Fundamentals of Fluorescence and the Fluorescence Microscope

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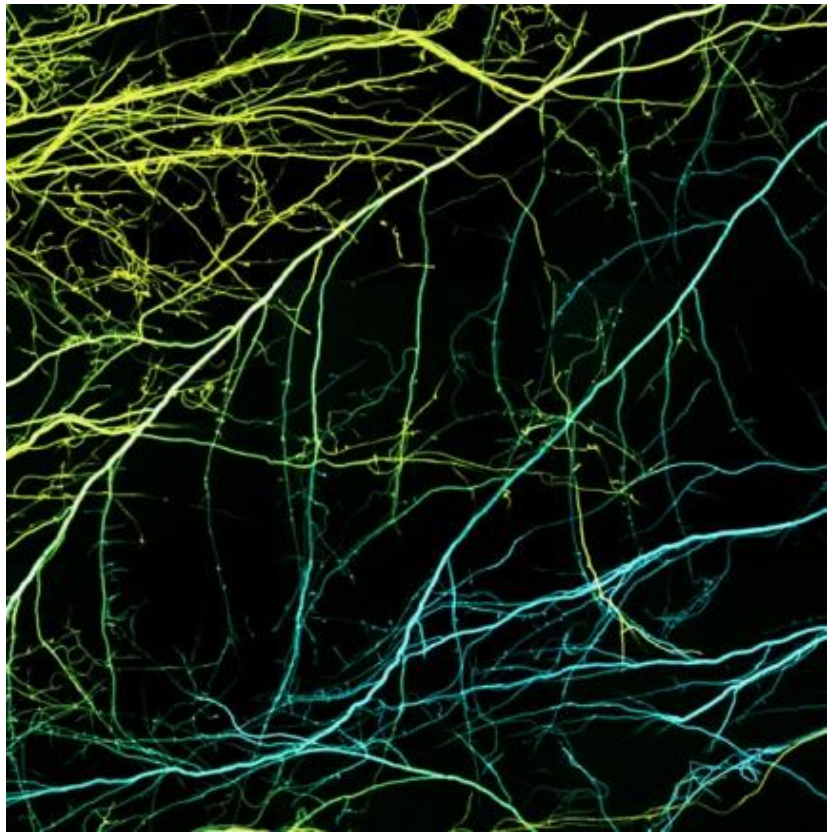
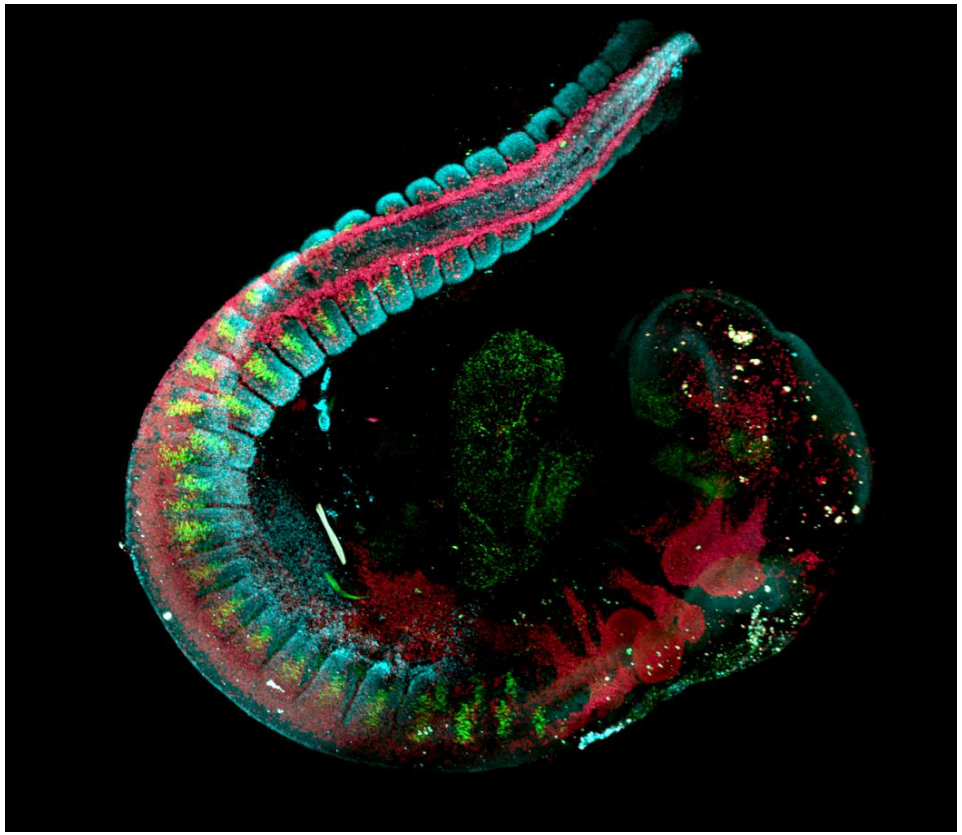
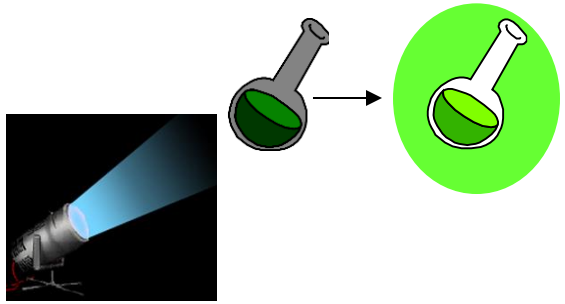




What is fluorescence?

Fluorescence occurs when specimens irradiated with **high energy** (short wavelength) light re-radiate light of **lower energy** (longer wavelength).



Fluorescence microscopy is used to study material that is **naturally fluorescent** (e.g. GFP) or that **can be made to fluoresce** when treated with chemicals (fluorochromes).

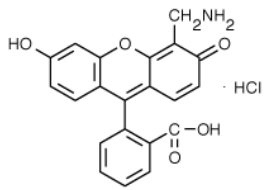
Categories of fluorescent molecules

- **Fluorochromes** (aka fluorescent probes, dyes) = molecules that undergo electronic transitions resulting in fluorescence;
- **Fluorophores** = fluorochromes conjugated to larger macromolecules (nucleic acid, lipid, enzyme, proteins). Can be:
  - Intrinsic (GFP, porphyrins);
  - Extrinsic (added to specimen to produce fluorescence).

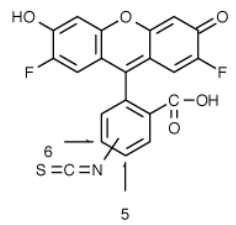
Fluorophore structure – typically a polyaromatic hydrocarbon or heterocyclic ring structure;

Minor changes can greatly enhance performance.

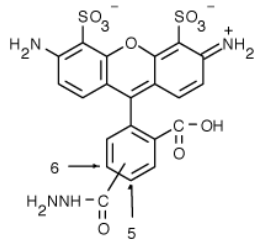
Fluorescein
pH sensitive
Unstable



Oregon Green
Enhanced stability
Enhanced intensity

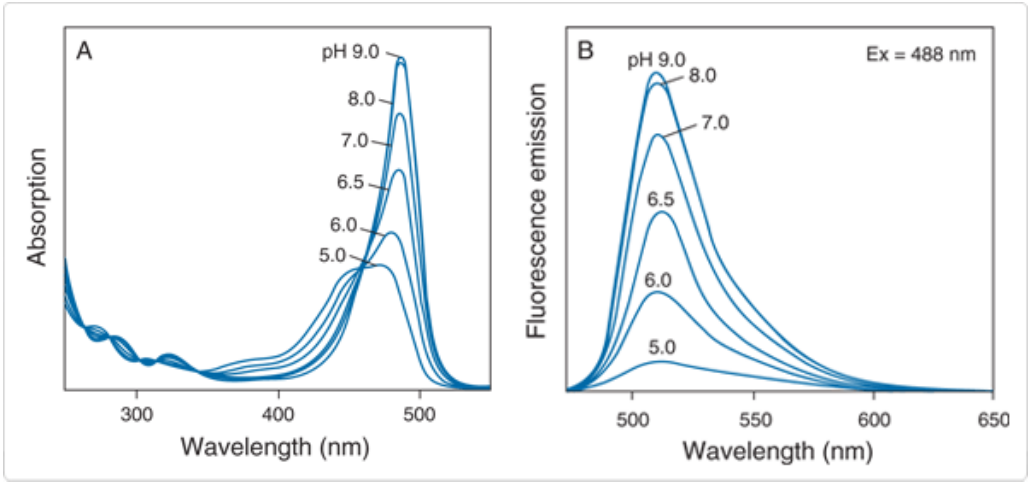


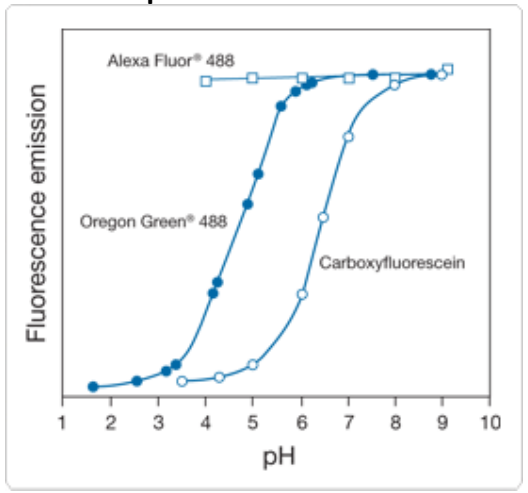
Alexa 488
Even greater stability
More Enhanced intensity



Comparison of FITC and its derivatives

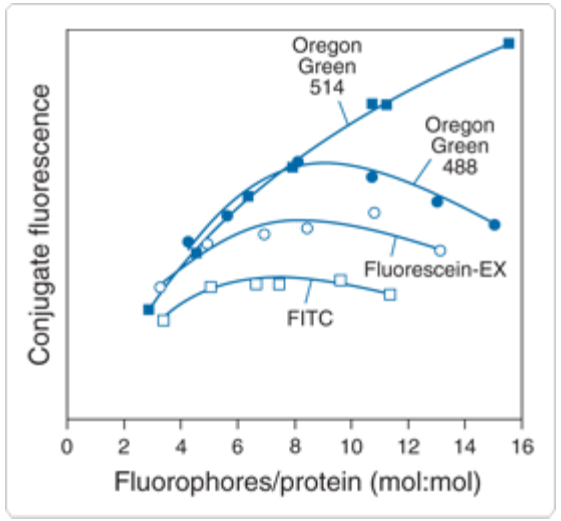
Fluorescein ← **pH-dependence** → Improved derivatives



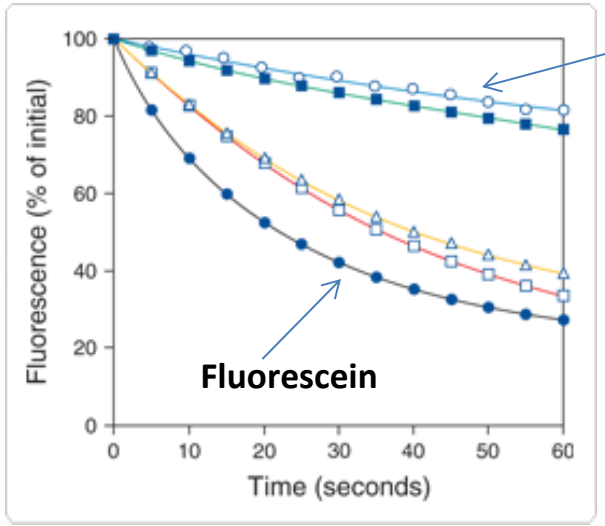


Conjugate fluorescence

(Quantum yield x fluorophores/protein)



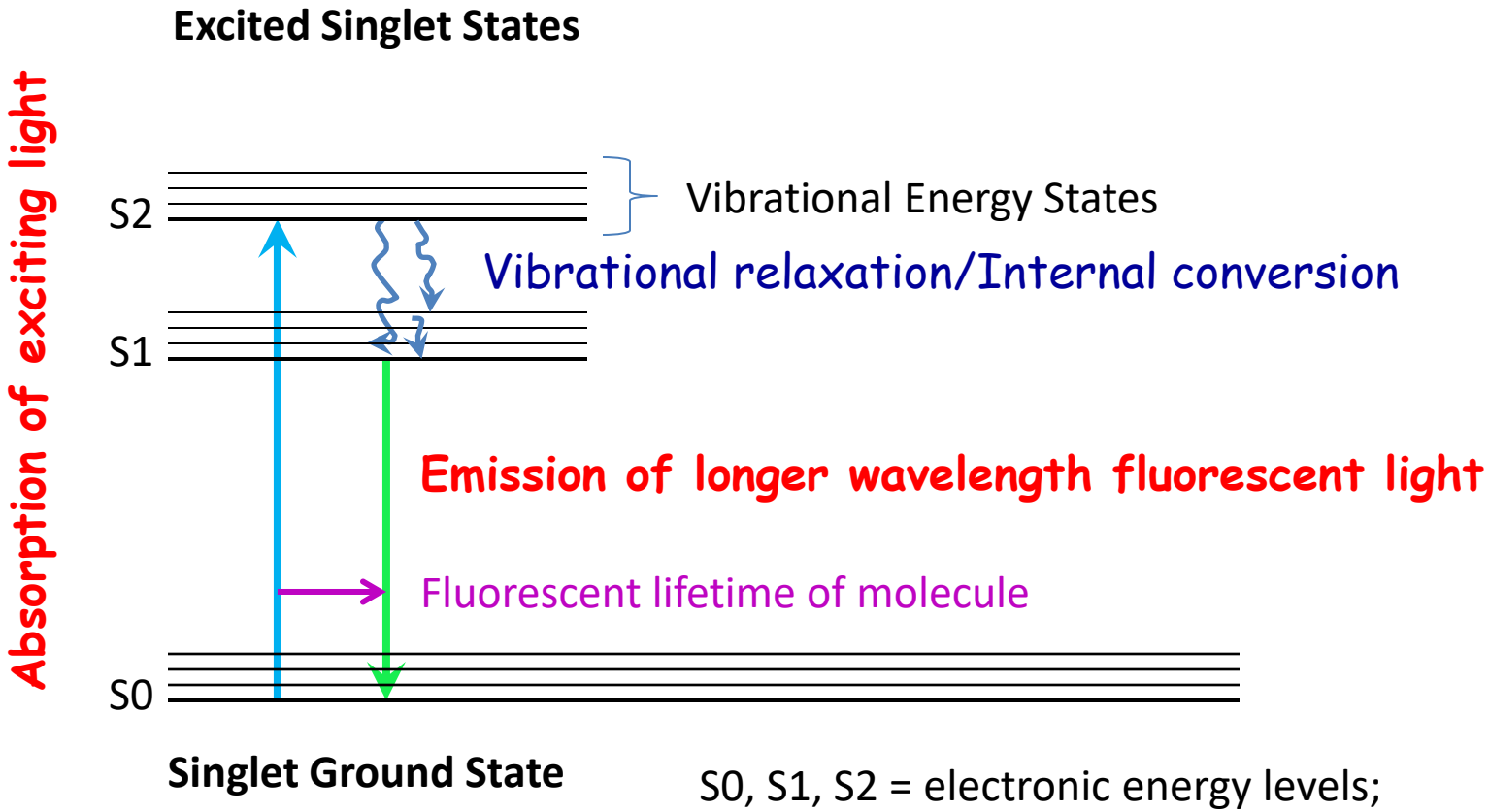
Photostability



http://www.lifetechnologies.com

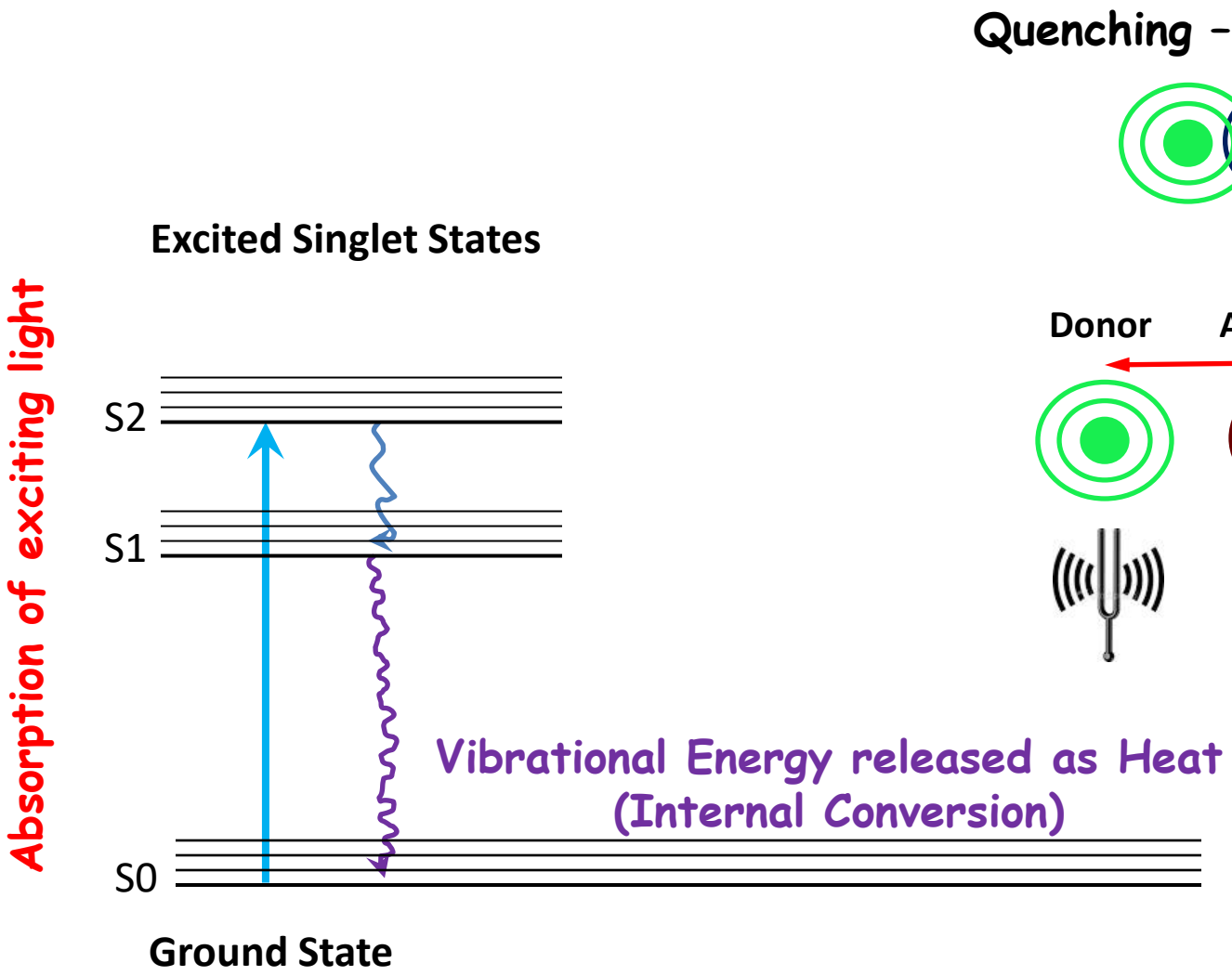
How does Photon Emission occur?

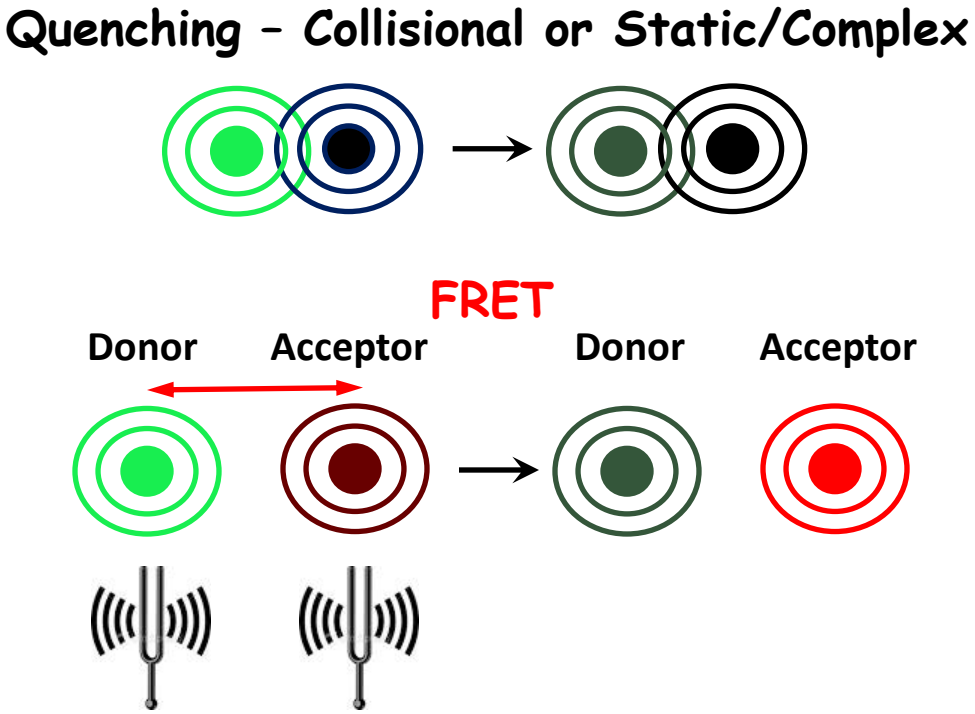
Jablonski diagrams show energy levels occupied by an excited electron within a fluorescent molecule



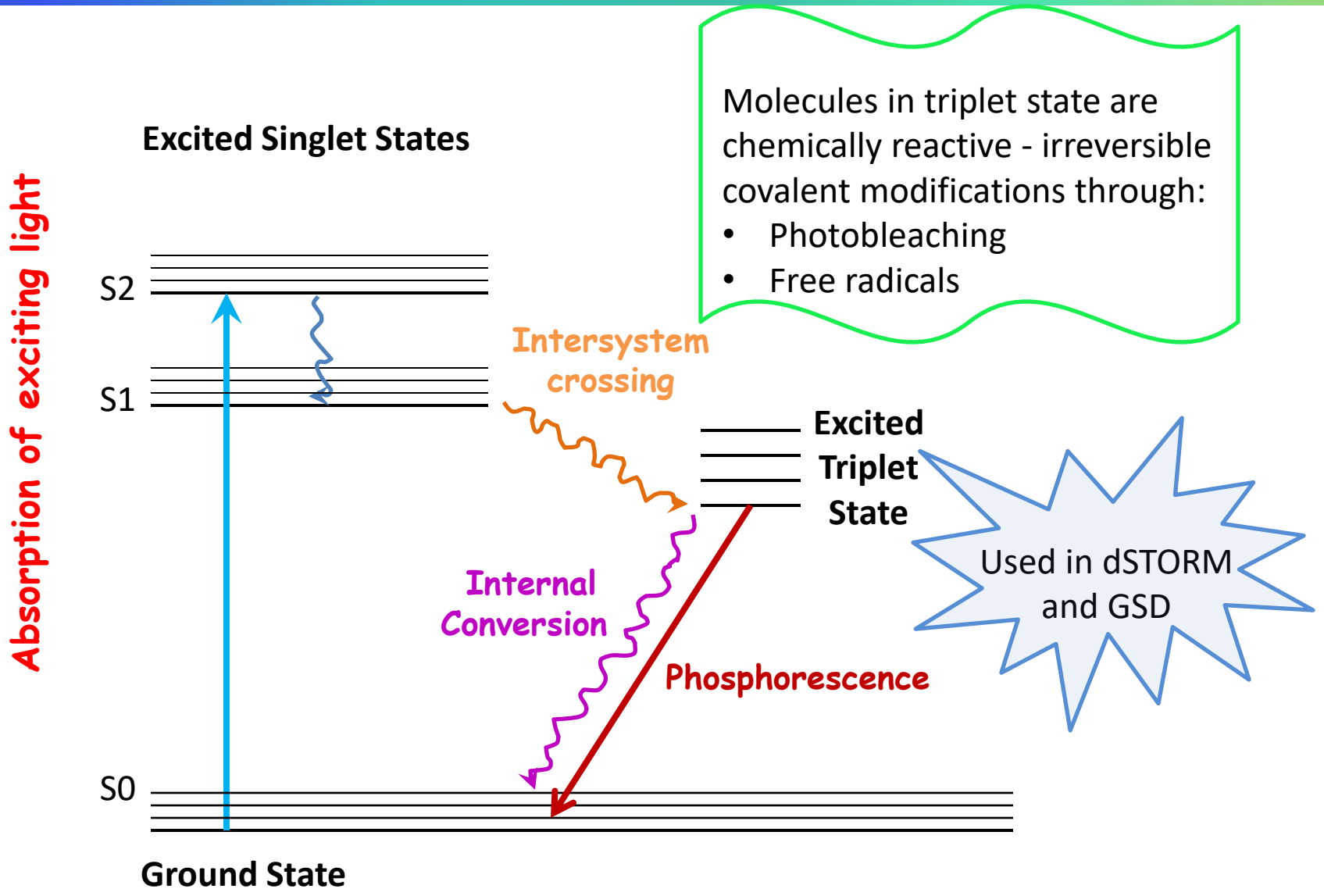
S0, S1, S2 = electronic energy levels;
Thin lines = vibrational/rotational energy levels.

Other possible relaxation pathways I





Other possible relaxation pathways II

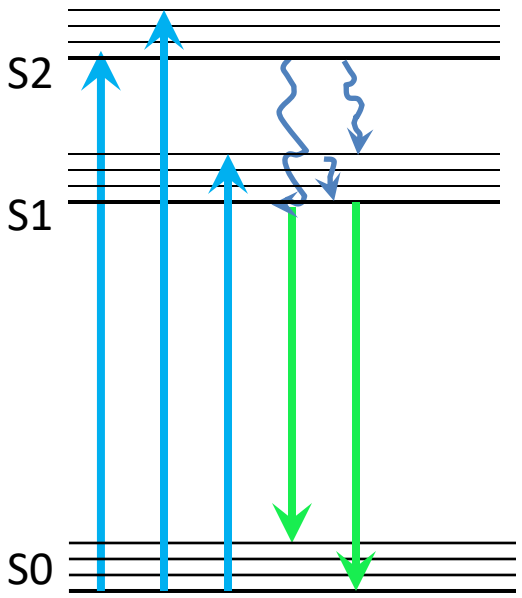


Timescale Range for Fluorescence Processes

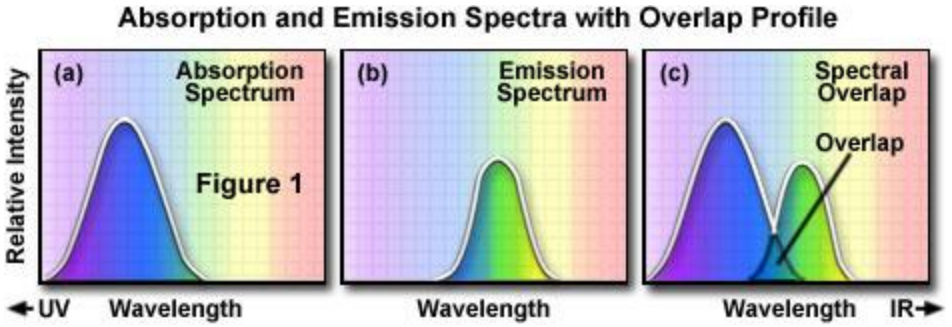
Transition	Process	Timescale (Seconds)	
S(0) => S(1) or S(n)	Absorption (Excitation)	10^{-15}	(femtoseconds)
S(n) => S(1)	Internal Conversion	10^{-14} to 10^{-10}	
S(1) => S(1)	Vibrational Relaxation	10^{-12} to 10^{-10}	(picoseconds)
S(1) => S(0)	Fluorescence	10^{-9} to 10^{-7}	(nanoseconds)
S(1) => T(1)	Intersystem Crossing	10^{-10} to 10^{-8}	
S(1) => S(0)	Non-Radiative Relaxation Quenching	10^{-7} to 10^{-5}	
T(1) => S(0)	Phosphorescence	10^{-3} to 100	
T(1) => S(0)	Non-Radiative Relaxation Quenching	10^{-3} to 100	

http://www.olympusmicro.com/primer/techniques/fluorescence/fluorescenceintro.html

Absorption and Emission occurs across a characteristic spectrum for each fluorochrome



- Irradiation with spectrum of wavelengths = transition to different energy states/vibration levels:
  Absorption spectrum
- Relaxation to different levels in the ground state:
  Emission spectrum



Fluorochromes may be **named by Ex. max.** (e.g. AlexaFluors, DyLights) or **Em. Max.** (e.g. Qdots, some Fluorescent Proteins).

http://micro.magnet.fsu.edu/primer/lightandcolor/fluoroexcitation.html

Sources of Autofluorescence

Analytical Biochemistry, **291**, 175 (2001)

Source	Organism/Tissue	Ex (nm)	Em (nm)
Flavins	CHO cells	380, 460	520
	Rat hepatocytes	468	525
	Neural cells (rat, bovine)	488	540-560
	Goldfish inner ear	450	540
	Periplaneta americana	<350	530
NAD(P)H	Rat cardiomyocytes	395	509
	S. cerevisiae	366	440-470
	CHO cells	360	440-450
Lipofuscins	Medulla (rat, human, rhesus monkey)	460-490	520
	Rat heart	450-490	550
	Muscle, myocardium, hepatocytes	360	540-560
	Human brain	435	481-673
	Rat liver	345	430
	Rat retina	390-490	>510
Collagen and elastin	Aorta, coronary artery (human)	476	>515
	Skin (human)	442	470-520

Autofluorescence @ ex/em= 488/530) of a typical 3T3 cell is equivalent to about 34,000 fluorescein molecules. Long Exposures are a problem.

Properties of Fluorophores

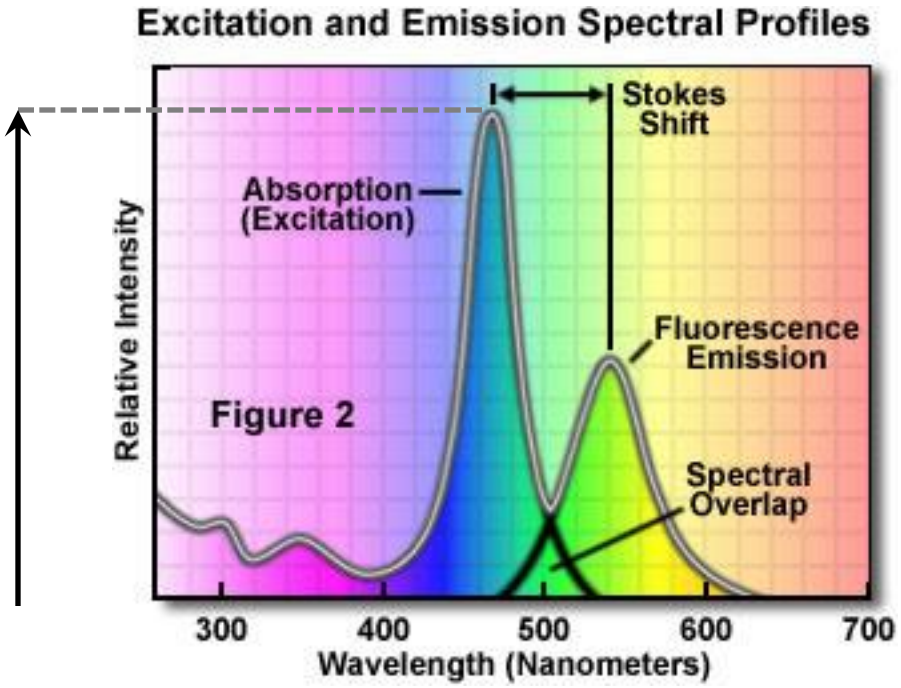
Molar extinction coefficient:
Potential of a fluorochrome to absorb photon quanta;

Quantum yield:
Probability that absorbed photon will be re-emitted as fluorescent photon

Useful fluorochromes typically have:

- High Molar extinction;
- High Quantum yield;
- High Stokes shift.

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



http://micro.magnet.fsu.edu/primer/lightandcolor/fluoroexcitation.html

Why use fluorescence microscopy?

Advantages of fluorescence:

- **Sensitivity (**high signal:background);
- **Specificity** (target single molecular species; light up single cells/sub-regions);
- Speed and **temporal** resolution (< 1 millionth of second) - kinetics;
- Compatible with **live cells**;
- **Multiple colours**/targets (cellular context);
- Local **environment** affects fluorescence emission (pH, ion concentrations, membrane potentials etc.).

Disadvantages of fluorescence:

- Not easily seen at low magnification;
- Fluorescence is **not permanent**;
- Requires a fluorescence microscope with appropriate lasers, filters etc.;
- Cell/tissue autofluorescence.

What technological improvements revolutionalized fluorescence microscopy?

- Brighter/more photostable fluorochromes;
- Better optics (high N.A. objectives);
- Better filters/dichroics;
- More sensitive detectors (e.g. EMCCDs, GaAsP detectors);
- Faster computers;
- Green Fluorescent Protein and other FP variants for studying living cells/organisms and investigating kinetics.

Commonly used fluorescence microscopy techniques

- **Epifluorescence** / widefield fluorescence microscopy;
- **Confocal** microscopy (point scanning or spinning disk);
- **Light-sheet** microscopy;
- **Multiphoton** microscopy;
- **Deconvolution** techniques (combined with any/all microscopies);
- **Total Internal Reflection** Microscopy;
- **Super-resolution** microscopy (STED, 3D-SIM, PALM/STORM/GSD).

Anatomy of the Basic Fluorescence Microscope

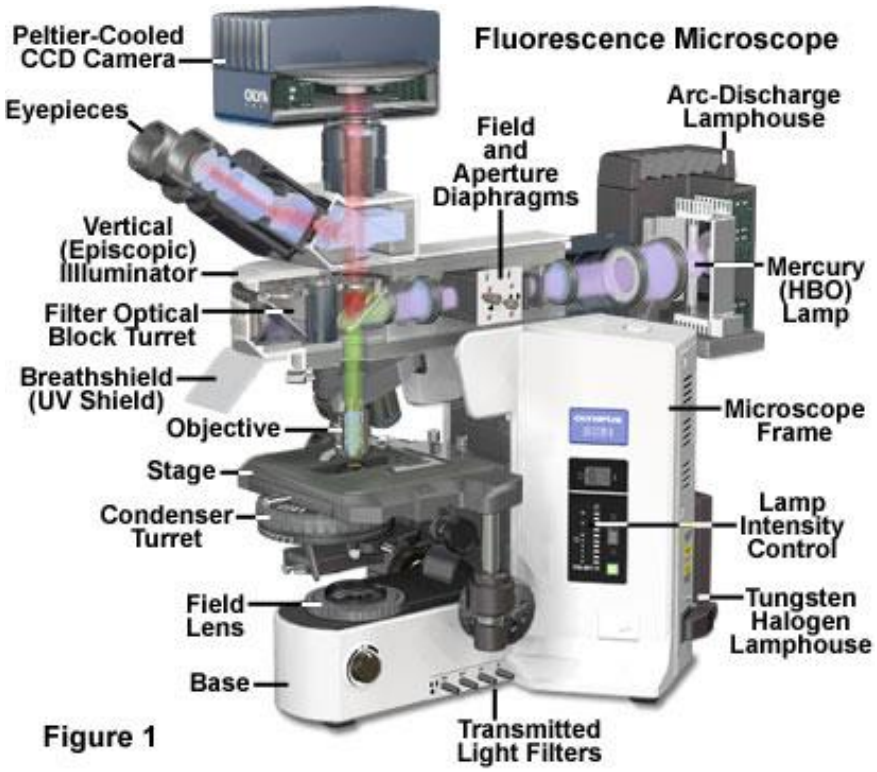


Figure 1

Epi-fluorescence microscopes aka:

- Reflected light fluorescence;
- Episcopic fluorescence;
- Incident light fluorescence.

Incident exciting light and emitted fluorescent emission follow the same pathway through the microscope i.e. **objective also acts as condenser**.

Microscope components for fluorescence imaging

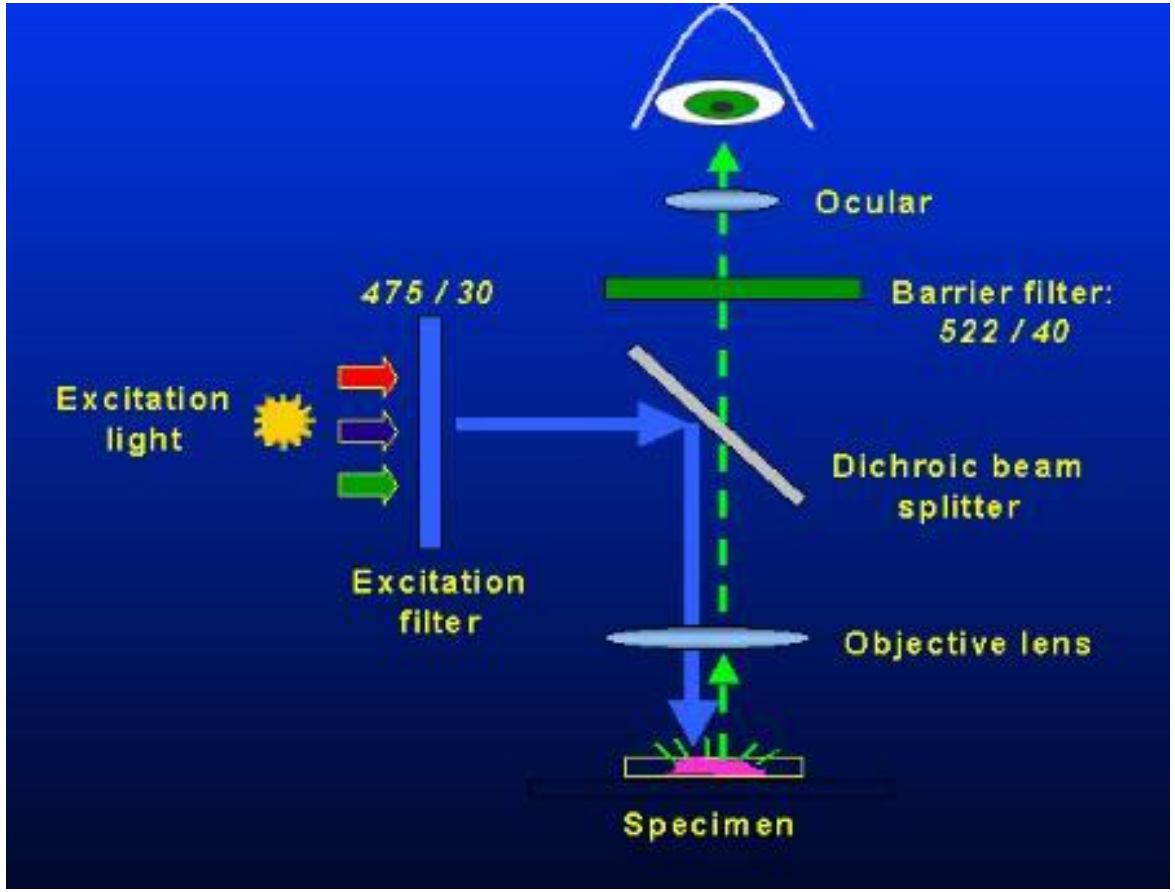
- ***Excitation light*** source (lamp, laser, LED) produces light of different colors;

- ***Excitation filter*** selects wavelengths to excite fluorochrome;

- ***Dichromatic beam splitter*** (mirror) typically reflects shorter wavelength light and transmits longer wavelength light;

- ***Emission/barrier filter*** selects wavelengths that are detected;

- ***Detector*** - sensitive to emitted wavelengths.



Reproduced from:
http://www.seas.upenn.edu/~confocal/epi-fluor.html

Fluorescent illuminators

Factors to consider?

- Bright;
- Evenly distributed across the field;
- Constant in amplitude over time;
- Spectral profile matches fluorophores to be imaged;
- Suitable (non-harmful) for live cell imaging;
- Cost (initial and ongoing).

Common fluorescent illuminators:

- Mercury arc lamps;
- Xenon arc lamps;
- Tungsten halide filament lamps;
- Metal halide arc lamps;
- Light Emitting Diodes (LEDs);
- Solid state excitation sources;
- Lasers.

Incandescent lamps

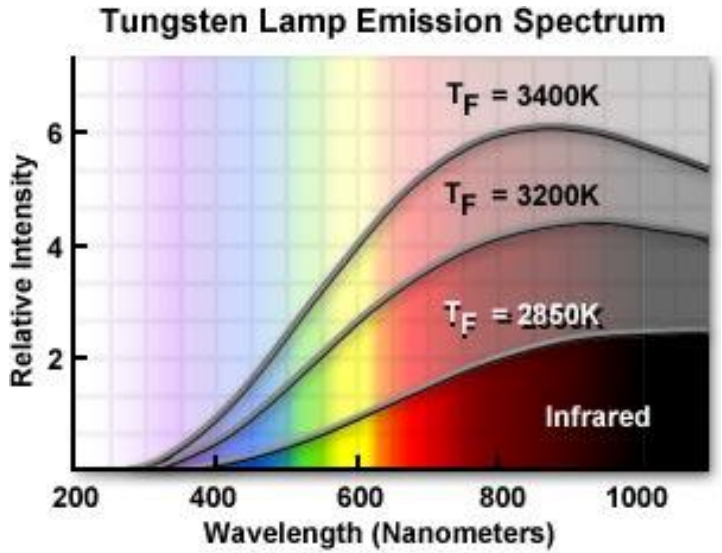
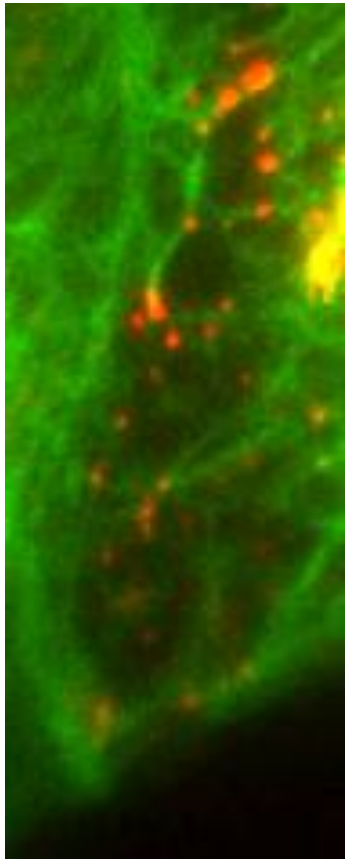


Figure 4

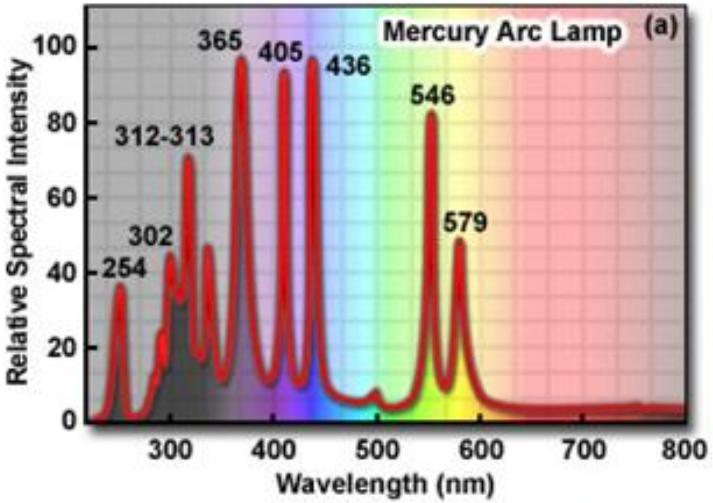
http://www.olympusmicro.com/primer/techniques/fluorescence/fluorosources.html

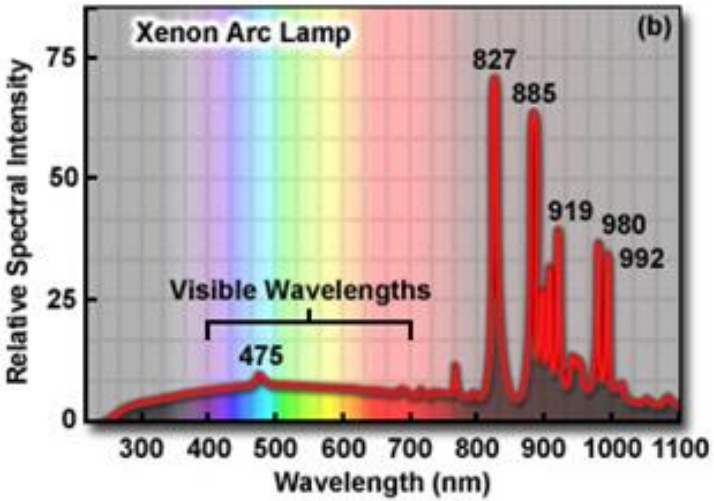
- Tungsten wire filaments plus inert argon gas;
- Bright, even illumination when used with ground glass filter;
- Continuous spectrum across visual range, peak in red and IR;
- Continuously variable power supply;
- Color balance depends on voltage;
- Cheap and convenient;
- Commonly used for brightfield, phase contrast and DIC;
- Can be used for fluorescence.

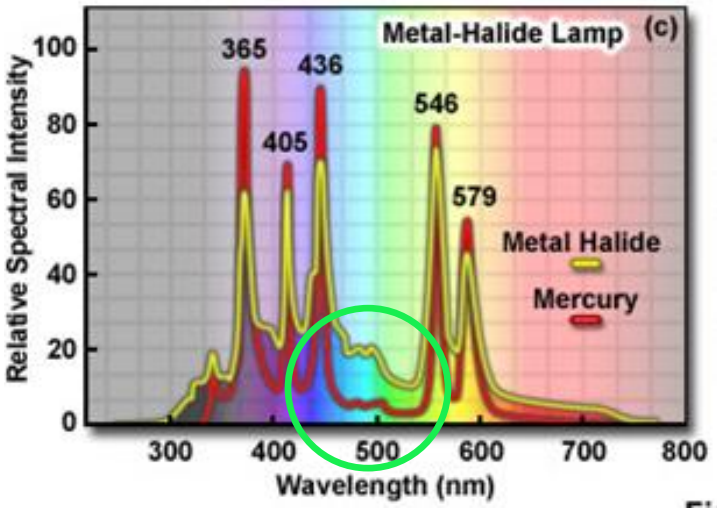


Alison North

Spectra of Arc lamps and Metal Halide lamps







- Intense peaks at specific wavelengths;
- Mercury high UV;
- Xenon high IR;
- Metal halide better for GFP range;
- UV and IR blocking filters required for live cell imaging.

http://zeiss-campus.magnet.fsu.edu/print/basics/fluorescence-print.html

Properties of Arc Lamps and Metal Halide lamps

Arc lamps:

- 10-100 x brighter than incandescent lamps;
- Good spectral flexibility when combined with appropriate filters;
- Require careful mechanical alignment;
- **Last only 200-300 hours**;
- **Flicker**;
- Brightness decreases with age;
- Mercury lamps – very bright 546 nm line and **UV lines**;
- Xenon lamps – uniform output, bright in blue-green and red range. **IR peaks**.

Metal halide lamps:

- Similar spectrum to mercury bulbs but **brighter between peaks**;
- **Long bulb life** (2000 hrs);
- Large electrode gap means more even illumination;
- Pre-aligned in factory;
- High initial cost – and liquid light guide requires replacement too!

Light-Emitting Diode (LED) Illuminators

- 20-50 nm wide spectral bandwidth;
- No unwanted UV or IR;
- Can be turned on/off < 1 msec;
- **Cool**;
- Stable (minimal intensity fluctuations);
- Last for > 10,000 hrs;
- Need multiple LEDs to cover full spectrum;
- Early LEDs were weak (green dyes);
- High initial cost, low ongoing;
- Now can combine up to 16 LEDs in one system (e.g. pE4000 from CoolLED), including UV and near IR wavelengths.

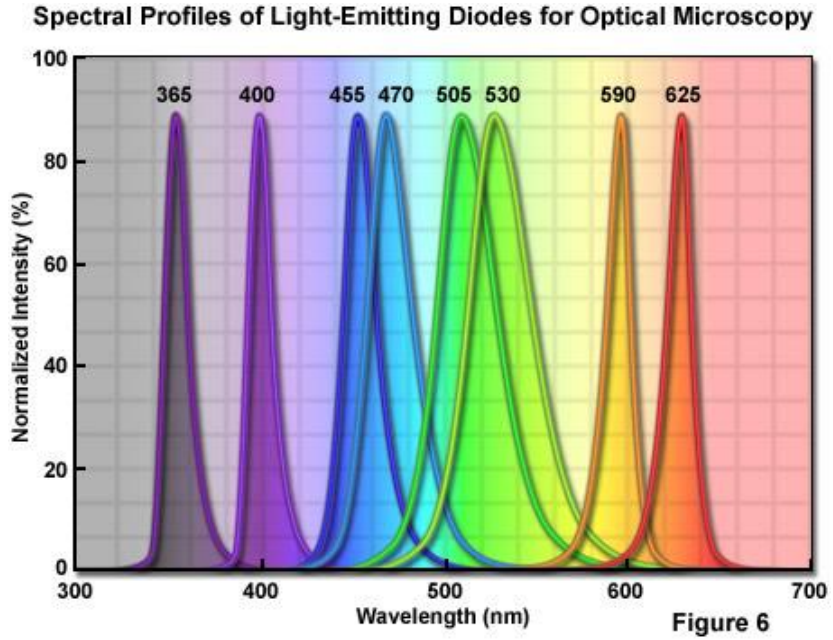
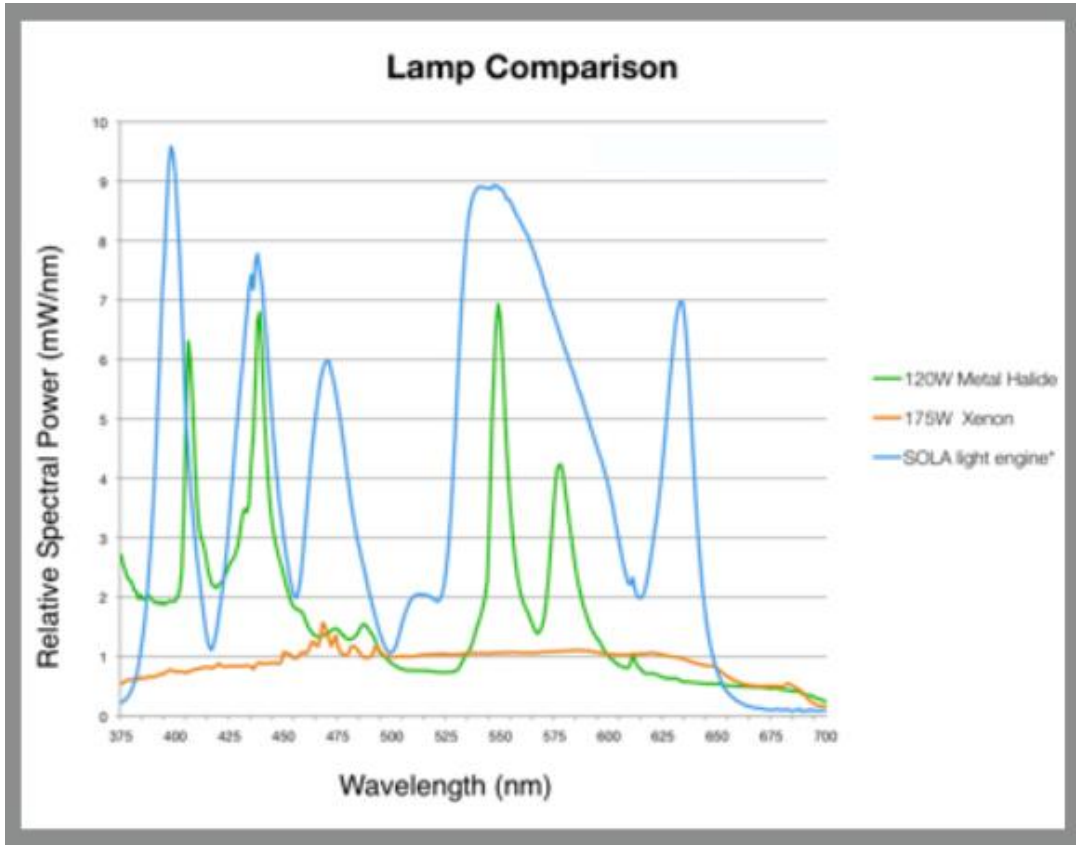


Figure 6

http://zeiss-campus.magnet.fsu.edu/articles/lightsources/lightsourcefundamentals.html

Solid State white-light excitation sources

- Multiple independent solid state sources for 2-7 discrete light bands (e.g. Lumencor SPECTRA), or white light output (SOLA), 380-680 nm, together with selectable filters;
- No UV or IR light;
- Fast switching and turn-off;
- Pre-aligned;
- Lasts ~20,000 hrs;
- Environmentally “green”.



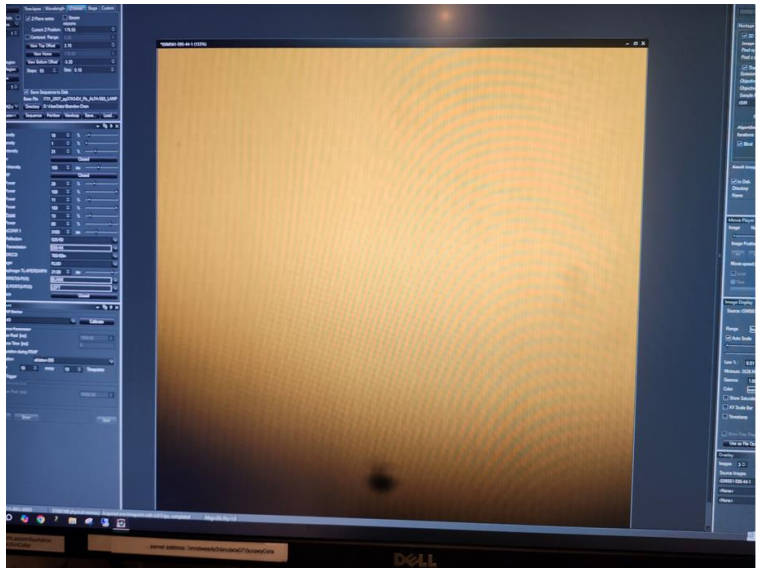
Aligning the fluorescence microscope for even illumination (typically Koehler illumination)

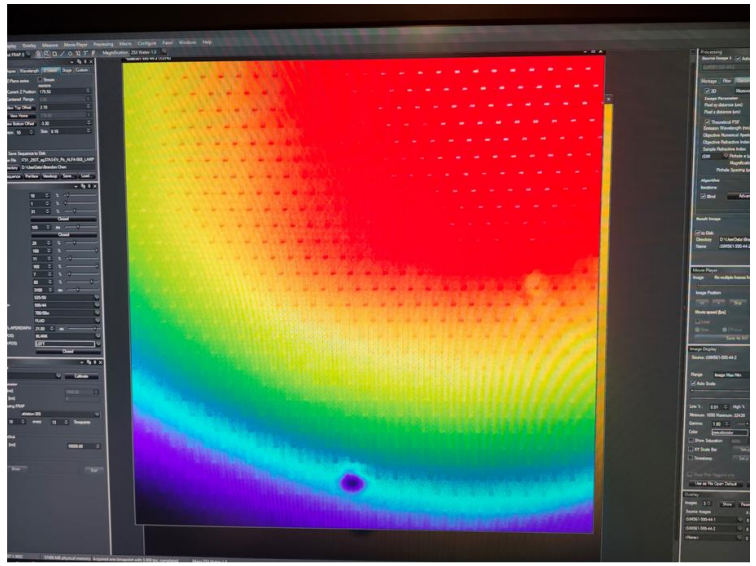
Most modern fluorescence microscopes use **Köhler illumination**:

- Light is focused at the back aperture of the objective and defocused at sample plane, to give even illumination.
- Light guides may improve evenness of illumination.

Note: it's important to image test samples e.g. plastic coloured slides (Chroma) to correct for uneven illumination.

iSIM – BIRC microscope, July 31 2025





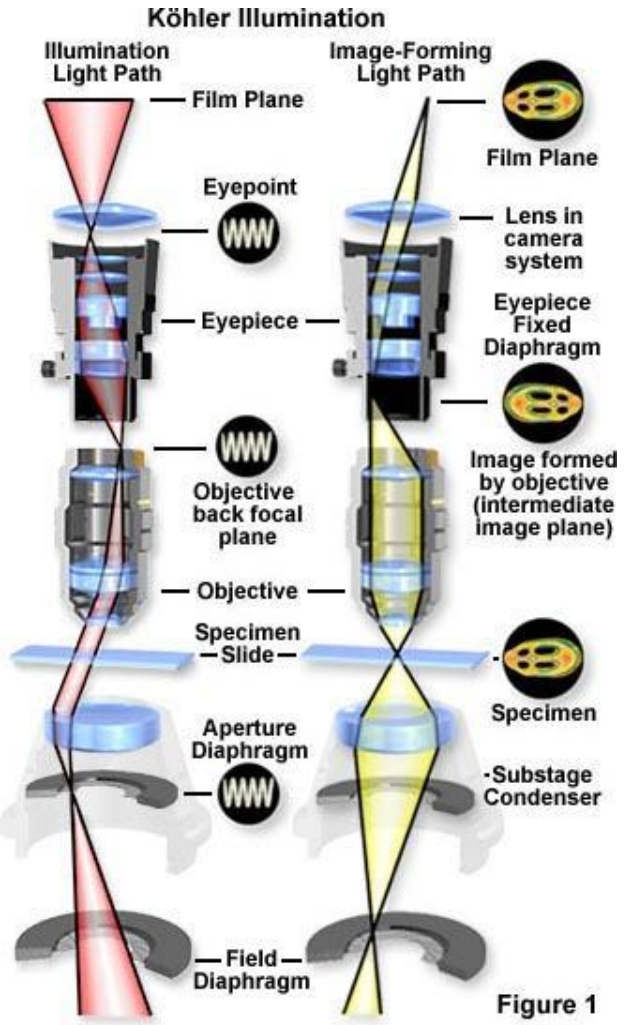


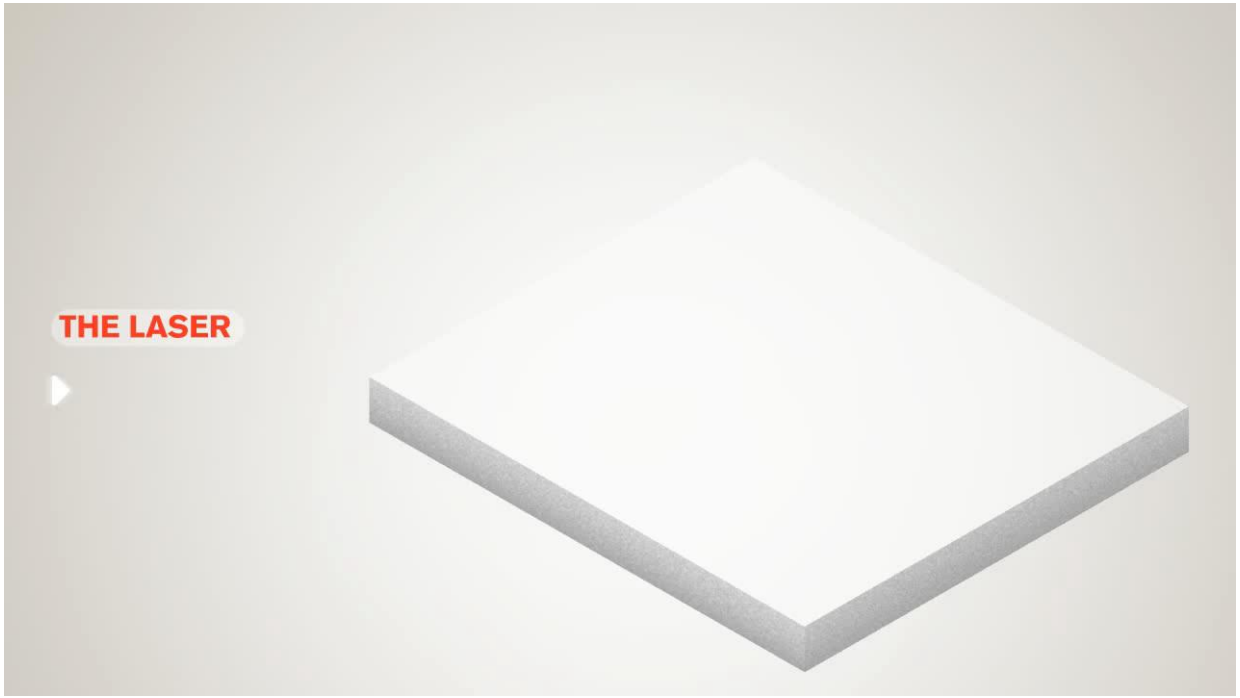
Figure 1

http://zeiss-campus.magnet.fsu.edu/articles/basics/kohler.html

Lasers

Light Amplification by Stimulated Emission of Radiation

- **Temporal coherence** = monochromatic (down to 1 nm bandwidth) and allows pulsing (femtosecond – multiphoton);
- **Spatial coherence** – can focus to tiny spot or maintain a collimated beam over long distances.



http://www.youtube.com/watch?feature=player_embedded&v=h7lhtHtKXIY

Filters for fluorescent microscopes

Excitation filters:

- Selectively pass only wavelengths required for excitation;
- Important with white light sources but even used with lasers (“clean-up” filters);

Dichromatic beamsplitter/dichroic mirror/polychroic:

- Reflect excitation (shorter) wavelengths and pass emission (longer) wavelengths;
- Polychroics - > one wavelength cut-off for simultaneous multi-colour imaging;

Emission or “barrier” filters:

- **Block out excitation light** that leaks through dichroic;
- Pass only desired emission wavelengths to the detector.

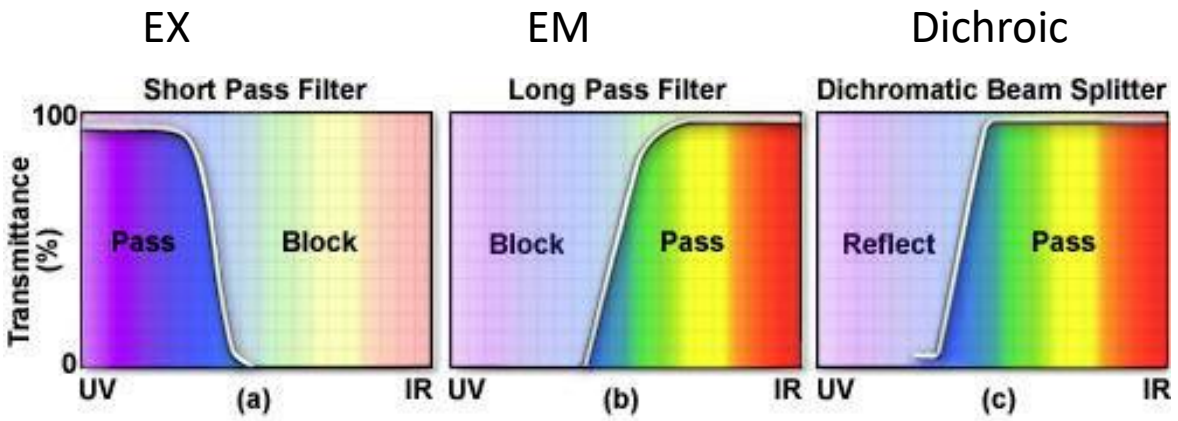
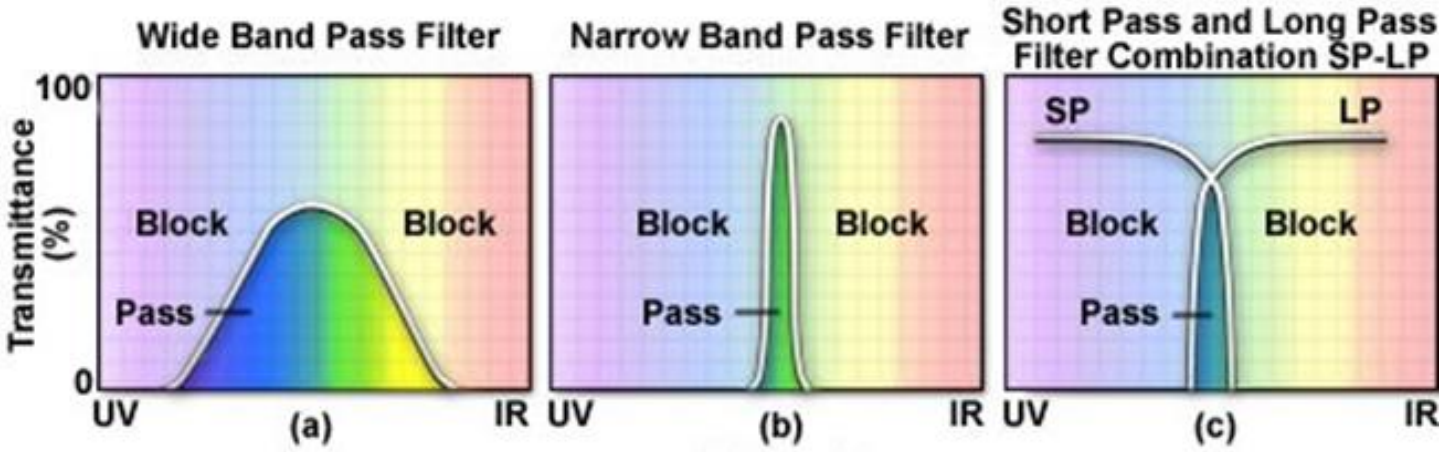


Figure 2

Multiple labelling requires bandpass filters



Bandpass filter nomenclature:

Center wavelength (CWL)/Bandwidth
at Full Width Half Maximum (FWHM)
e.g. 500/50 = 500 +/- 25

Filters may be described by % **transmittance** and by Optical Density (OD: logarithmic) = efficiency of **blocking**.

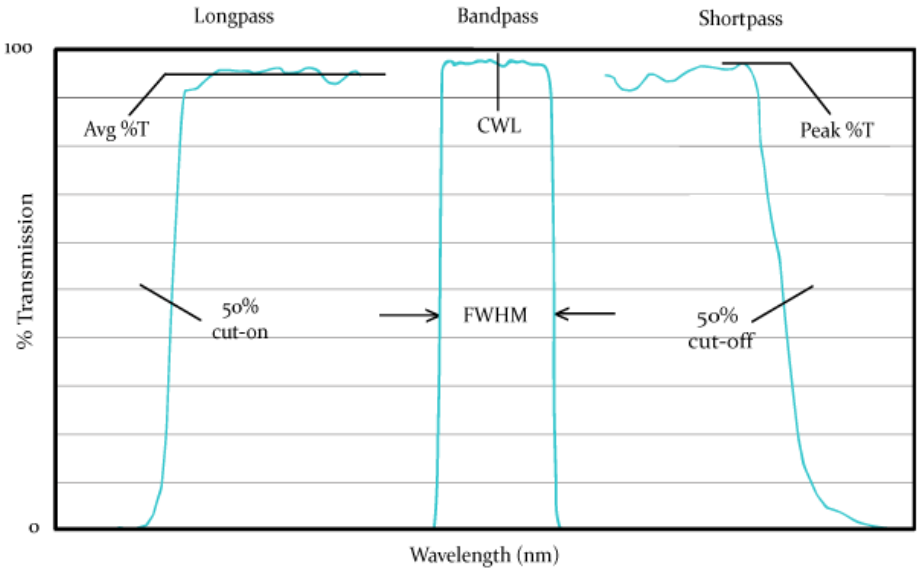
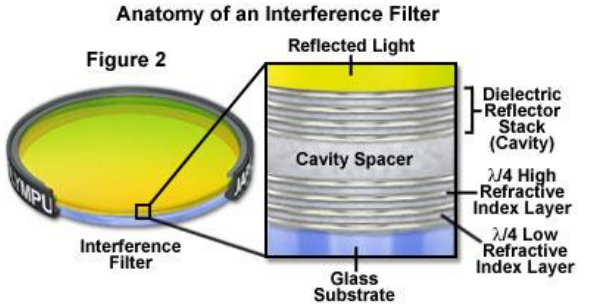


Figure 5: Nomenclature for transmission characteristics

http://www.chroma.com/knowledge/fluorescence-filters/filter-characteristics

Fluorescence filter design

- Formerly coloured **glass** or coloured gelatin sandwiched between glass;
- Now mostly **interference filters** – dielectric coatings on glass;
- Newer **hard-coating** interference filters – more durable and allow more dielectric layers – sharper cut-on and cut-off boundaries;
- Older-style filters/dichroics burned out and **needed replacing** every few years!



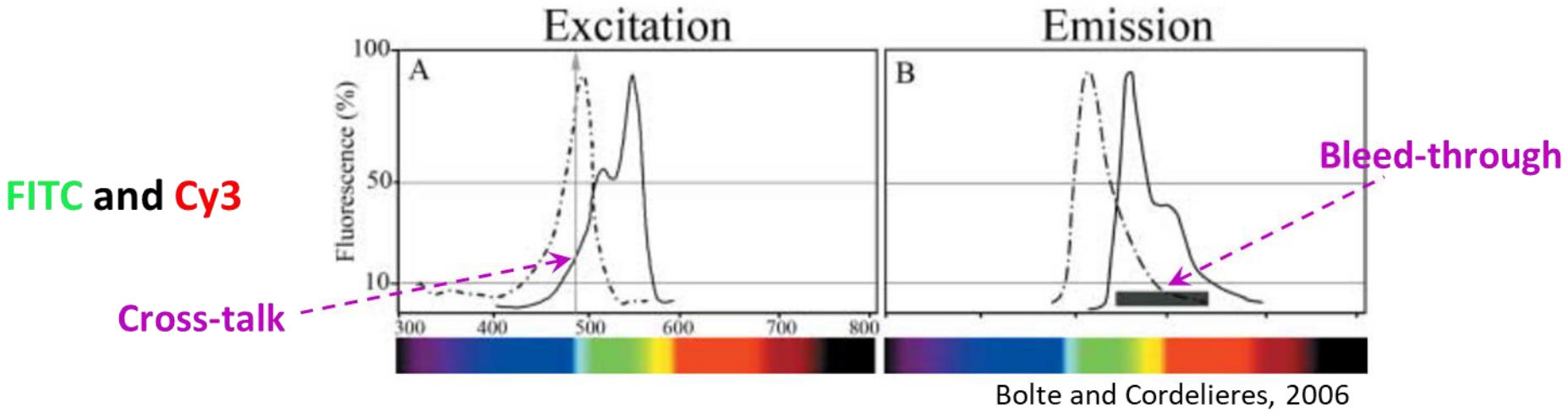
Newer filters can have **> 200 alternating dielectric layers** – constructive interference improves reinforcement and transmission.
More layers give sharper cut-offs.

Note: if you switch illumination sources (e.g. metal halide lamp to LEDs or lasers) you will also need different filter sets.

Bleed-through and cross-talk

Excitation cross-talk: Laser line used to excite fluorochrome A also excites fluorochrome B.

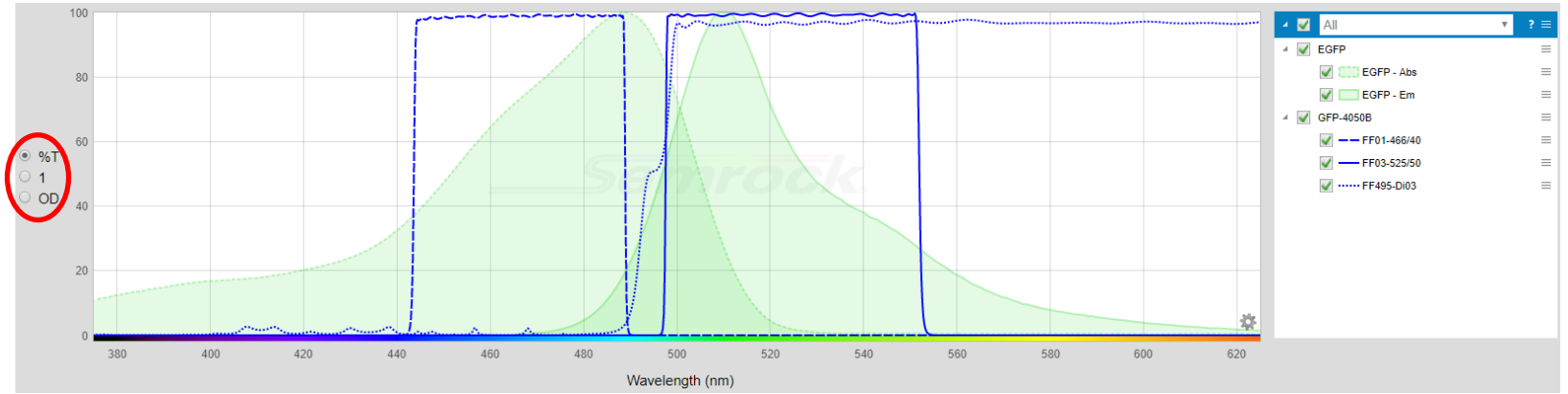
Emission cross-talk (bleed-through): Undesired light makes it through to the detector: this term sometimes refers to excitation light (e.g. laser) bleeding through to the detector, but more commonly to a signal from e.g. fluorochome A being detected through the filter for fluorochrome B.



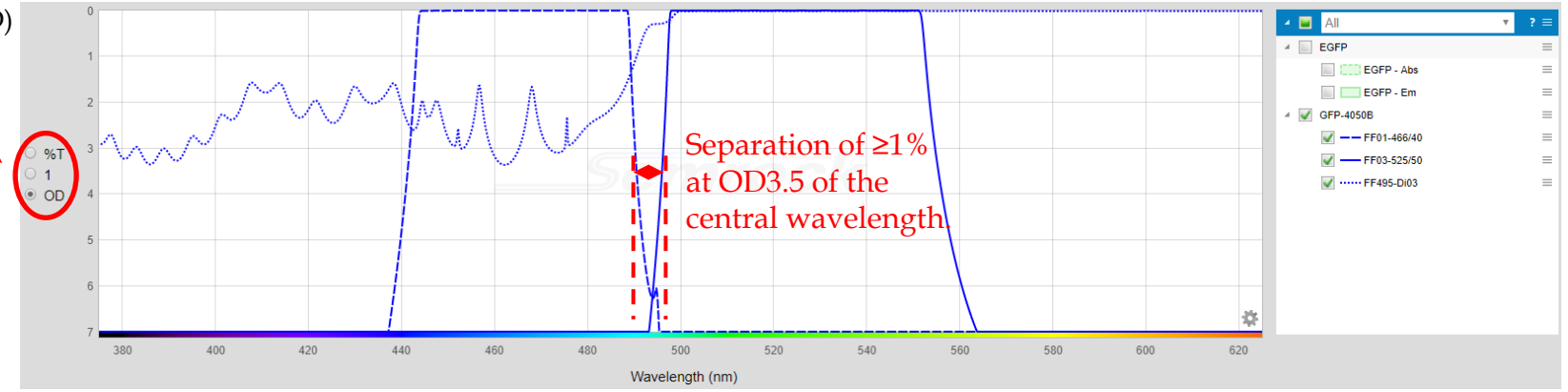
Bolte and Cordelieres, 2006

Avoiding Laser Bleed-Through

Transmission Plot



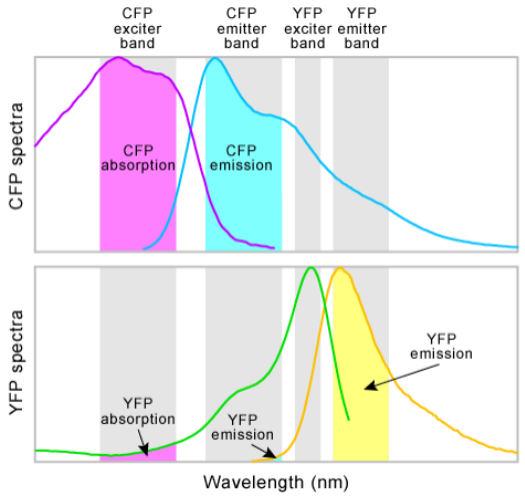
Optical Density (OD) Plot



Semrock

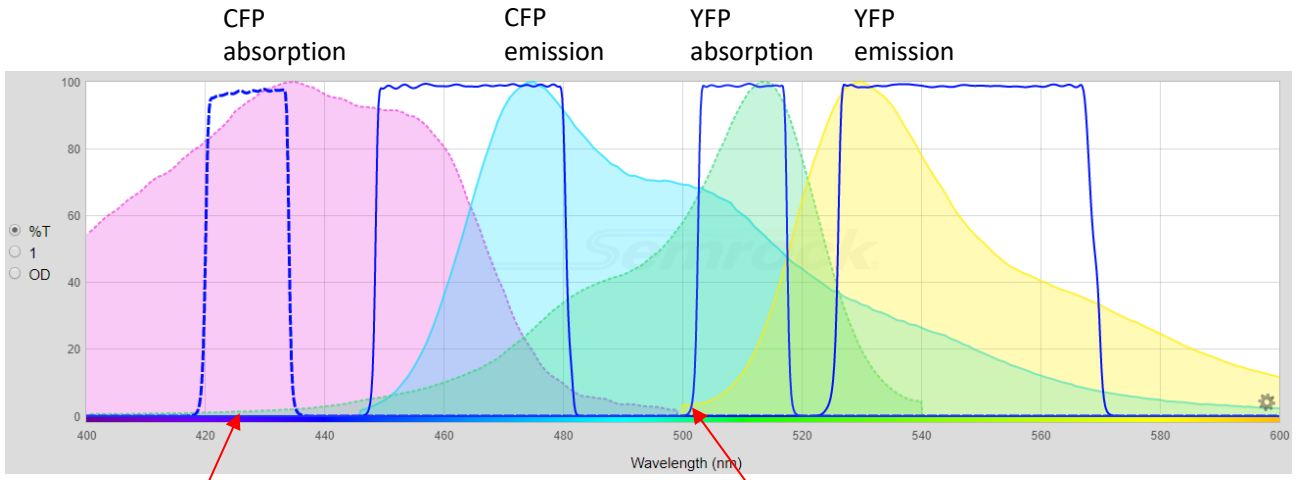
Be cautious about combining Ex/Em/dichroics from different companies!

Reducing Cross-Talk through Filter Selection



Example: Imaging CFP in a sample co-labeled with YFP

Bleedthrough occurs when the emission of one fluorophore is detected in the emission filter passband that is reserved for a different fluorophore



Reduce YFP absorption in the CFP excitation band

Reduce CFP absorption in the YFP excitation band

Ask yourself – do you REALLY need to label with 5 fluorophores at the same time?

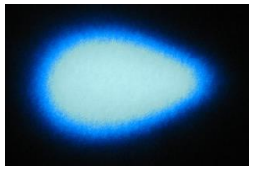
Dichroic Flatness

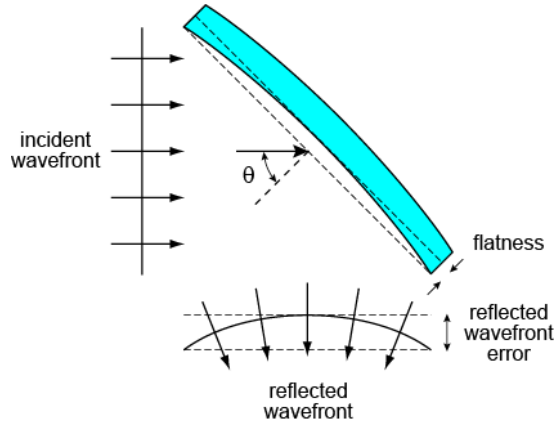
Reflecting excitation light

Wavefront distortion in the excitation beam leads to sub-optimal focusing both in terms of location and spot size.

Reflected laser beam with high-flatness dichroic

Reflected laser beam showing astigmatism due to standard dichroic

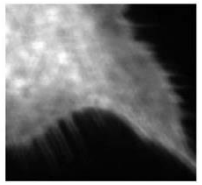




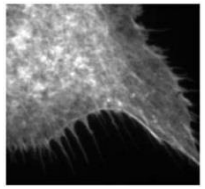
Reflecting emission/imaging

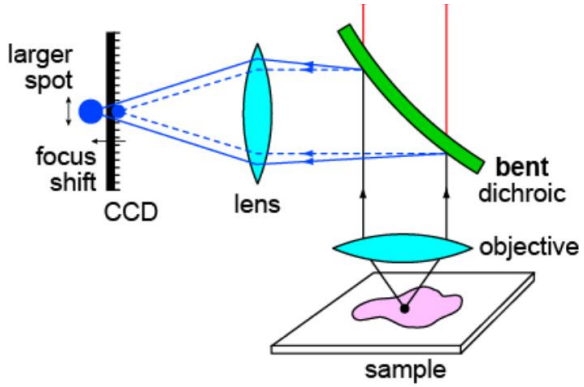
Optical aberrations on the emission side can degrade image quality, and compromise the microscopy technique.

Imaged with a standard flatness dichroic



Imaged with a high-flatness dichroic





- Thick dichroics must be specifically requested if surface quality is critical!
- Filter cube itself is more stringent for some types of microscopy.

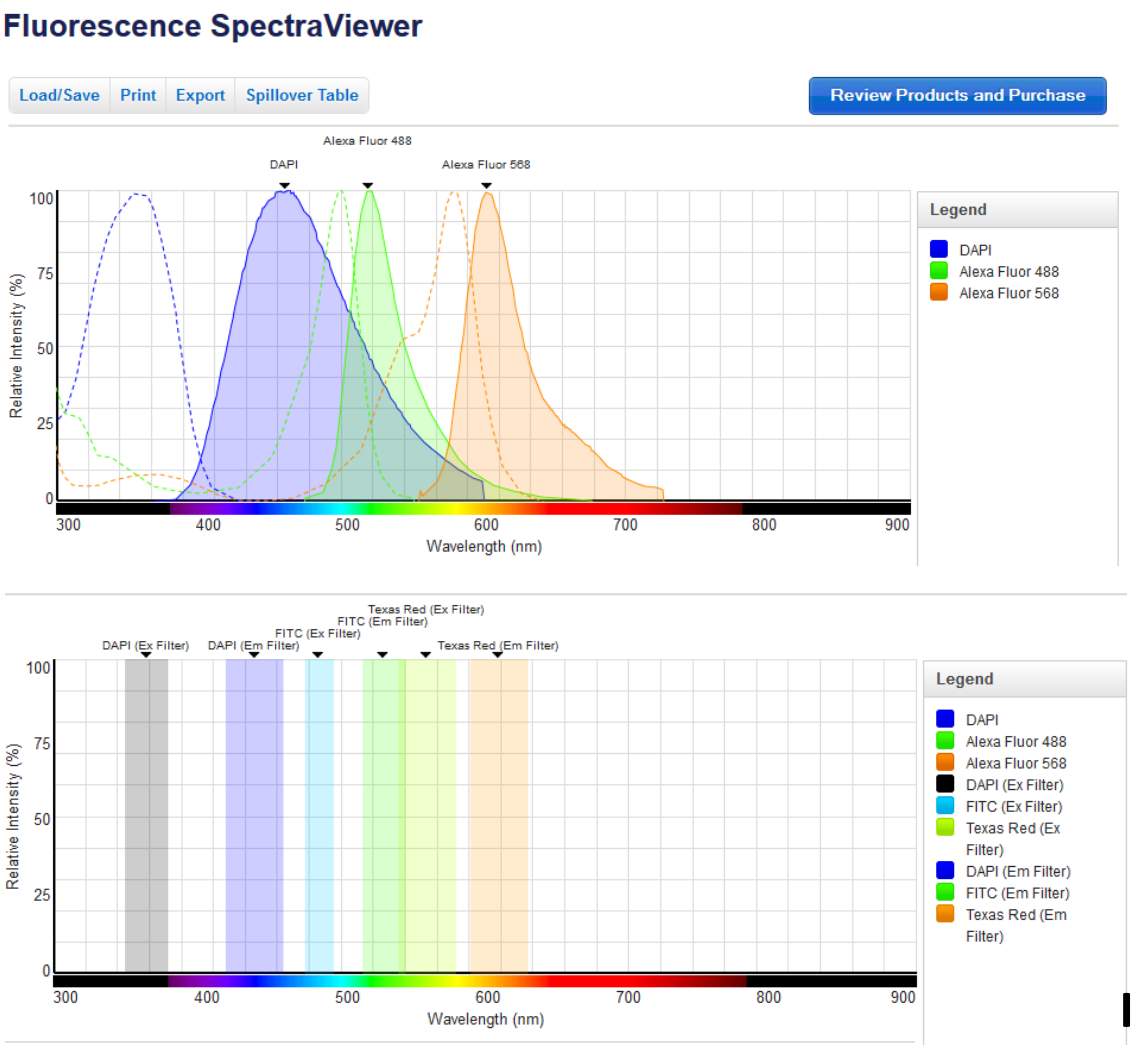
Use Fluorescence SpectraViewers to choose filter sets

Filter selection: (a) maximize signal/background and (b) minimize cross-talk

Dyes:

- DAPI
- AF 488
- AF 568

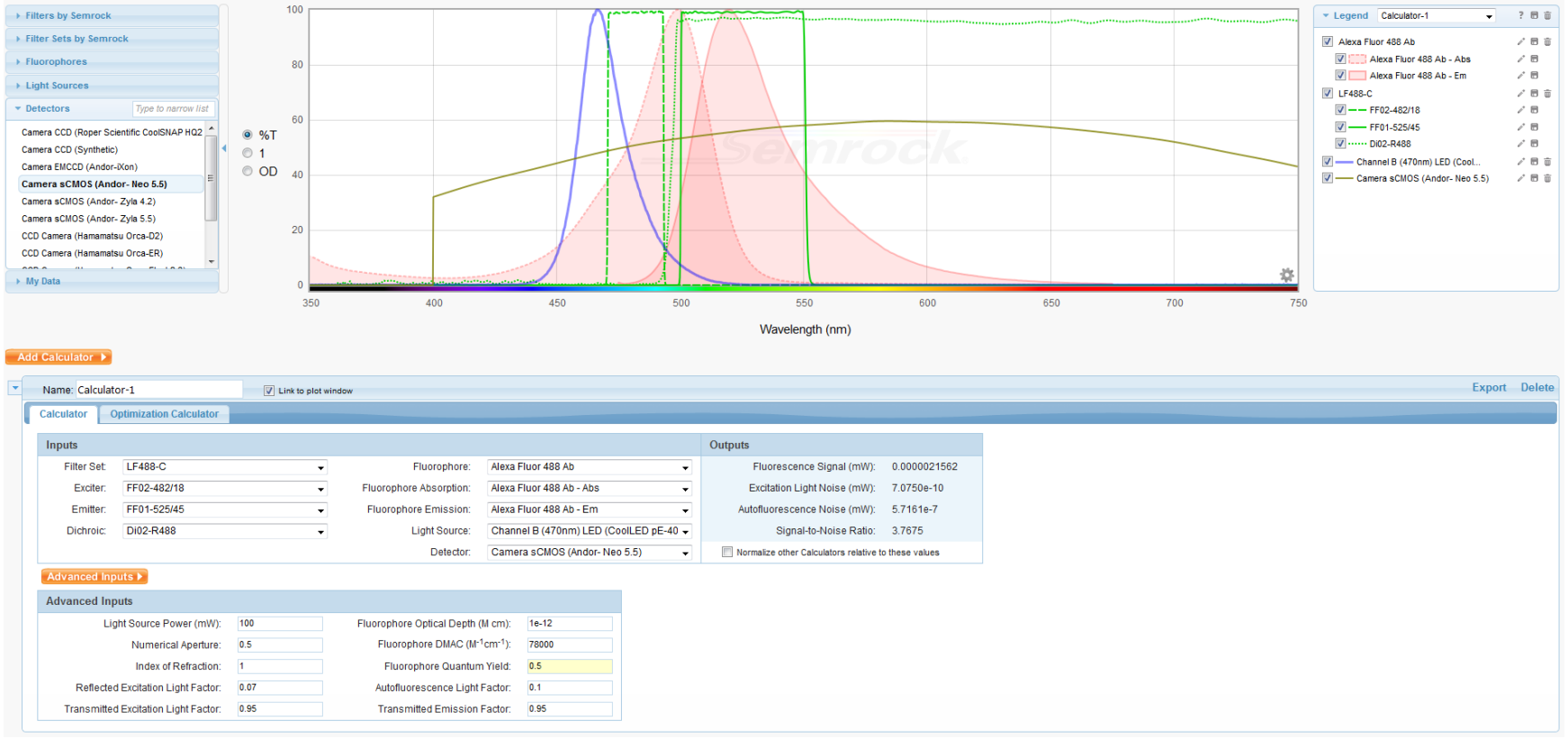
Ex and Em Filter sets



Invitrogen

Websites like Semrock's "Searchlight" tailors the filters to specific illumination devices and detectors

http://searchlight.semrock.com/



When selecting a filter/dichroic to purchase, call the companies – Chroma, Semrock, Omega – and ask for advice. They can custom design them and may have additional options that are not posted online. And they are super helpful!

Other online resources include Talley Lambert's FPbase

FPbase info tools explore sign in Search

FPbase

A fluorescent protein database

FPbase is a free and open-source, community-editable database for fluorescent proteins (FPs) and their properties. The primary objective is to aggregate structured and searchable FP data that is of interest to the imaging community and FP developers. Each protein in the database has a dedicated page showing amino acid sequence, accession IDs (e.g. GenBank, UniProt), evolution lineages and mutations, fluorescence attributes, structural data, references that introduced or characterized the protein, and more. Excerpts from primary literature can be entered to store key information about a protein that is otherwise difficult to capture within the current database schema. (For more technical information about the structure of the FPbase database, please refer to the database schema page.)

FPbase additionally includes a variety of tools to:

- explore the lineage and directed evolution of FPs
- view relationships between FP properties
- compare spectra with common filters and light sources
- calculate FRET relationships
- create and share curated protein collections
- build a spectra viewer customized for the specifications on your microscope

FPbase was designed and created in 2018 by Talley Lambert at Harvard Medical School.

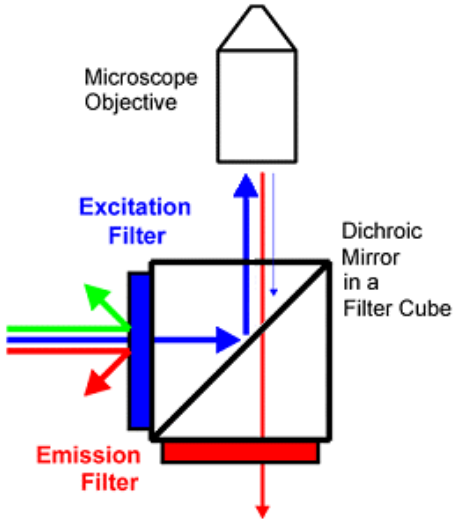
https://www.fpbase.org

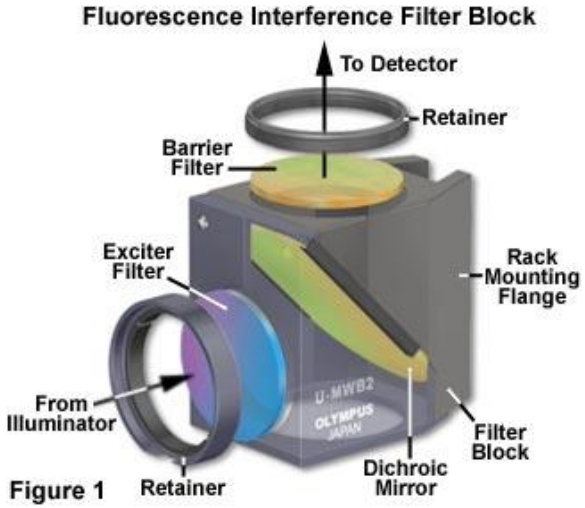
Combined filter cubes or separate filters and dichroics?

- **Ex + Em filters** + **dichroic** in **filter cube** or
- House in separate **filter wheels**;

Decide according to need for:

- Low cost/simplicity;
- Signal registration;
- Flexibility (e.g. FRET);
- Speed of imaging (live cells).





http://www.fluorescence.com/tutorial/fm-optic.htm

http://www.olympusmicro.com/primer/techniques/fluorescence/filters.html

Conventional filter sets typically separate ~ 4 fluorochromes

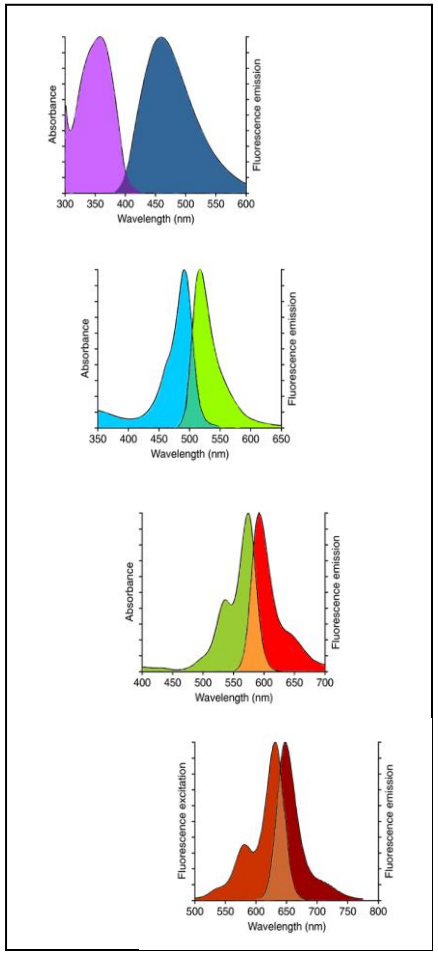
Em Range:

Blue (DAPI)

Green (AF488)

Red (Rhod Red-X)

Far Red (Cy 5)



DAPI, Hoechst
AF/DyLight 405
Pacific/Marina Blue
CF 405M

Fluorescein
Cyanine 2
Oregon green
AF/DyLight 488

Rhodamine
Texas Red
Cyanine 3
AF/DyLight 555, 568, 594

Cyanine 5
Alexa Fluor/DyLight 633/647
SiR Hoechst

Note: Filters in the red region may be chosen to maximize reds shifted towards orange or deeper red

The trend is moving towards the NIR region to avoid autofluorescence, scattering and phototoxicity – **make sure your instruments can excite and detect these wavelengths!**

Detecting cross-talk and avoiding it by dye selection

Suspect cross-talk if:

- Two fluorochromes show identical staining patterns;
- Double labelled sample is brighter in one channel than in single labelled controls.

Reduce cross-talk by:

- Dyes with widely separated spectra;
- Match fluorochromes to available filter sets and laser lines;
- Optimize staining to balance signal intensities;
- Use sequential scanning.

How to choose the best fluorochromes for your experiment

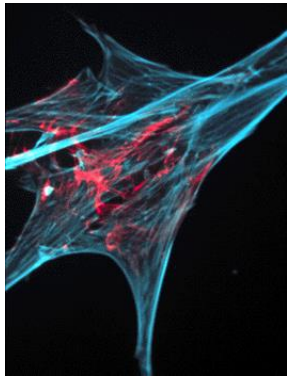
- **Brightness**;
- **Photostability** (PE and many other Flow Cytometry dyes are bad!);
- **Narrow excitation/emission spectra** and good separation from other dyes;
- **Resolution** (short wavelengths);
- Spectral separation from **autofluorescence** (long wavelengths);
- **Spectra match available laser lines and filters**;
- **Multiphoton excitation** properties;
- **Chromatic aberrations** for fluorochromes with widely-separated spectra;
- **Camera** for imaging (far-red capability?);
- Requirement for **direct visualization** (far-red invisible to your eye).

New generation fluorochromes are brighter and more photostable

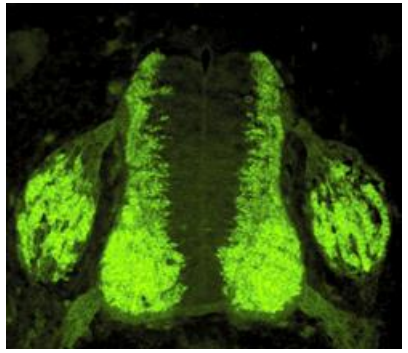
Generations of fluorescent markers:

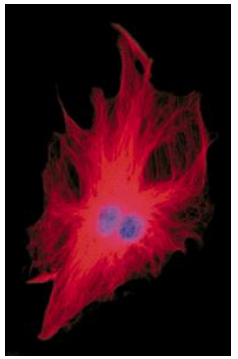
1st Generation **1942** – Coons – Fluorescein and Rhodamine dyes;
2nd Generation 1993 – cyanine dyes Cy2, Cy3, Cy5, also AMCA and Texas Red;
3rd Generation **1999** – Alexa Fluor and DyLight dyes;
4th Generation 2003 – Quantum dots (Q-dots);

More recently… SiR dyes; Abberior dyes; “Janelia Fluor” dyes (Luke Lavis lab).



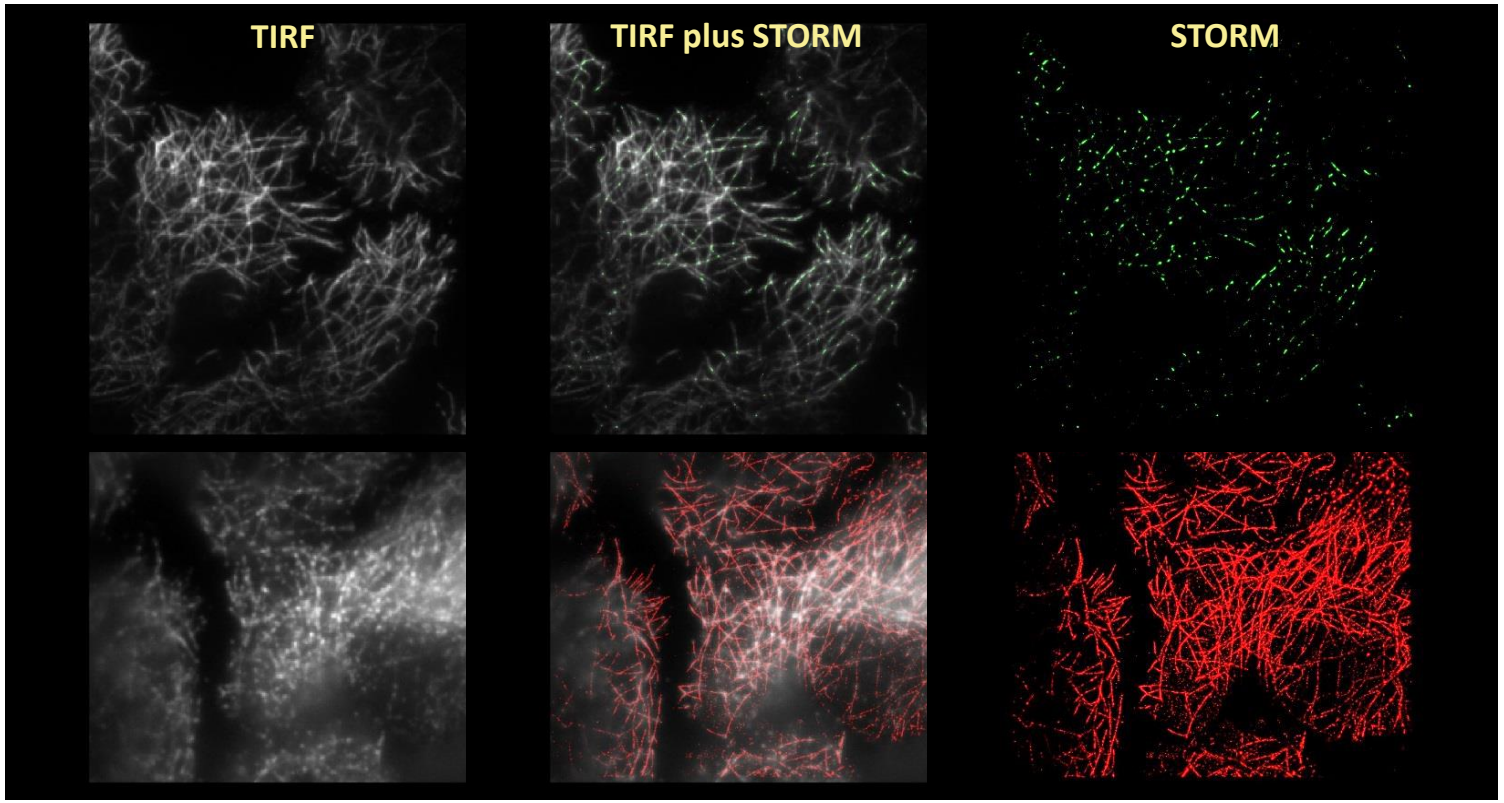
Modified from Mary Dickinson





Many of the super-resolution techniques are critically dependent on the choice of fluorochrome

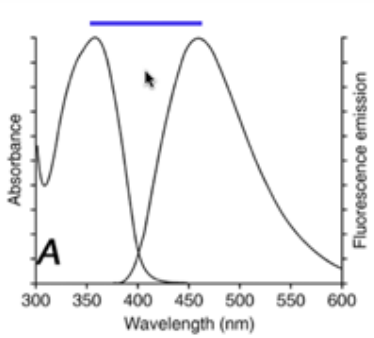
Tubulin in MDCK cells labelled with secondary antibodies conjugated to Atto 488 or Alexa 647 and imaged by STORM microscopy



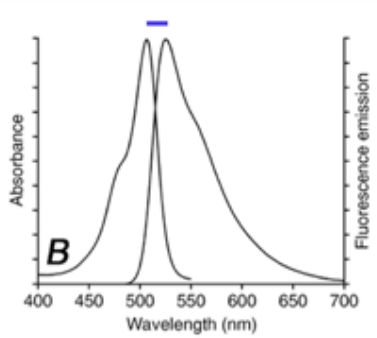
Challenges in choosing fluorochromes

DAPI has a big Stokes shift…

DAPI



Syto 11

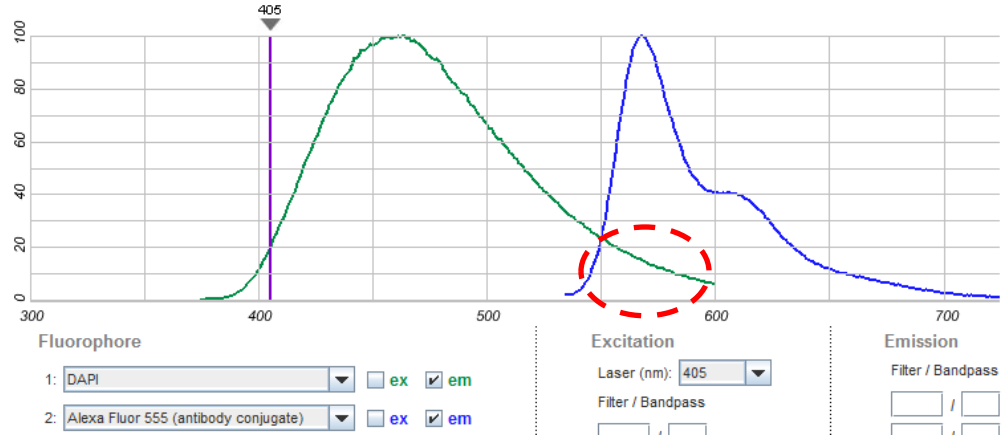


but also a very long Em tail

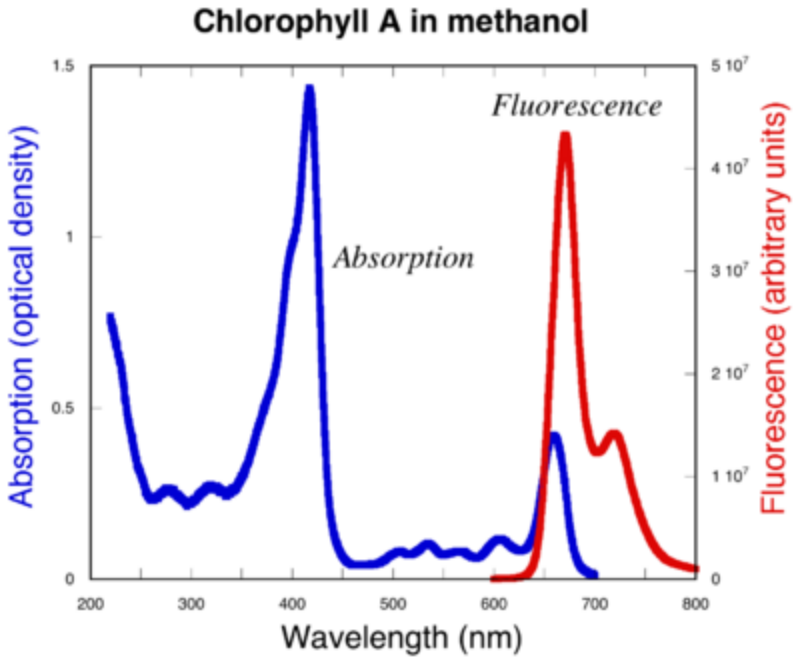
Fluorescence Spectra Viewer

Now you can plot and compare spectra and check the spectral compatibility for many fluorophores offered by Molecular Probes.
To answer any questions you have, see our User Guide.

DAPI AF555



Chlorophyll autofluorescence is a major problem for imaging plants



http://biologywiki.apps01.yorku.ca/index.php?title=Main_Page/BIOL_4160/Fluorescence_and_Reaction_Centers

Antifades

Antifades reduce light-induced damage (bleaching) of fluorophore
(antioxidants, oxygen scavengers, singlet oxygen quenchers).

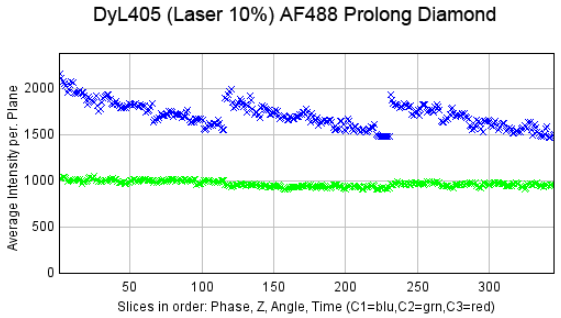
Properties of antifades to consider:

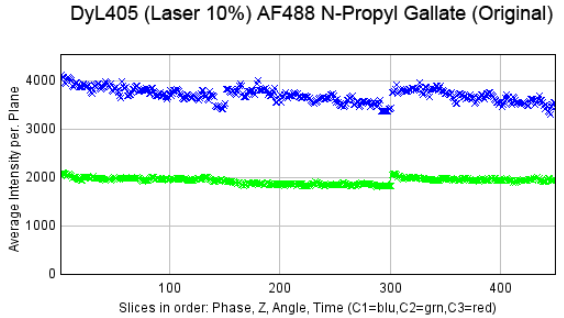
- Bleaching resistance;
- Quenching of initial fluorophore intensity;
- Long-term storage.

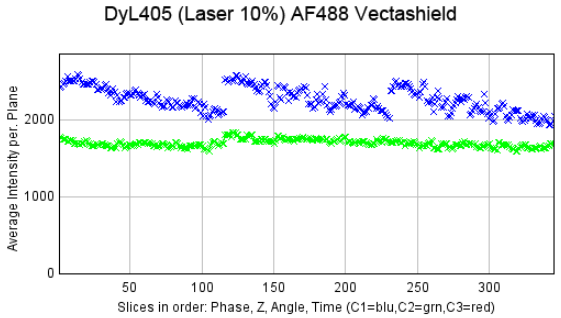
Anti-fade	Compatible	Incompatible	Long-term stability
None	**GFP**, Alexa 546	FITC, PE, Texas Red	Yes
p-phenylenediamine	FITC, Rhod	Cyanine dyes (Cy2, Cy3)	No!!!
SlowFade	FITC, Alexa 488	Alexa 350, DAPI	Yes
n-propyl gallate	Cyanine dyes	?	?
DABCO	FITC, Rhod	AF 350, AF 488?	?
Vectashield		Cy2, **AF 647**	No

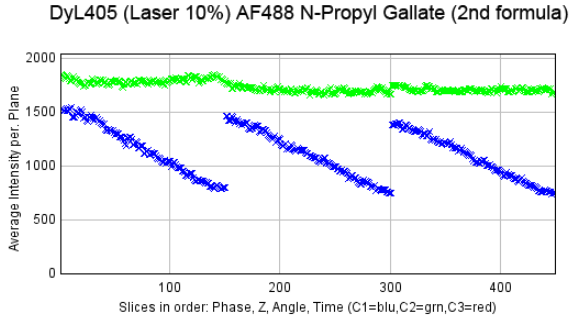
Note – original Vectashield drives AF 647 into a dark state – great for STORM, terrible otherwise!
But VECTASHIELD PLUS claims signal retention across the spectrum from blue to far red.

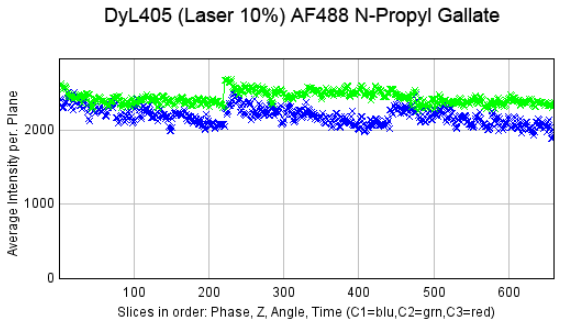
Blue-emitting dyes are particularly susceptible to photobleaching and the choice of antifade is critical

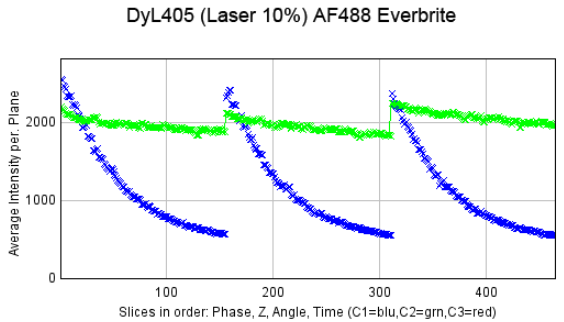












Bleaching profiles of **DyLight 405**-labelled cells in different mounting media/ antifades.

Graphs show 3D-SIM images acquired at 3 different angles. Bleaching profiles were calculated using SIMcheck software.

Spectral unmixing can be used to separate fluorochromes with overlapping spectra

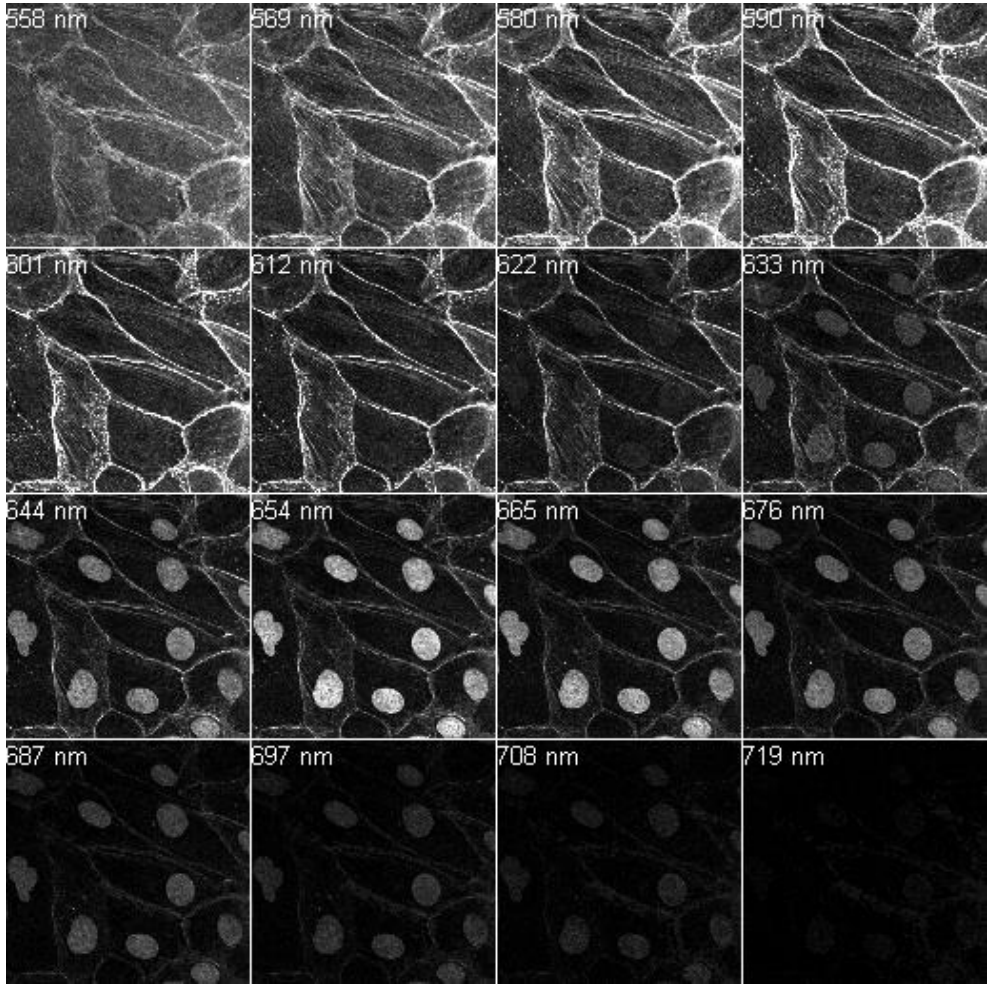
Spectral imaging systems perform "**emission fingerprinting**" by splitting emitted fluorescent light into many small bandwidths.

To separate individual emissions:

- Acquire **spectral signature for each dye**;
- **Digital unmixing** using reference spectra.

Applications:

1. Separation of multiple dyes with highly overlapping emission spectra (e.g. GFP and YFP).
2. Separation of true signal from broad-band autofluorescence.

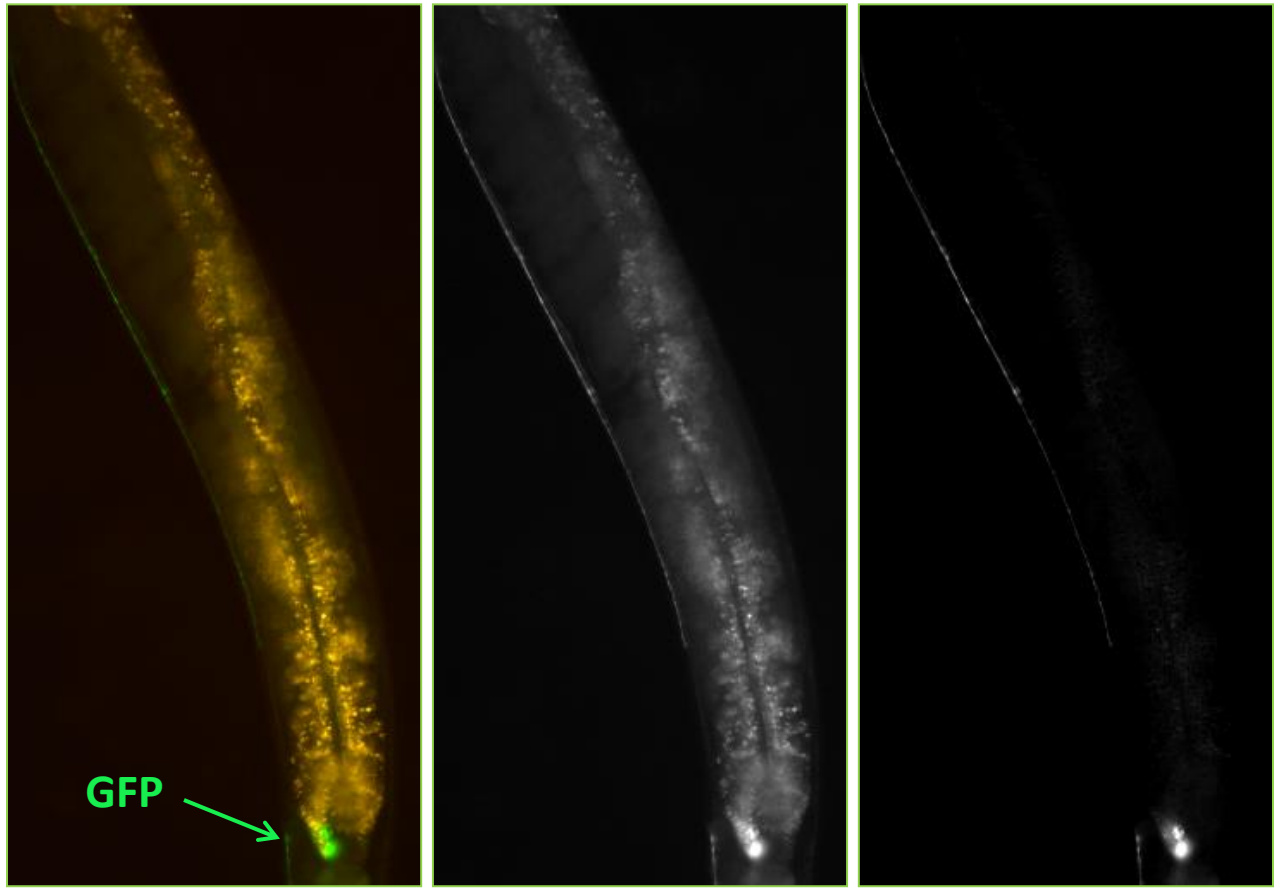


Lambda stack from 558 nm to 719 nm
Zeiss LSM 510 confocal microscope

Separation of GFP from autofluorescence using spectral unmixing (widefield/CRI Nuance camera)

C. elegans

Max Heiman
Formerly RU



RGB image (longpass emission)

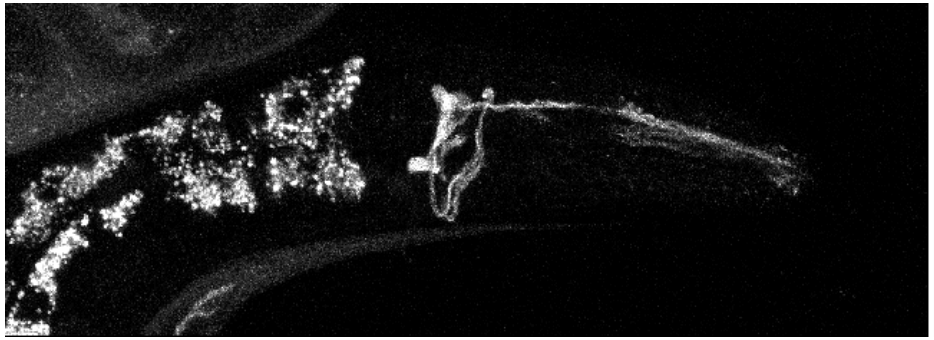
Monochrome image (bandpass emission)

Nuance unmixed GFP image

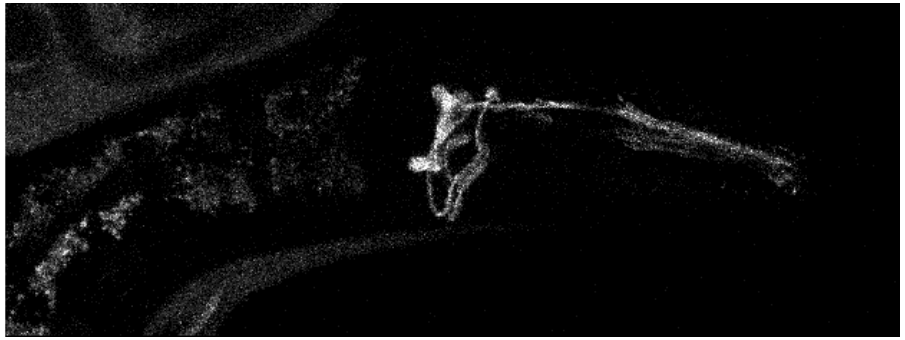
Using Time-Gated detectors to subtract autofluorescence (e.g. Leica HyDs)

***C. Elegans* – GFP plus autofluorescence**

No gating

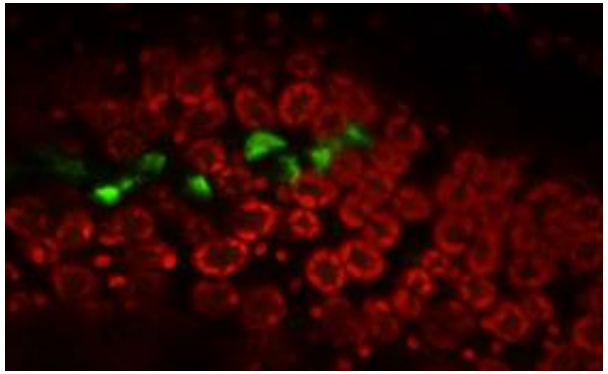


Plus gating

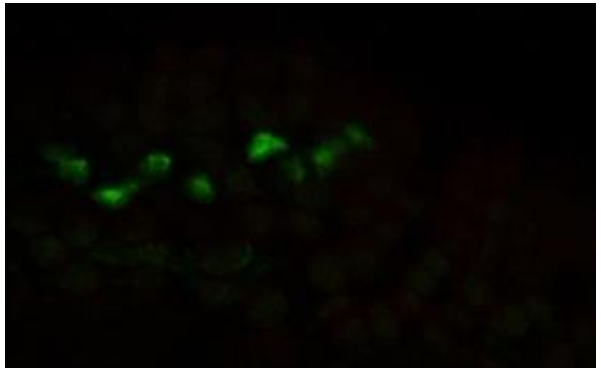


Plant autofluorescence

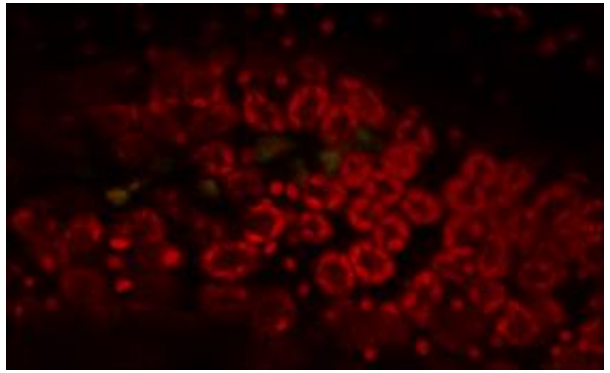
No gating



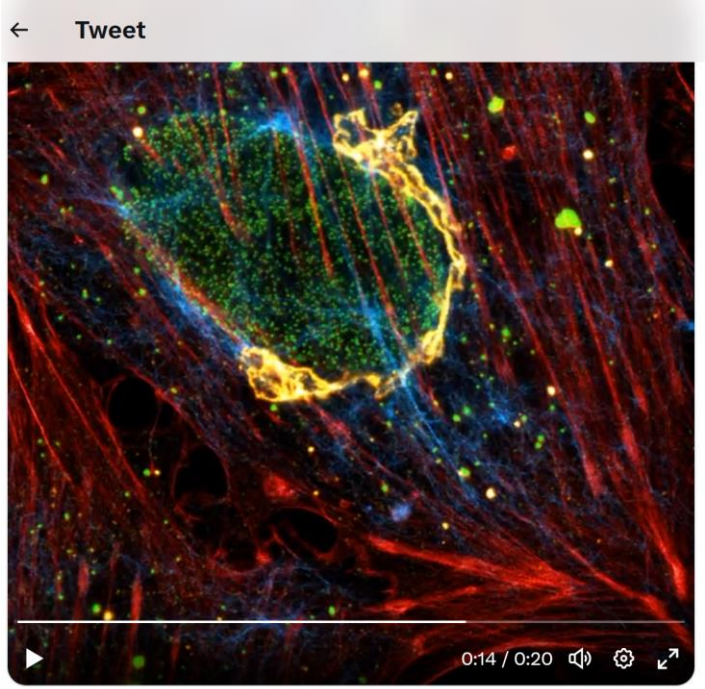
Red HyD gating



Green HyD gating



Abberior “TIMEBOW” module for Fluorescence Lifetime Imaging also allows you to use more dyes in STED



4-colour STED imaging using just the 775 nm depletion laser and the following dyes:

Phalloidin – Abberior STAR RED

Nuclear Pores – Abberior STAR ORANGE

Giantine – CF594

Vimentin - Abberior 460L

- TIMEBOW uses Picoquant’s hardware to enable detection of a fluorochrome whose average **fluorescence lifetime** falls within a certain range;
- Thus dyes with overlapping spectra can now be separated by their lifetime;
- Works in both confocal and STED mode;
- **Increases the number of STED dyes that can be combined with the 775 nm depletion laser, e.g. 2 orange + 2 red dyes.**

Minimizing Autofluorescence

- **Red or far red fluorophore** (exception – plant tissue);
- **Narrow** band pass Em filter;
- **Thin** physical or optical **sections**;
- **Spectral unmixing** or "**gating**" technology to "remove" autofluorescence;
- **Chemical methods** of reducing autofluorescence.

Lipofuscins: CNS tissue, worse in older animals, quench using CuSO4 or Solvent Black (0.1-0.3% solution in 70% ethanol);

Elastin: 0.5% Pontamine sky blue 5BX;

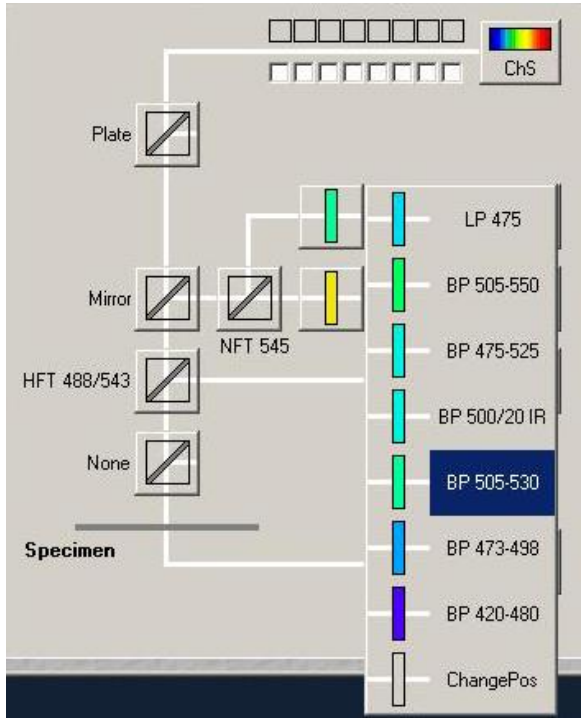
Glutaraldehyde-induced: freshly dissolved 1 mg/ml sodium borohydride.

Choose your filters and dichroics carefully

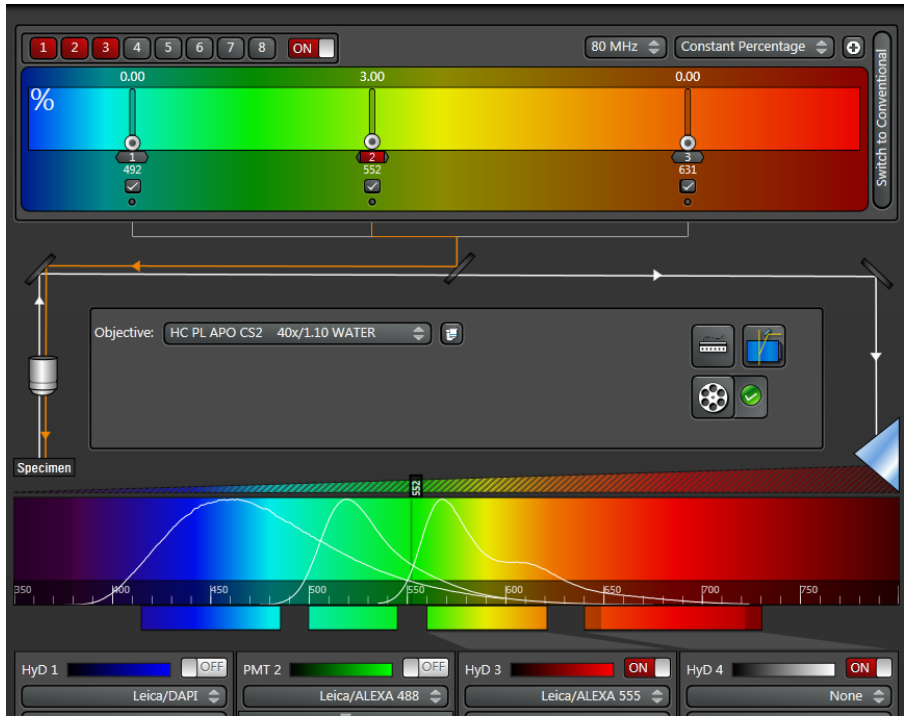
Select between longpass, broad bandpass and narrow bandpass filters, and between dichroics or polychroics, according to requirement for:

- Signal intensity;
- Specificity and clean spectral separation for multiple labelling;
- Speed of imaging;
- Minimize autofluorescence.

Older confocals with fixed width filters

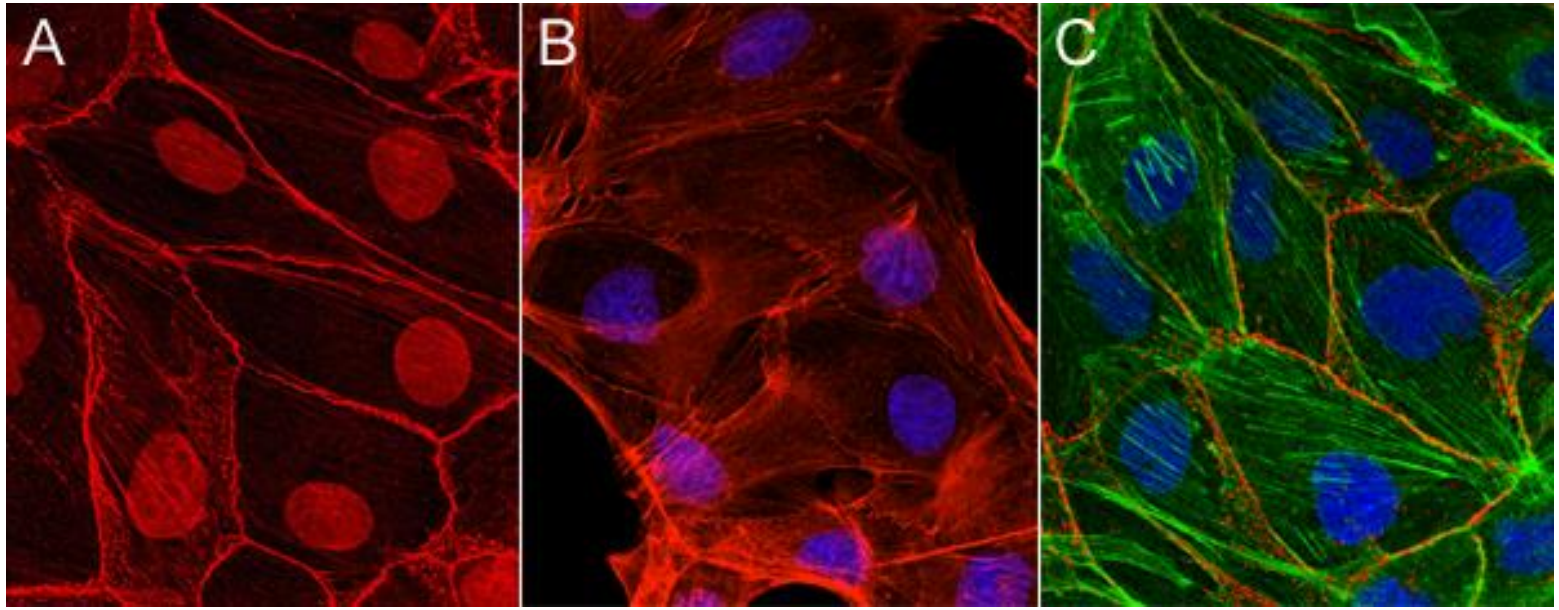


Modern spectral confocals…



Know your filter sets…

How many different fluorochromes are present in this sample?



A: 560 nm long pass Em filter.
B: 560-615 band pass Em filter (red channel); **650 nm long pass** filter (blue channel).
C: spectral unmixing of **~11 nm spectral bands** across a total range of 552-723 nm.

Three: Tetramethyl Rhodamine (actin filaments), Rhodamine Red-X (desmosomes) and To-Pro3 (nuclei)

Increase Signal and Decrease Background

For good labelling need to do BOTH:

Increase signal with:

- Brighter Dyes;
- Filters;
- Appropriate antifades;
- Signal amplification.

Decrease background with:

- Non-specific dye blockers;
- Faster exposure times;
- Greater dye dilutions;
- More washing.

Detectors for fluorescence microscopy

Common detectors:

- Cameras – CCDs, EMCCDs and sCMOS;
- PMTs – newer GaAsP, Hybrid and SilVIR detectors ;
- Avalanche Photodiodes (APDs).

Eye:

Good for:

- **Rapid scanning** of samples;
- High resolution;
- True “colour” detector.

Bad for:

- Live cell imaging;
- Far red/infrared emitters (e.g. Cy5);
- Techniques requiring post-acquisition computation to reveal details (e.g. super-resolution, deconvolution, spectral unmixing).

Other components in the optical path that can affect fluorescence

Objective lens

- Transmittance of certain wavelengths (e.g. UV, NIR);
- N.A. and magnification;
- Aberrations;

Immersion medium

- Balance signal intensity (high N.A.) against spherical aberrations;

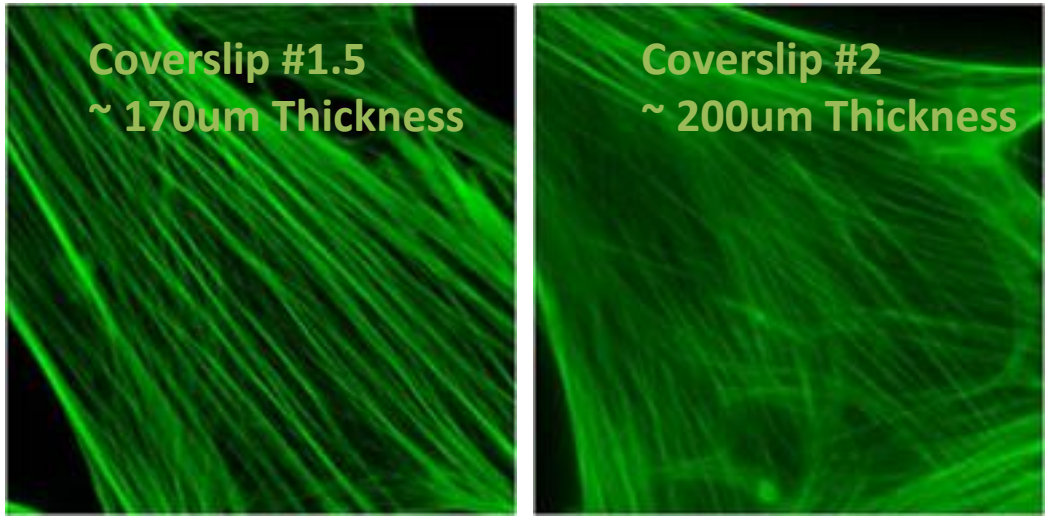
Coverslip thickness (no. 1 ½)

Additional optics in the path including:

- UV and IR blocking filters;
- Neutral density filters;
- Optovar lens (auxilliary magnification);
- Elements of the transmitted light path (Wollaston prism, analyzer);
- F-aperture - close down for greater contrast.

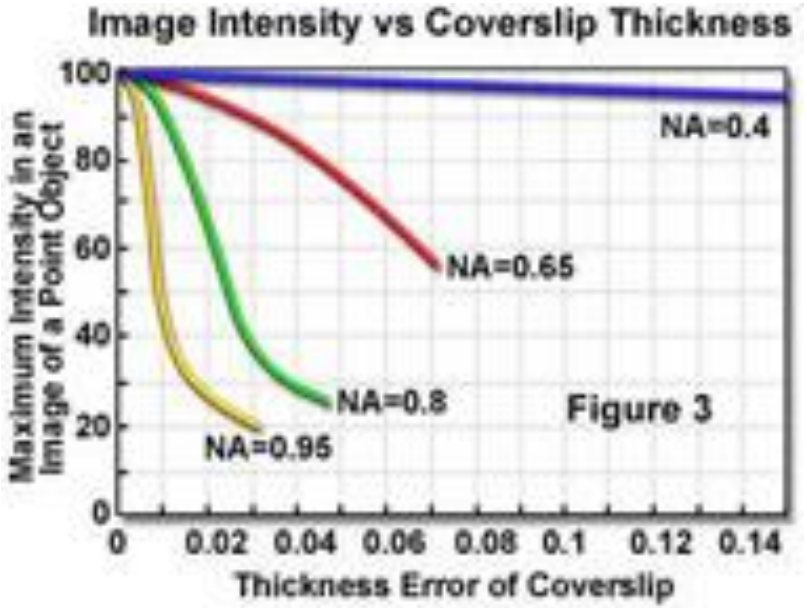
Impact of Coverslip Thickness on Image Quality

Plan Apo 40x/0.95



Michael Davidson/FSU

Always use a no. 1 ½ coverslip!!



Visible effects in image are:

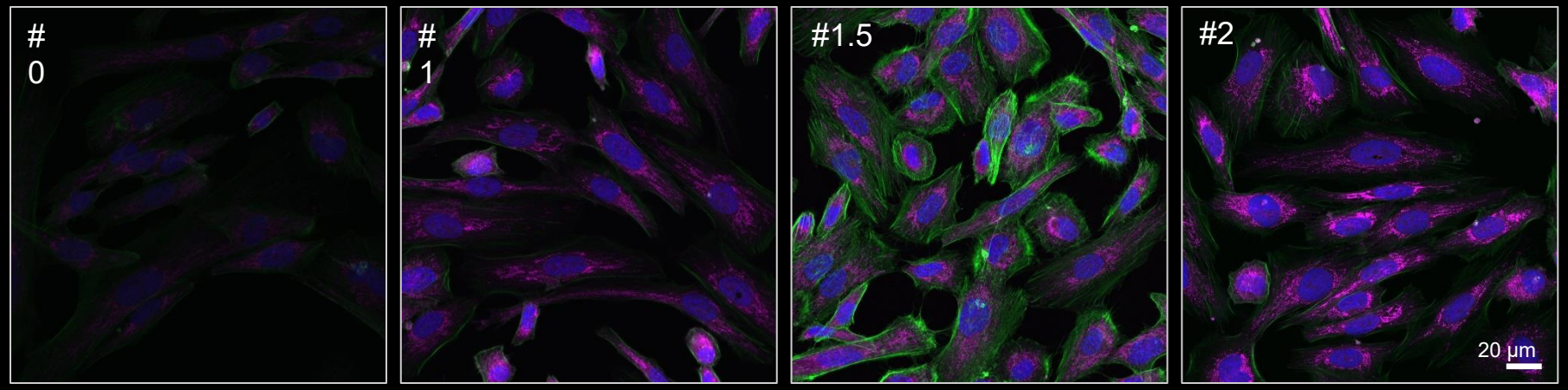
- Optical aberrations (spherical);
- Decreased image contrast;
- Loss of signal;
- Degraded point spread function;
- Limited optical resolution.

http://www.meditec.zeiss.com/4125681F004CA025/Contents-Frame/C66A2521E524C891852575A200721A7C

Fantastic supporting data from Jacqueline Leung

(Rocky Mountain Labs, Montana)

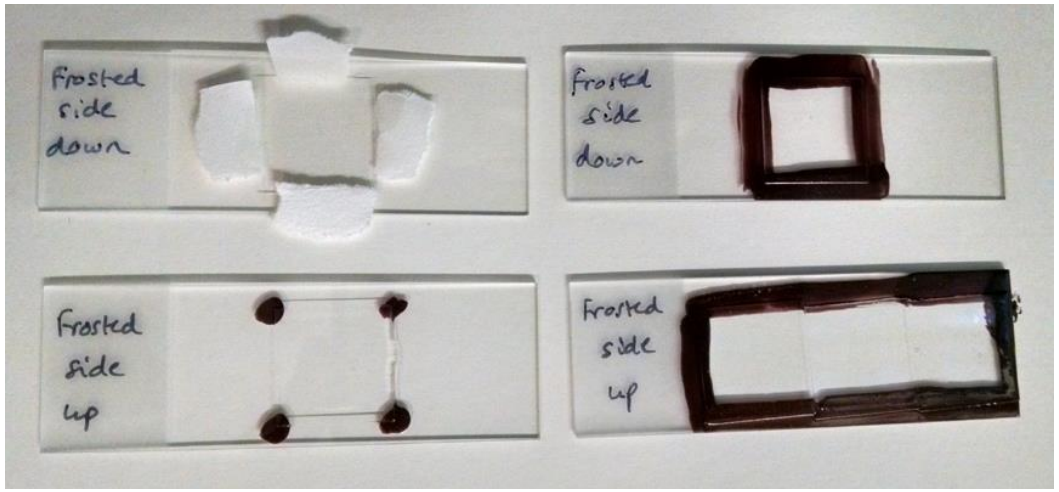
What happens if you don't use a #1.5 thickness coverslip?



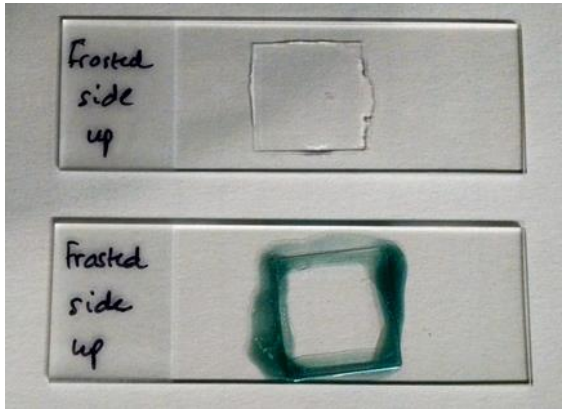
[40X/0.95 Korr obj; all images captured and processed using identical settings]

How could you possibly mess up mounting a coverslip?

1st step



Final



- Slide frosted side down;
- Wick away excess mounting medium;
- One coverslip in middle;
- Seal all around with nail polish;
- Use quick-dry, metallic, thick, coloured nail polish.

Mountants

Mountants must be aqueous to preserve fluorescence:

Harden to a solid (typically PVA-based e.g. Moviol, gelvatol):

- Good for long-term storage and wide-field imaging;
- Terrible for 3-D imaging (confocal or deconvolution);

Do not harden and require sealing (typically glycerol-based):

- Worse for long-term storage and wide-field imaging;
- Good for 3-D imaging;

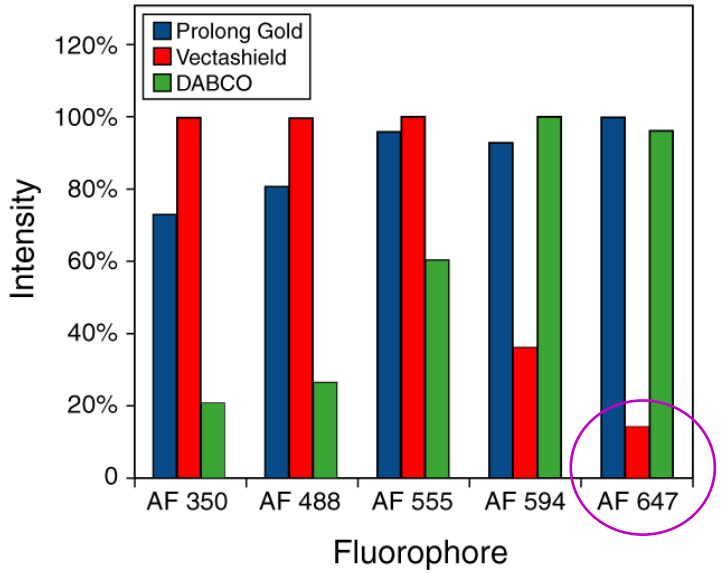
Consider refractive index for super-resolution (3D-SIM, STED).

There is no mounting medium/anti-fade that is compatible with both Mol. Probes “Q-Dots” and Alexa Fluor dyes.

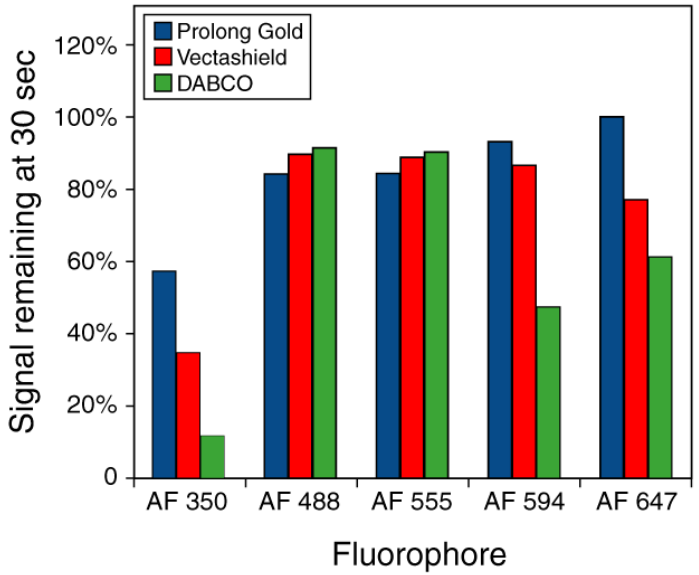
ProLong® Gold – a useful mountant from Molecular Probes / Invitrogen / Thermofisher

Check product information for compatability of the included anti-fade reagent with YOUR fluorescent dye!

Prolong® Gold Improves Initial Intensity



Prolong® Gold Reduces Photobleaching

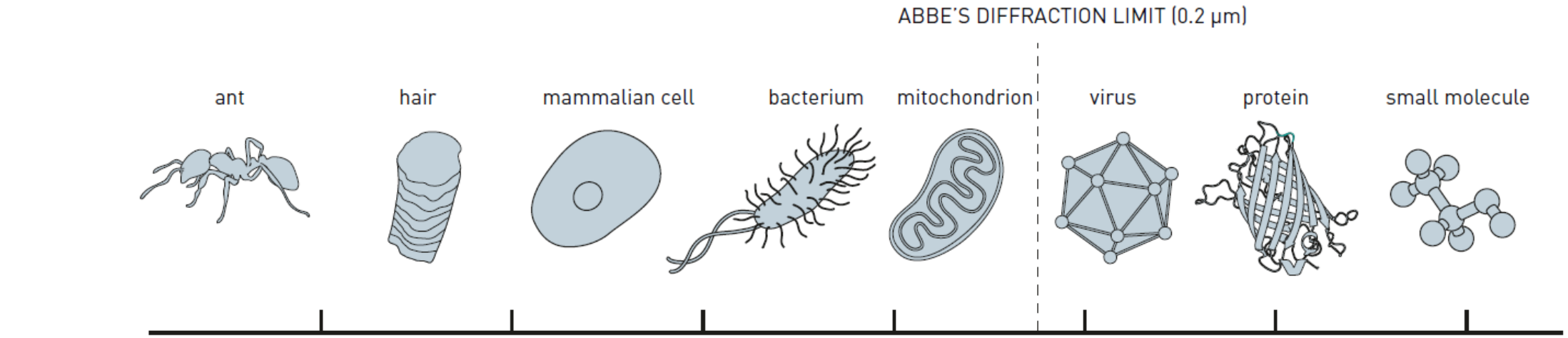


ProLong Diamond and **SlowFade Diamond** also preserve the fluorescence of Fluorescent Proteins (e.g. EmGFP, TagRFP, mCherry) and certain blue-emitting dyes.

Graphs provided by Mike Ignatius, Mol. Probes/Invitrogen

What is “resolution” and why is it important?

- Resolution is the ability to see structures at a high level of detail and to be able to distinguish neighboring features as separate.
- Resolution is typically limited by the optics of the microscope and the wavelength of the illumination.



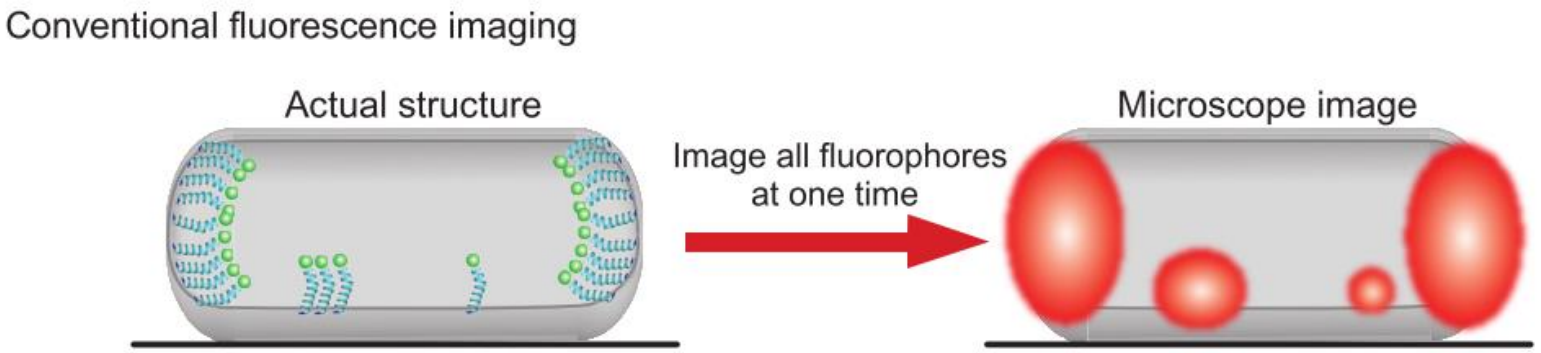
Light microscope:
Live cells

Electron microscope:
Dead cells

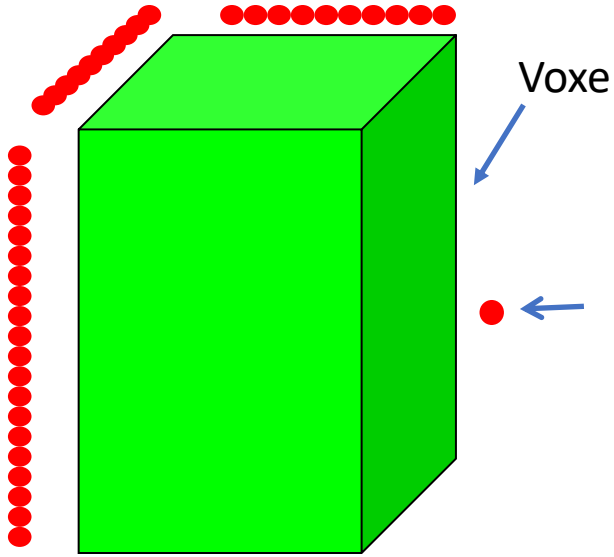
Super-resolution microscopes:
Live cell imaging with continually improving resolution

Resolution of conventional microscopes

A point source of light will be blurred by the microscope optics as the light rays pass through, as though you are painting over it with a thick paintbrush.



McEvoy et al. BMC Biology 2010, 8:106



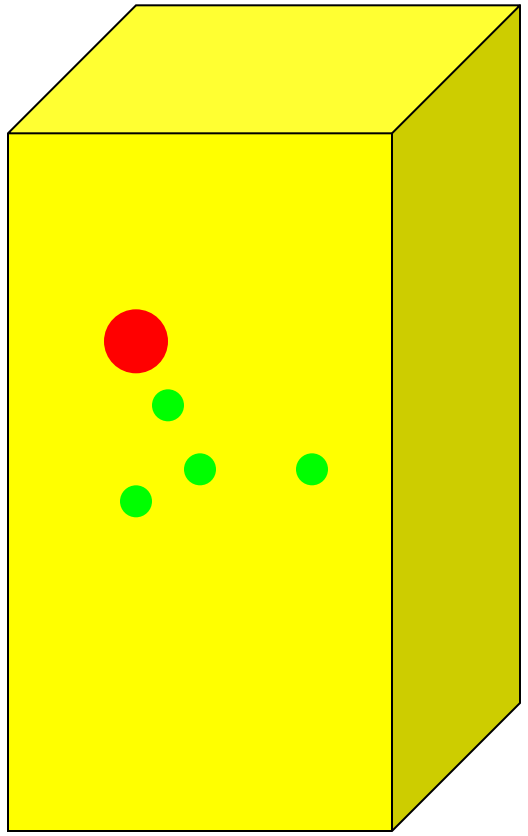
The spatial resolution of a microscope is limited by diffraction to about
0.2 - 0.25 microns in lateral (xy) axis and
0.5 - 0.6 microns in axial (z) direction.

Resolution vs. Biology

Resolution limits affect your ability to see where things are and how big they are:

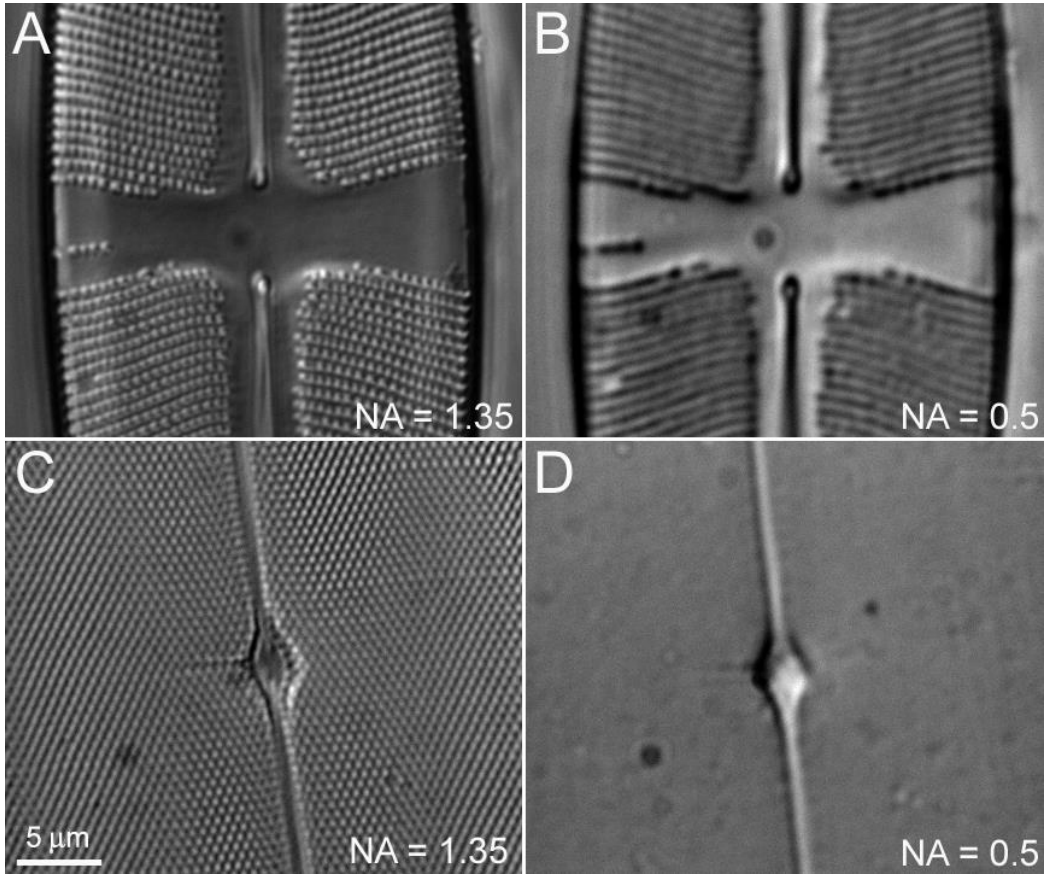
- Anything smaller than the diffraction limit appears to be the same size:

- Fluorophores up to 200 nm apart or more will be localized to the same voxel.



1 voxel (3D pixel)

Numerical aperture (N.A.), rather than magnification, determines the resolution of the objective lens



Images of diatoms, all acquired using a 100x objective lens!

N.A. is a measure of the light-gathering power of the lens.

N.A. = $\eta \sin\alpha$

Resolution depends on wavelength and N.A.

The Rayleigh equation for resolution is:

$$R = 0.61\lambda/N.A.$$

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

Where:

R is the size of the distance between two minute points lying close together in the specimen:

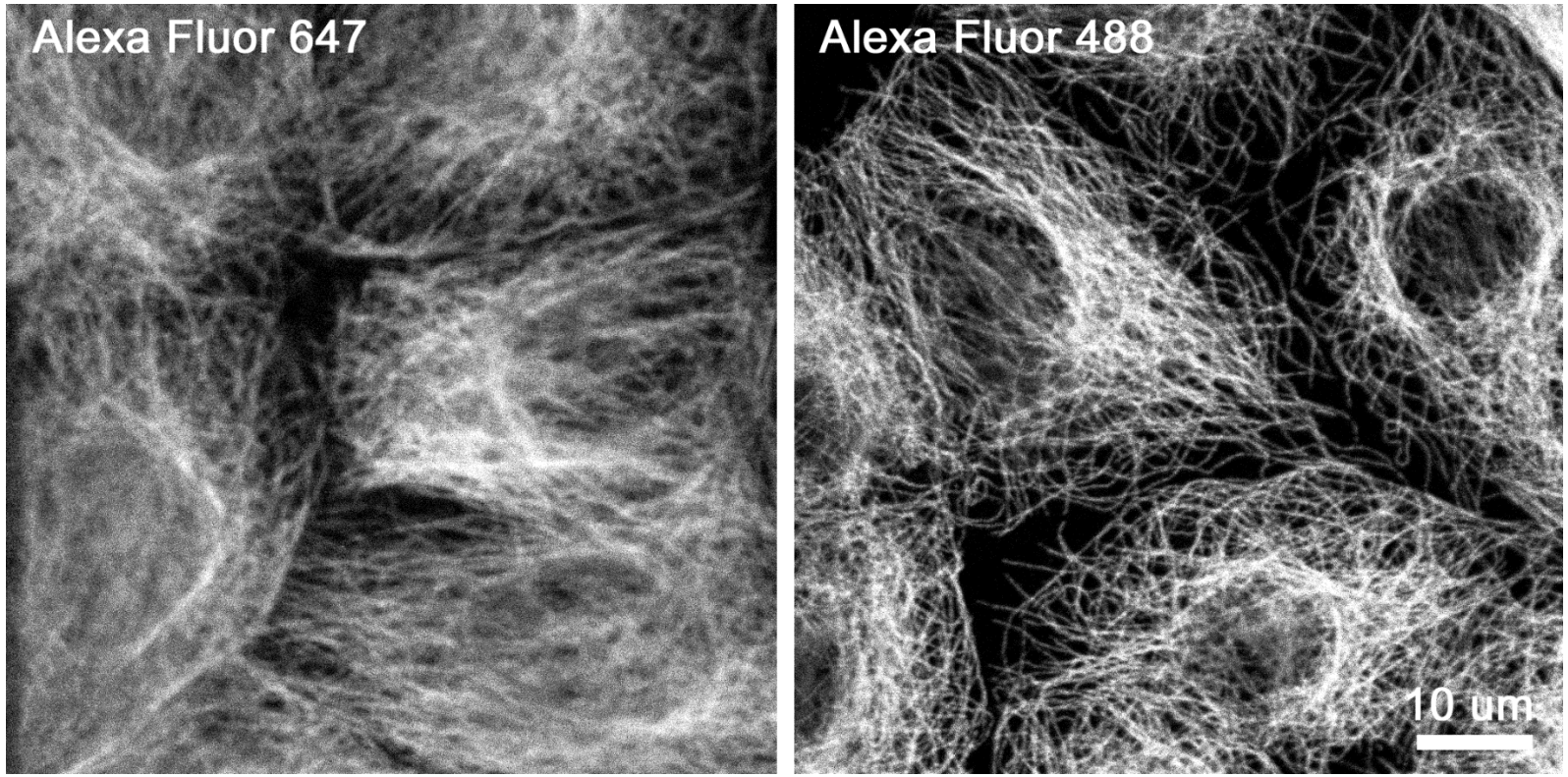
λ is the wavelength of the light being used;

N.A. is the numerical aperture.

The resolvable distance, R, is smaller for shorter wavelengths of light.

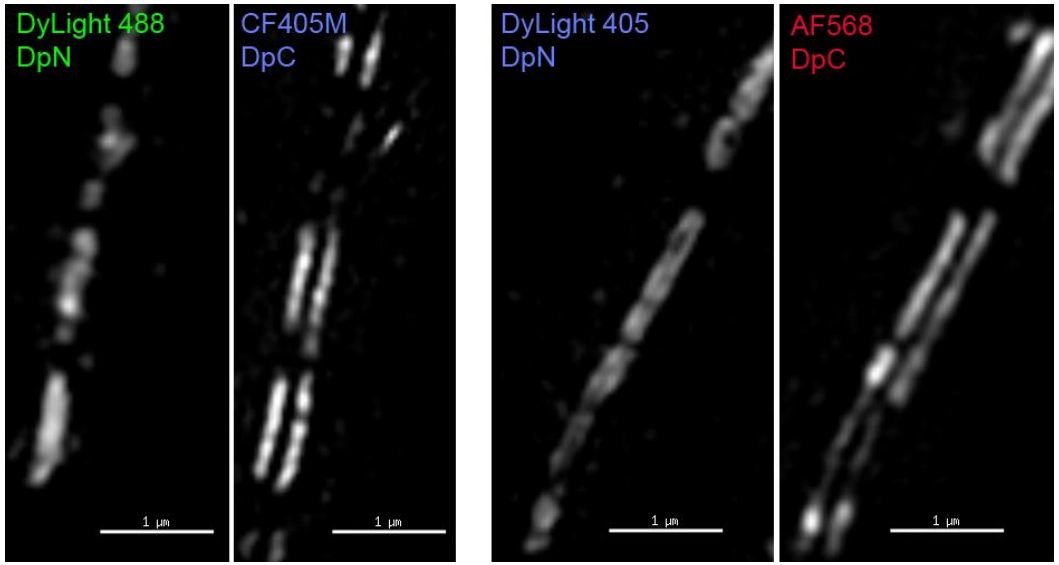
Figures reproduced from the Nikon MicroscopyU:
http://www.microscopyu.com

But will I actually notice a difference in resolution with different colours?



MDCK epithelial cells fixed and stained for tubulin with the same primary antibody followed by different colour secondary antibodies

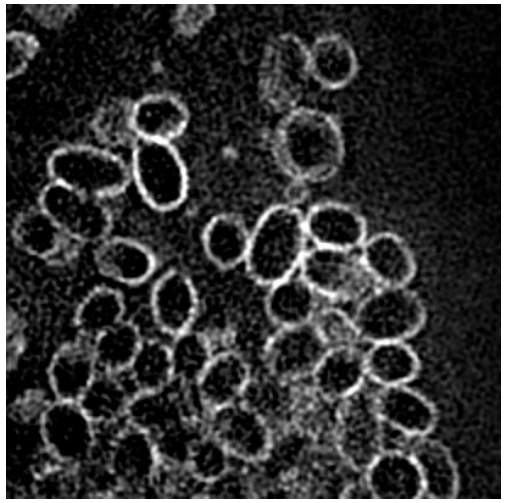
Resolution is demonstrably improved by using blue-emitting dyes



Desmosomes immunolabelled using antibodies directed against the N-terminus or the C-terminus of Desmoplakin and imaged by 3D-SIM super-resolution microscopy

North, Sharma et al., 2024. J. Microscopy

Imaging *Orientia Tsutsugamushi* by super-resolution microscopy



Microscope Systems	FWHM (nm, n = 30)	
	Mean	Stdev
3D-SIM (Dylight 405)	92.8	9.9
3D-SIM (AF488)	109.2	13.1
iSIM (AF488)	161.1	13.2
Airyscan confocal (AF488)	186.8	18.2
STED (Settings 1, STAR-RED)	144.7	26.1
STED (Settings 2, STAR-RED)	186.0	30.7

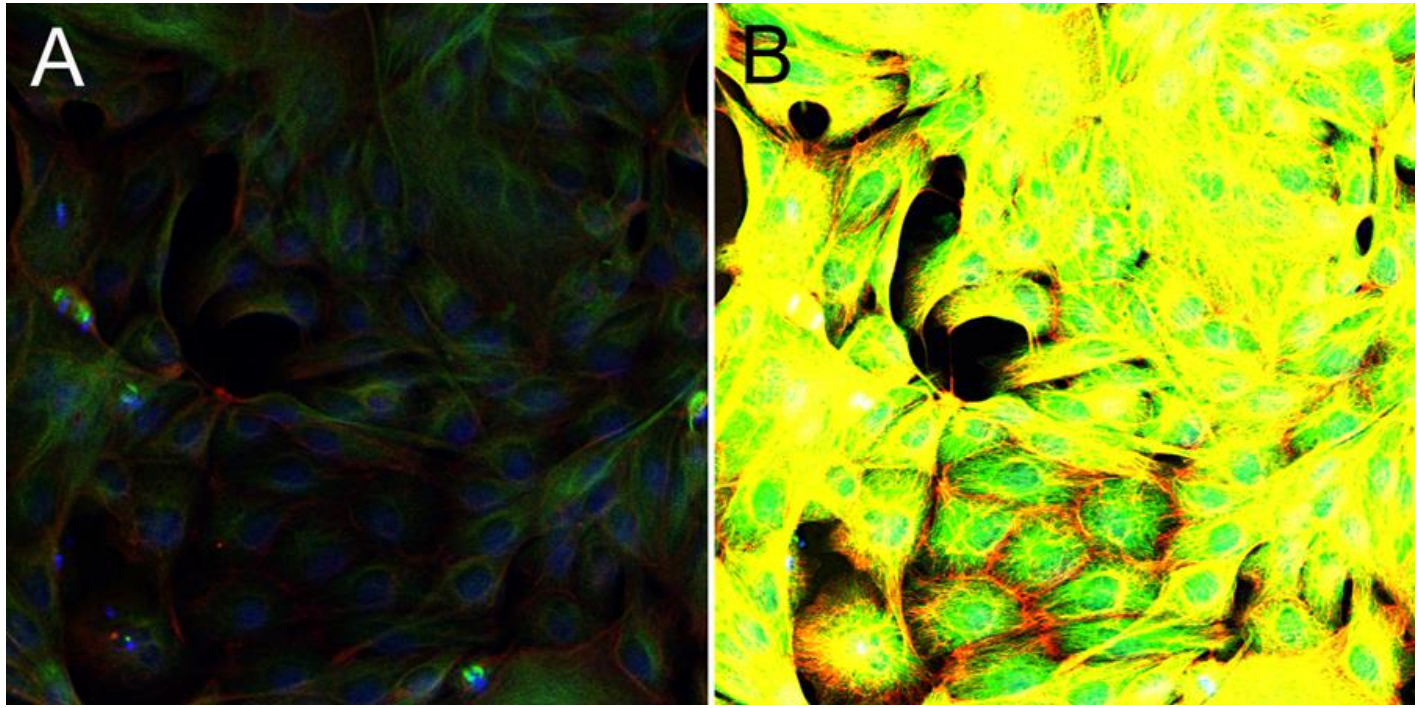
N.A. also determines Intensity

$I \propto N.A.^4 / magnification^2$ (I = intensity)

- 60x 1.4 N.A. objective gives brighter signal than 60x 0.9 N.A. objective;
- 60x 1.4 N.A. objective gives brighter signal than 100x 1.4 N.A. objective.

Two images with identical acquisition conditions (laser power, gain, offset etc.):

(A) 10x/**0.25** NA objective;
(B) 10x/**0.45** NA objective.



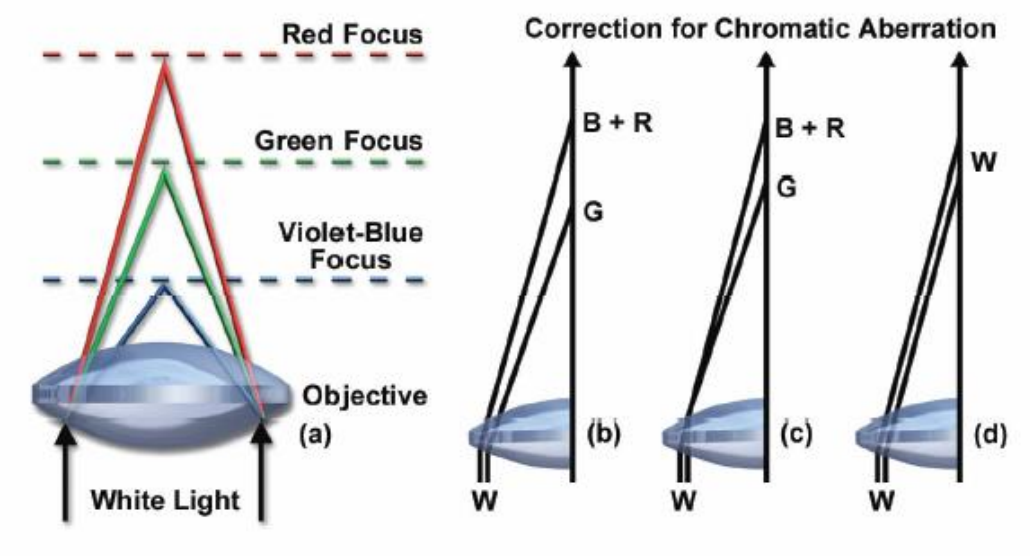
Different classes of objective lenses show different degrees of correction for aberrations

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

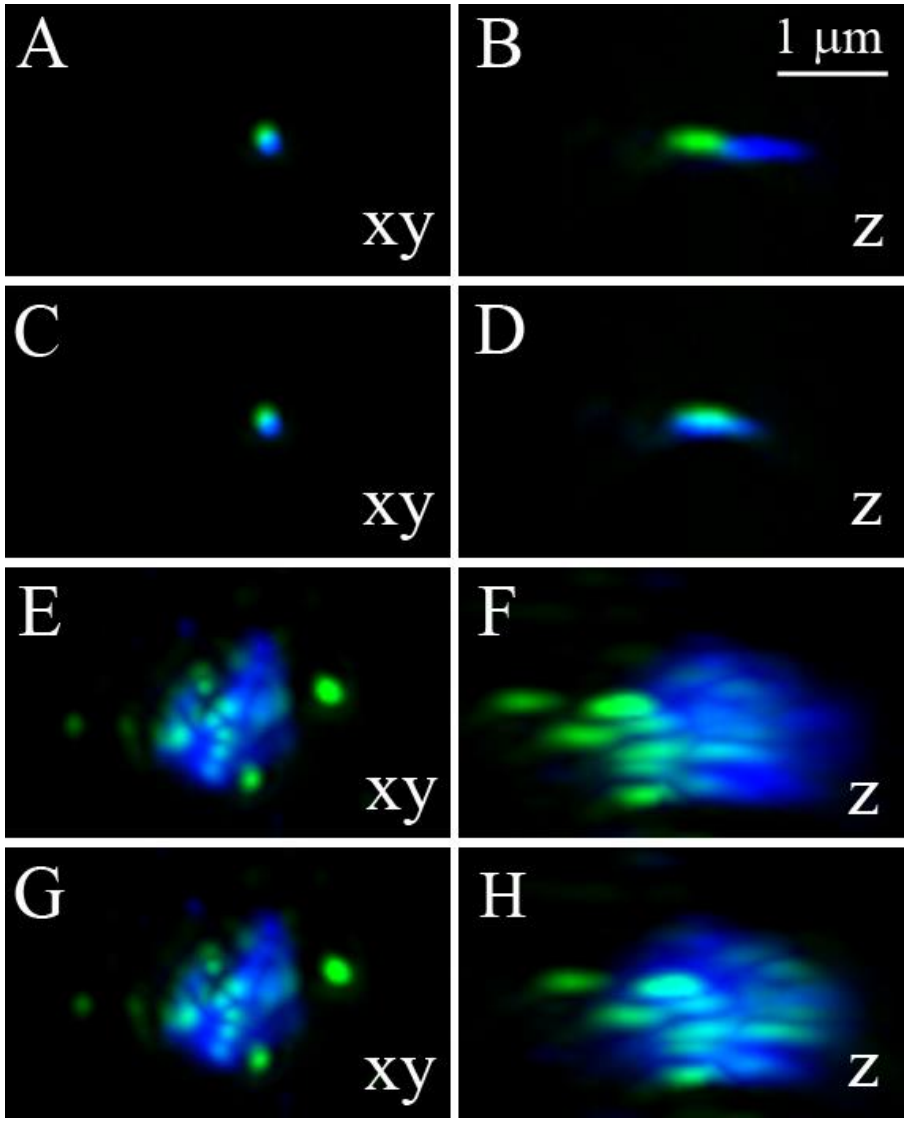
(a) Simple lens
(b) Achromatic lens (B and R corr)
(c) Fluorite lens
(d) Apochromatic lens

In (d): ***"For all practical purposes chromatic aberration may be considered eliminated."***



From: Microscope: Basics and Beyond. Mortimer Abramowitz

Minor chromatic aberrations may be seen even with the best of objective lenses



Olympus 100x/1.40 NA **S-Apochromat** DeltaVision microscope

A-D; 0.1 mm tetraspeck beads (imaged through the DAPI and FITC channels)

E-H; *S. pombe* nucleus labelled with DAPI (DNA; blue) and for specific nuclear structures (AF 488; green)

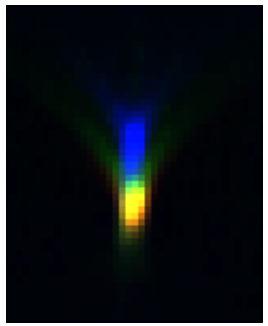
A, B, E and **F**; raw data

C, D, G and **H**; images corrected for chromatic aberration shifts

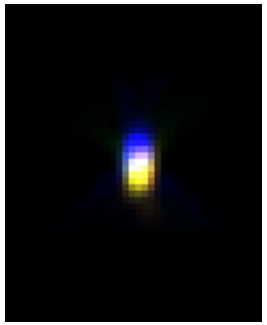
Axial chromatic shifts can even be exacerbated by the brand of immersion oil

Axial (xz) view of Tetraspeck 200 nm beads (widefield/deconvolved)

Cargille 1.516 oil

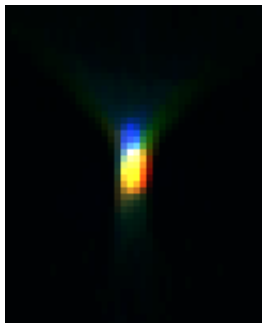


Olympus oil

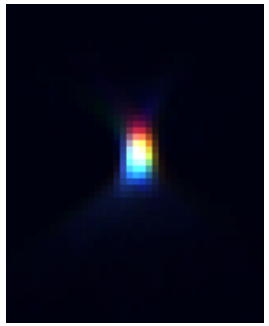


Old style Olympus 60x/1.42 objective
405, 488, 632 channels

Cargille 1.516 oil



Olympus oil



2 um

New Olympus "X-line" 60x/1.42 objective
405, 488, 632 channels

Chromatic shifts in multicolour imaging

Hmmm … why is one colour always shifted in the same relative direction?

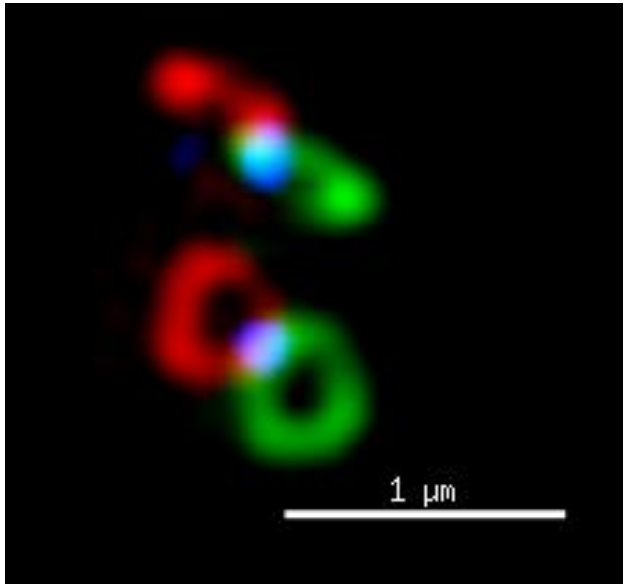
Lateral chromatic shifts can be caused by:

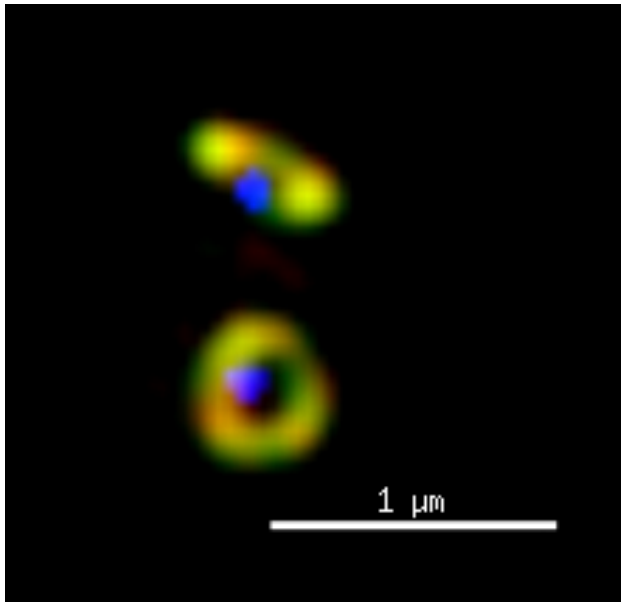
- Poor filter cube registration;
- Significant wedge in dichroic or filters;
- Filters shifted within the filter wheel;
- Imperfect alignment in multiple camera systems;

Axial chromatic shifts caused by:

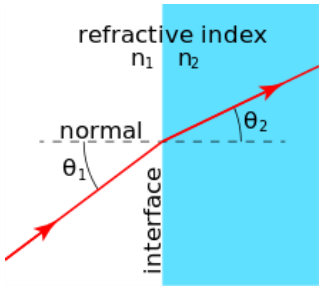
- All of the above;
- Chromatic aberrations.

Use sub-resolution multicolour beads to test and apply corrections!



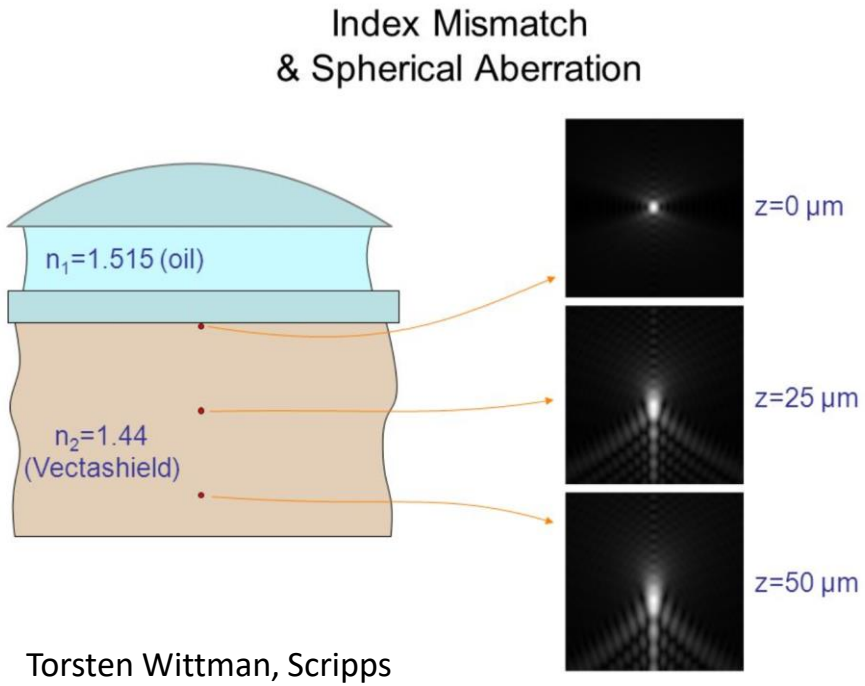


Refraction, Refractive index mismatch and Spherical Aberration



Wikipedia

Refractive Index matching will minimize aberrations



Torsten Wittman, Scripps

RI mismatch RI matching

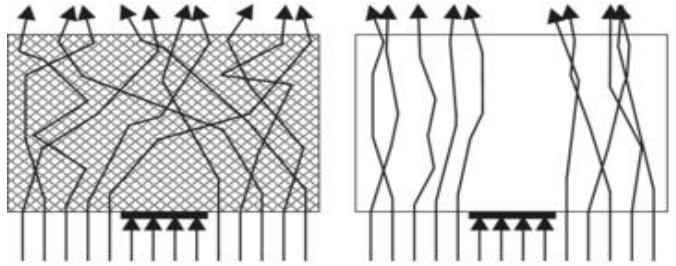


Figure 1 from Refractive index matching improves optical object detection in paper

J M S Saarela et al 2008 Meas. Sci. Technol. 19 055710
doi:10.1088/0957-0233/19/5/055710

Next demo!

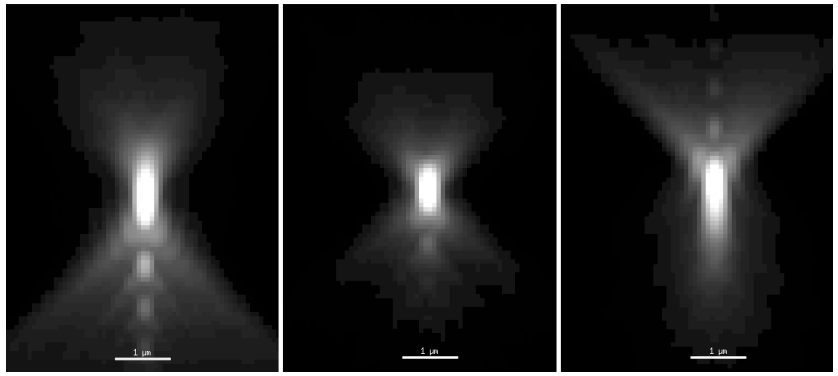
Spherical aberrations

Spherical aberrations can arise from:

- Wrong coverslip thickness;
- Refractive index mismatch;
- Increasing distance from coverslip.

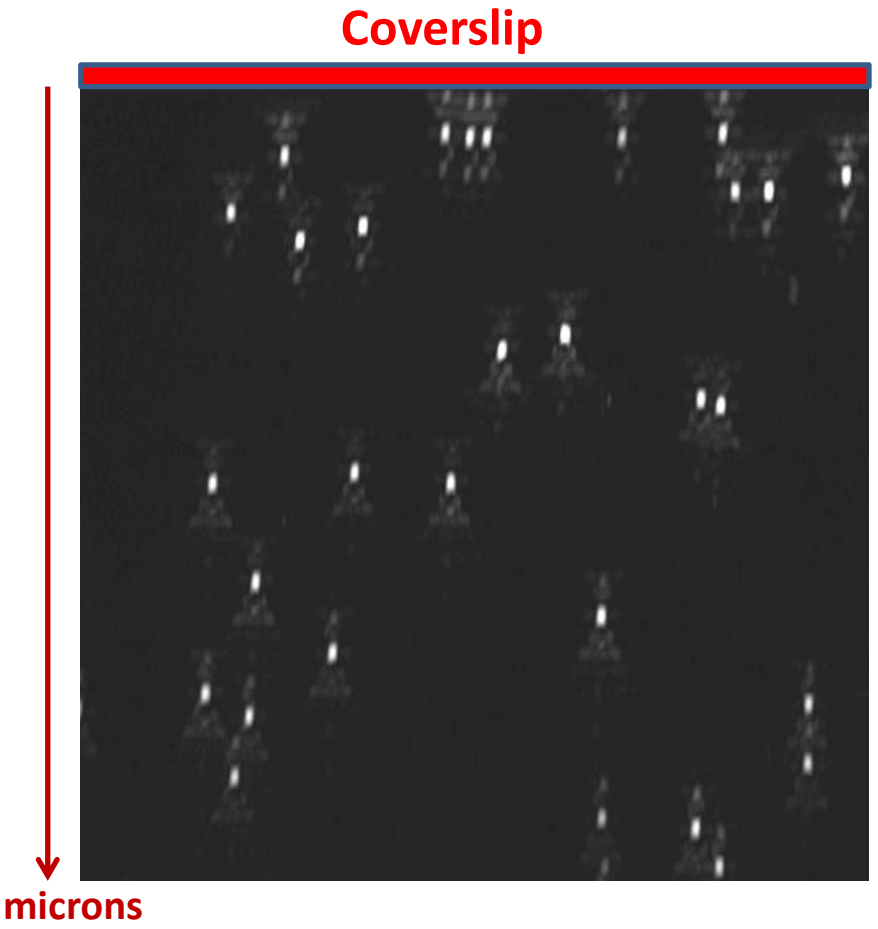
Refractive index of immersion oil was:

Too low **Correct** **Too high**



XZ views of bead psfs

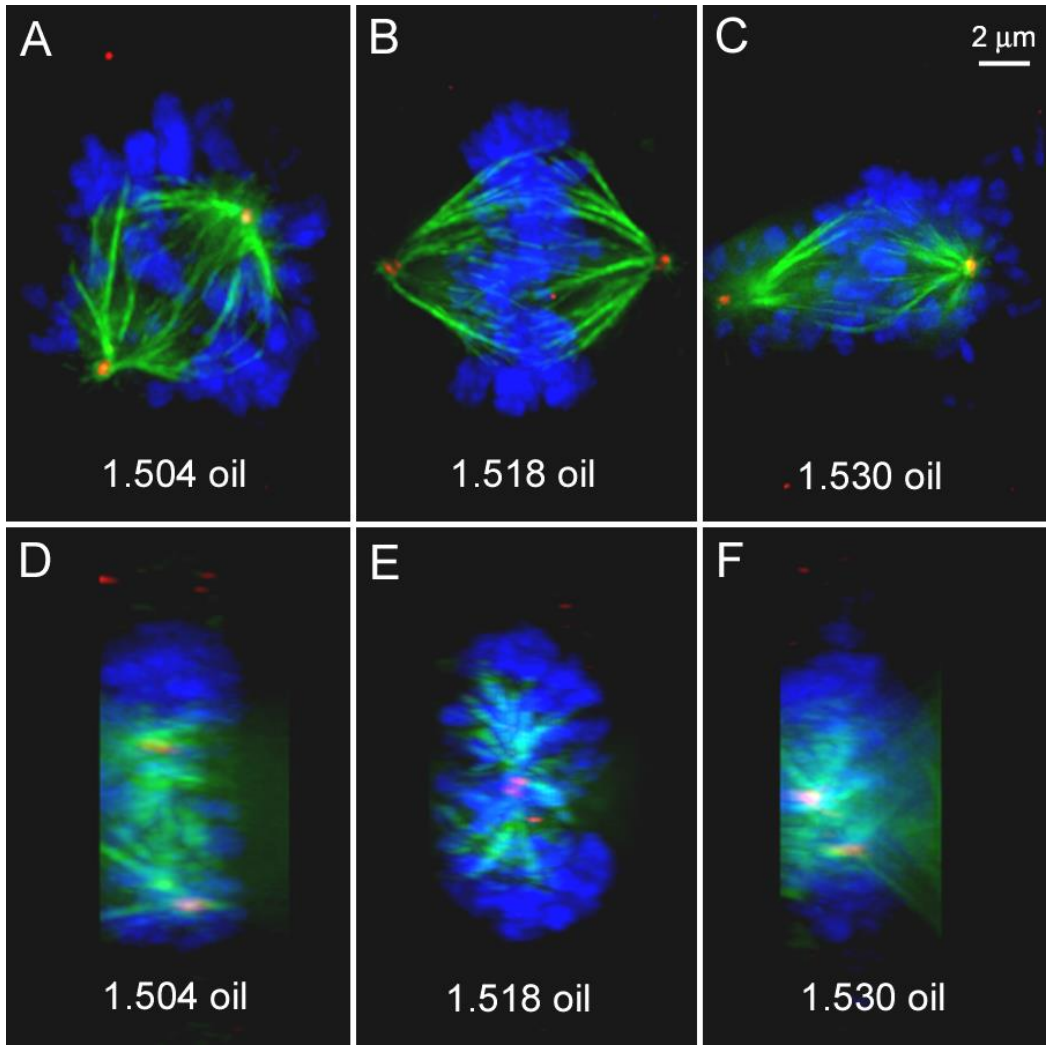
Oil immersion r.i. mismatch differs with increasing distance from the coverslip



Spherical aberrations in cells resulting from refractive index mismatch

XY view

XZ view

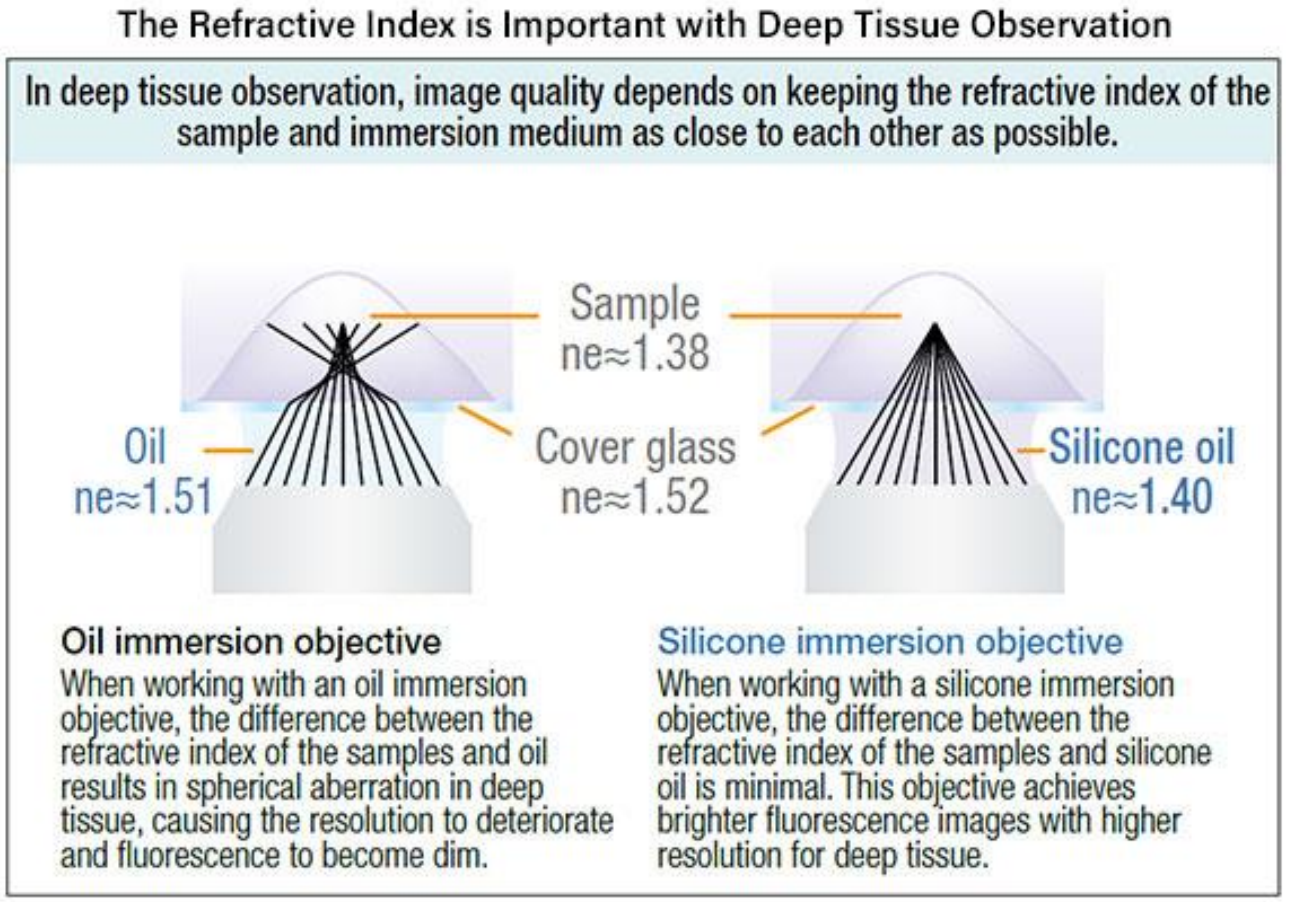


Alison North (BIRC) and Lily Copenagle
Rockefeller University

Effects are **most striking in XZ view.**

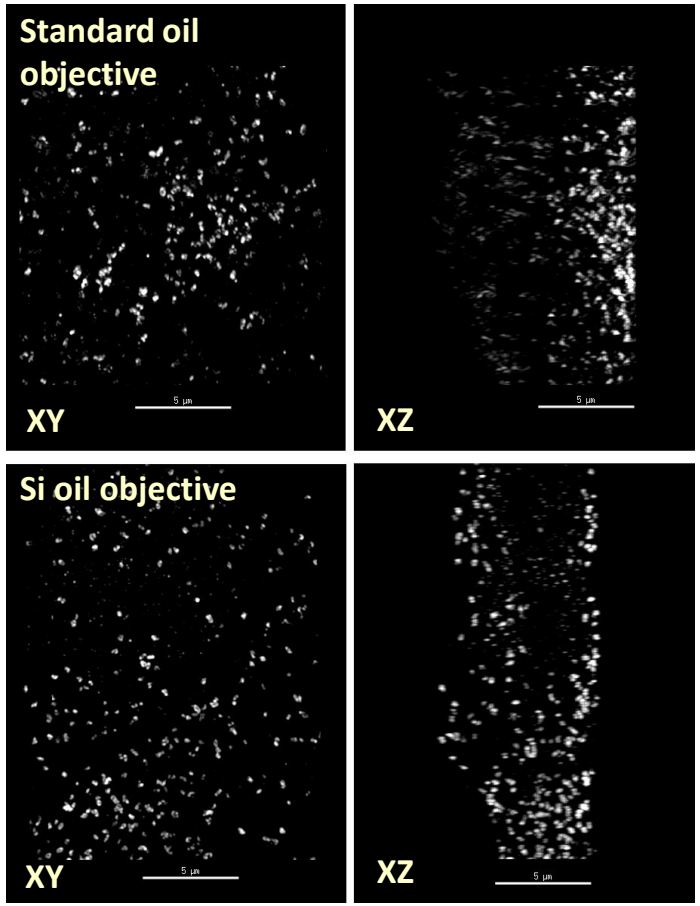
How can we address this issue?

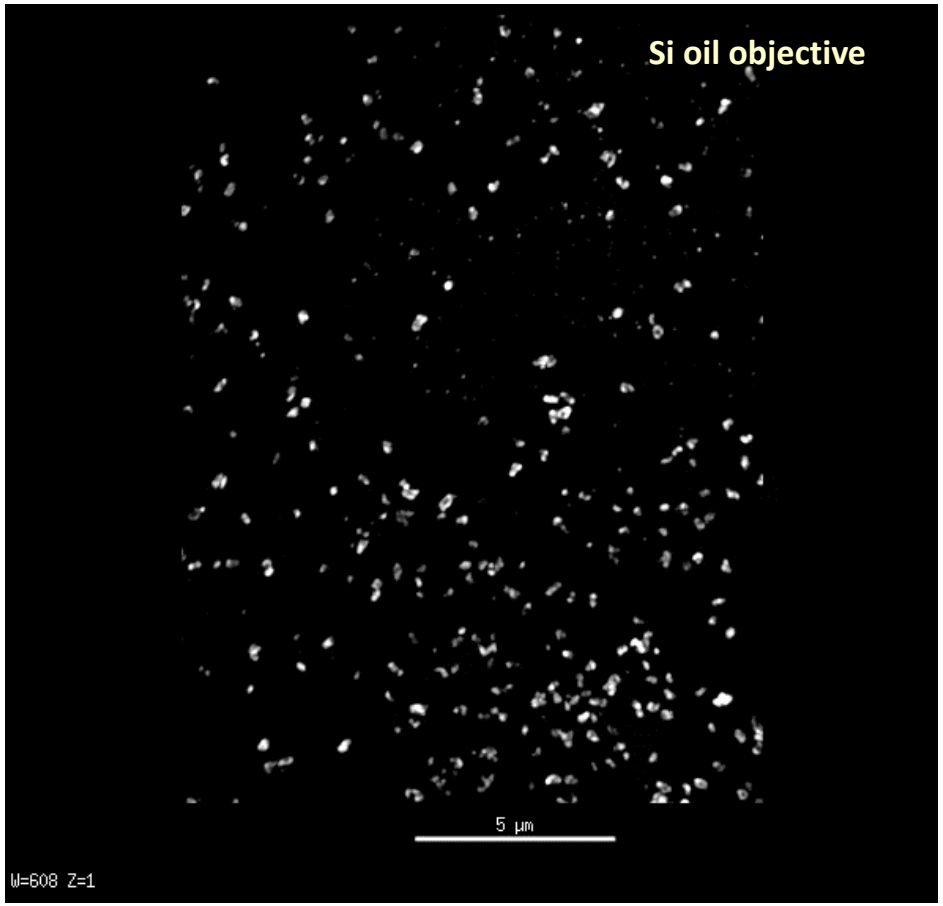
- Silicone oil (or glycerol) objectives can be used to image biological samples;
- Silicone oil provides the best match between refractive index of immersion medium (1.40) and refractive index of live organisms (1.38).



Does it really help?

Peroxisomes in cells (in PBS) imaged on the OMX using a PlanApo 100x/1.40 oil objective plus **standard 1.516 oil** or with a 100x/1.35 NA Si oil objective plus **Silicone oil**





Sample provided by Heinrich Hoffman, Rice lab, RU

How do we know what is non-specific background and what is specific low-level signal?

School bus analogy:

Consider you are an alien looking down at planet Earth from afar and trying to decide the function of the yellow blobs we call school buses…..

Time for the Practicals!

Working with arc lamps and metal halide lamps

- Turn arc lamps on/before and off/after other electronics;
- Precaution - plug mercury lamps into separate electric circuit;
- Align new arc lamps ~ 15 mins after turning on, then leave the lamp on for several hours “burn in” period. Alignment needs re-checking over time;
- Allow lamp to cool for > 30 mins before switching back on;
- Never open up lamp housing while hot;
- If lamp explodes, evacuate room immediately;
- Dispose of old lamps appropriately;
- Metal halide bulbs do not need aligning. Note - liquid light guide must be changed approx. once for every 2 bulb changes.

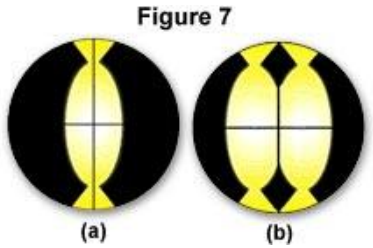


Figure 7