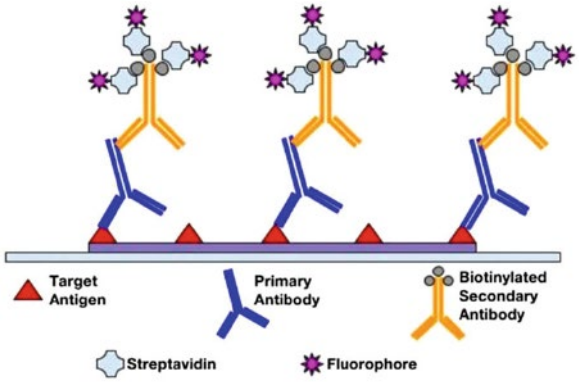
1 Direct immunofluorescence



Amplification of signal with polyclonal secondary antibodies labeled with a fluorophore



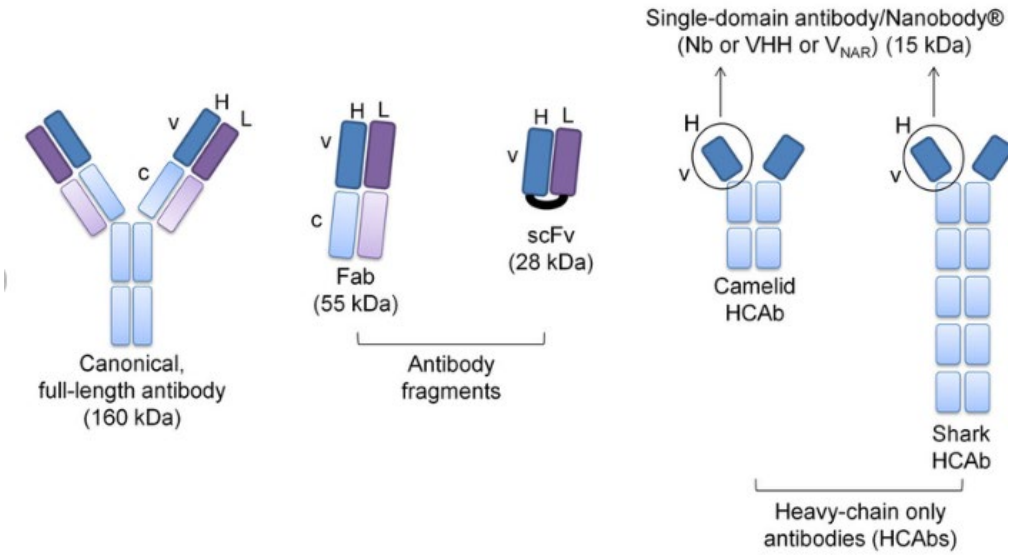
Indirect immunofluorescence

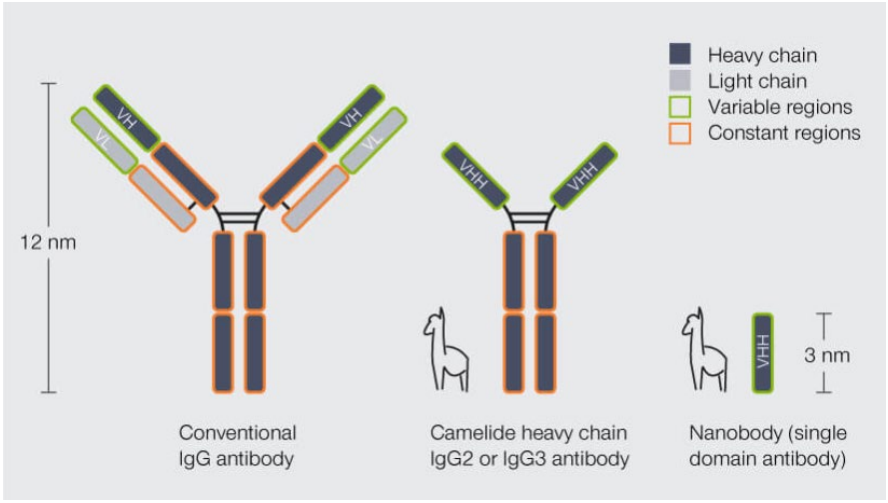


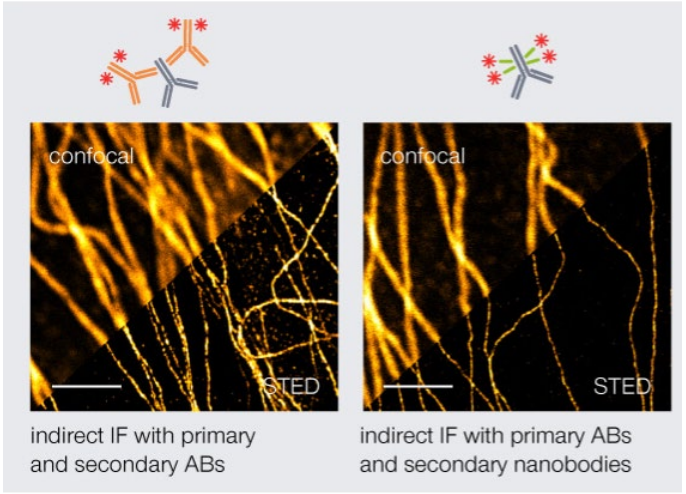
Amplification of signal by a secondary antibody conjugated with multiple fluorophore–protein complexes

Kim k., et al., 2019 https://doi.org/10.1007/978-1-4939-8935-5_26

...And nanobodies
Staining for SuperResolution



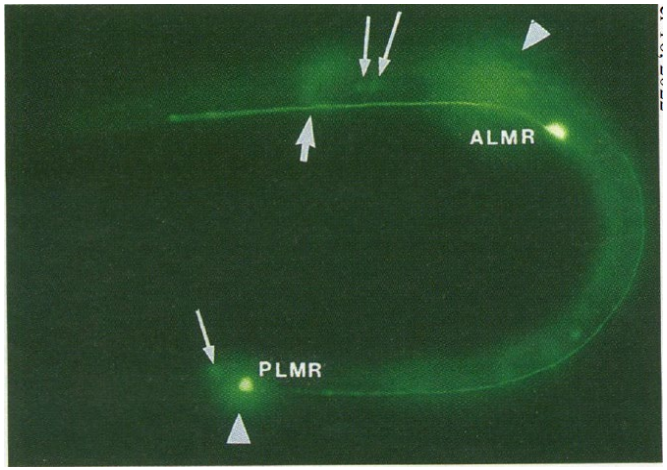




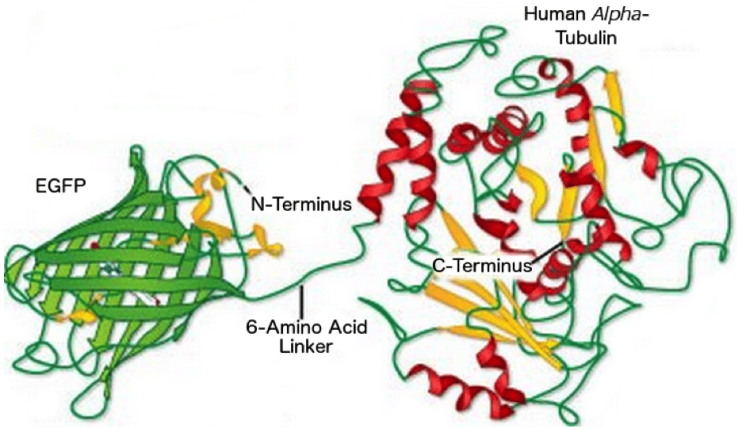
https://abberior.rocks/knowledge-base/what-makes-camelides-so-special/

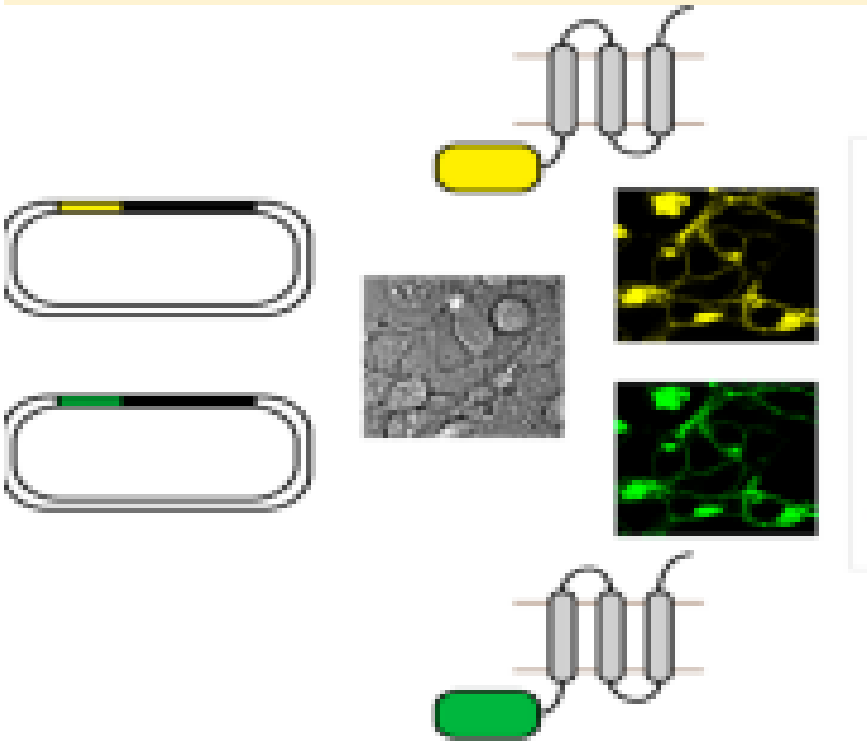
Fluorescence as a genetic tag

- The Nobel prize winning discovery of green fluorescent proteins (GFP) in the 1960s and their subsequent development as a genetic tag, the field has evolved to permit targeted live cell imaging.



Chalfie et al, 1994





Biochemistry 2018, 57,6741-6751

Why fluorescence?

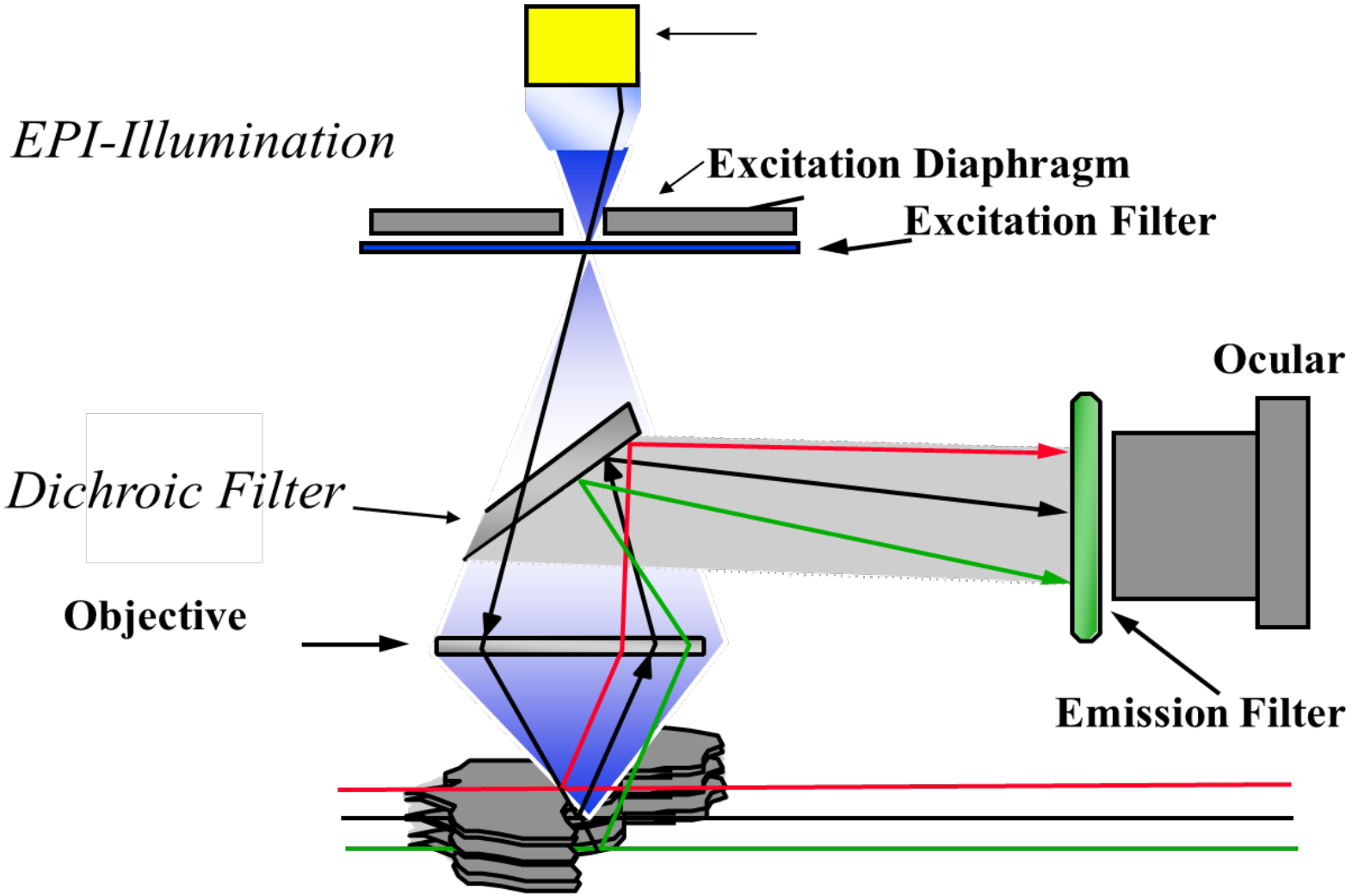
Advantages of fluorescence:

- **High Sensitivity** (high signal to background)
- **High Specificity** (target single molecular species; light up single cells/sub-regions)
- Speed and **temporal** resolution Compatible with **live cells**
- Variety of fluorophores with different emission maxima
- **Versatile** variety of techniques available for different applicatio**ns**
- Local **environnent** affects fluorescence emission (pH, ion concentrations, membrane potentials etc.).

Disadvantages of fluorescence:

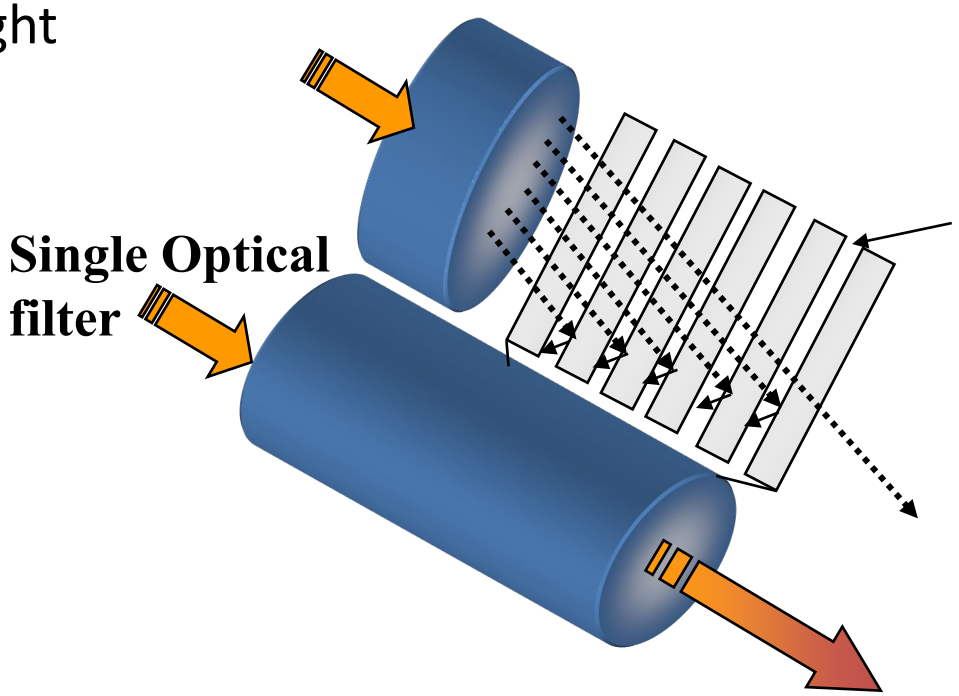
- Not great for low magnification
- There is a shelf life for the samples
- Requires a fluorescence microscope with appropriate lasers, filters etc.
- Some techniques can get expensive
- Autofluorescence.

The anatomy of fluorescent Microscope



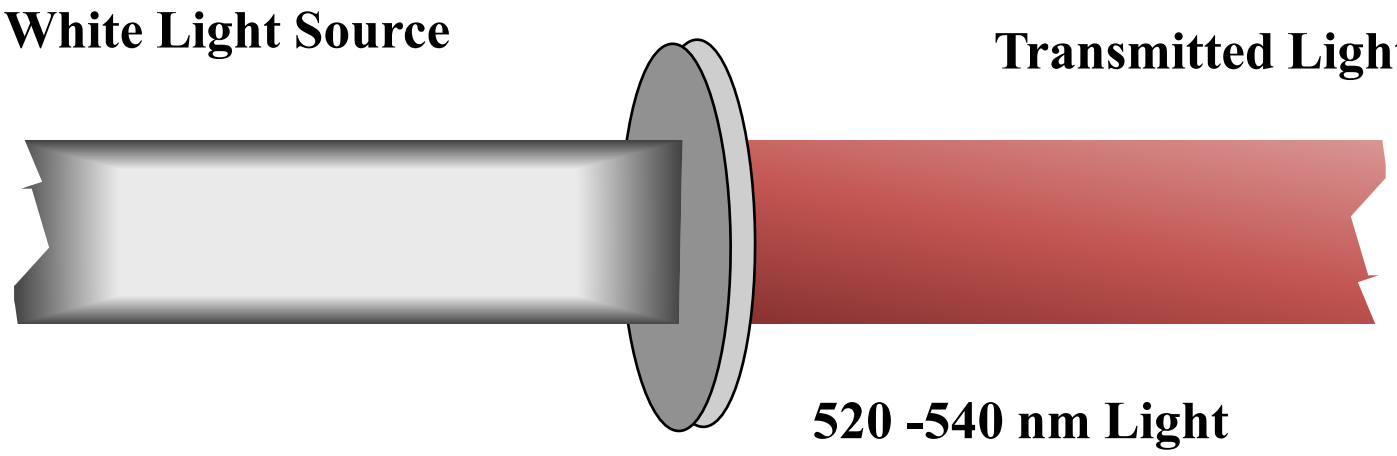
Optical filters

- Small amounts of incident light are reflected at the interface between two material of different RI
- Thickness of the material will alter the constructive or destructive interference patterns - increasing or decreasing certain wavelengths
- Optical filters can thus be created that "interfere" with the normal transmission of light

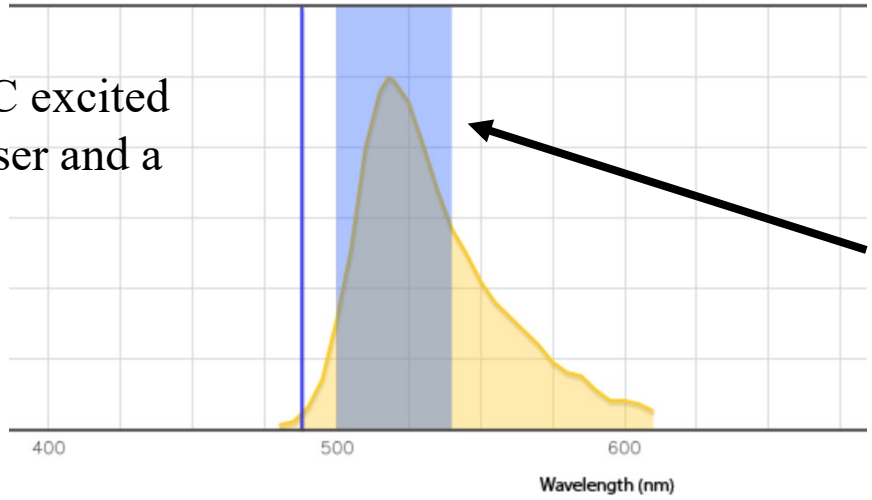


Standard Band Pass Filters

530 nm/20 BandPass Filter

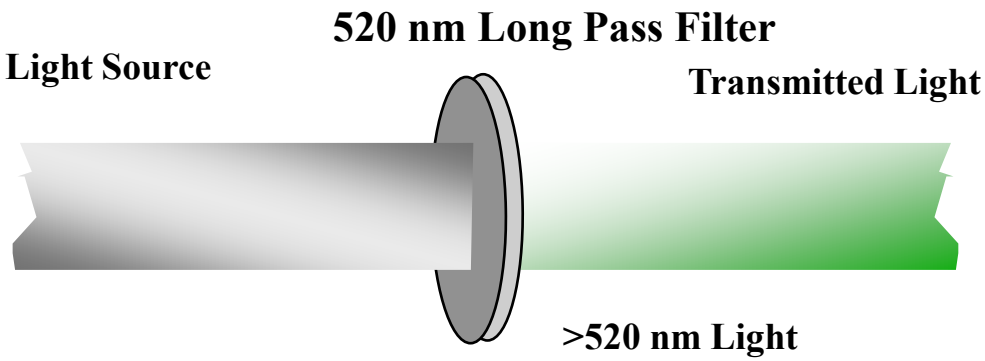


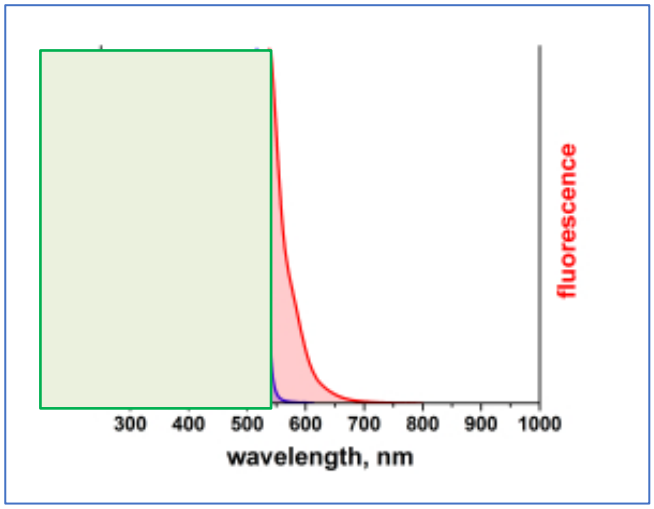
Example: FITC excited by a 488nm laser and a 530/20 filter



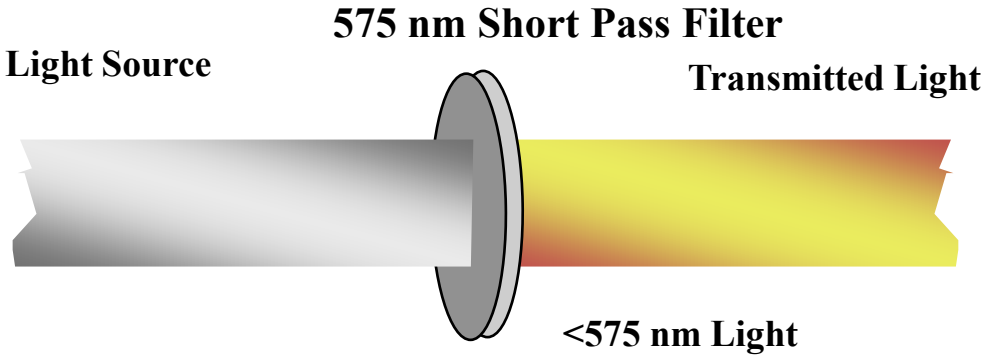
This is the "band" of light

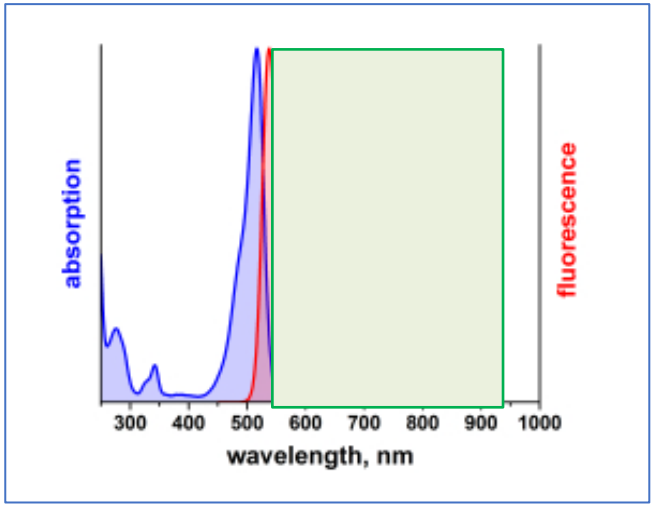
Standard Long Pass Filters





Standard Short Pass Filters



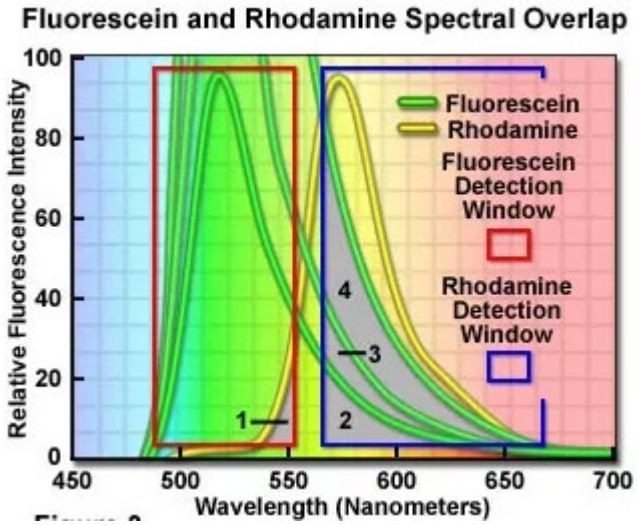


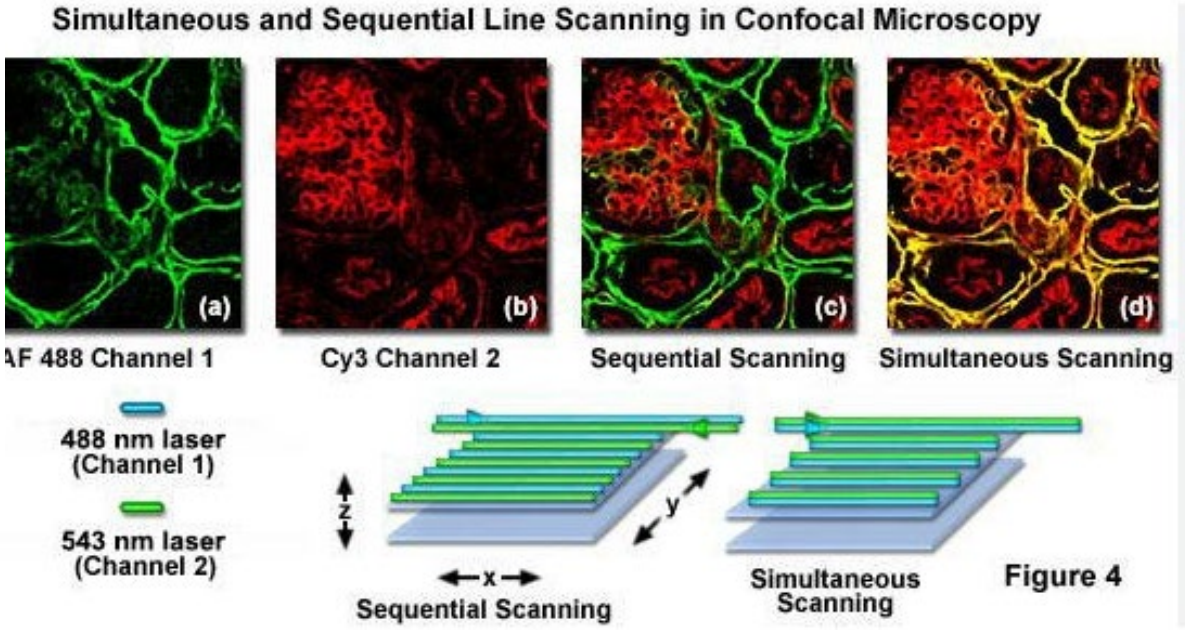
Useful sites about dyes and filters

- BioLegend: https://www.biolegend.com/spectraanalyzer
- BD: http://www.bdbiosciences.com/us/s/spectrumviewer
- Thermo: https://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html
- Chroma: https://www.chroma.com/spectra-viewer
- Sigma: https://www.sigmaaldrich.com/labware/learning-center/spectral-viewer.html
- Evrogen: http://evrogen.com/spectra-viewer/viewer.shtml
- Expedeon: https://www.expedeon.com/resources/applications/flow-cytometry/new-fluorescence-spectra-viewer/
- Fluorophoresorg: http://www.fluorophores.tugraz.at/substance/
- Semrock: https://searchlight.semrock.com

Fluorescence Artifacts

Bleed-through



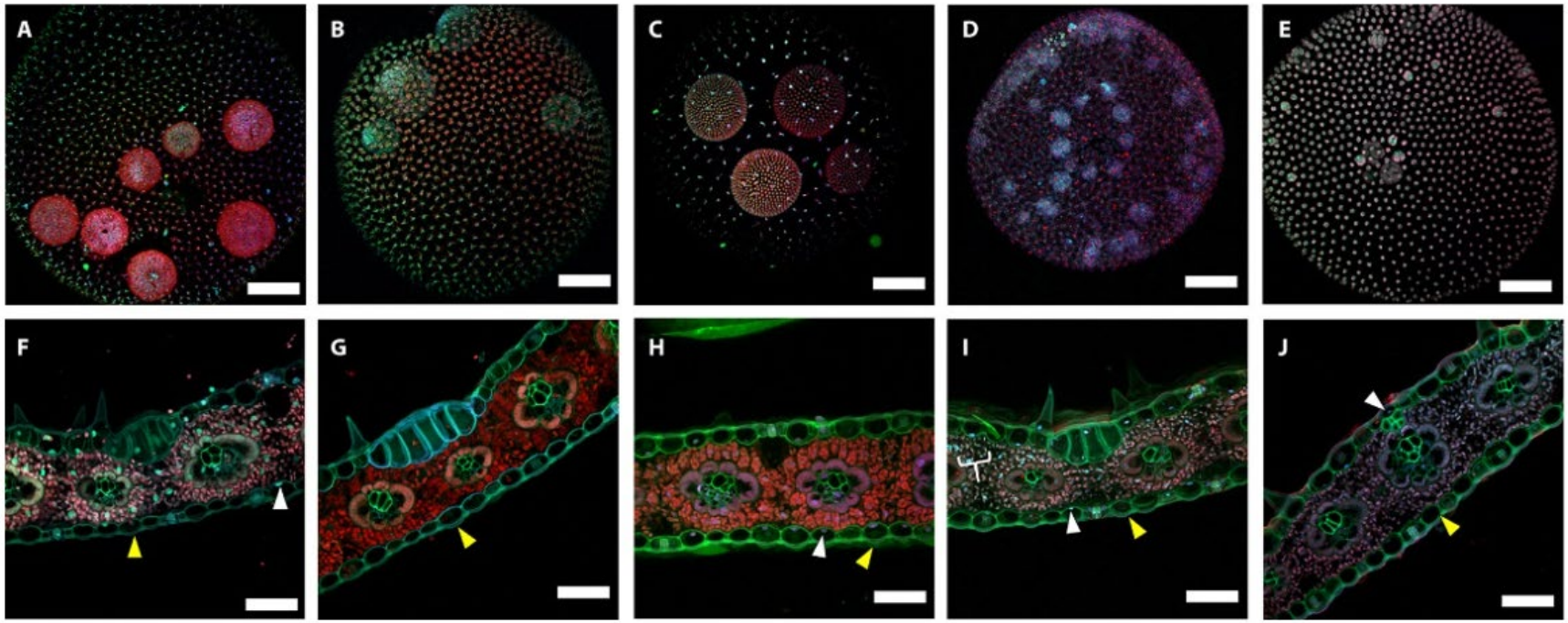


https://evidentscientific.com/en/microscope-resource/knowledge-hub/techniques/confocal/bleedthrough

What you can do

- Select fluorophores with minimal spectral overlap
- Use narrow-band emission filters
- Apply sequential acquisition instead of simultaneous detection
- Use spectral detection + linear unmixing
- Lower excitation laser power to reduce bleedthrough into neighboring channels

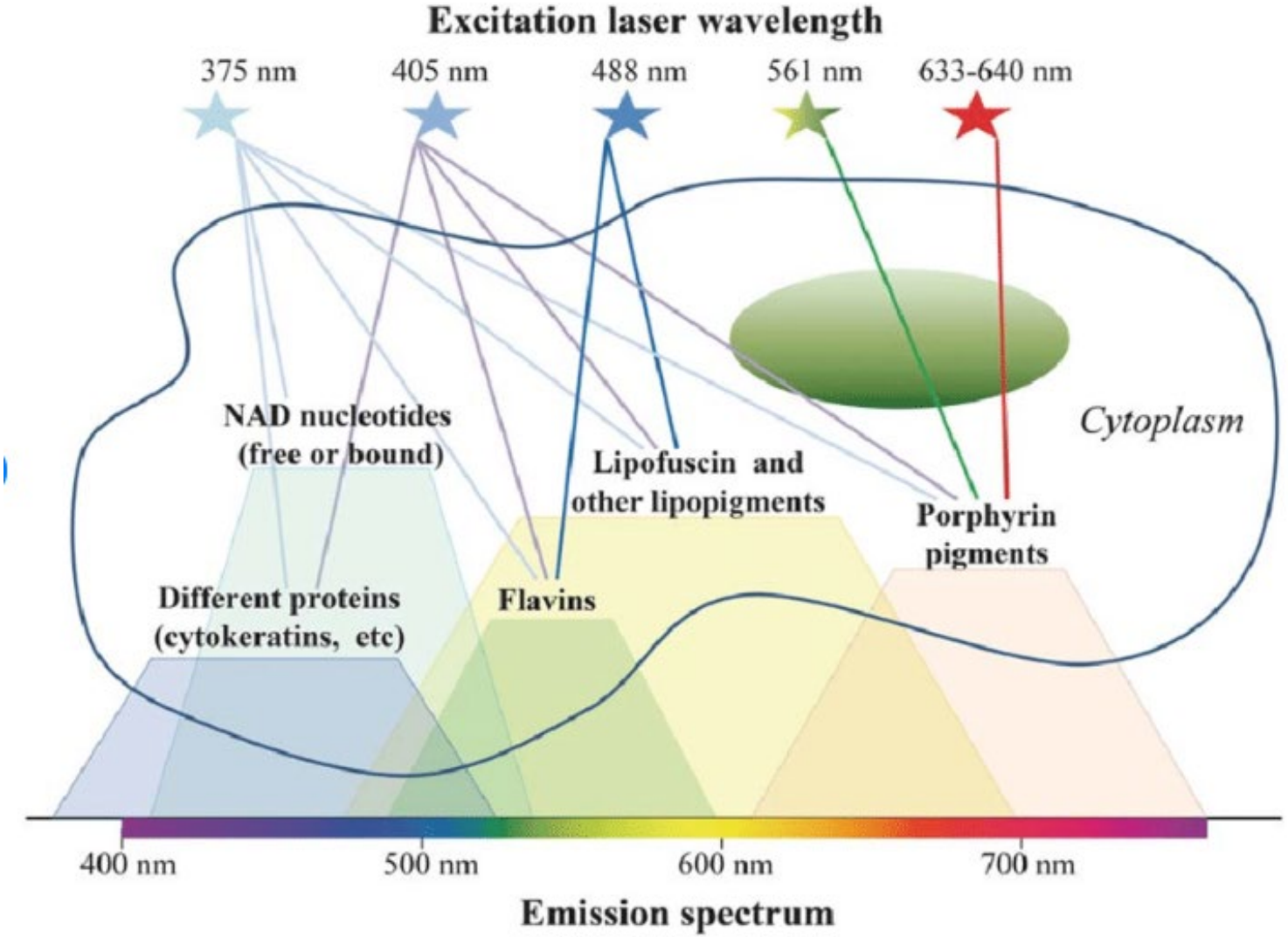
Autofluorescence



Comparison of the five fixation treatments in Volvox colonies and Zea mays leaf tissue. Images display

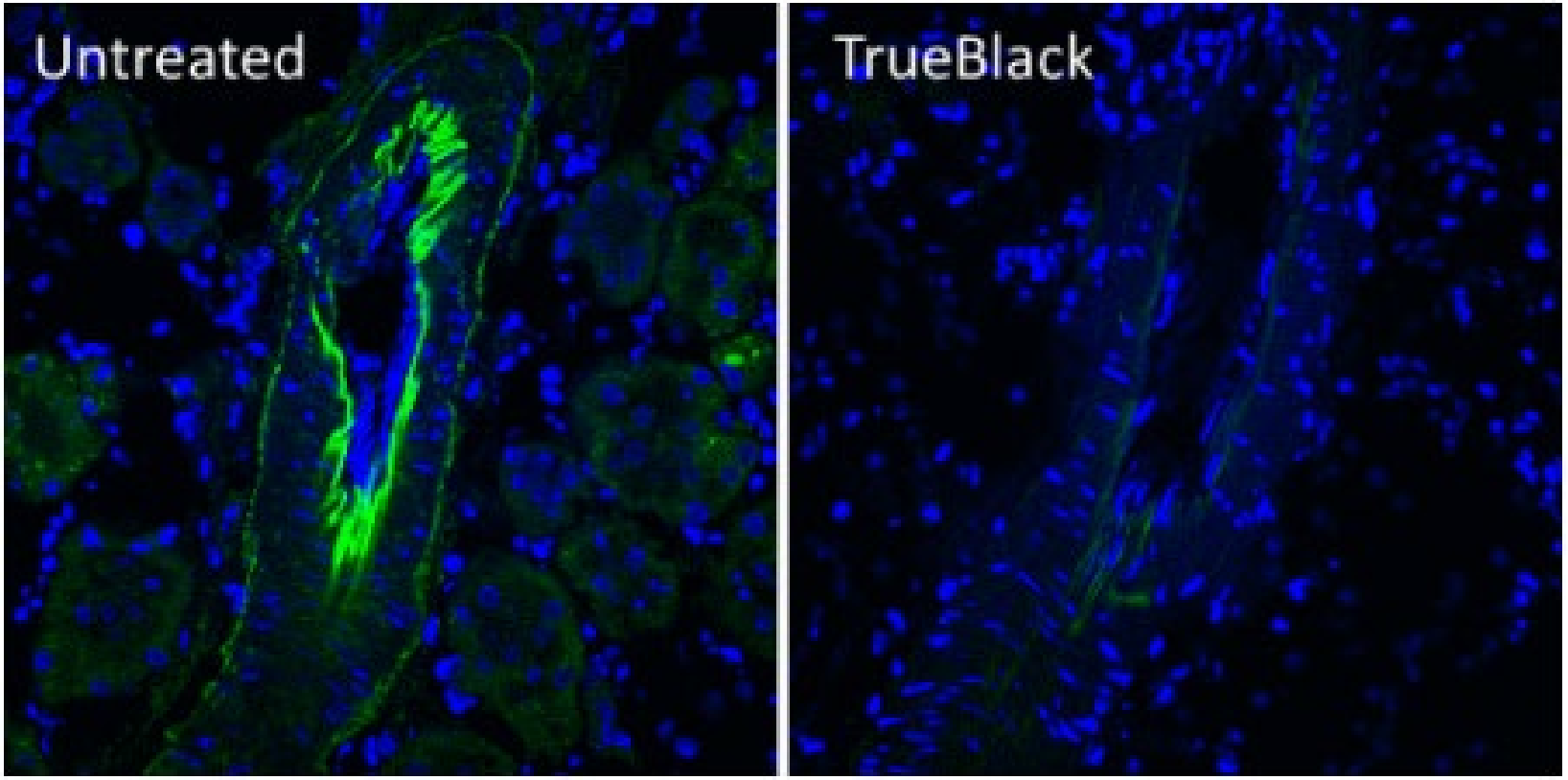
Pegg et al Applications in Plant Sciences 2021 9(6): e11437

Autofluorescence



Shilova et al., 2017

Autofluorescence

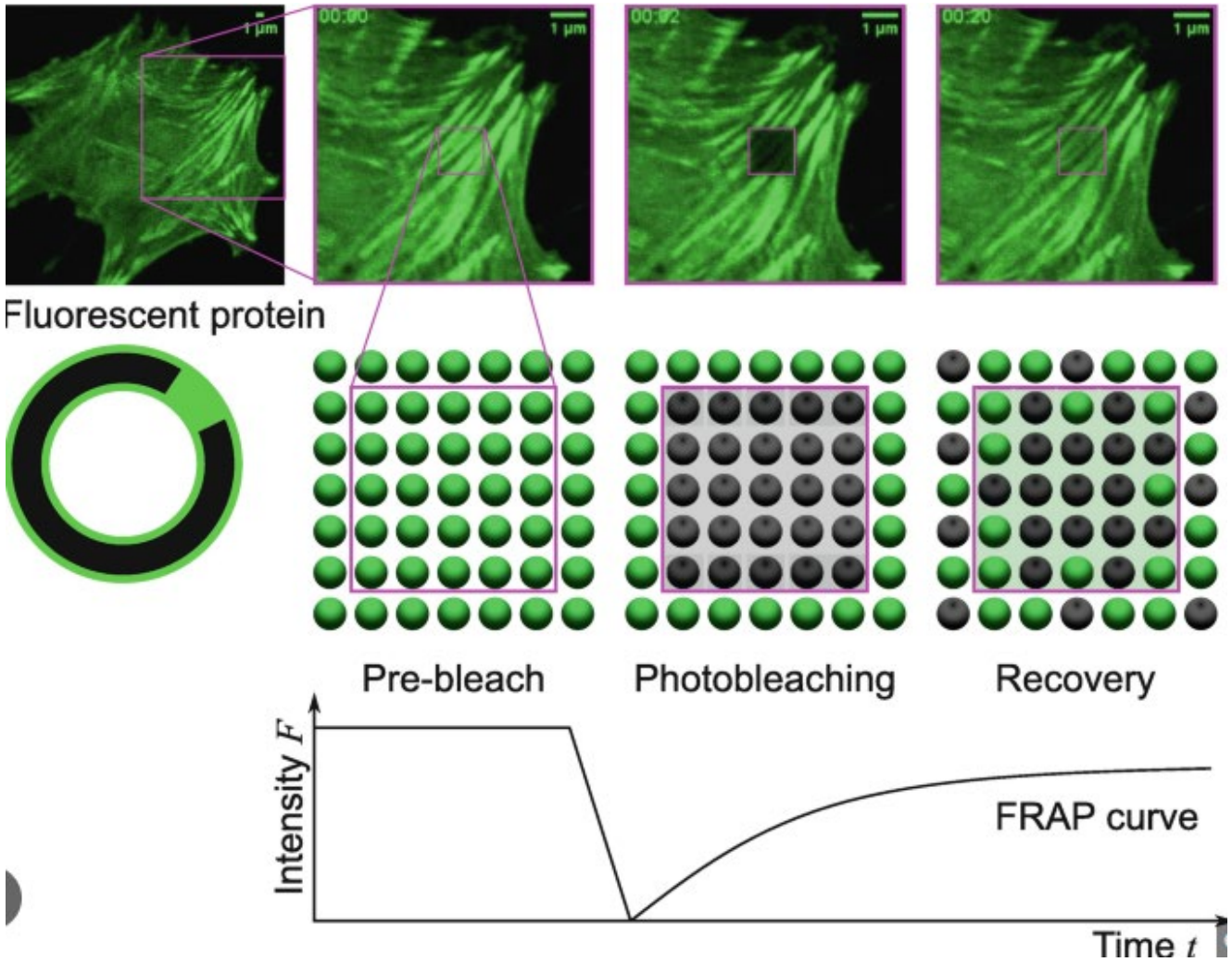


https://biotium.com/product/trueblack-lipofuscin-autofluorescence-quencher/

What you can do

- Choose fluorophores with emission spectra well separated from autofluorescence background
- Use longer-wavelength dyes (e.g., far-red/near-infrared)
- Apply spectral unmixing to mathematically separate autofluorescence from signal
- Use fluorescence lifetime imaging (FLIM) if available
- Minimize fixation or clearing methods that increase autofluorescence

Photobleaching



What you can do

- Minimize excitation intensity (optimize laser power and exposure time)
- Use more photostable fluorophores (e.g., Alexa Fluors, Atto dyes)
- Apply anti-fade mounting media (e.g., ProLong, Vectashield)
- Use neutral density filters to reduce illumination
- Use imaging techniques that limit exposure (e.g., confocal ROI scanning, TIRF, light sheet microscopy)
- Collect data quickly; reduce repeated imaging of the same area

Fluorescent Microscopy Techniques

Fluorescent Microscopy techniques

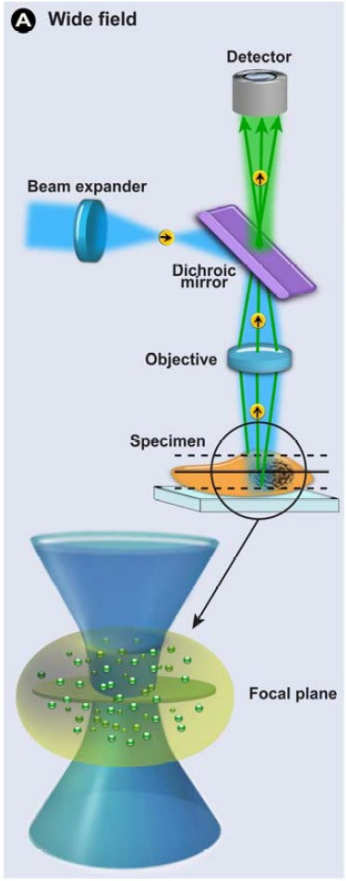
Diffraction limited

- Widefield microscopy (epifluorescent microscopy)
- Confocal microscopy
- Two-photon microscopy
- Light sheet microscopy
- TIRF
- FRAP
- FRET

Super resolution

- SIM
- STED
- PALM/STORM

Widefield Microscopy



Ishikawa-Ankerhold HC. et al.
Molecules 2012, 17, 4047-4132

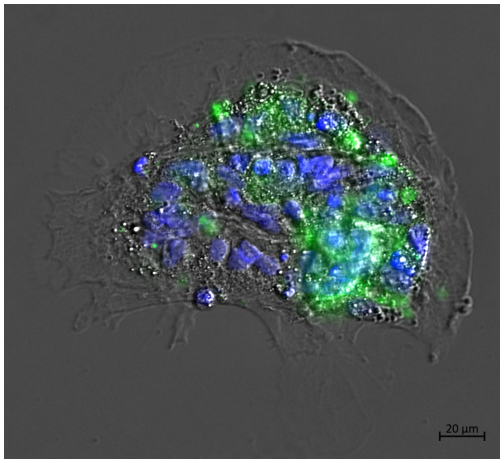
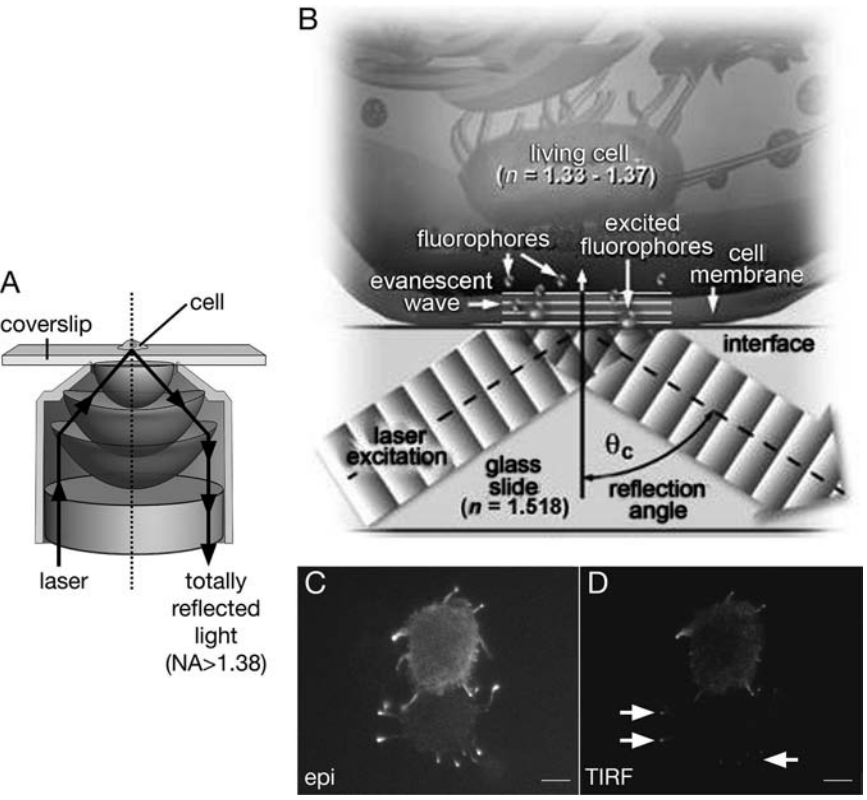


Image captured on CD7 Zeiss incubated microscope
With a 20x/0.8 dry lens, Rockefeller University C.Pyrgaki

- Least expensive technique to implement
- Good lateral resolutions
- Fast temporal resolution
- Amenable to further processing to improve axial resolution.
- Not ideal for thick or densely labelled samples because all the out of focus light that is integrated in the image

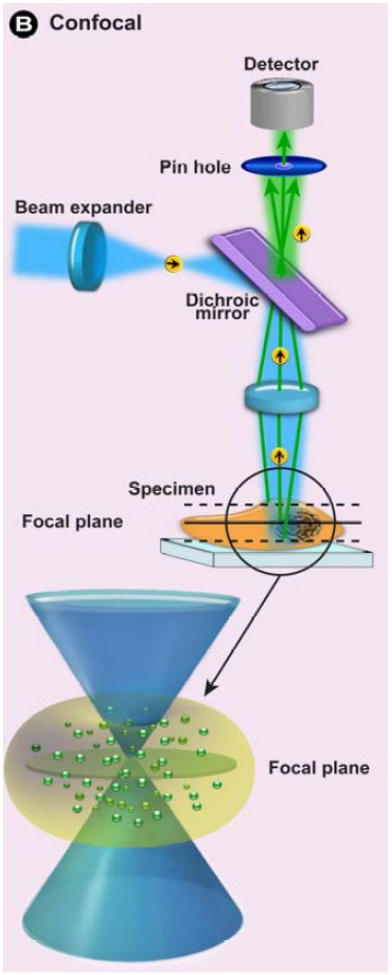
TIRF (Total internal reflection)



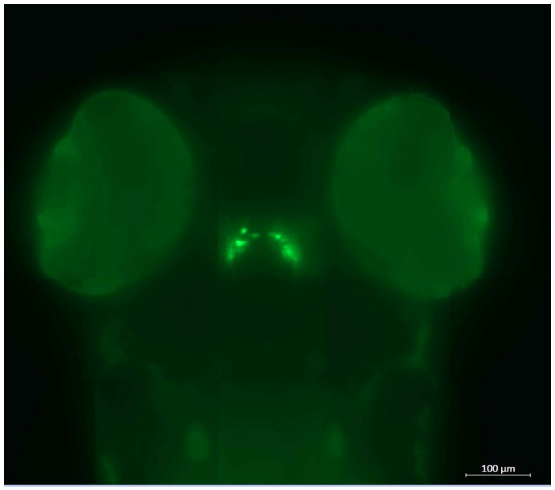
- Increased z resolution several times as compared to the z-resolution of a confocal.
- Reduced background better signal to noise ratio
- Relatively inexpensive as compared to techniques like confocal or two photon microscopy
- Able to observe a very short distance from the coverslip (100nm).
- Quantification is not a trivial task when it comes to TIRF

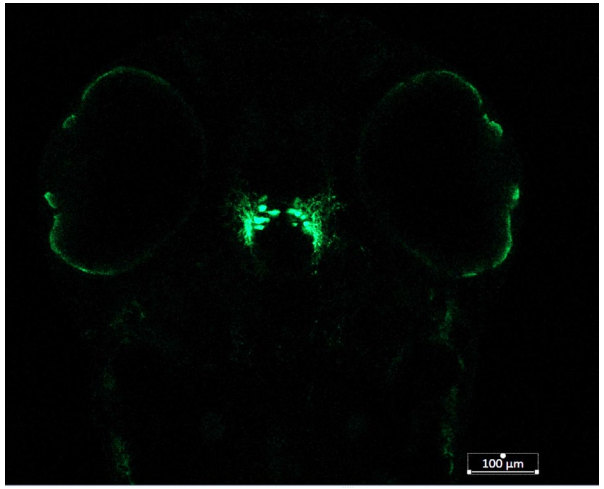
Combs CA and Shroff H
Current Protocols in Neuroscience 2.1.1-2.1.24

Confocal Microscopy



Ishikawa-Ankerhold HC. et al. Molecules 2012, 17, 4047-4132

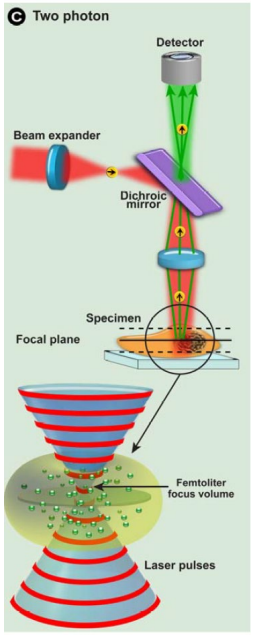




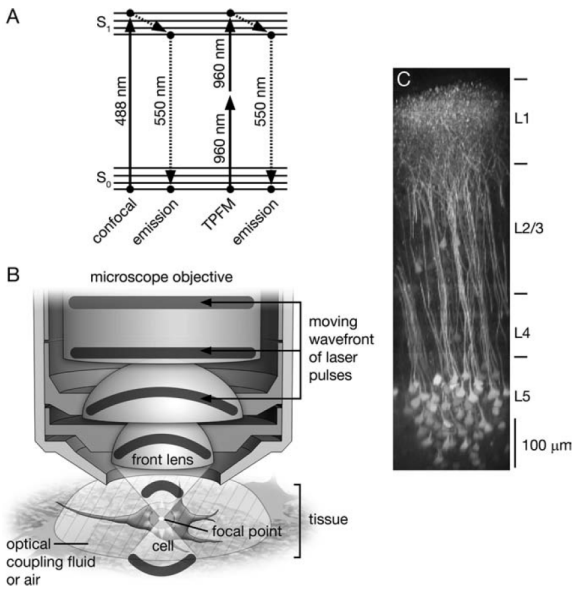
Zebrafish neurons imaged on an LSM980 with a 40X/1.2 water imersion lens. LED illumination and axiocam on the left and 488nm Laser and GaAsp detector on the right.

- Optical sectioning that allows thicker or densely samples to be imaged
- Suitable for live and fixed samples.
- Can easily handle 3D, 4D experiments.
- Slow (beam must be swept through each pixel in the field of view)
- Photobleaching and phototoxicity throughout the cone of illumination

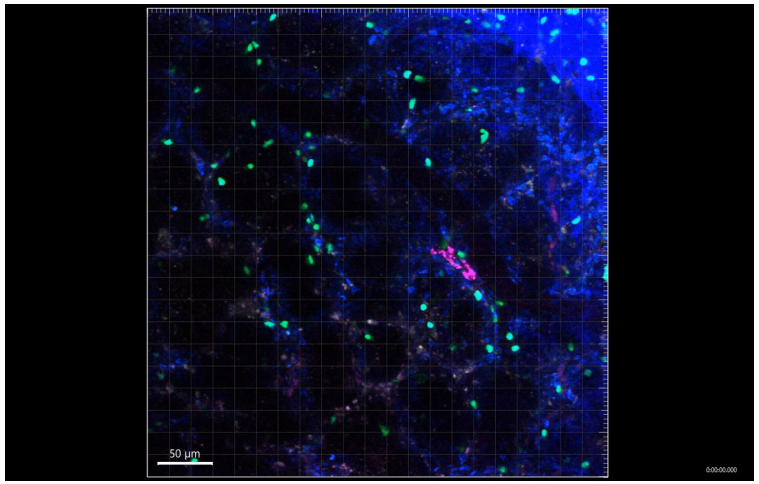
Multiphoton Microscopy



Ishikawa-Ankerhold HC. et al.
Molecules 2012, 17, 4047-4132



Combs CA and Shroff H
Current Protocols in Neuroscience 2.1.1-2.1.24

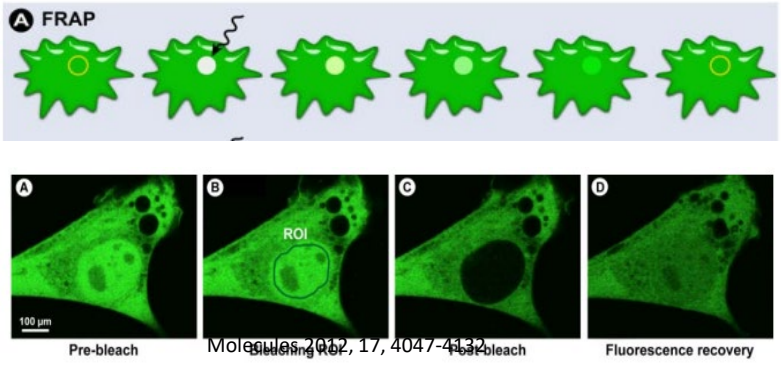


T-Cells moving in the small intestine of mouse model of colon cancer
Dr. Bernardo Reis

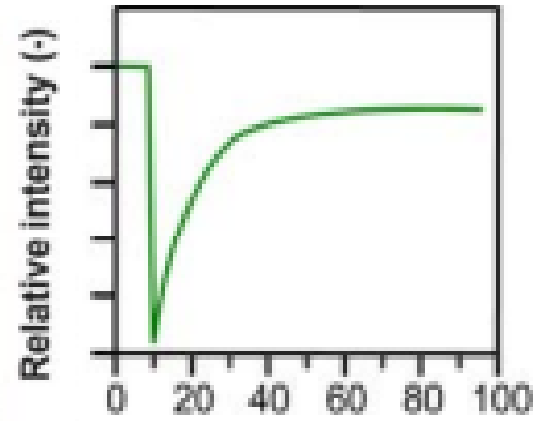
- Optical sections inherently no need for a pinhole
- No loss of light that would get rejected by the pinhole in confocal imaging
- Useful for imaging deep into live and fixed samples and whole organisms.

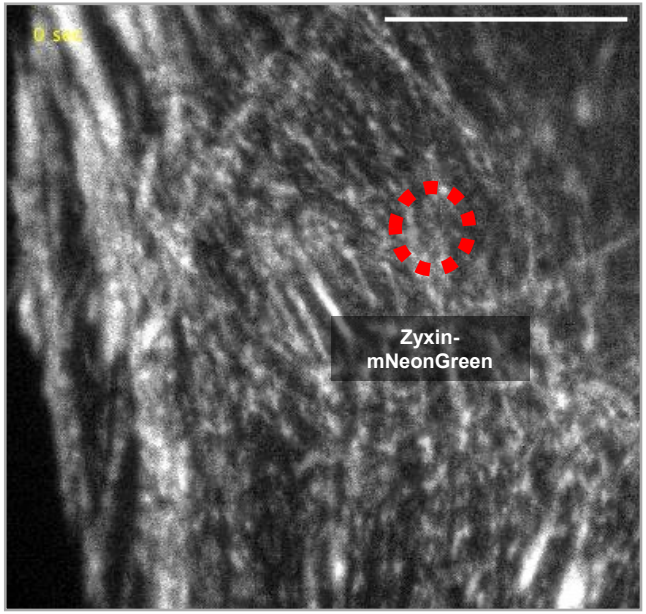
- Photobleaching only at the focal point
- Expensive to implement because of the need for pulsed and tunable lasers
- Trickier to design multi-dye experiment due to excitation overlap

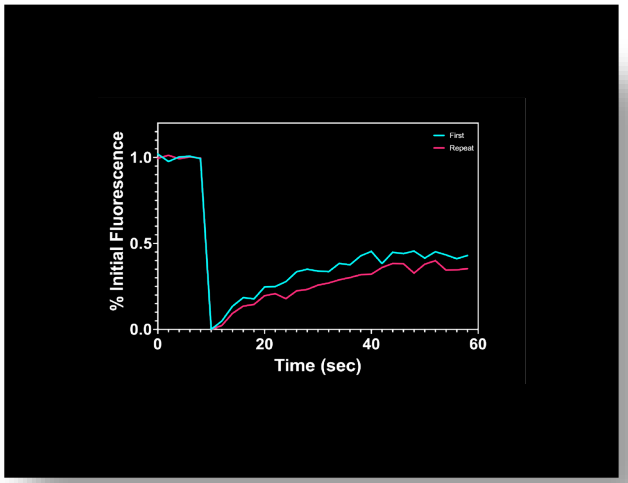
FRAP (Fluorescence Recovery after Photobleaching)



Molecules 2012, 17, 4047-4132

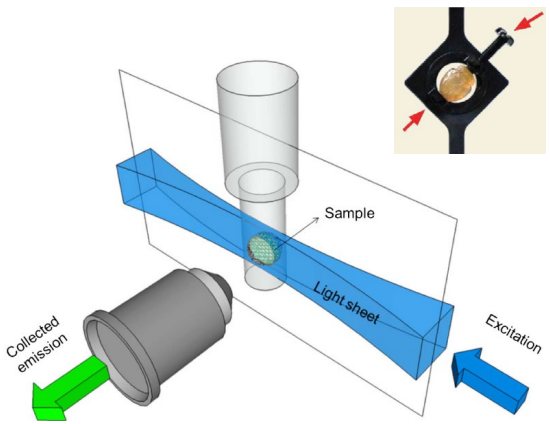






Images acquired with 63x/1.3 Glycerol lens on the iSIM from VisiTech Courtesy of Donovan Phua

Light Sheet Microscopy



Vol. 10, No. 1 / March 2018 / Advances in Optics and Photonics

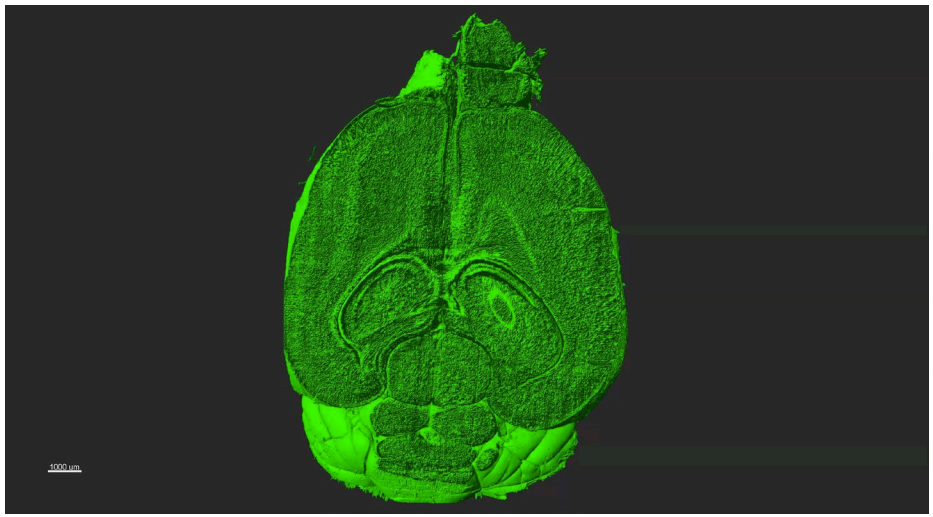
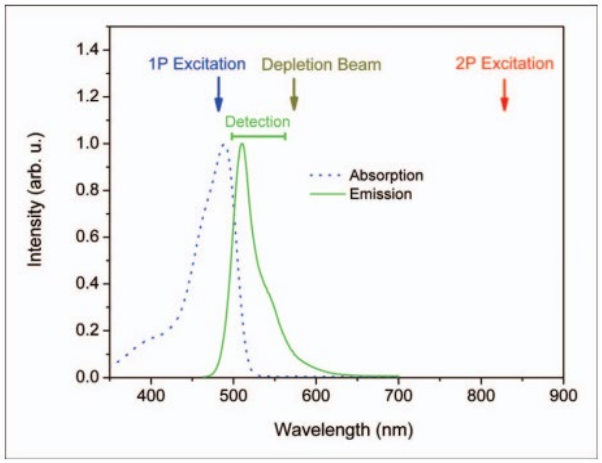


Image Courtesy of Marc Schneeberger Pane captured with a 4x/0.3 lens on a LaVision Ultramicroscope. BIRC Rockefeller University

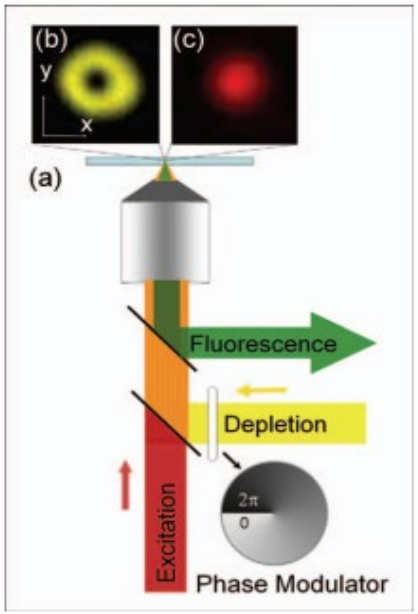
- Fast 3D imaging
- Optical sectioning
- Can be adapted to a variety of sample sizes
- Low photobleaching and phototoxicity
- Great for live imaging

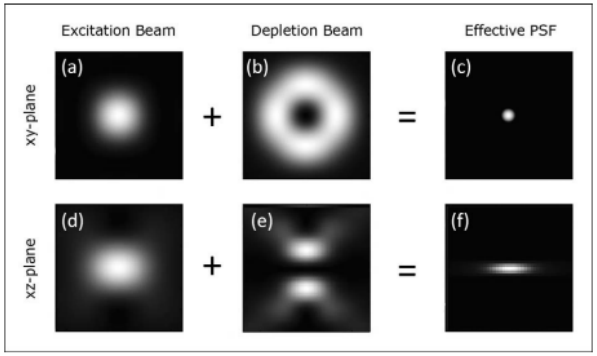
- Large data sets
- Shadows/streak effect on the sample
- Special sample preparation for thicker samples

STED stimulated emission depletion



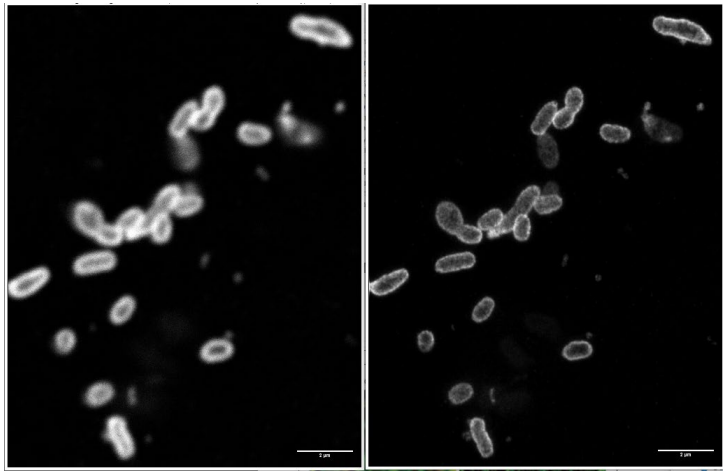
- No need for post-processing (although decon helps)
- Suitable for live cell and 3D imaging
- Pretty straight forward at the user's end
- Easy to implement two-color STED
- High cost of the system (high power depletion lasers, time gating HyD detectors)
- Photobleaching and phototoxicity
- Challenges of maintaining the shape of the STED beam as one moves deeper in a sample.



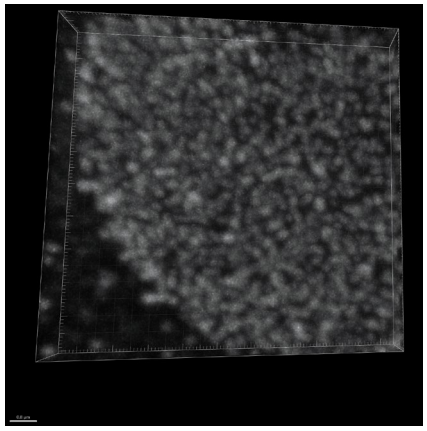


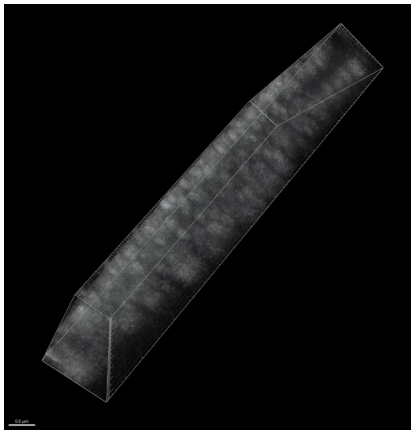
Leung BO, Chou KC. Review of Super-Resolution Fluorescence Microscopy for Biology. *Applied Spectroscopy*. 2011;65(9):967-980

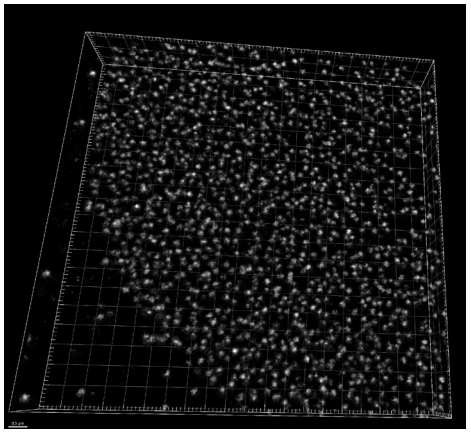
STED images

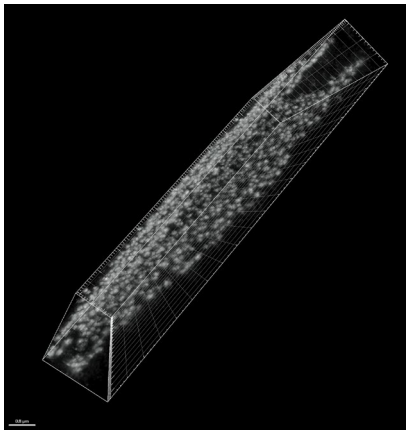


Cell wall of *Orientia tsutsugamushi stained with Star Red*
Images captured with 100x/1.45 Olympus lens on an Abberior facility line (2D STED) Image by C.Pyrgaki



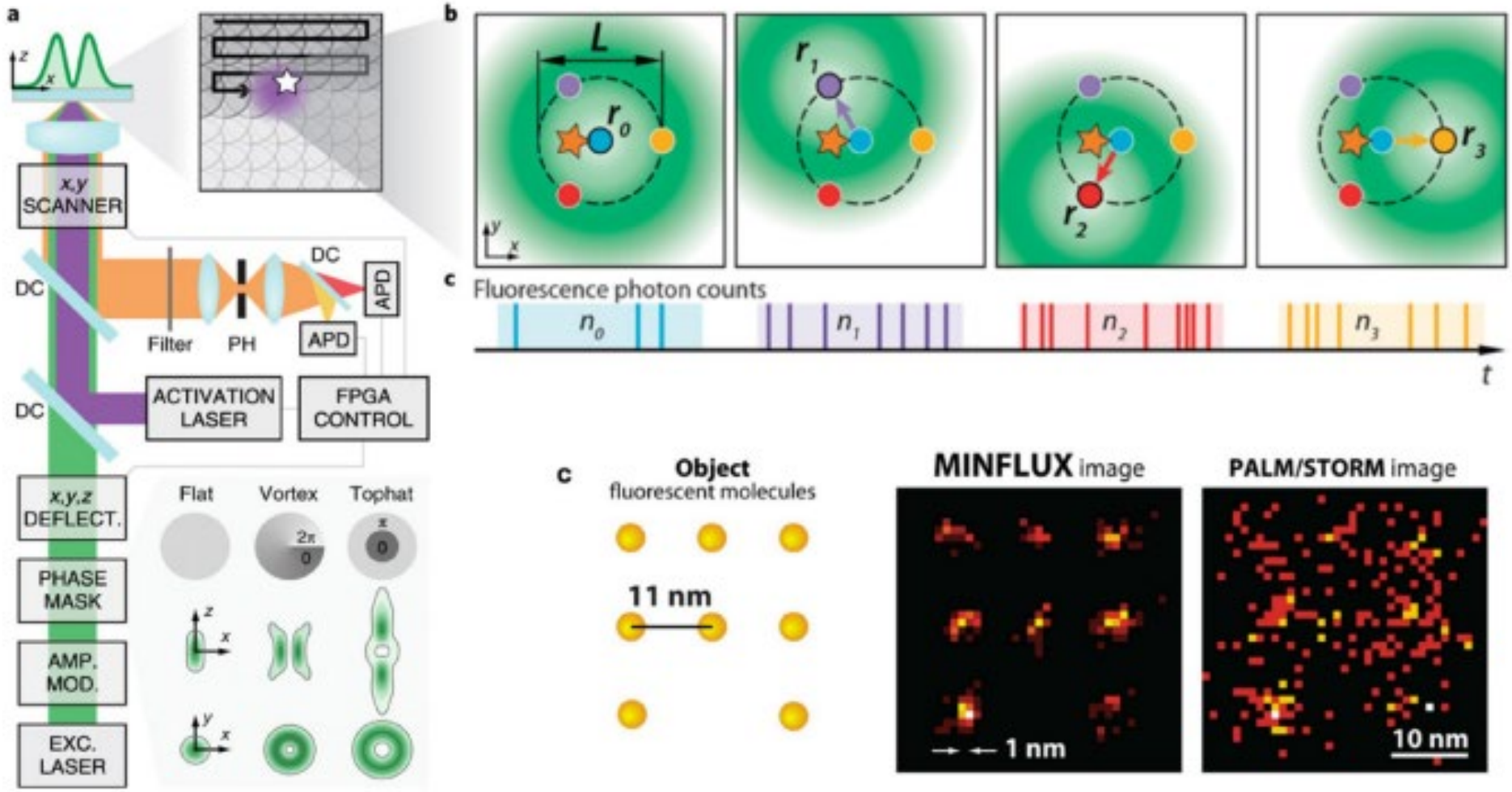






Neucleopores stained with StarRed. Images captured with 100x/1.45 Olympus lens on an Abberior facility line (3D STED)

Minflux Principle



Future of fluorescence microscopy

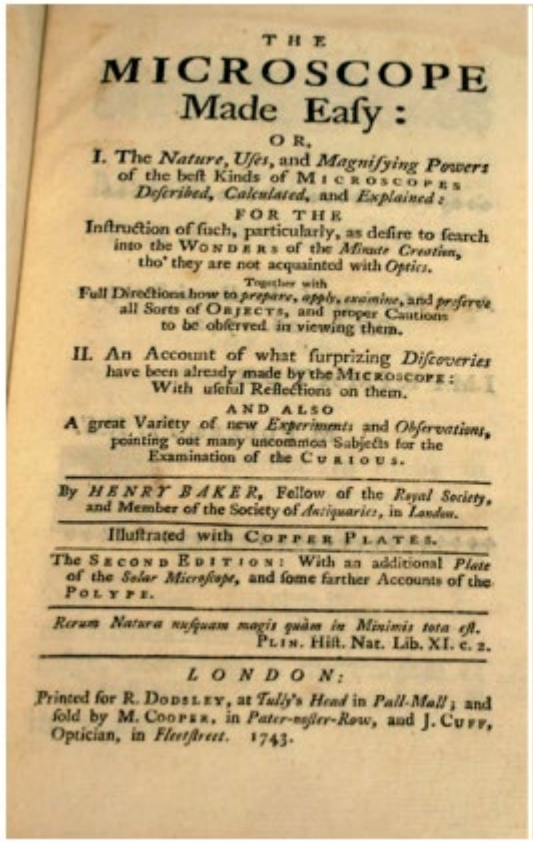
- Brighter and more photostable fluorophores
- Fluorophores with longer stoke's shifts
- Fluorophores in the NIR
- Nanobodies and small protein tags
- Instruments with smaller footprint/portable
- Open-source software and hardware
- New techniques pushing the limits of resolution even further (Minflux)

Summary

- Consult with the experts early in the process of designing your experiment
- Don't cut corners when it comes to sample prep
- Match technique to question
- Understand trade-offs
- Image aiming for the best quantifiable image

If you have questions feel free to reach out:

Pyrgakc1@mskcc.org



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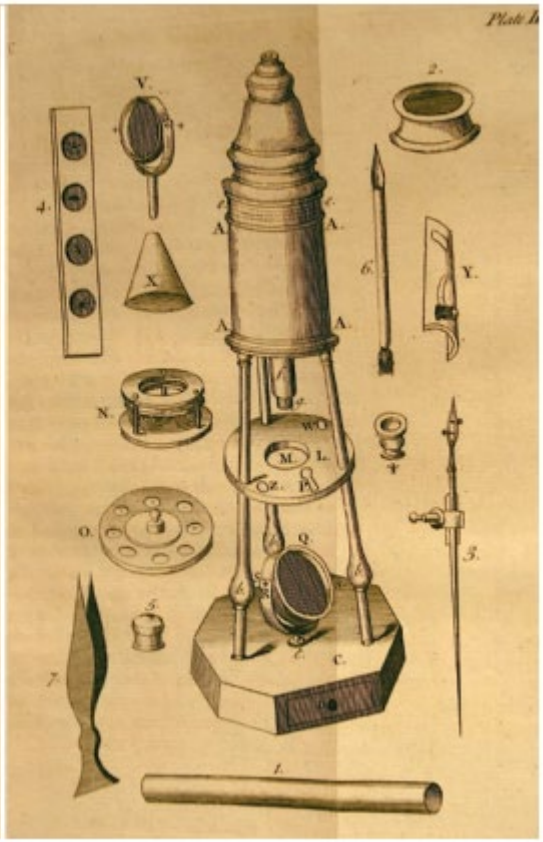
PLIN. Hiſt. Nat. Lib. XI. c. 2.
LONDON:
Printed for R. DODSLEY, at *Tully's Head* in *Pall-Mall*; and ſold by M. COOPER, in *Pater-noſter-Row*, and J. CUFF, Optician, in *Fleetſtreet*. 1743.



"When you employ the microscope, shake off all prejudice, nor harbor any favorite opinions; for, if you do, 'tis not unlikely fancy will betray you into error, and make you see what you wish to see".

Henry Baker from “The microscope made easy”1753

Q&A