MICROSCOPIA OTTICA IN BIOLOGIA CELLULARE [675SM]

aa 2023/2024, 2nd semester

Lesson 4

Aula exCLA, edificio C1, 15:00-18:00

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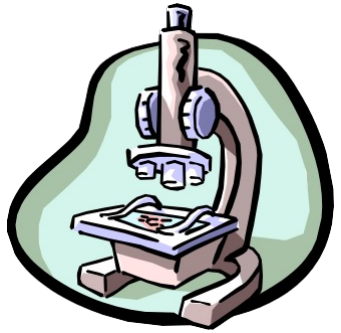
UNIVERSITÀ DEGLI STUDI DI TRIESTE
Dipartimento di Scienze della Vita

MICROSCOPIA OTTICA IN BIOLOGIA CELLULARE [675SM]

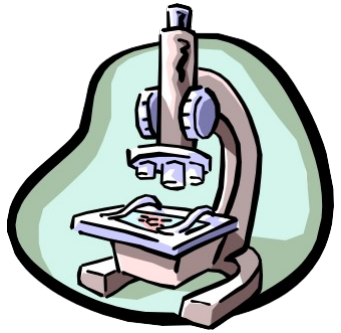
date	lesson/lab	aula	time
06/03/24	intro	Aula Ex-Cla, C1	15-16
13/03/24	lesson1	Aula Ex-Cla, C1	15-18
20/03/24	Lesson2+lab	sala microscopia F2, C1	15-18
27/03/24	lesson3	Aula Ex-Cla, C1	15-18
10/04/24	lesson4	Aula Ex-Cla, C1	15-18
17/04/24	lesson5	Aula Ex-Cla, C1	15-18
24/04/24	Lesson6+lab2	Aula Ex-Cla, C1	15-18
08/05/24	lab2	sala microscopia F2, C1	15-18
15/05/24	lab3	CIMA center, groupI	15-17
22/05/24	lab3	CIMA center, groupII	15-17

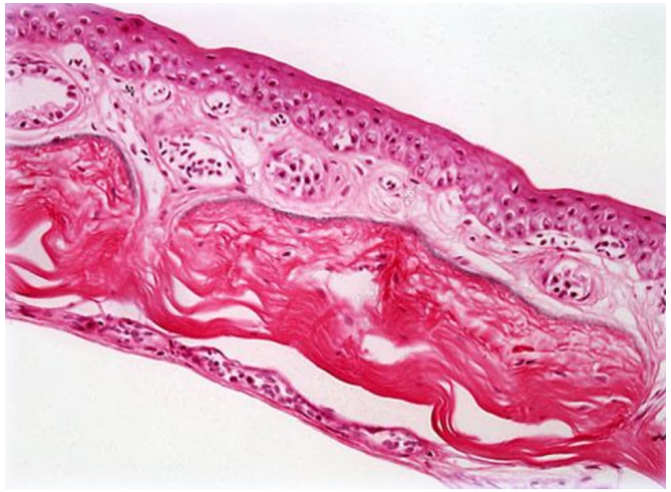
12 h lab + 16 h lessons



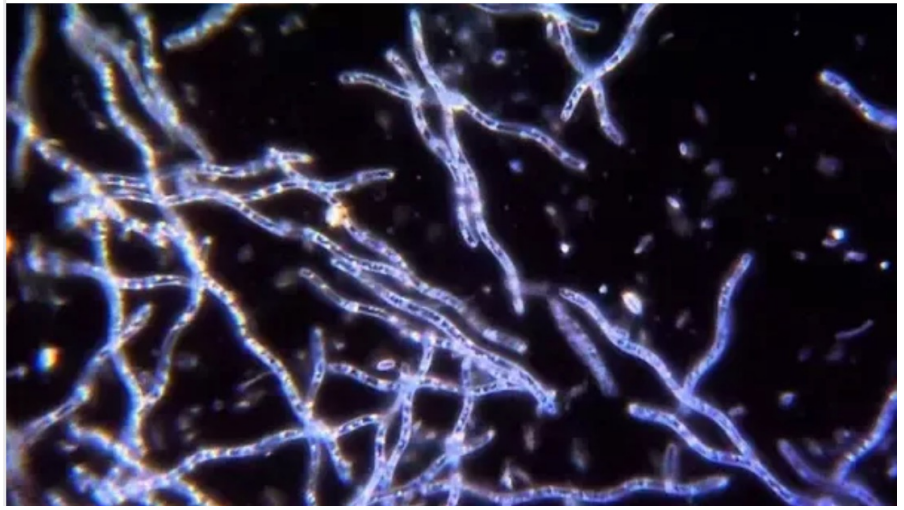
WHICH CONTRASTING TECHNIQUE?

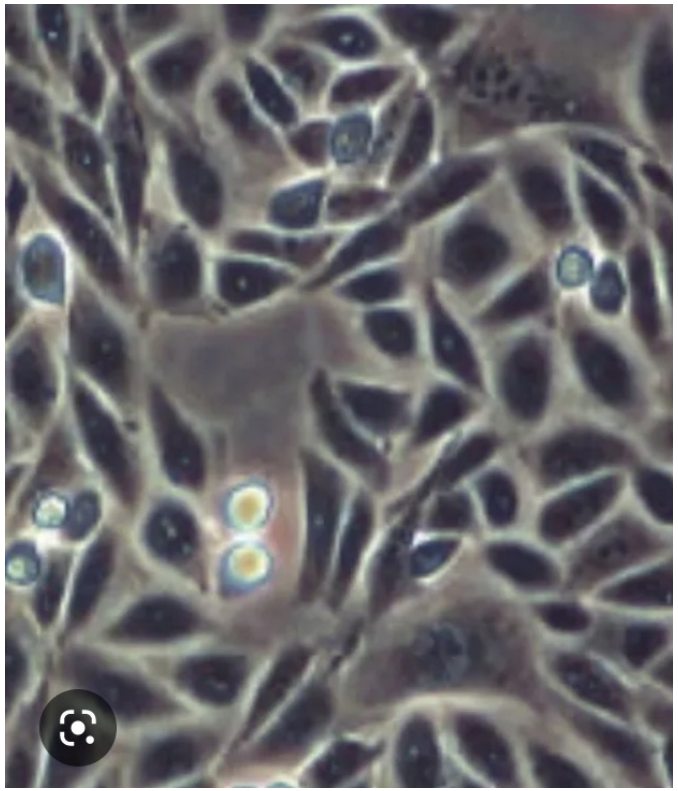
Which contrasting technique?





Amphibian Skin





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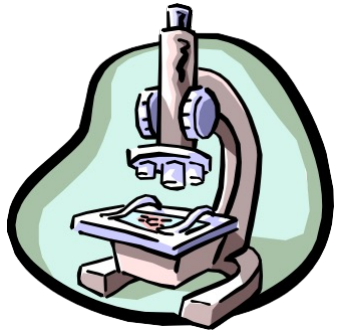
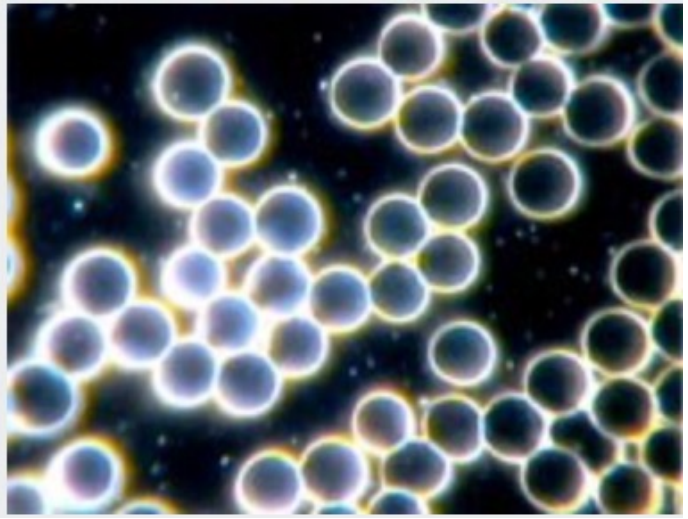
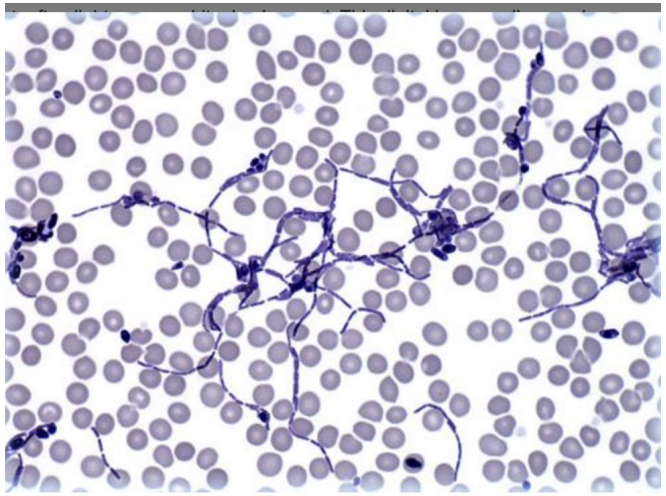
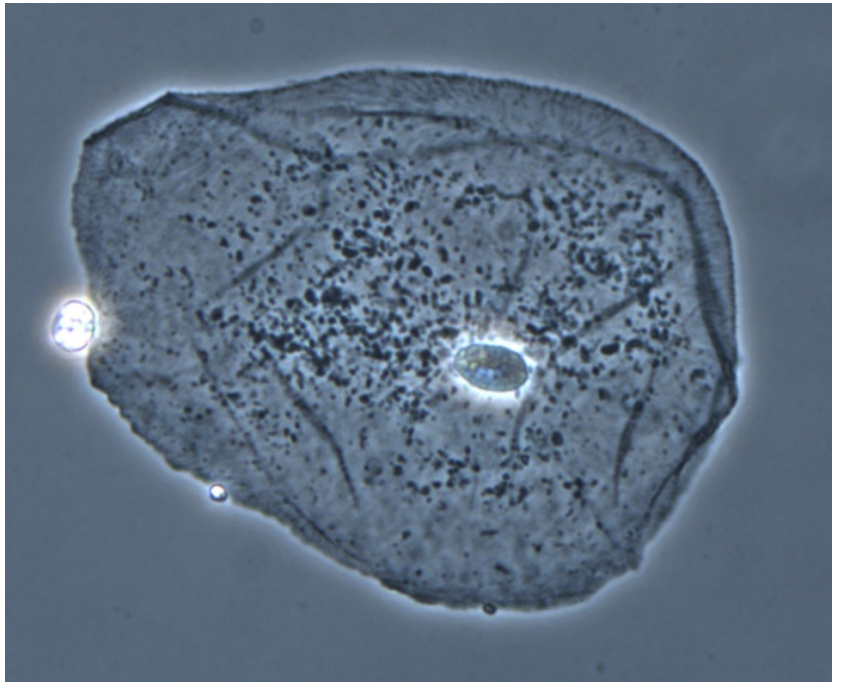






Bacteria, Yeast, and Blood



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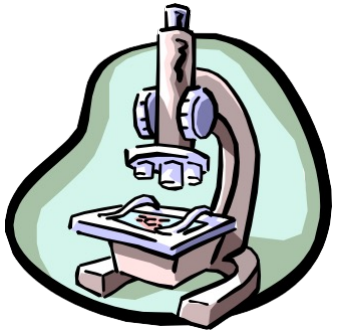

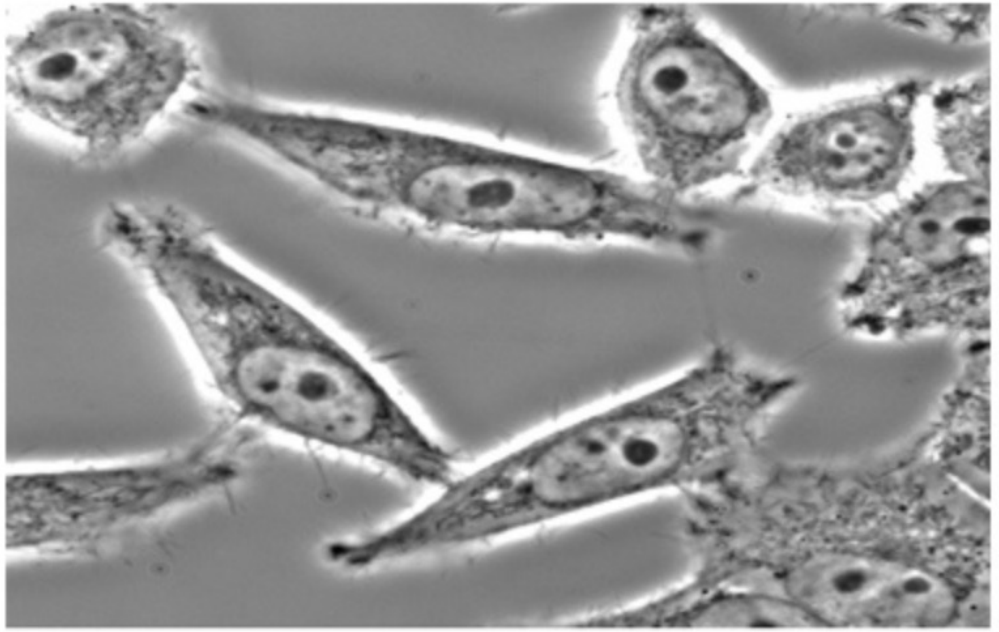

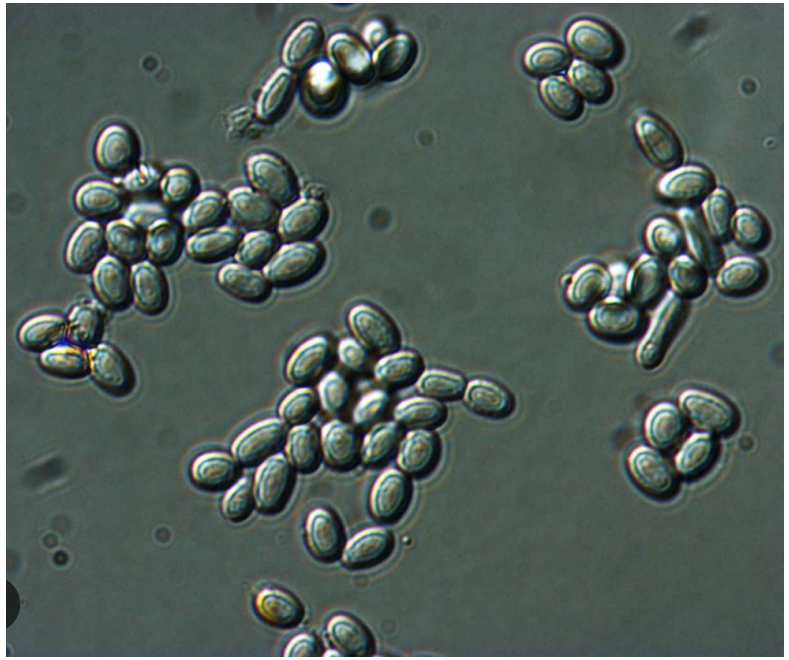

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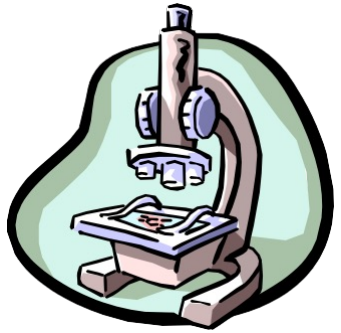

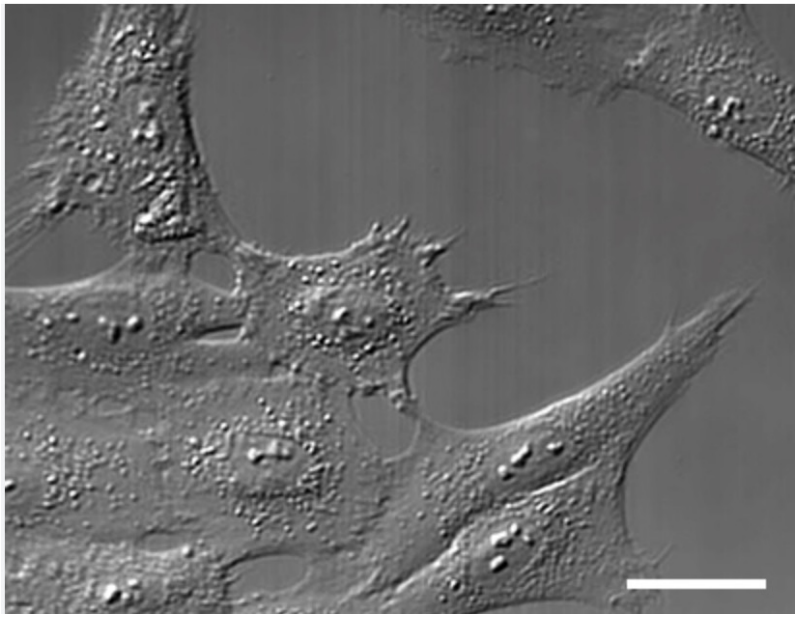

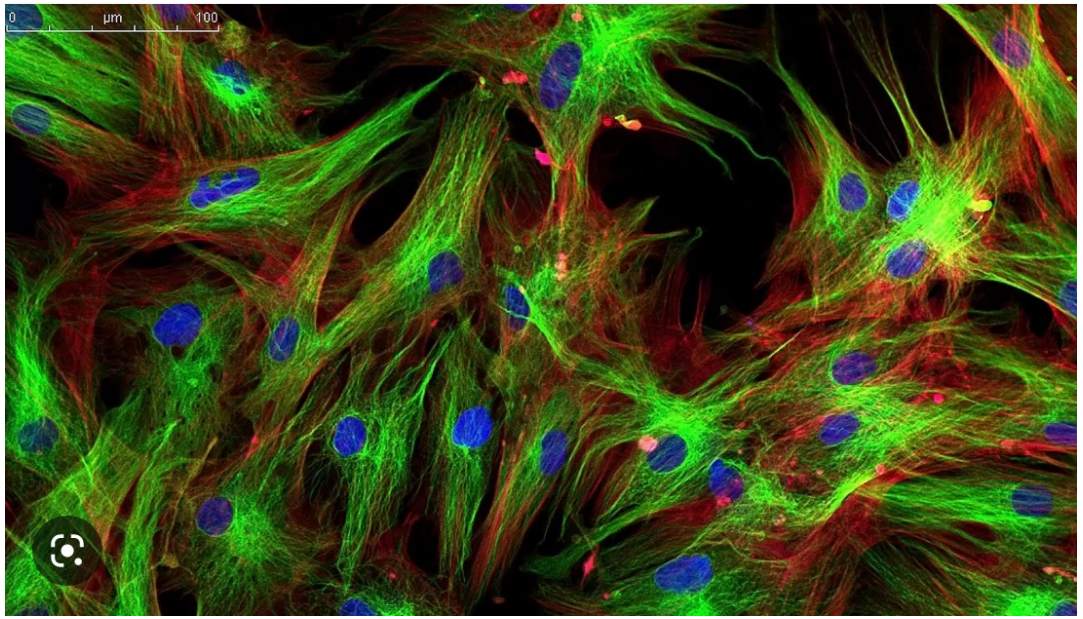

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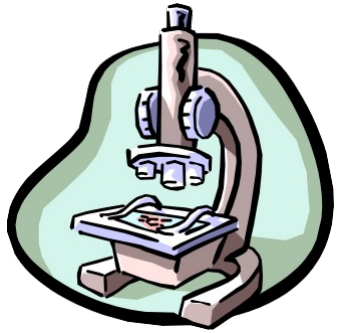
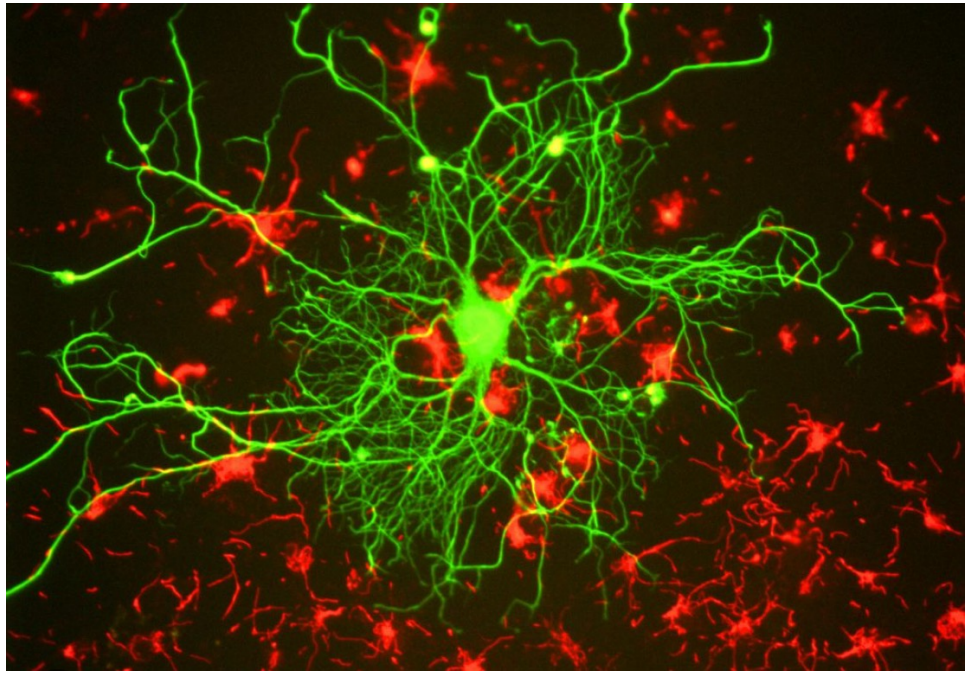
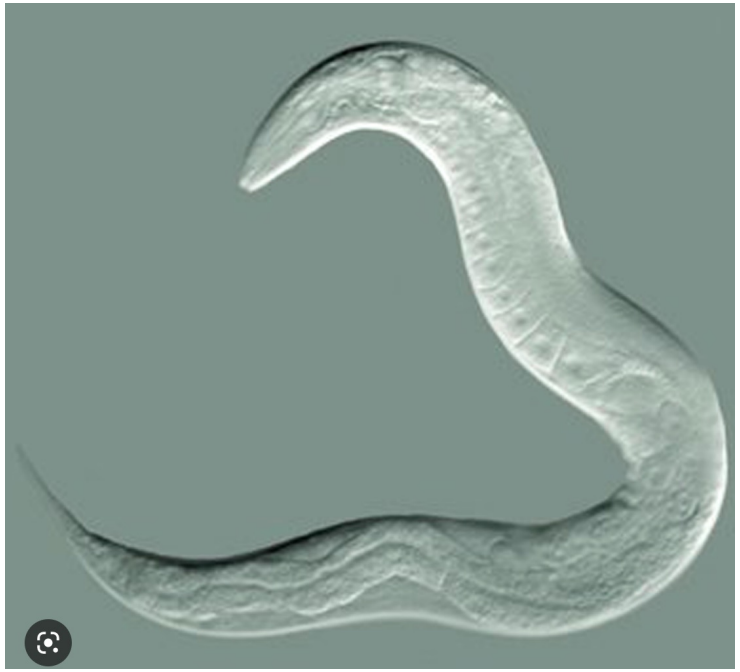
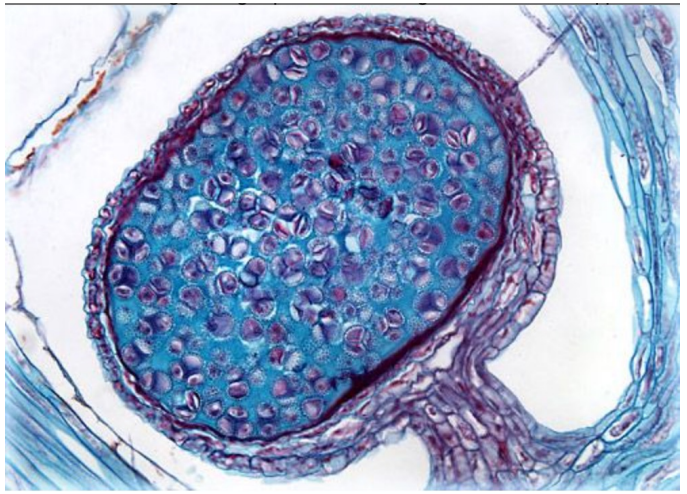


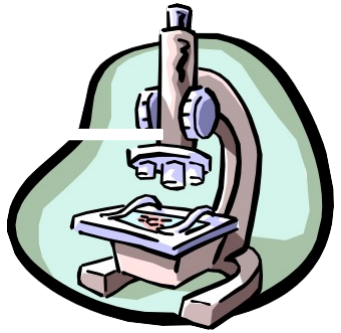






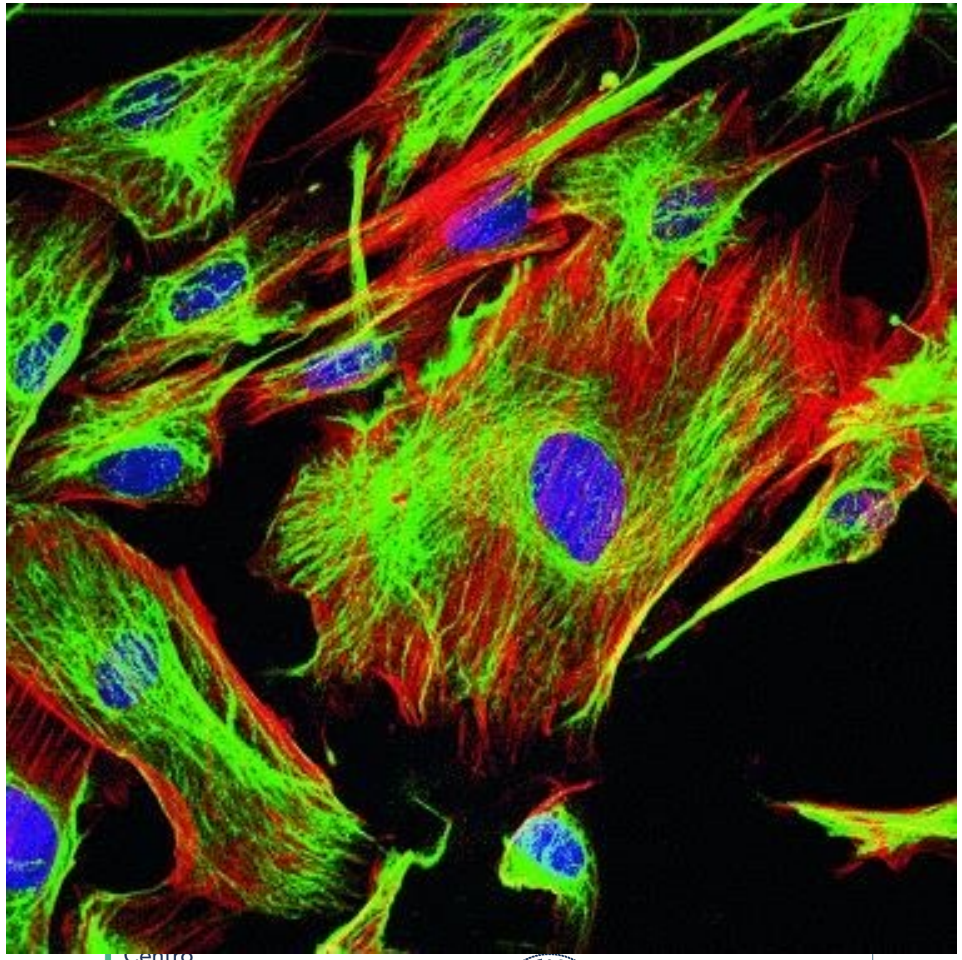
Clubmoss (*Lycopodium*) Strobilus

WHY FLUORESCENCE MICROSCOPY?

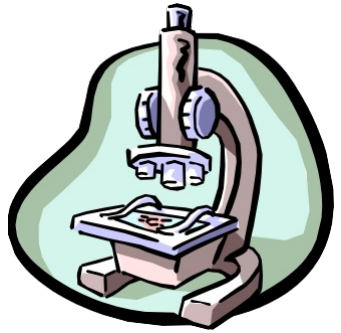


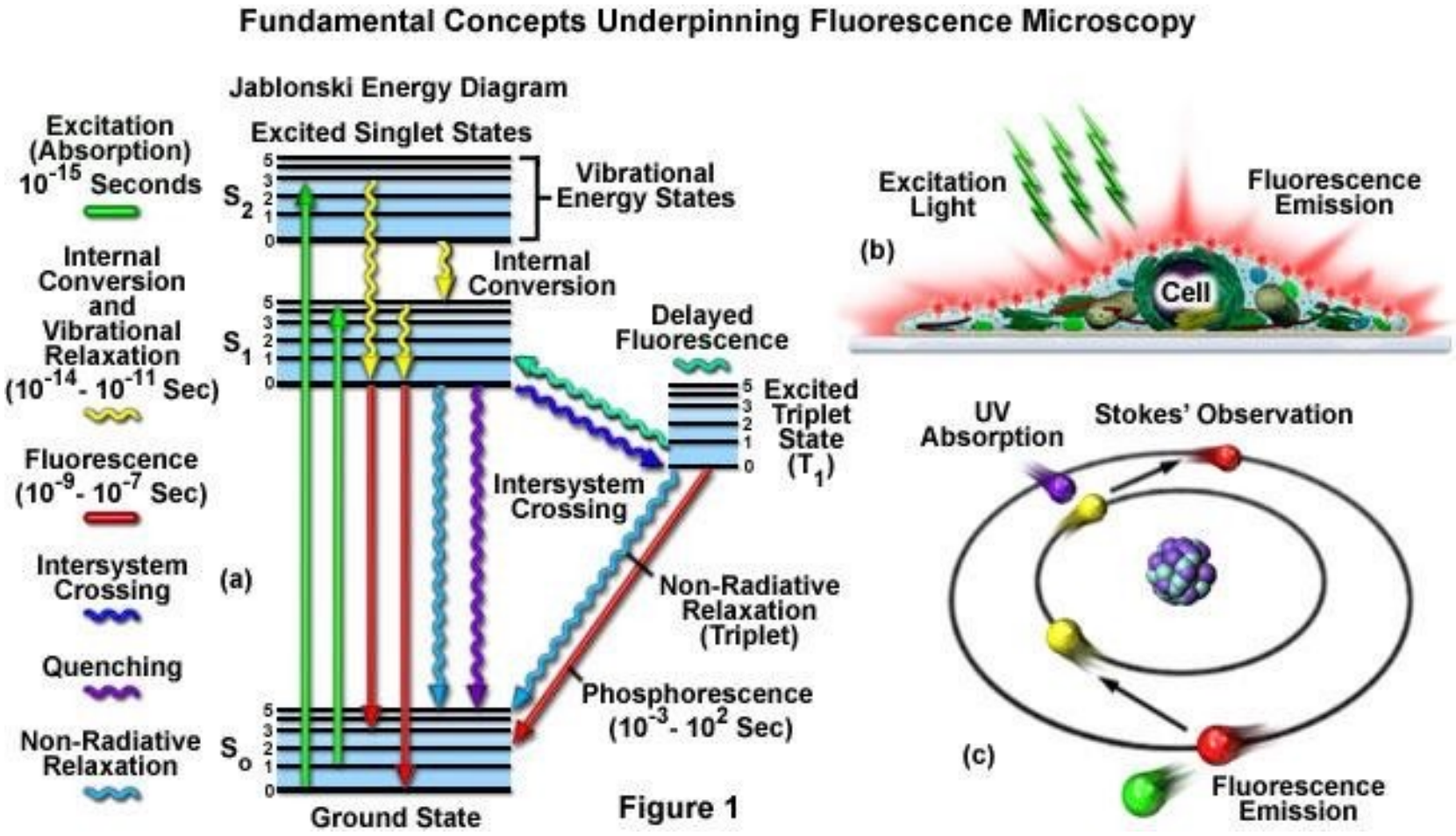
High resolution

High contrast
High specificity
Quantitative
Live Cell Imaging

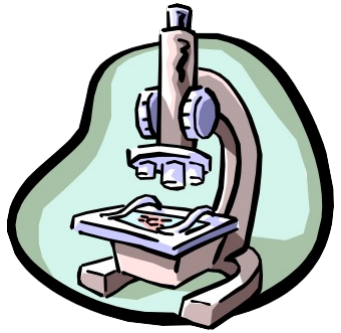


FLUORESCENCE PRINCIPLE

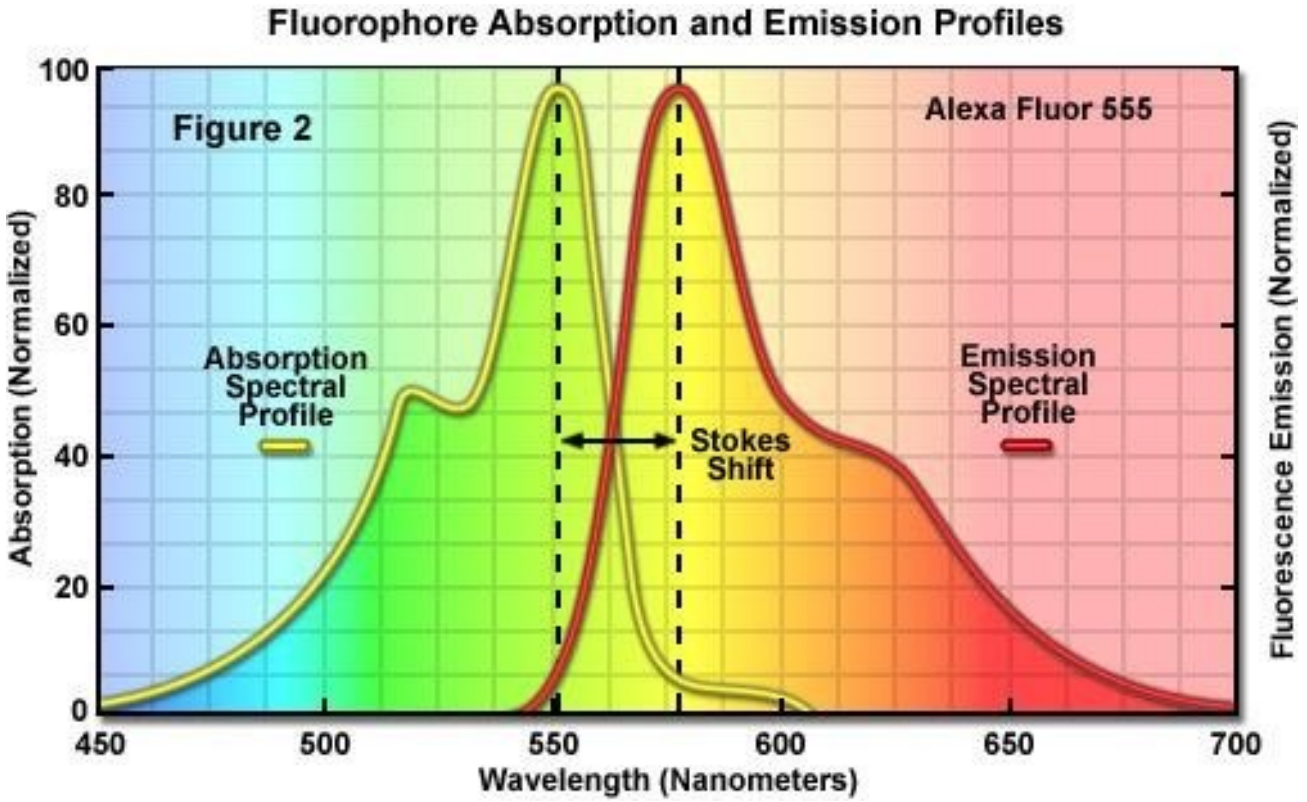


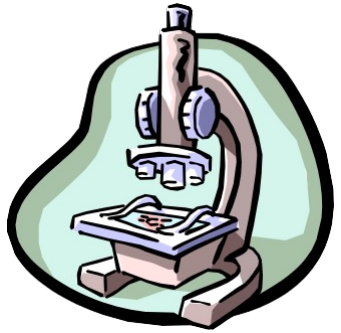


BASIC CONCEPTS



- excitation light radiates specimen
- weaker emitted light to make up the image is separated
- use fact that the emitted light is of lower energy and has a longer wavelength
- The fluorescent areas can be observed in the microscope and shine out against a dark background with high contrast





Molecules absorbing the energy of electromagnetic radiation will jump to a higher energy level. When certain excited molecules return to the ground state they emit radiation. This phenomenon is known as fluorescence. Fluorescent molecules are known as fluorochromes or fluorophores.

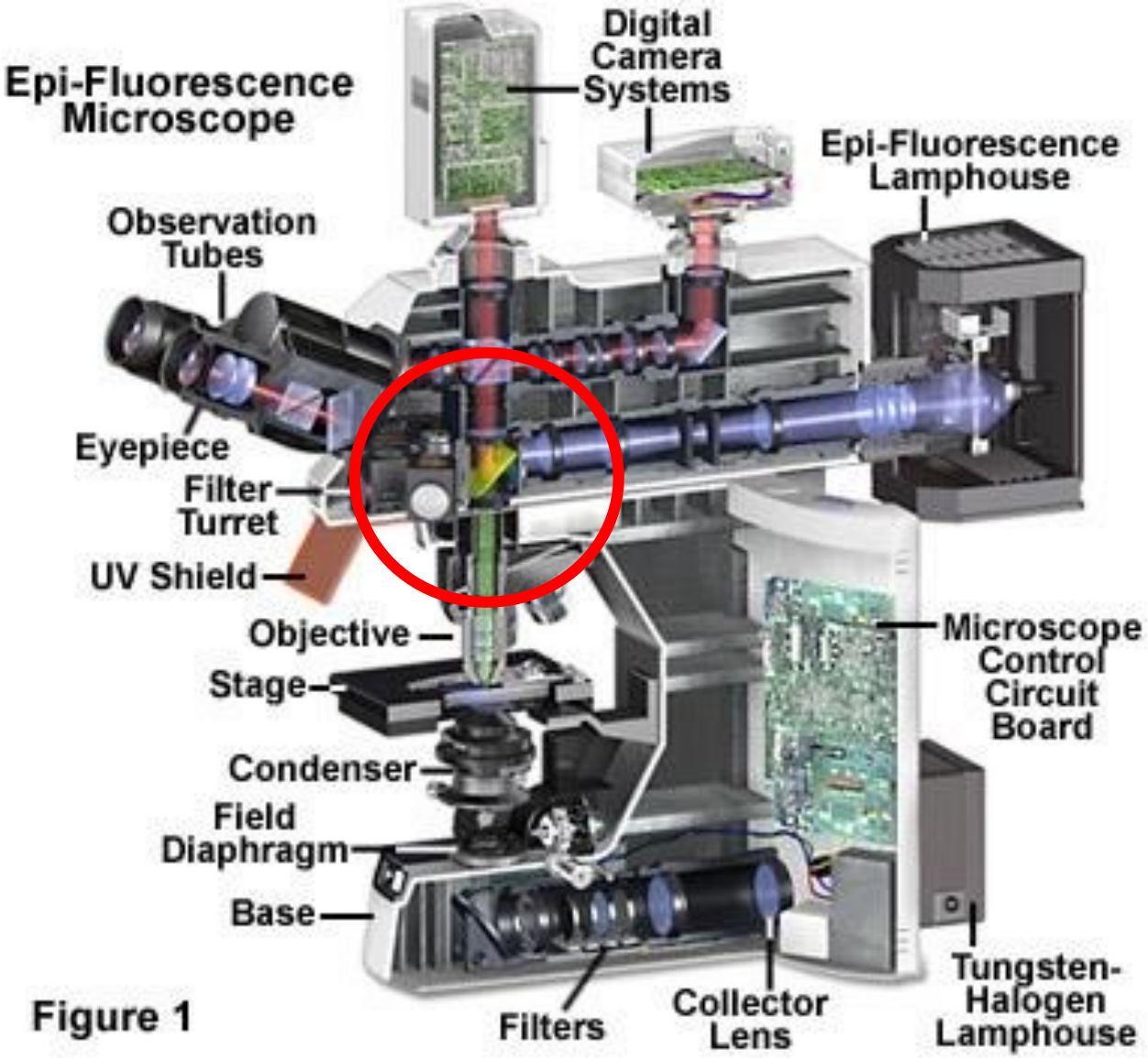
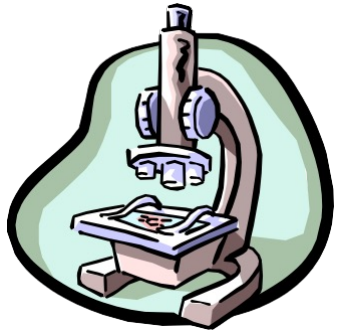


Figure 1



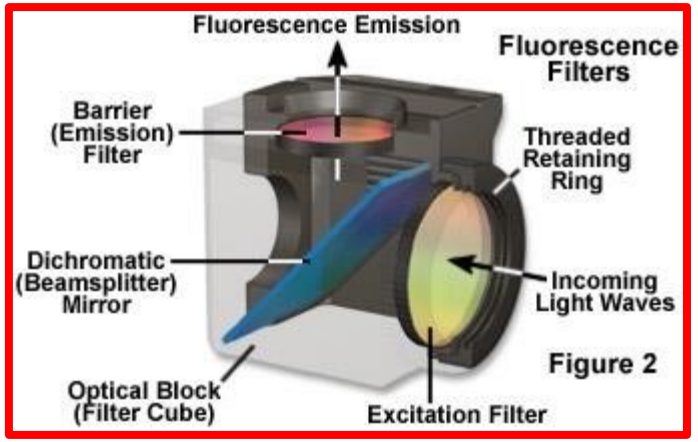
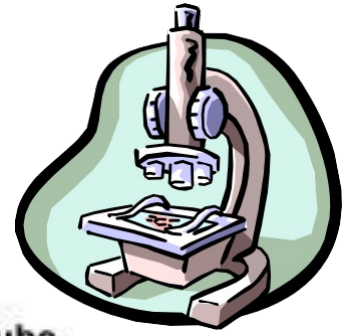
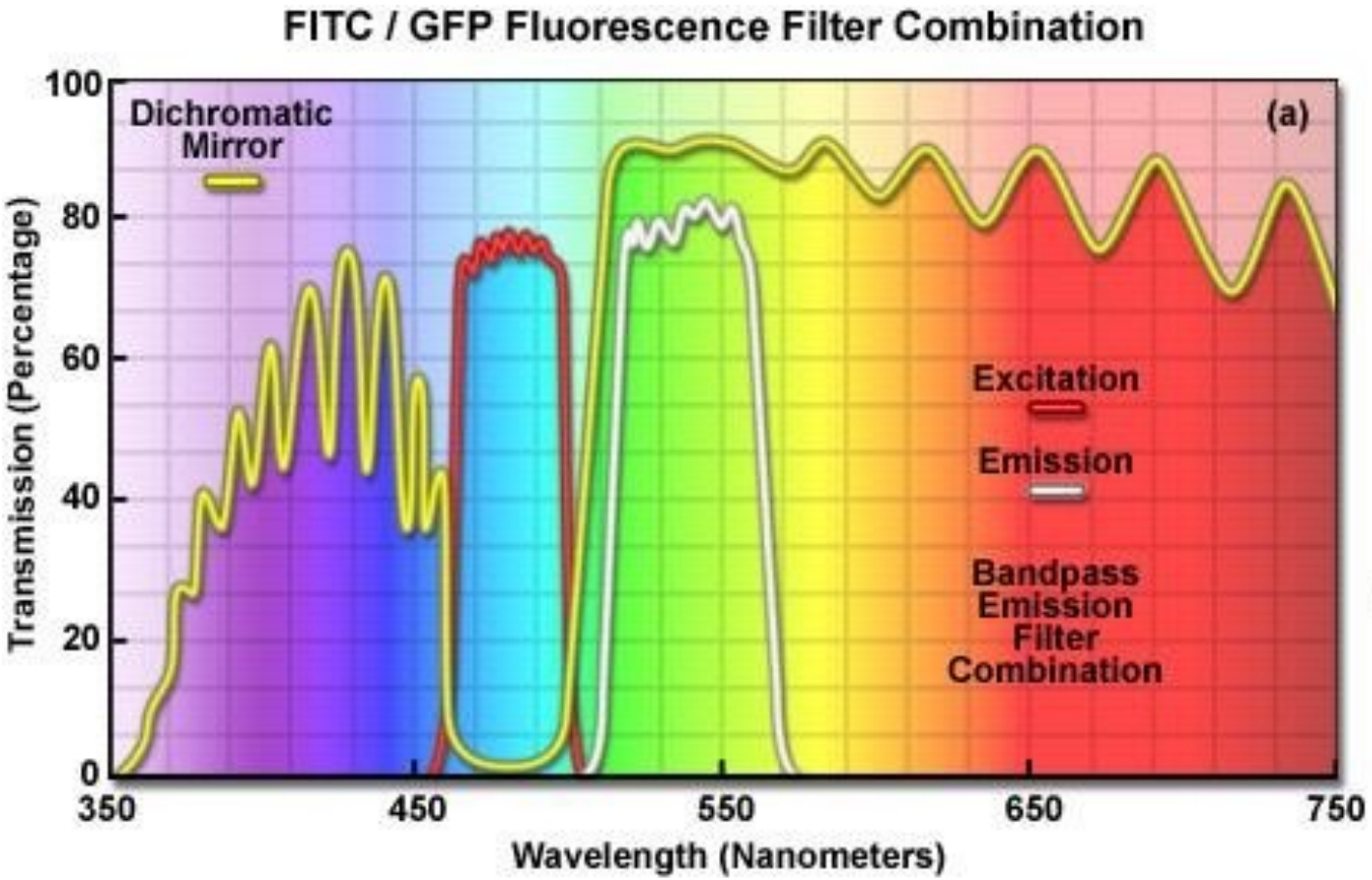


Figure 2




FITC / GFP Fluorescence Filter Combination
Dichromatic Mirror
(a)
Excitation
Emission
Bandpass Emission Filter Combination
Transmission (Percentage)
100
80
60
40
20
0
350
450
550
650
750
Wavelength (Nanometers)

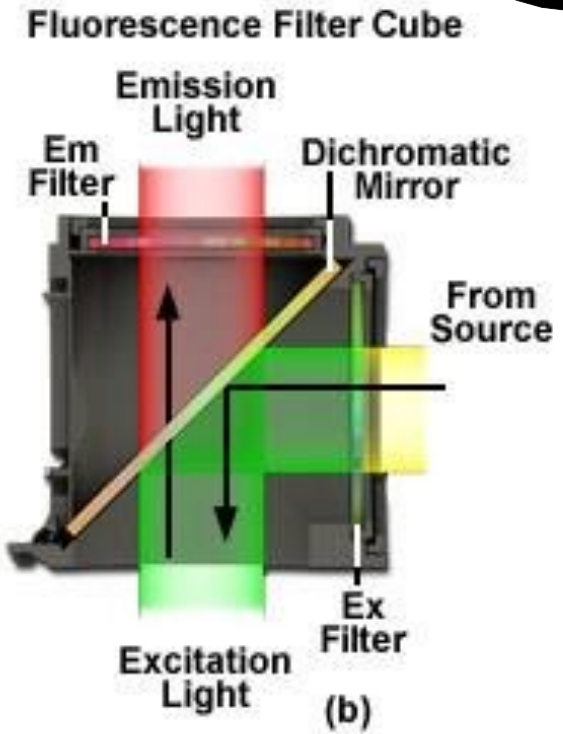
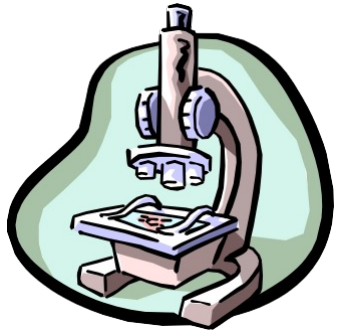

Fluorescence Filter Cube
Emission Light
Em Filter
Dichromatic Mirror
From Source
Ex Filter
Excitation Light
(b)

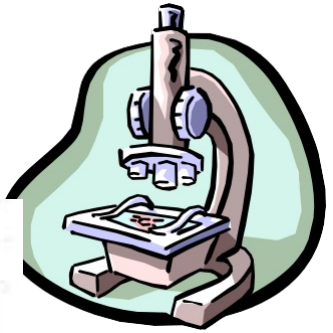
Figure 4

CHROMOPHORES

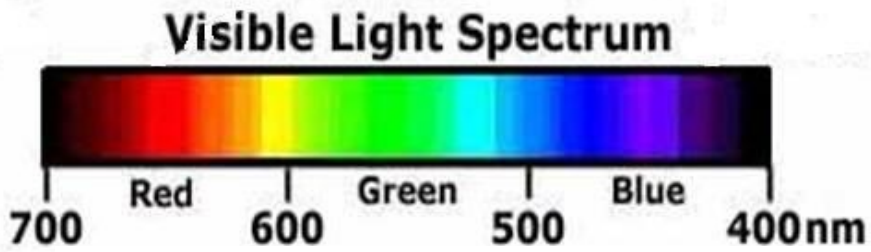


- Chromophores are components of molecules which absorb light
- e.g. from protein most fluorescence results from the indole ring of tryptophan residue
- They are generally combined aromatic groups, planar or cyclic molecules with several π-bonds
- Used alone as dyes or conjugated to macromolecules (antibodies!!)

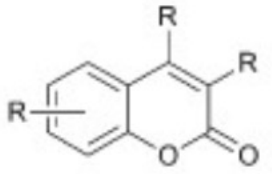
FLUOROPHORES (Fluorochromes, chromophores)



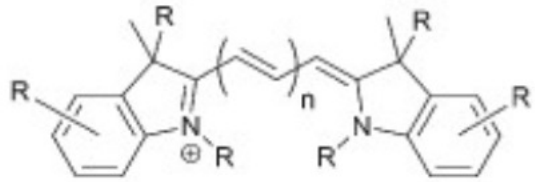
Probe	Ex (nm)	Em (nm)	MW	Notes
Reactive and conjugated probes				
Hydroxycoumarin	325	386	331	Succinimidyl ester
Aminocoumarin	350	445	330	Succinimidyl ester
Methoxycoumarin	360	410	317	Succinimidyl ester
Cascade Blue	(375);401	423	596	Hydrazide
Pacific Blue	403	455	406	Maleimide
Pacific Orange	403	551		
Lucifer yellow	425	528		
NBD	466	539	294	NBD-X
R-Phycoerythrin (PE)	480;565	578	240 k	
PE-Cy5 conjugates	480;565;650	670		*aka* Cychrome, R670, Tri-Color, Quantum Red
PE-Cy7 conjugates	480;565;743	767		
Red 613	480;565	613		PE-Texas Red
PerCP	490	675		Peridinin chlorphyll protein
TruRed	490,675	695		PerCP-Cy5.5 conjugate
FluorX	494	520	587	(GE Healthcare)
Fluorescein	495	519	389	FITC; pH sensitive
BODIPY-FL	503	512		
TRITC	547	572	444	TRITC
X-Rhodamine	570	576	548	XRITC
Lissamine Rhodamine B	570	590		
Texas Red	589	615	625	Sulfonyl chloride
Allophycocyanin (APC)	650	660	104 k	
APC-Cy7 conjugates	650;755	767		PharRed



Coumarin

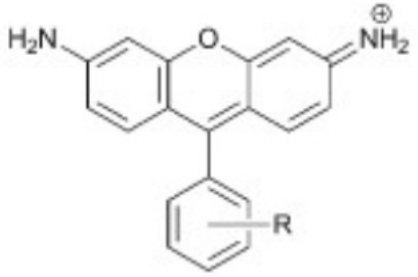


Cyanine

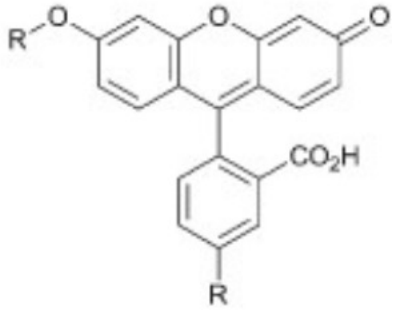


n = 1-3

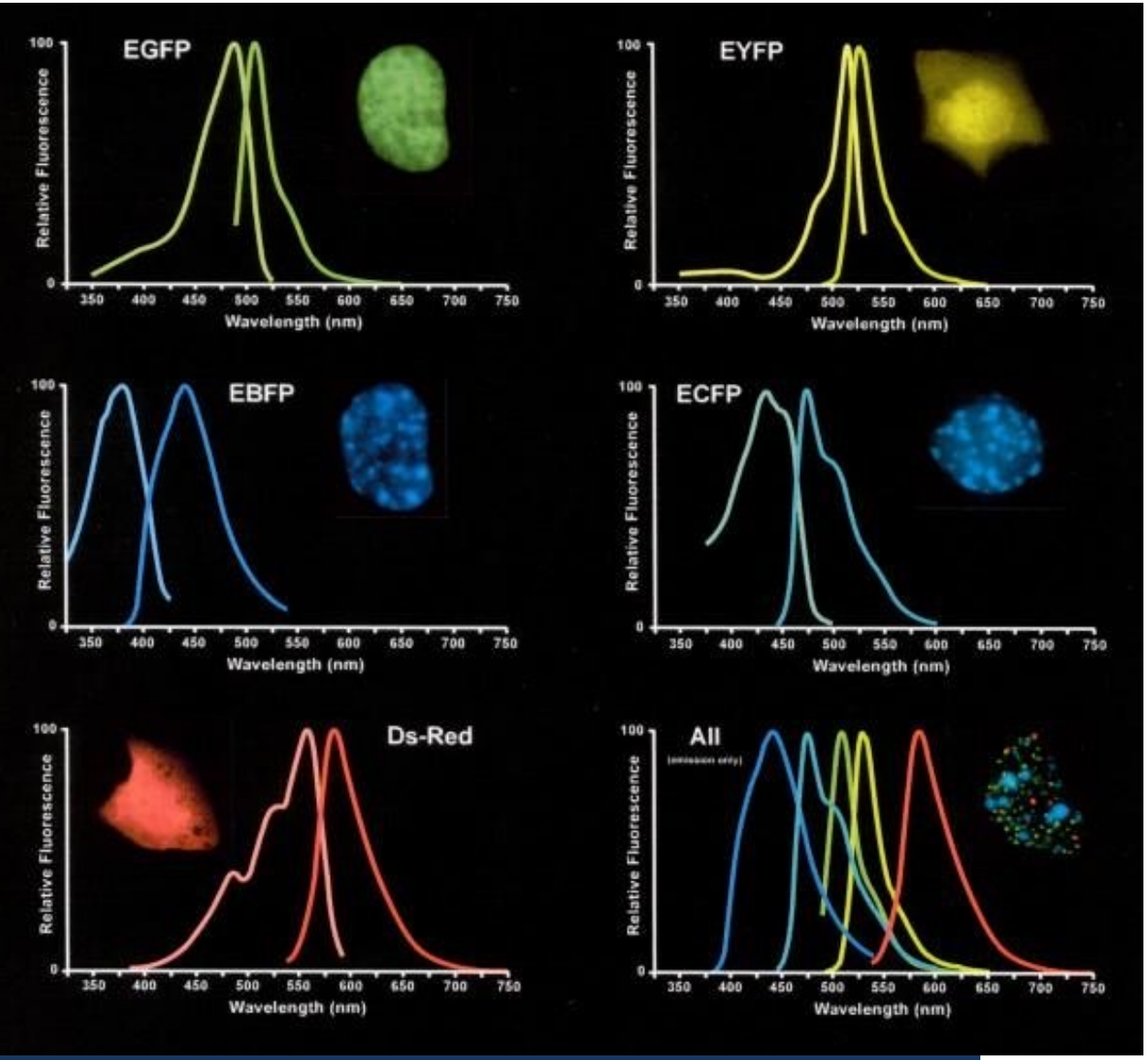
Rhodamine

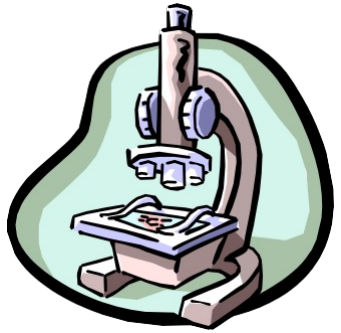


Fluorescein



FLUORESCENT PROTEINS





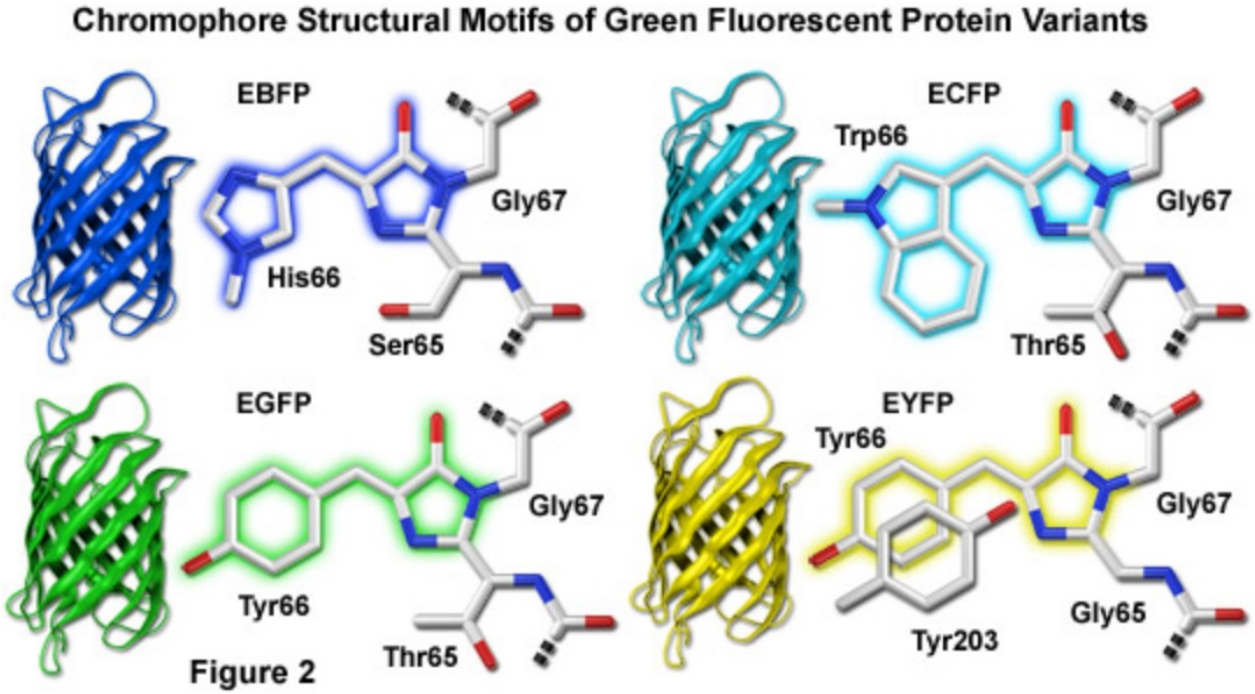
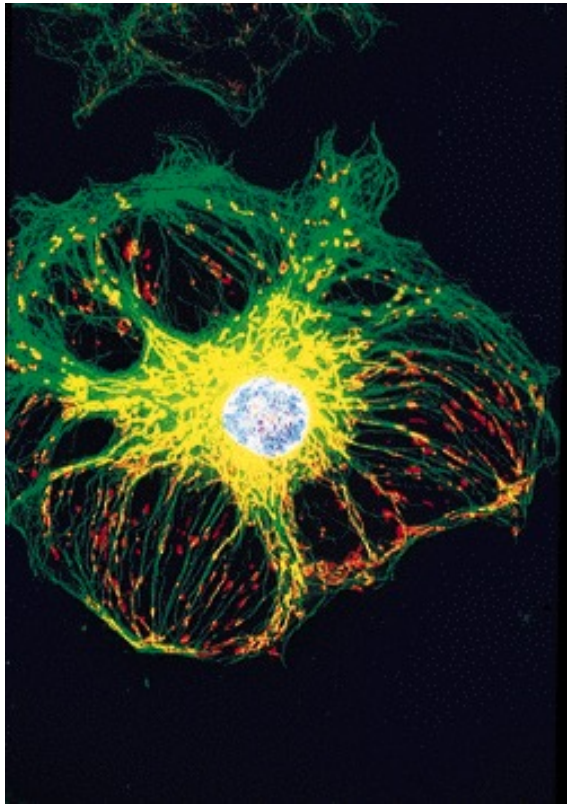
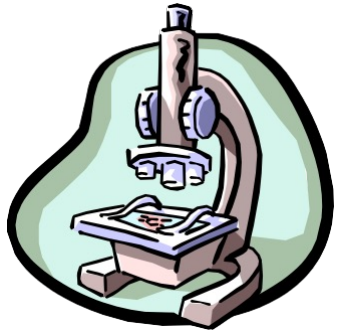


Figure 2

MULTICHANNEL FLUORESCENCE

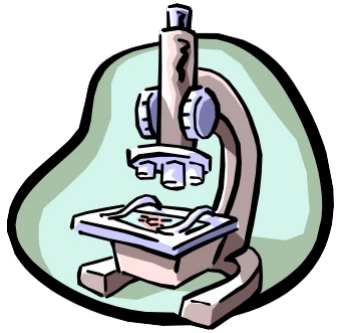


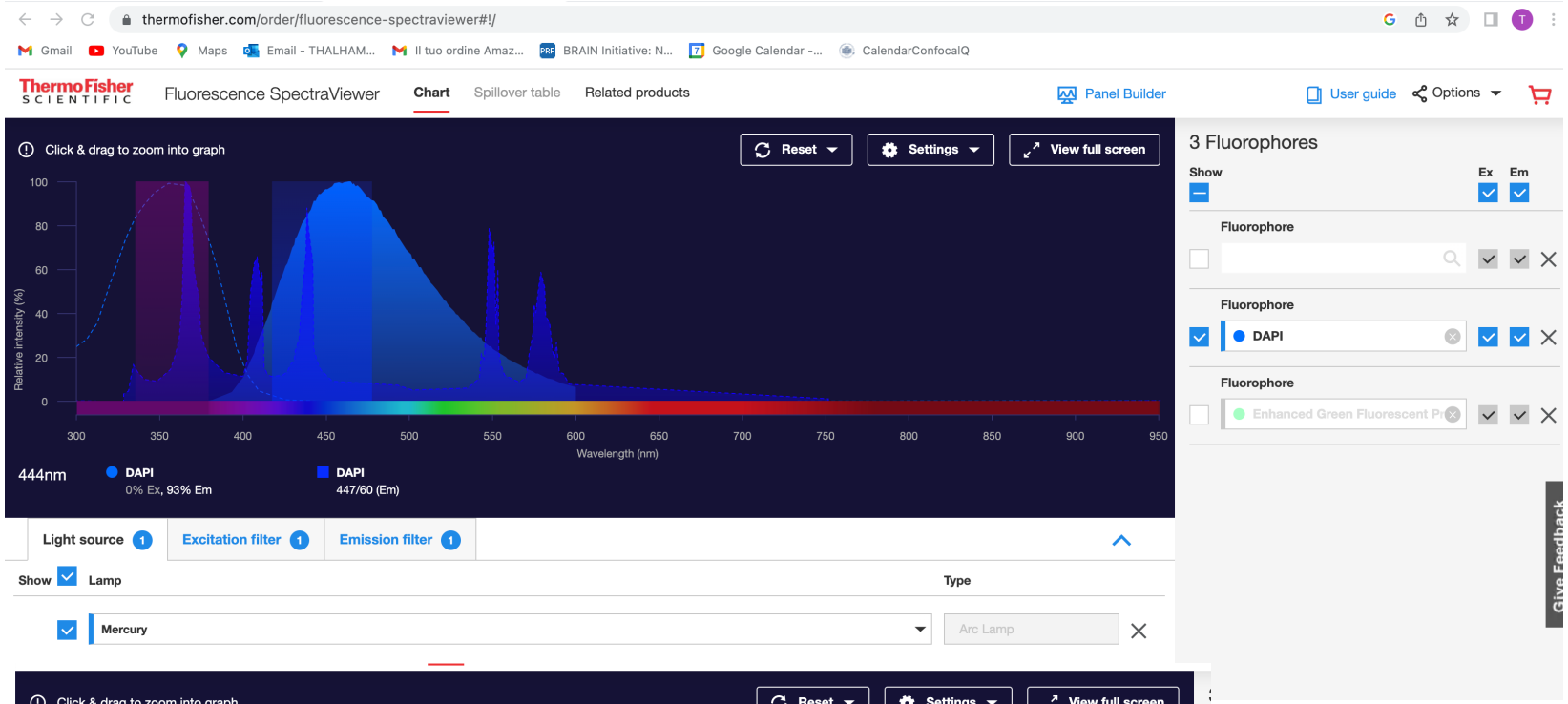
Arterial edothelial cell
Ch1(Green) FITC Tubulin
Ch2(Red) mitotracker
Ch3(Blue) DAPI

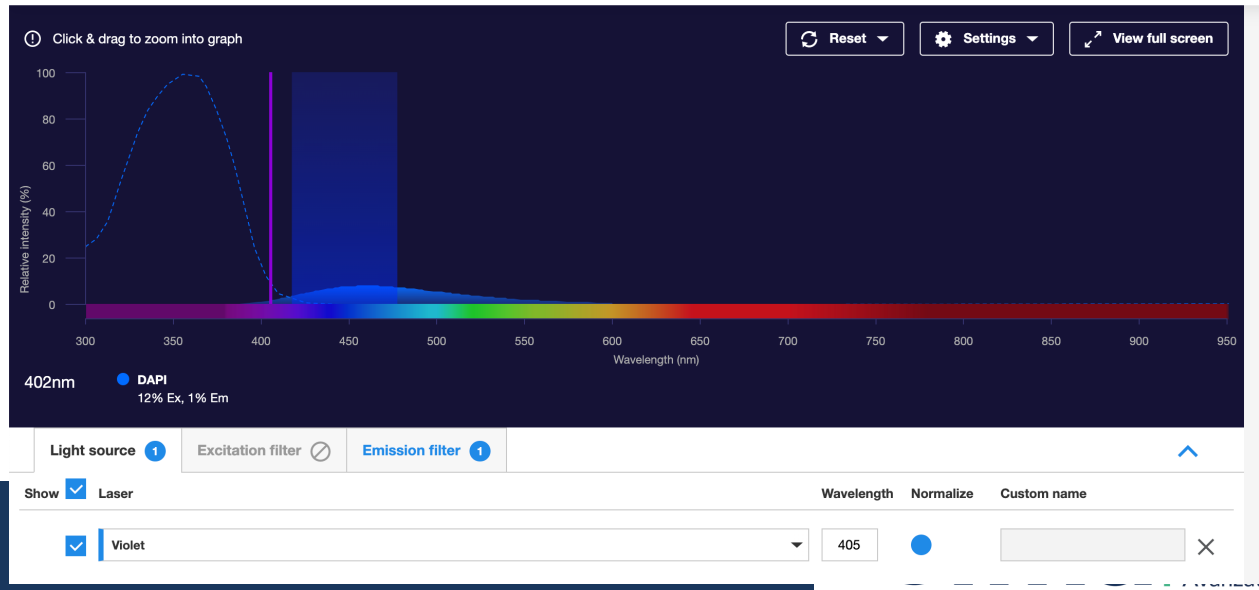


- Direct coupling to macromolecules
- Fluorescent dyes and substrates
- Fluorescent fusion proteins
- Fluorescent Antibodies

FILTER COMBINATIONS



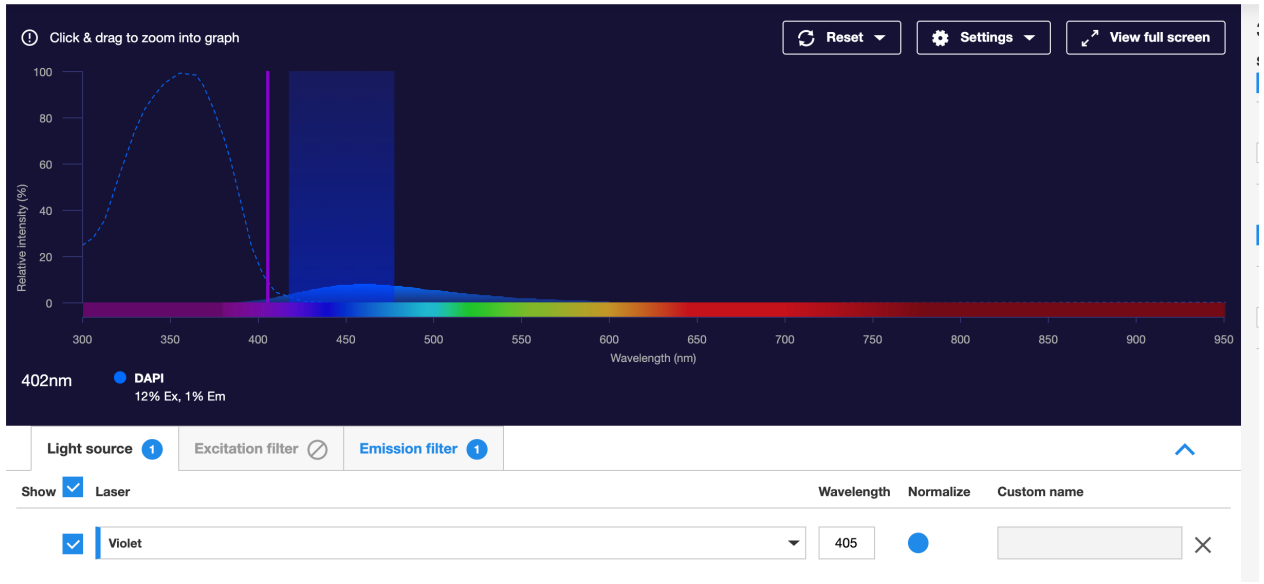


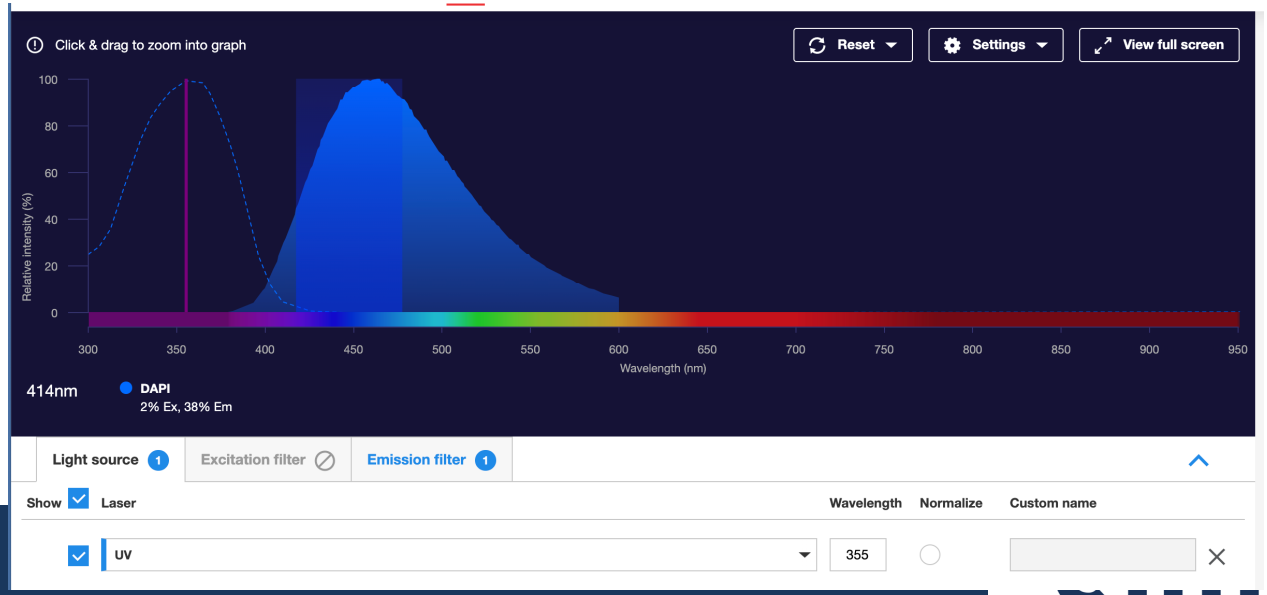


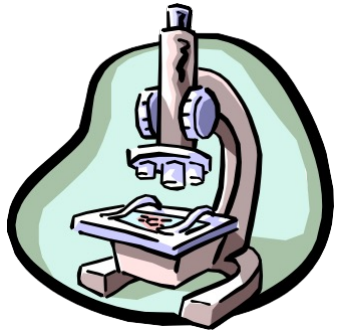
https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of light source!!

FILTER COMBINATIONS







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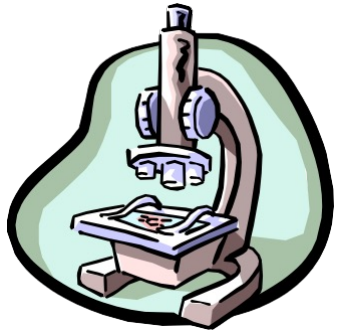
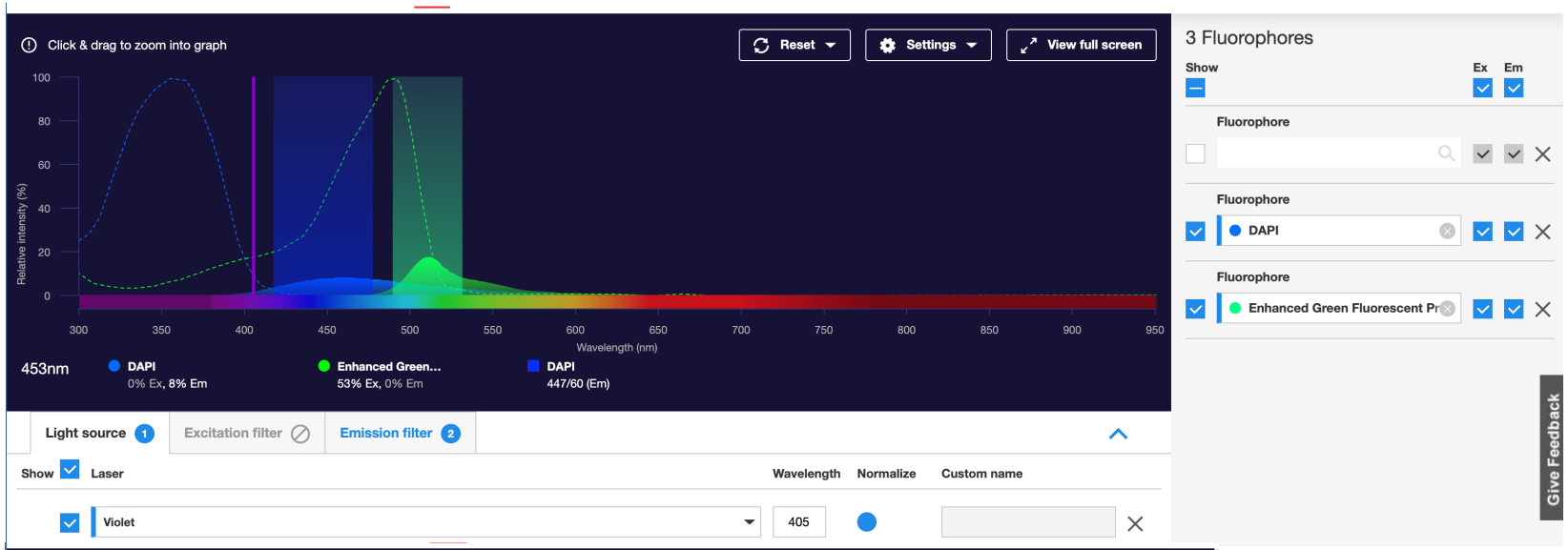
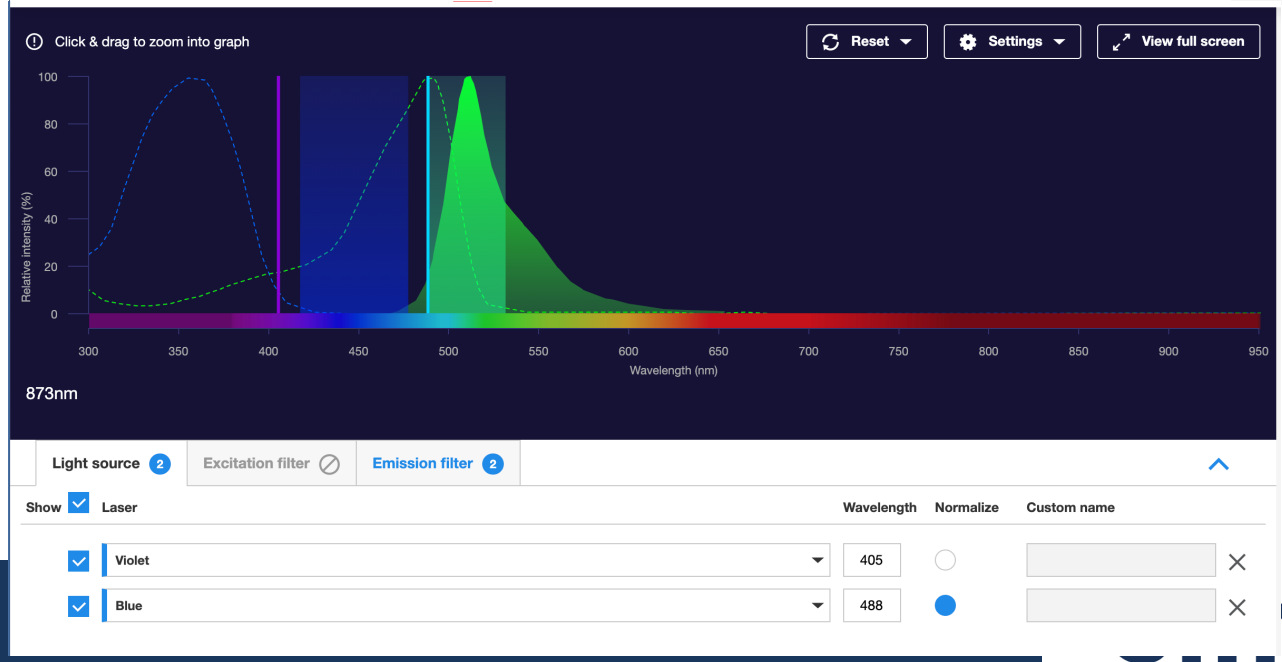
Beware of light source!!

FILTER COMBINATIONS







https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of light source!!

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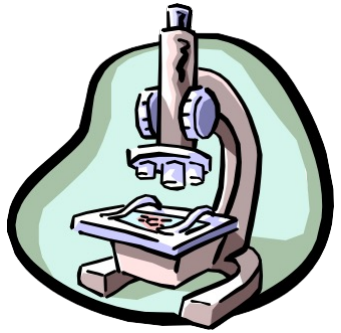
FILTER COMBINATIONS



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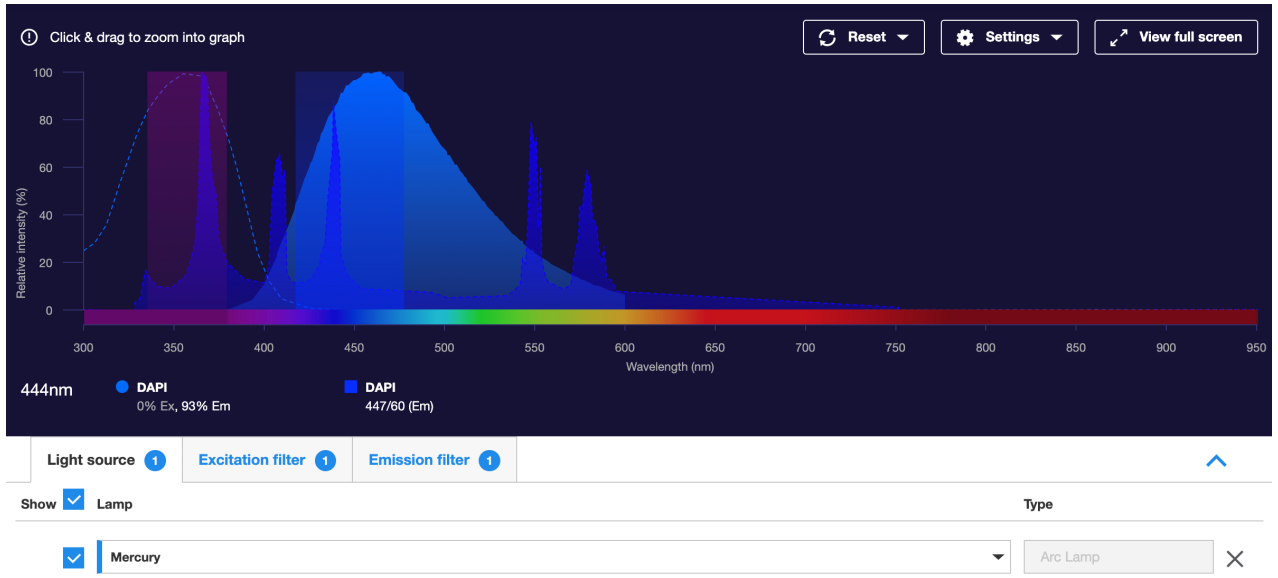
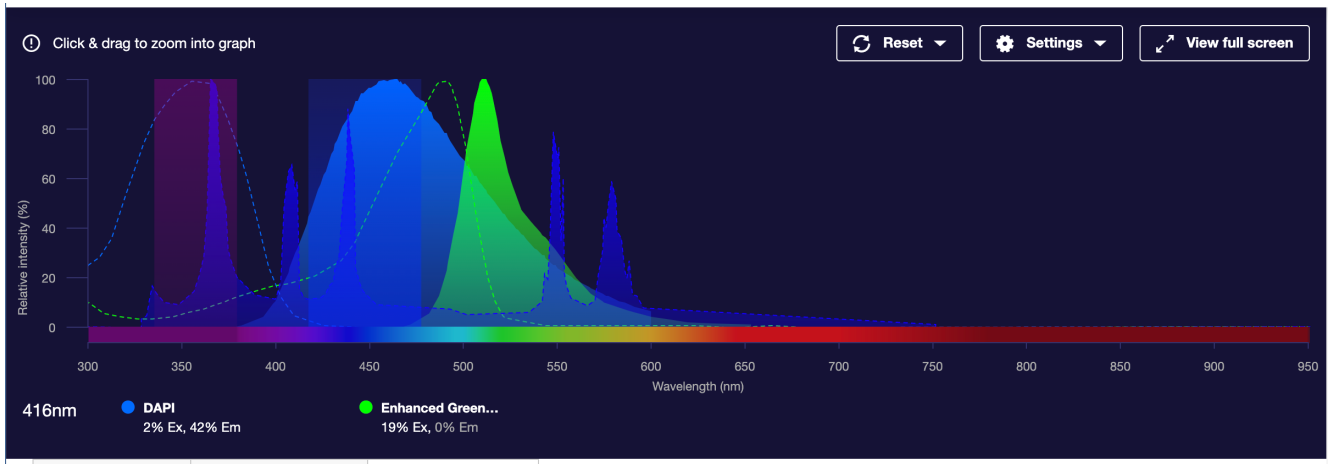
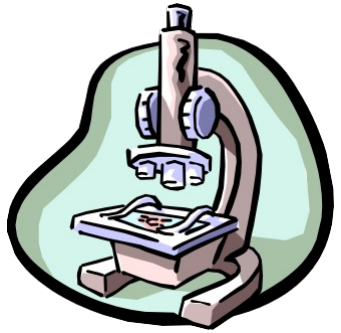
Beware of light source!!

FILTER COMBINATIONS



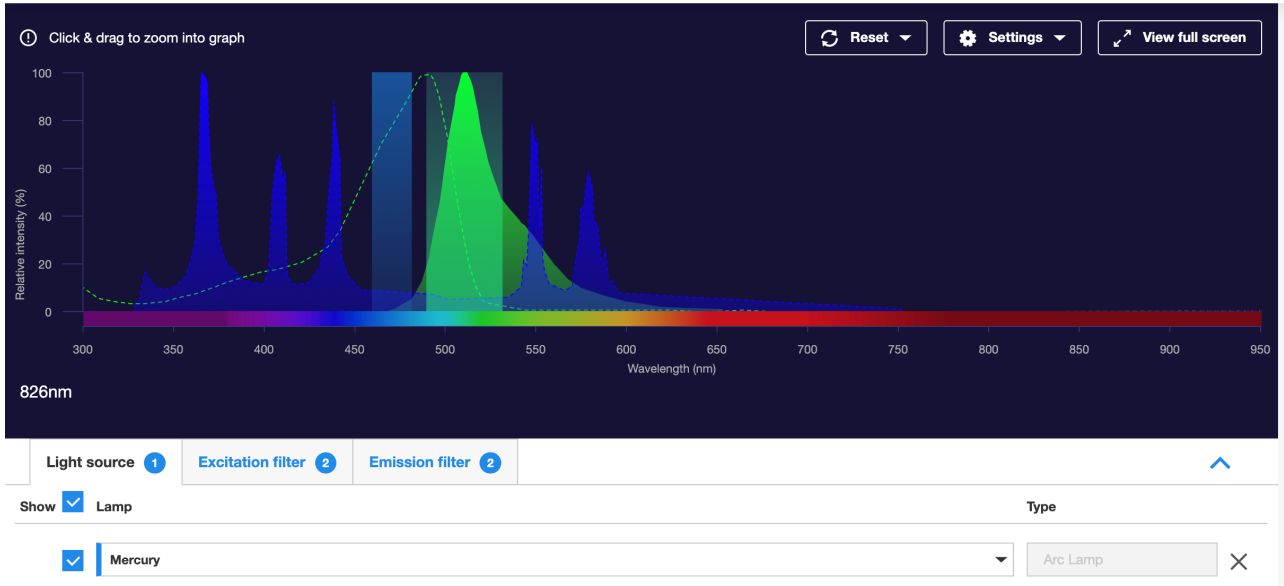


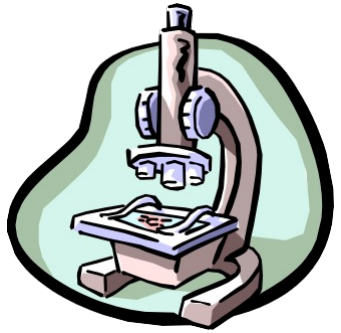


https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Check for crosstalk!!

FILTER COMBINATIONS

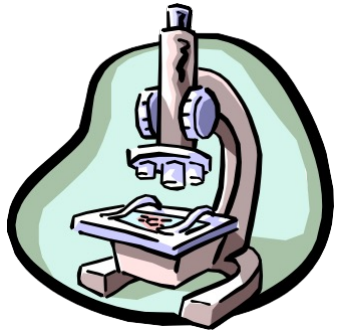


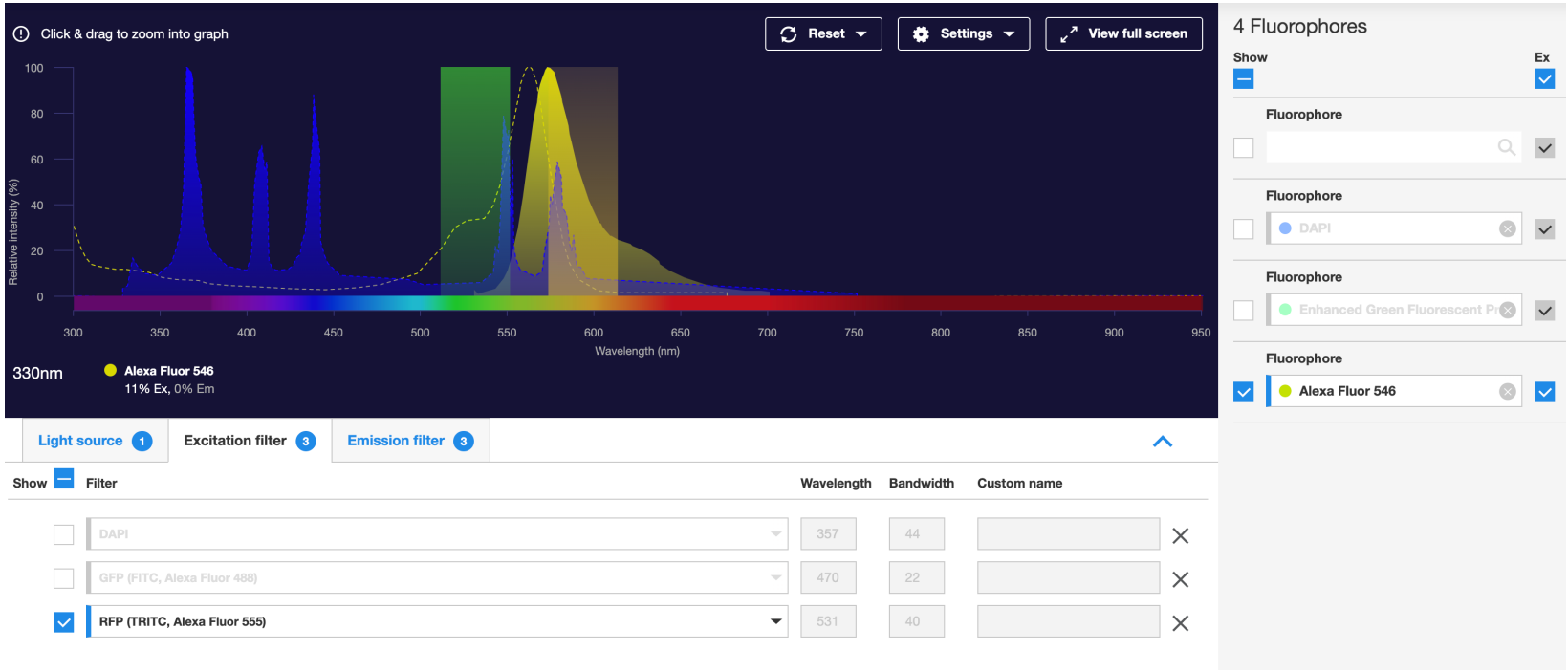


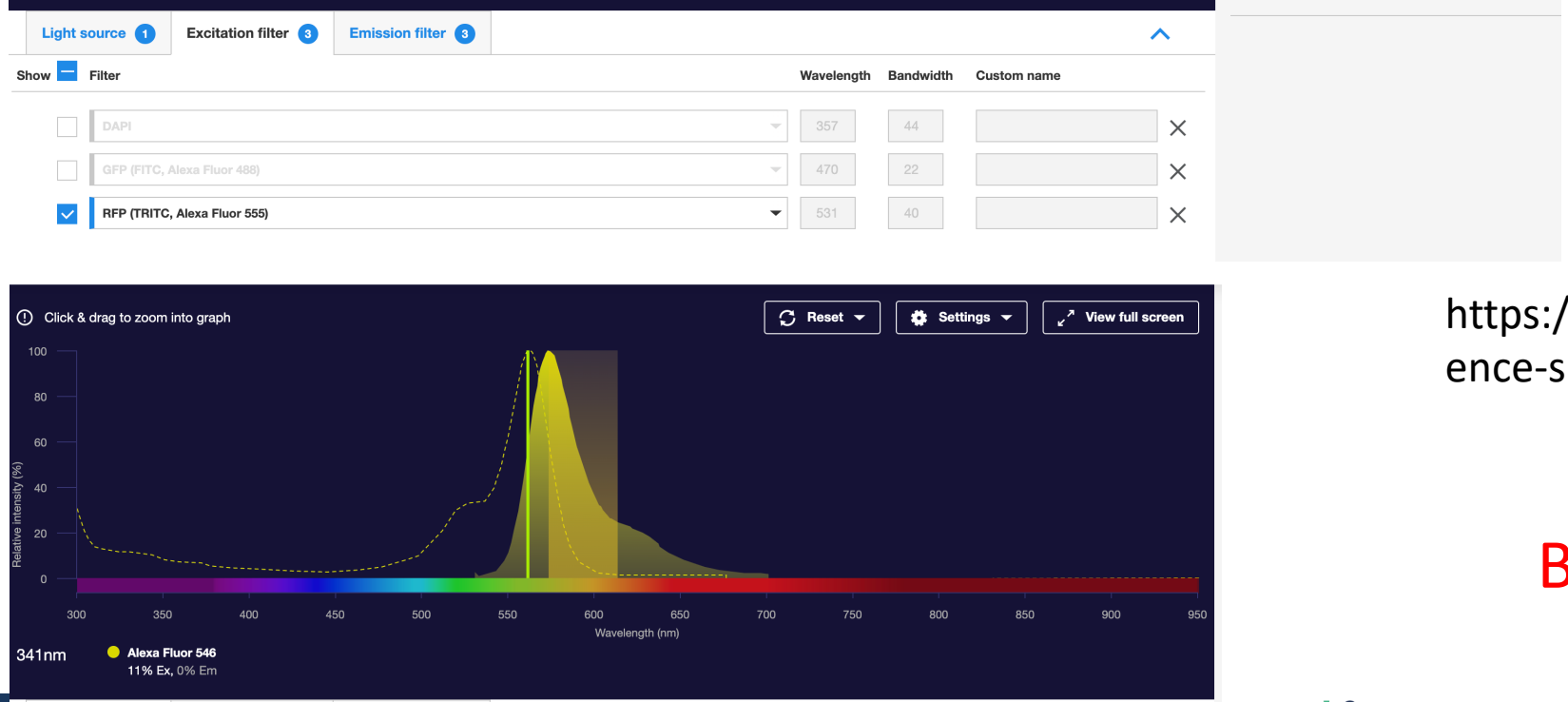
https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Check for cross-talk!!

FILTER COMBINATIONS







https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of filter!!

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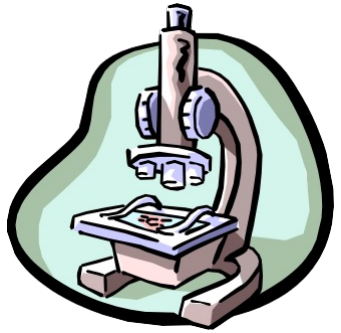
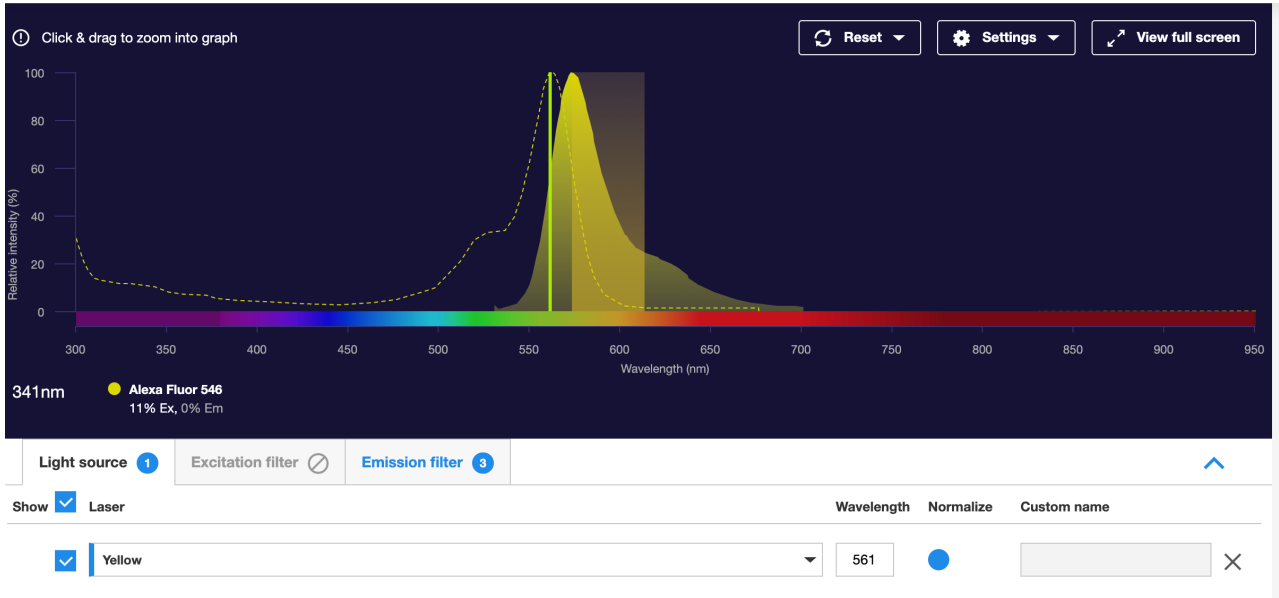
FILTER COMBINATIONS

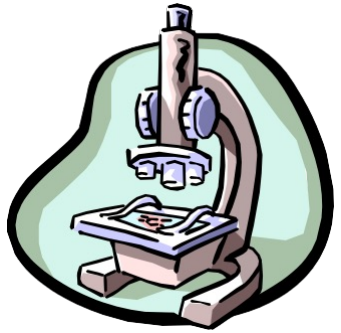


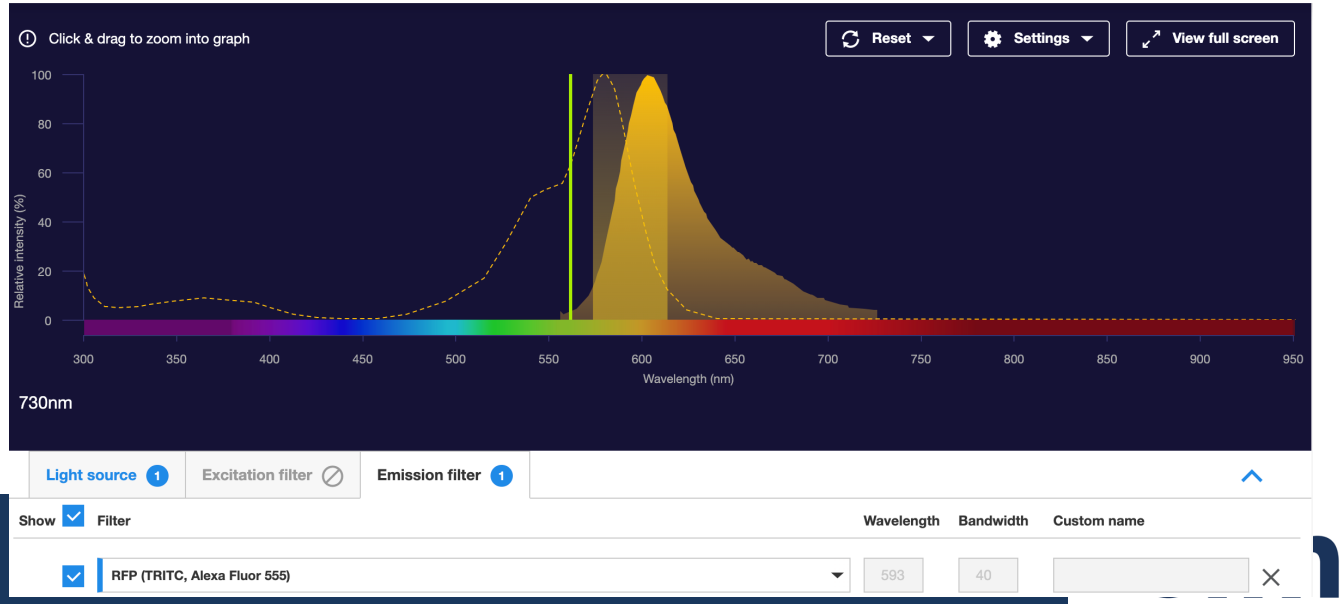


https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of crosstalk!!

FILTER COMBINATIONS

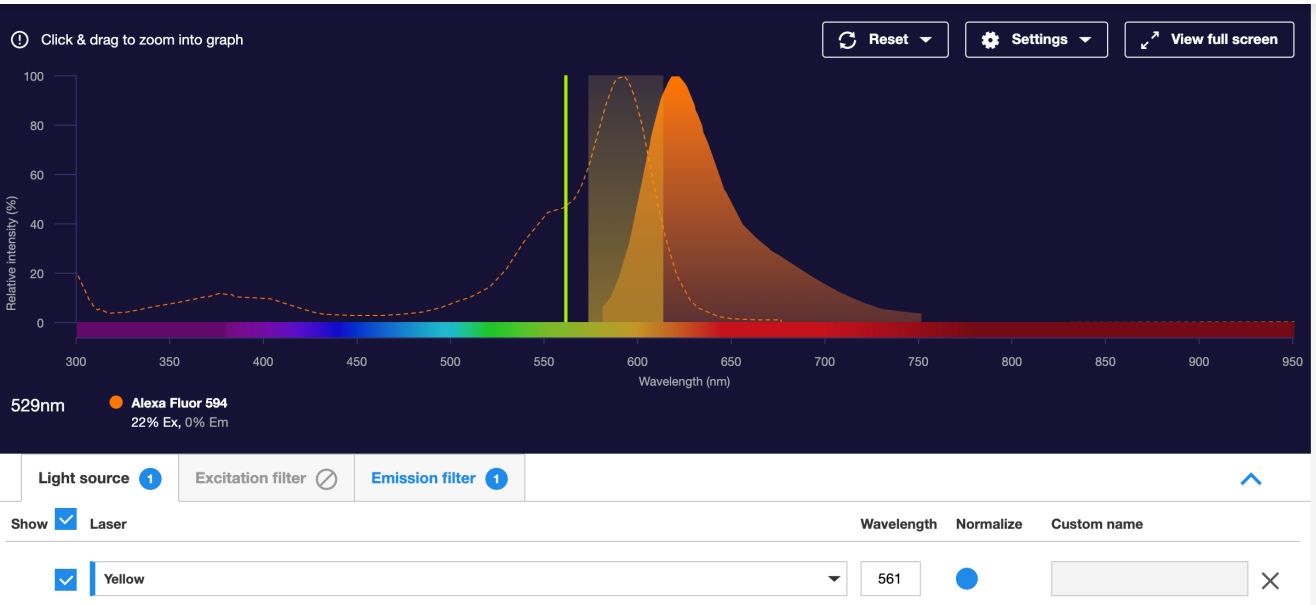


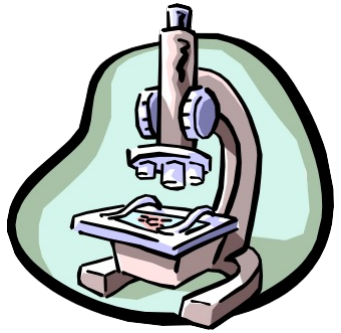


https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of fluorophore!!

FILTER COMBINATIONS





https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of fluorophore!!

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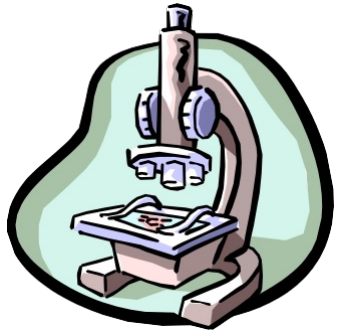
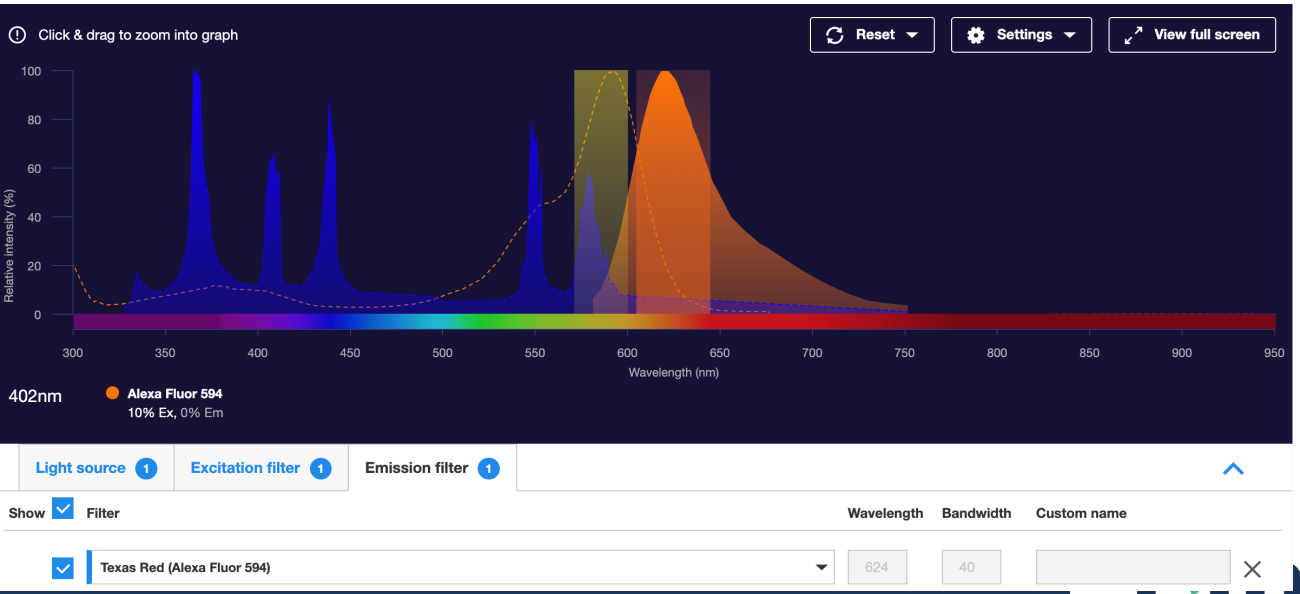
FILTER COMBINATIONS

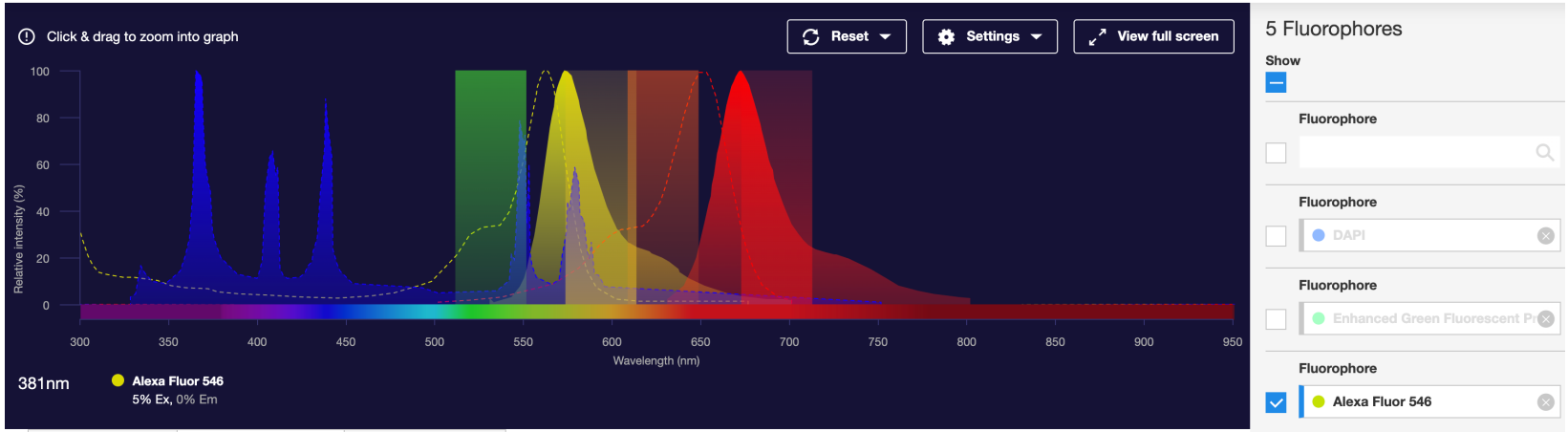




https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

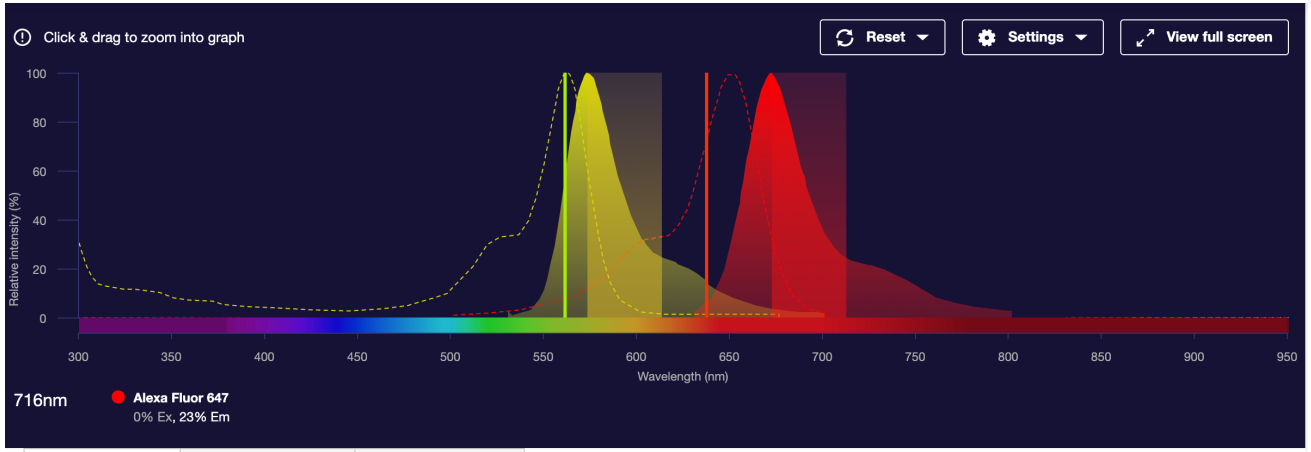
Beware of filter cube!!

FILTER COMBINATIONS



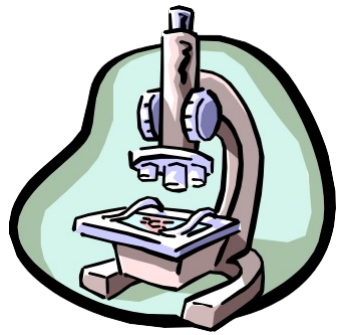
Light source 1 | Excitation filter 2 | Emission filter 2

Show	Filter	Wavelength	Bandwidth	Custom name
☑	RFP (TRITC, Alexa Fluor 555)	531	40	
☑	Cy5 (Alexa Fluor 647)	628	40	



Light source 2 | Excitation filter | Emission filter 2

Show	Laser	Wavelength	Normalize	Custom name
☑	Yellow	561	●	
☑	Red	637	○	



https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Farred is not visible to eye!!

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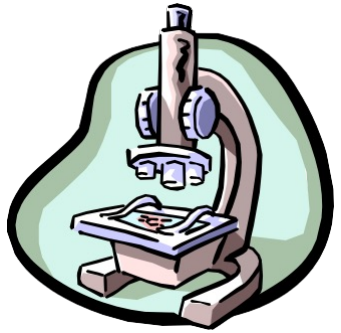
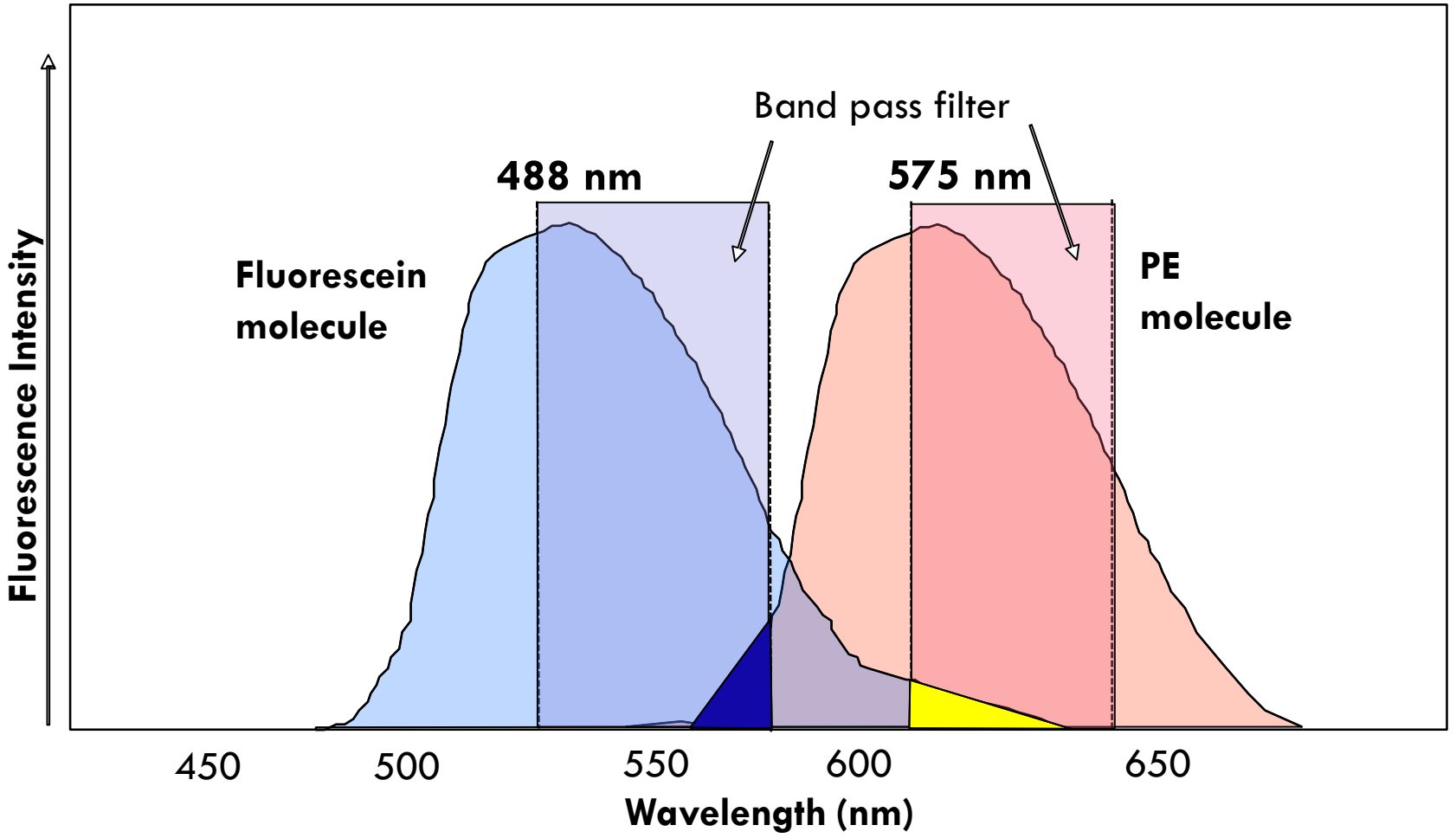
FLUORESCENCE OVERLAP

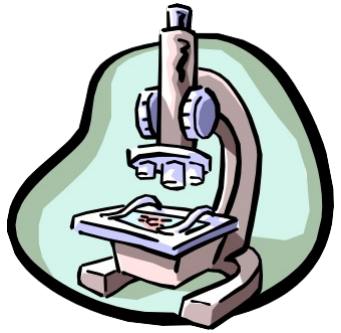




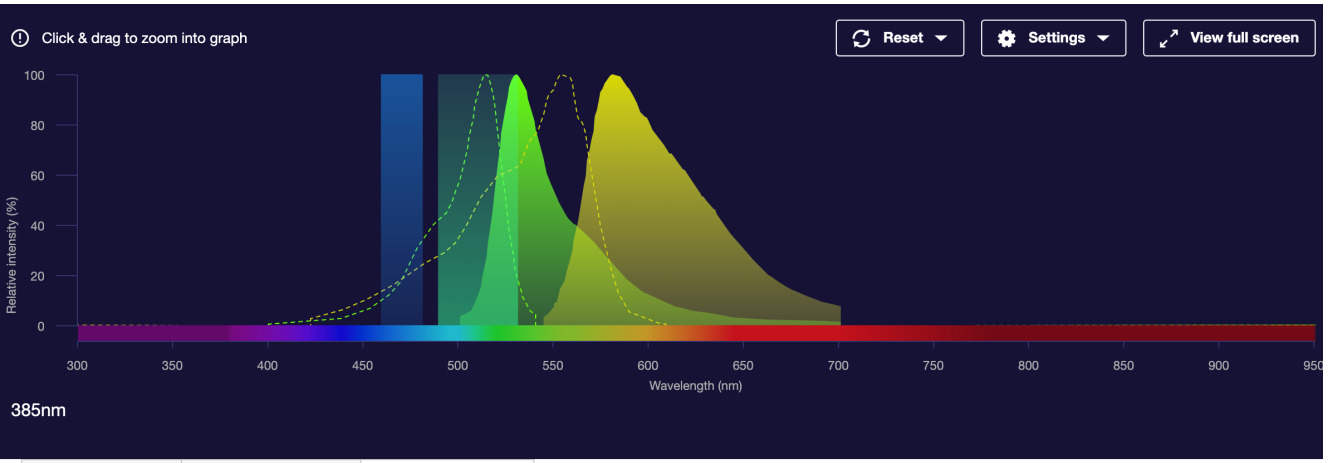
Overlap of FITC fluorescence in PE PMT

Overlap of PE fluorescence in FITC PMT

FLUORESCENCE OVERLAP

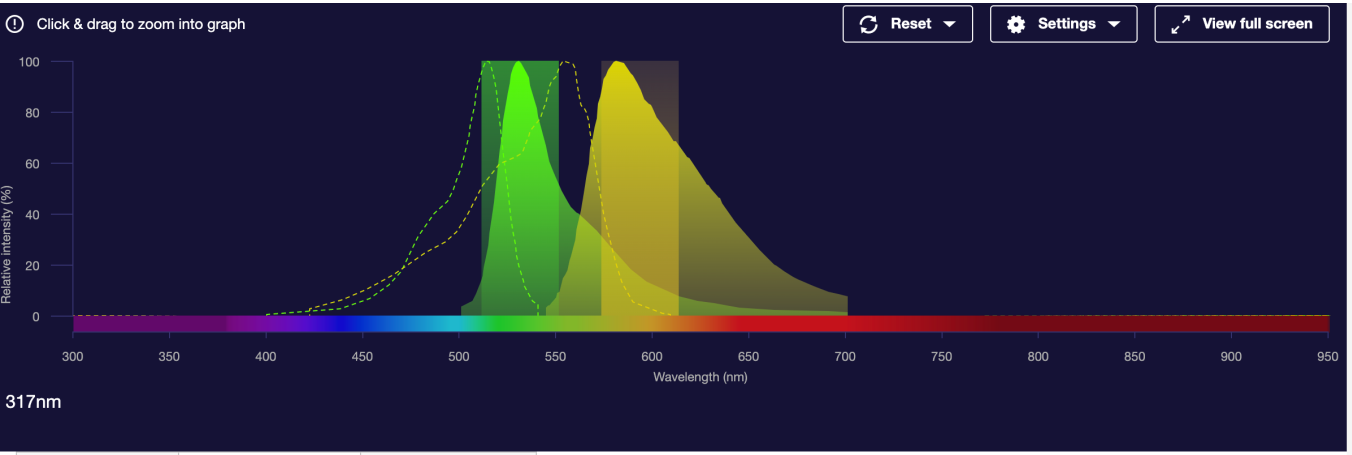


FLUORESCENCE OVERLAP



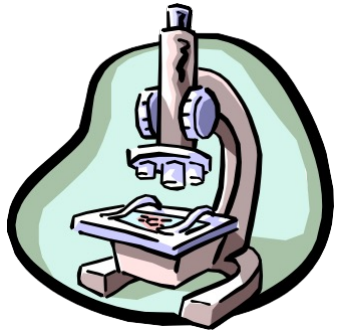
Light source 1 | Excitation filter 2 | Emission filter 2

Show	Filter	Wavelength	Bandwidth	Custom name
☑	GFP (FITC, Alexa Fluor 488)	510	42	



Light source 1 | Excitation filter 2 | Emission filter 2

Show	Filter	Wavelength	Bandwidth	Custom name
☐	GFP (FITC, Alexa Fluor 488)	470	22	
☑	RFP (TRITC, Alexa Fluor 555)	531	40	



Overlap!!

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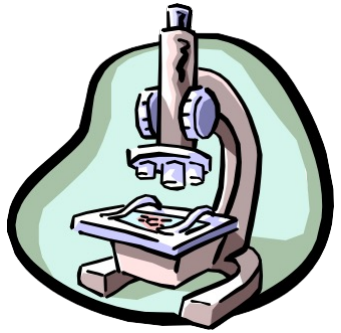

Advanced Microscopy Techniques

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5. Lesson 4 – Confocal, super-resolution and 2-photon microscopy

5.1. TIRF microscopy

5.2. Confocal microscopy

5.3. 2-photon microscopy

5.4. Superresolution microscopy

- 5.4.1. SIM microscopy
- 5.4.2. STED microscopy
- 5.4.3. PALM microscopy
- 5.4.4. STORM microscopy

5.5. FRET microscopy

5.6. FRAP microscopy

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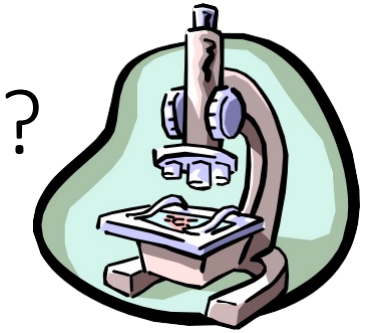
lab3

WHICH IMAGING TECHNIQUE SHOULD I USE?

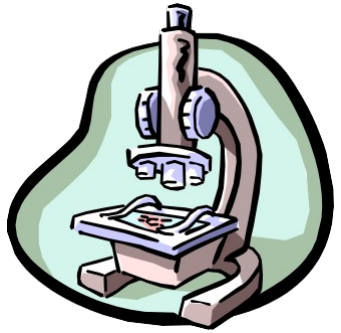


Sample Thickness

1-5 μm	TIRF (for samples at the coverslip)	Fast
1-20 μm	Wide-field (+deconvolution)	Fast
	Spinning Disk Confocal	Fast
10-100 μm	Line-scanning confocal	Fast
>20 μm	Point scanning Confocal	Slow
>50-100 μm	2-photon confocal	Slow

Sensitivity

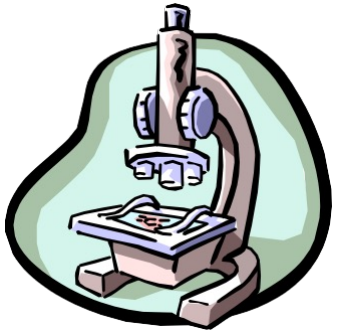
NEAR FIELD IMAGING (NSOM)

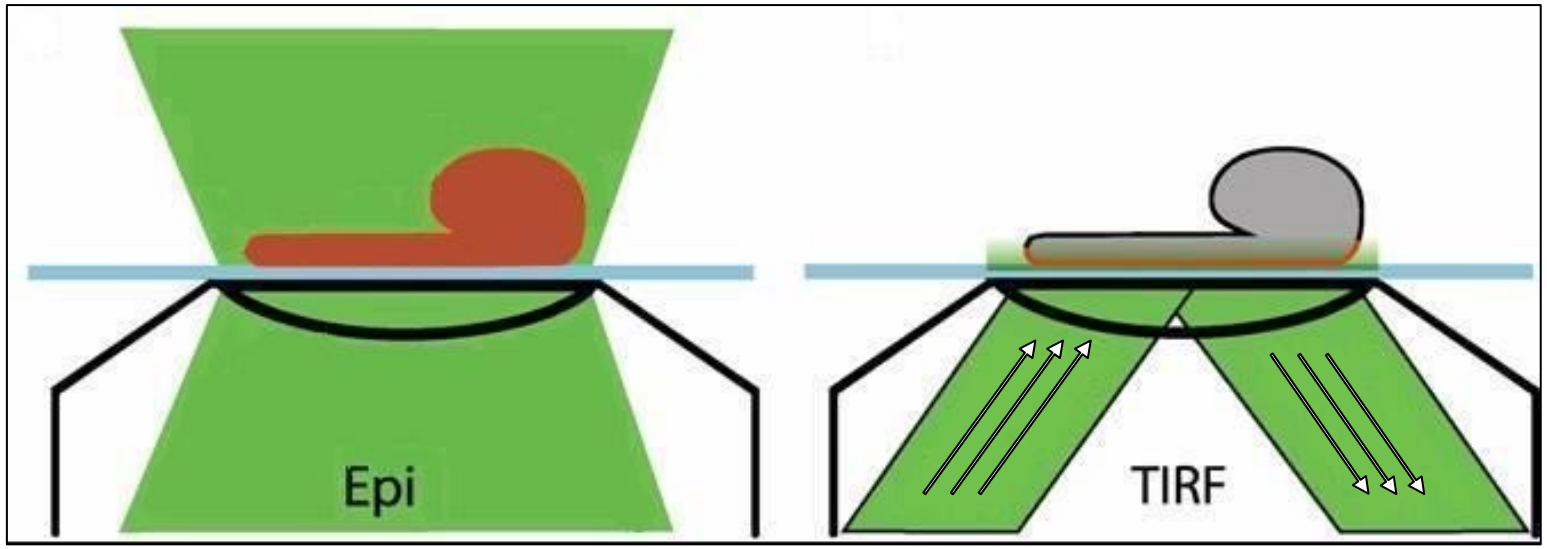


-place microscope distance less than 1 wavelength from sample

-20-50 nm resolution

problem: cannot image into sample because of wavelength restriction

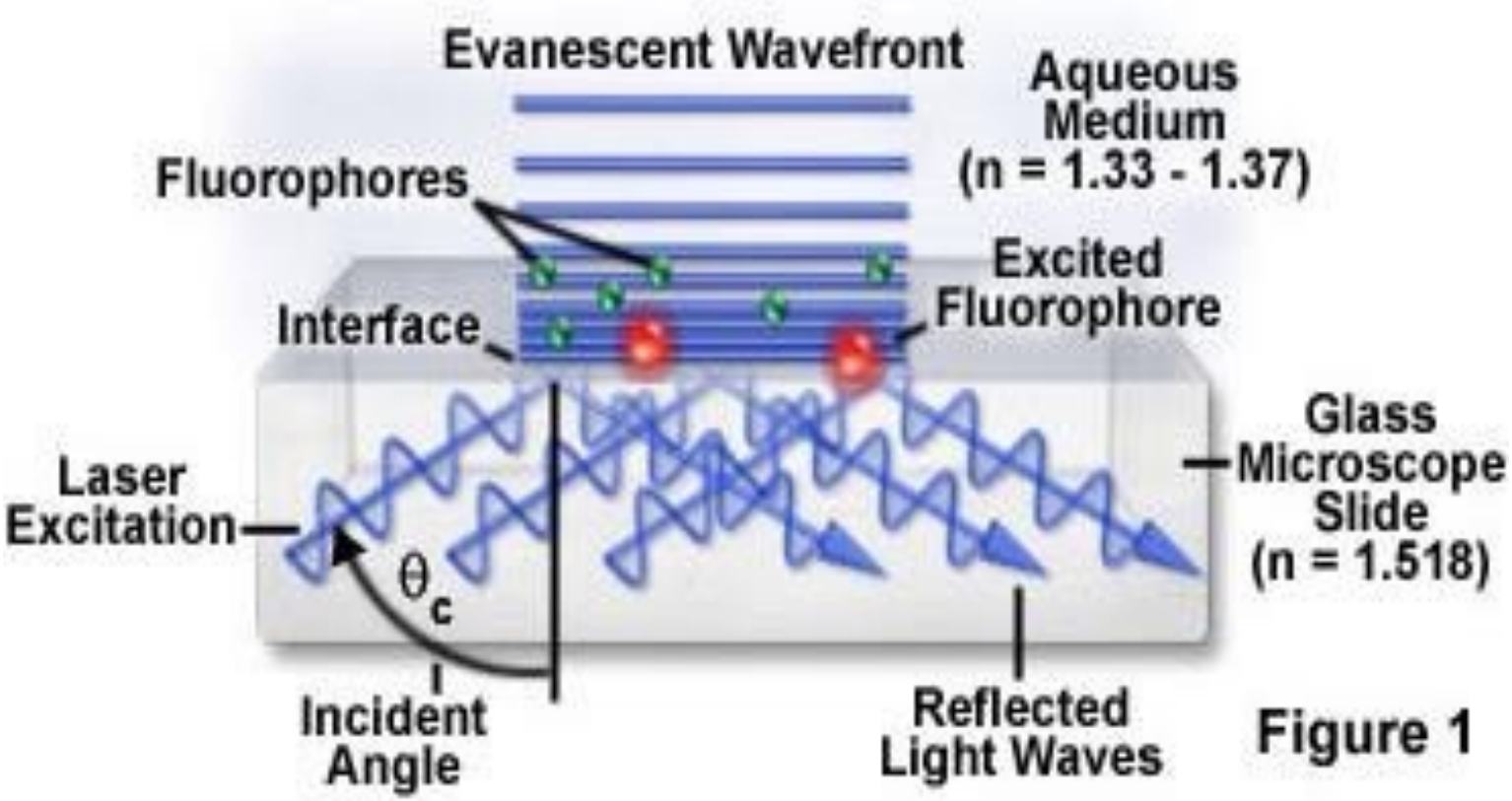


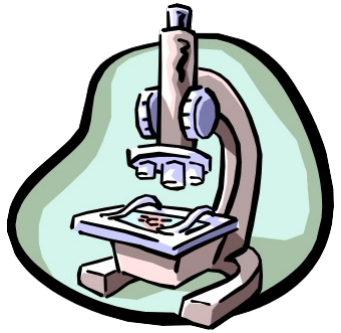


You need:

- TIRF objectives with high NA
- TIRF condensor, where you are able to change the angle of illumination
- Glass coverslips

TIRF



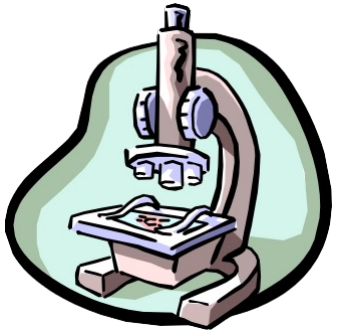


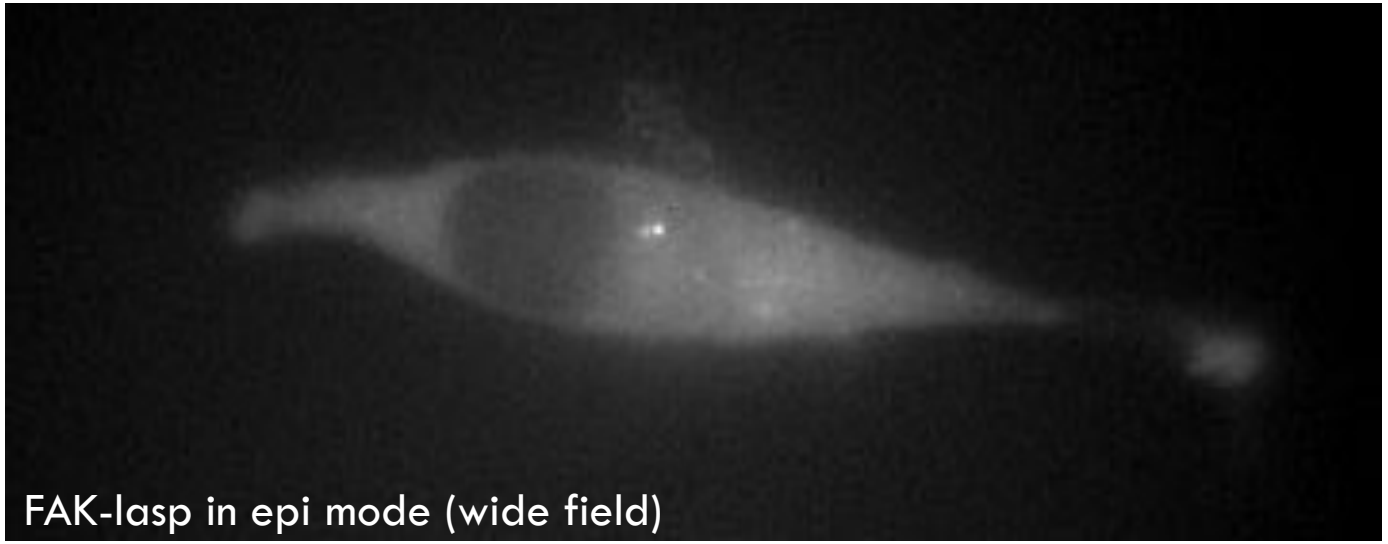
micro.magnet.fsu.edu

Result: very thin section at the bottom of the sample 150-200nm

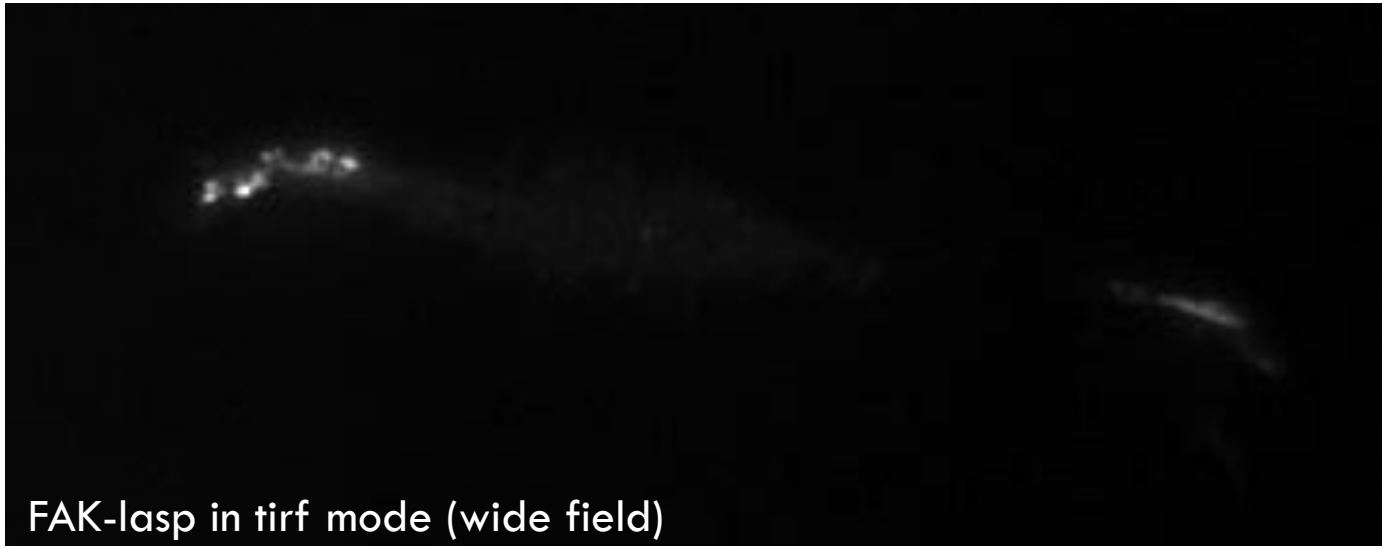
Use: to study membrane dynamics (endocytosis, focal adhesions, receptor binding) *Nikon TE 2000*

TIRF vs EPI





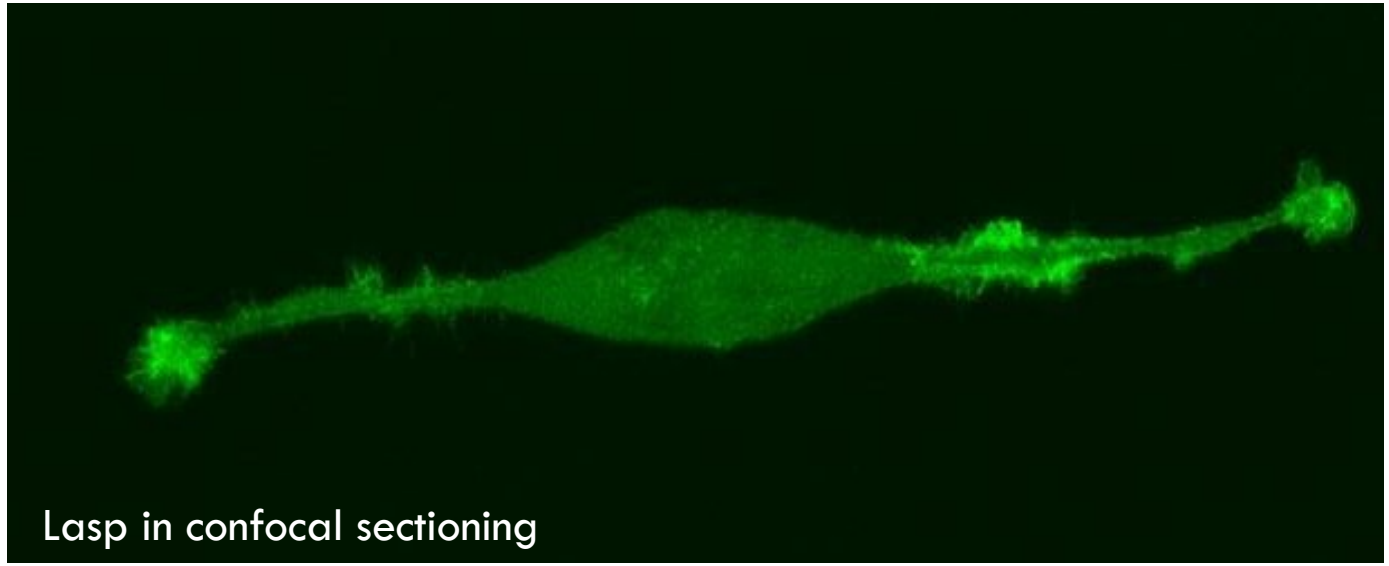
FAK-lasp in epi mode (wide field)

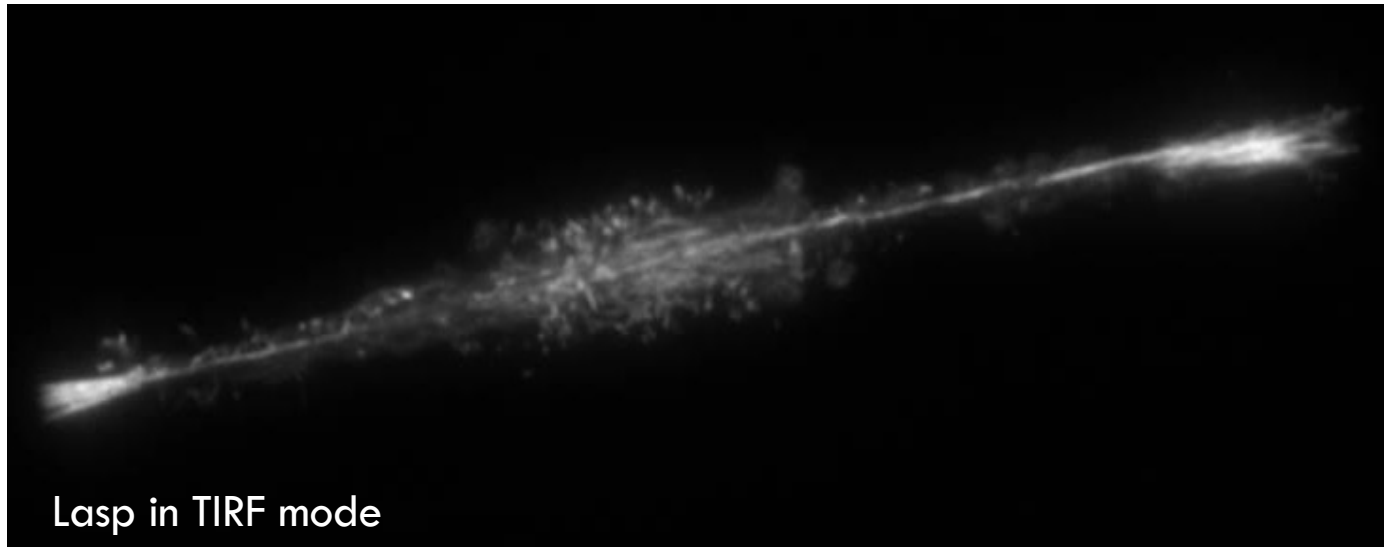


FAK-lasp in tirf mode (wide field)

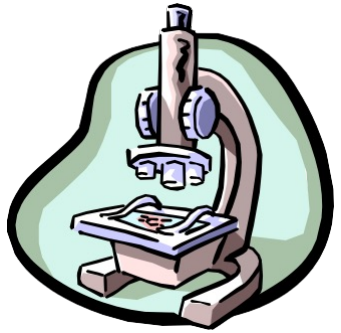
Heather Spence, R10

TIRF vs EPI

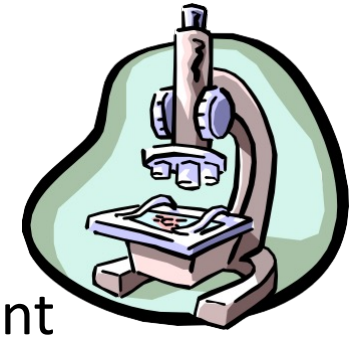




Heather Spence, R10



PRINCIPLE OF CONFOCAL MICROSCOPY



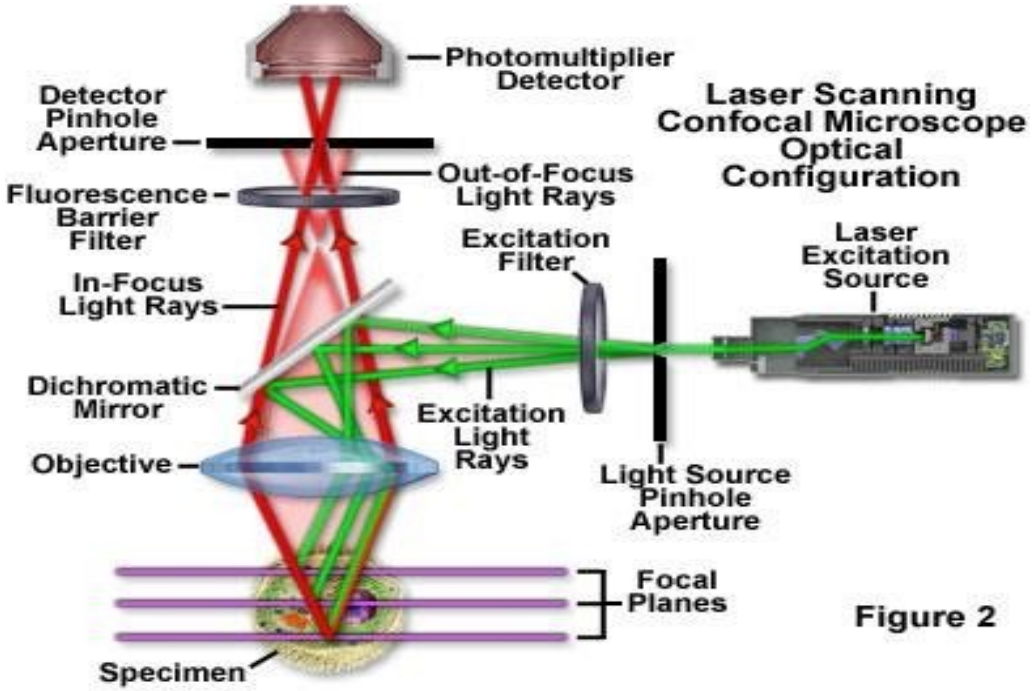
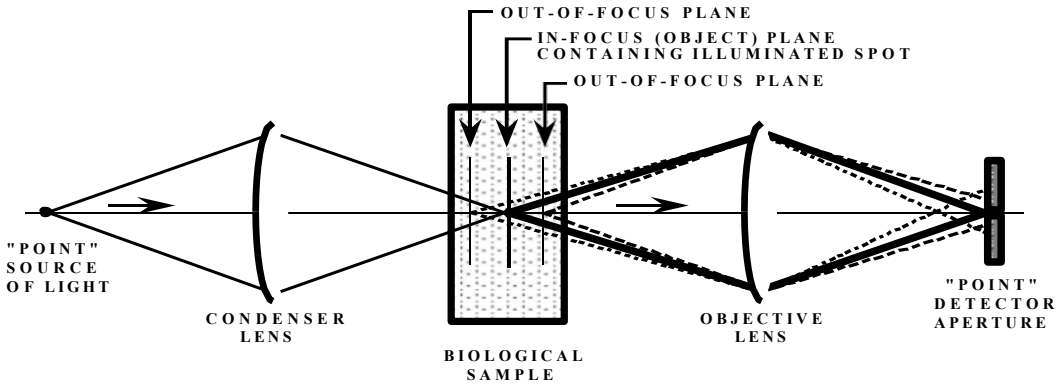


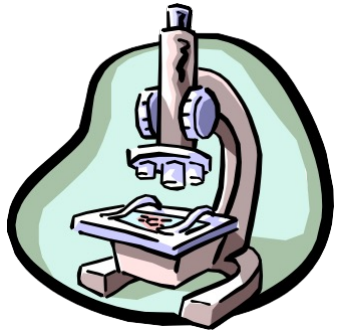
Figure 2



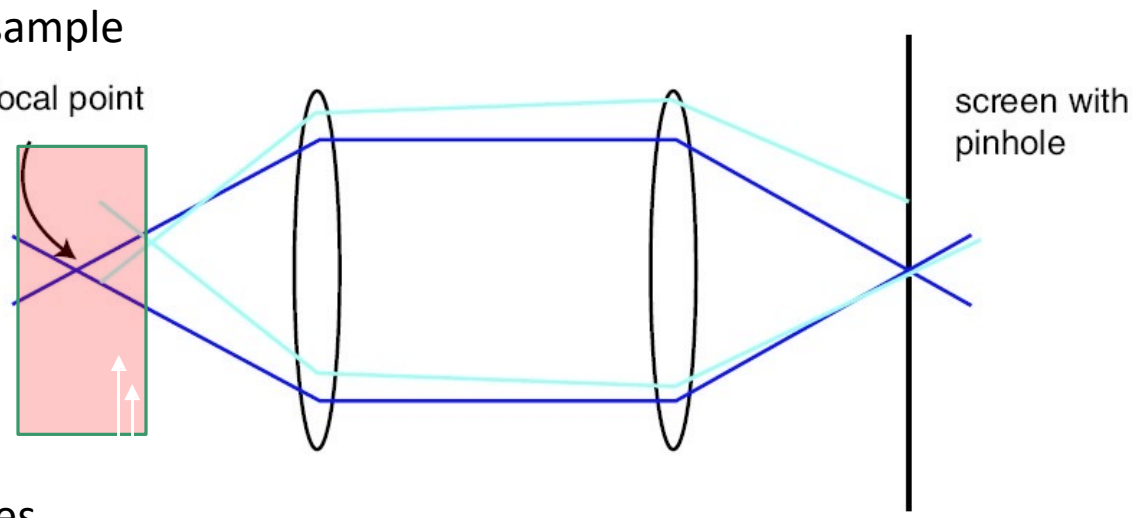
two pinholes are used:

- One pinhole is placed in front of the illumination source to allow transmission only through a small area
- only a point of the specimen is illuminated at one time
- Fluorescence excited at the focal plane is imaged onto a confocal pinhole placed right in front of the detector
- Only fluorescence excited within the focal plane of the specimen will go through the detector pinhole
- Need to scan point onto the sample

CONFOCAL DETECTION



Sample 3D -> Detector 2D
A pinhole allows only in-focus light through



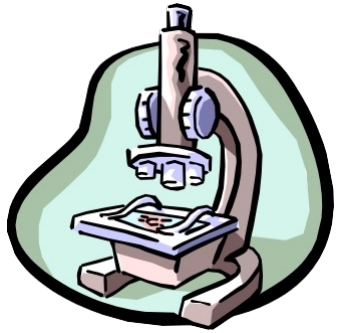
Focused Light creates fluorescence which gets to detector

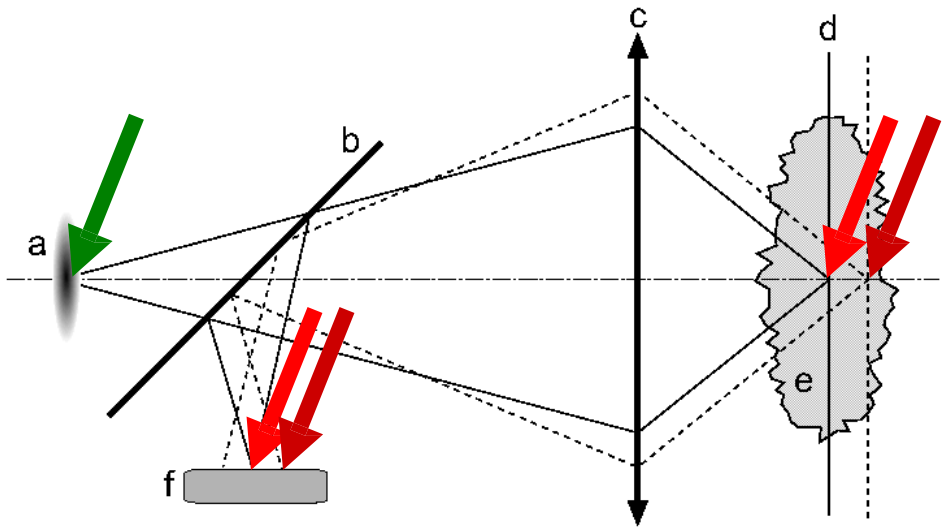
Light mostly gets rejected

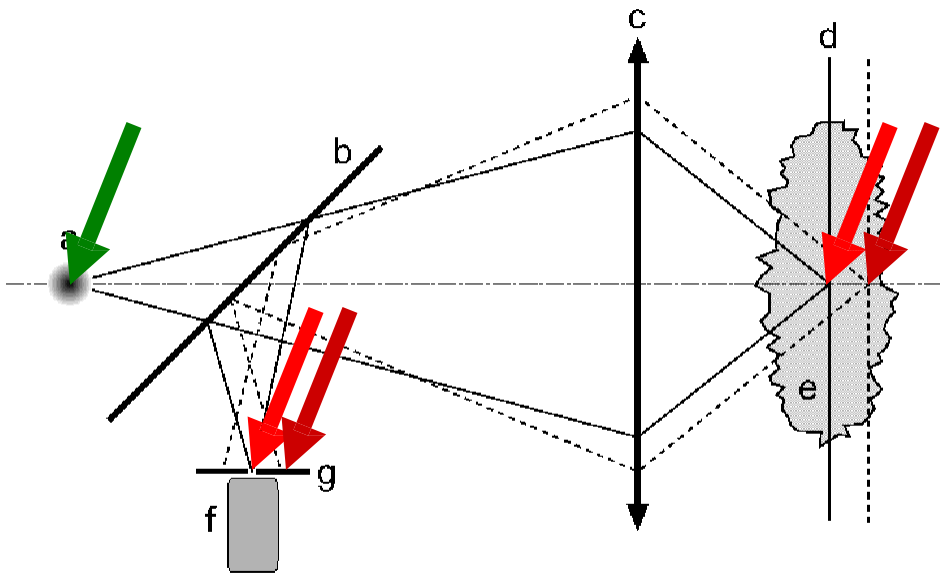
Smaller the pinhole, better out-of-focus discrimination but lose more signal.

Scan sample in x, y, z and reconstruct entire image

WIDE-FIELD vs CONFOCAL MICROSCOPY







a – light source

b – dichroic filter

c – objective lens

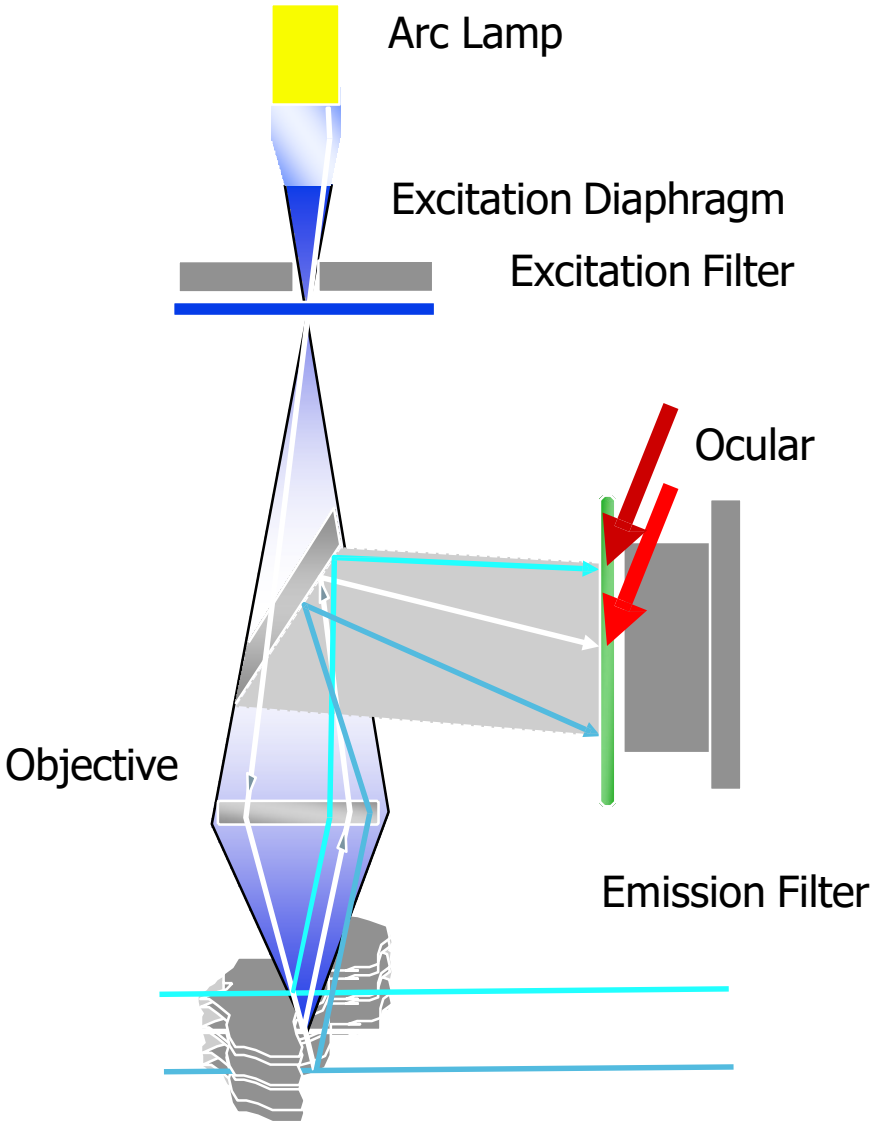
d – focal plane

e – specimen

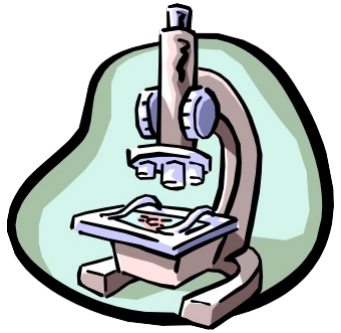
f – light detector

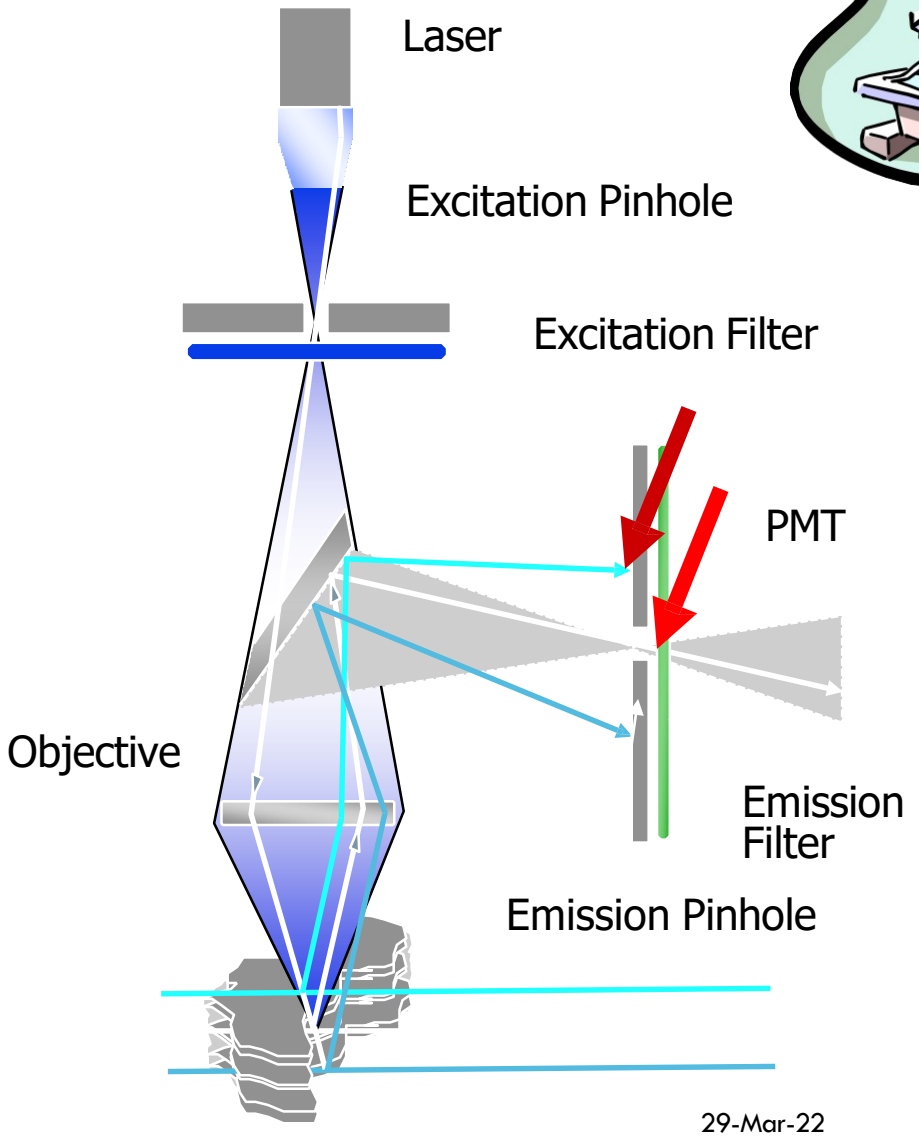
g – confocal aperture

Fluorescence Microscope

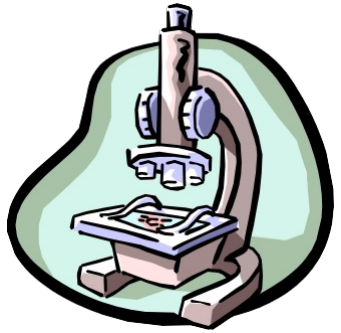


Confocal Fluorescence Microscope



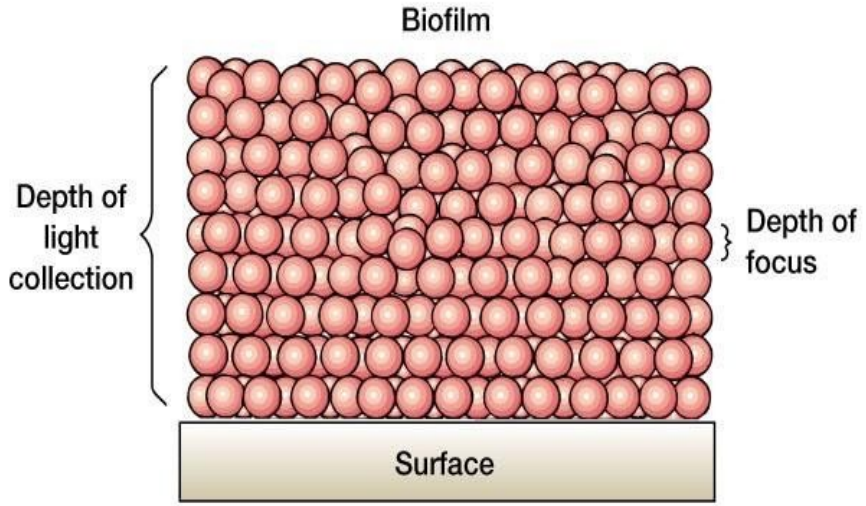


WHEN TO USE CONFOCAL?



- Confocal is not a magic bullet
  - It is extremely wasteful of photons
  - Laser-scanning confocal is 100 – 200-fold less sensitive than widefield

Conventional light microscope



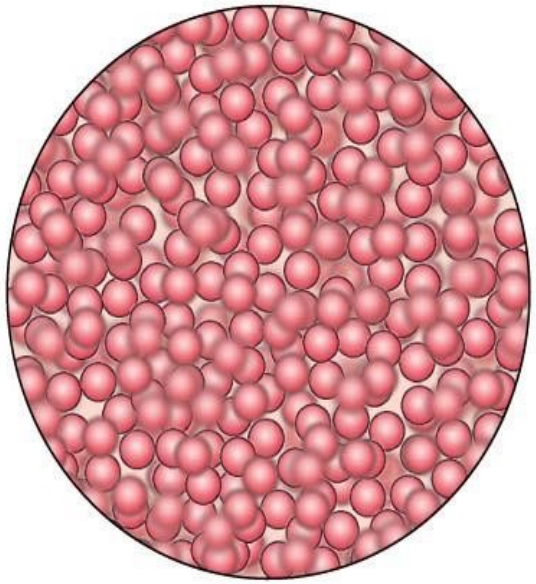
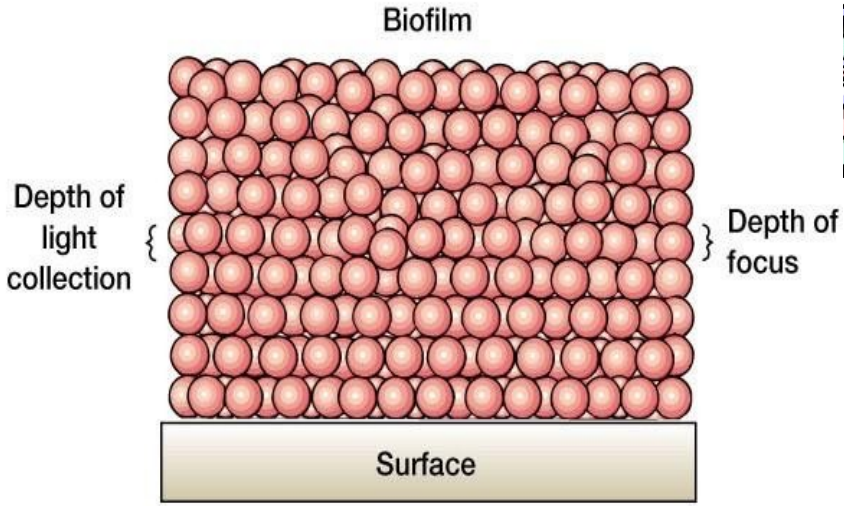


Image in field of view

(a)

Confocal scanning laser microscope



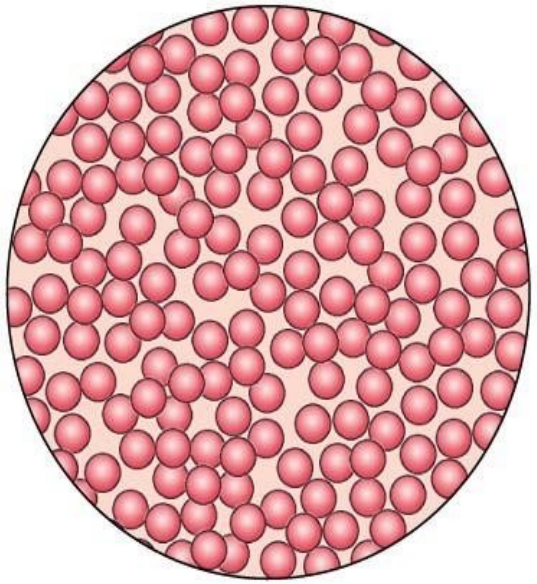
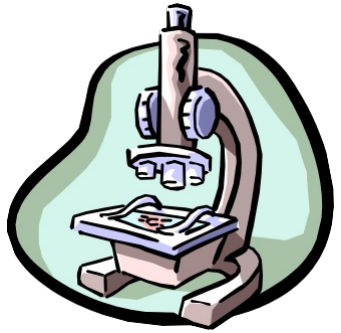


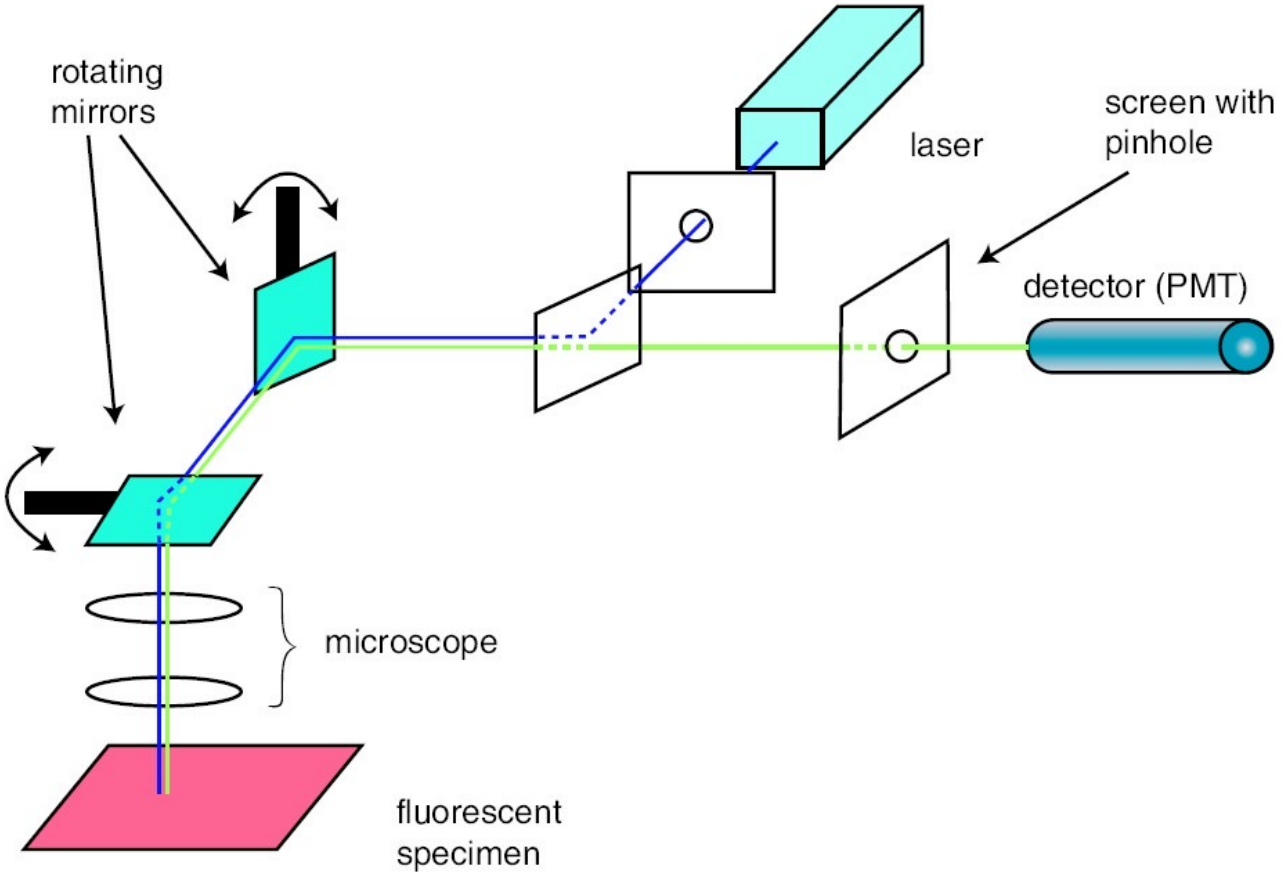
Image in field of view

(b)

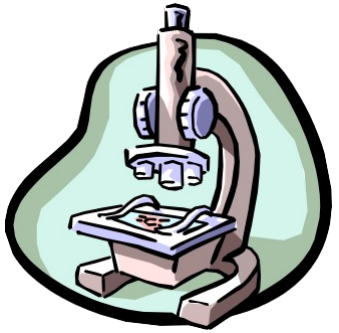
CONFOCAL MICROSCOPY

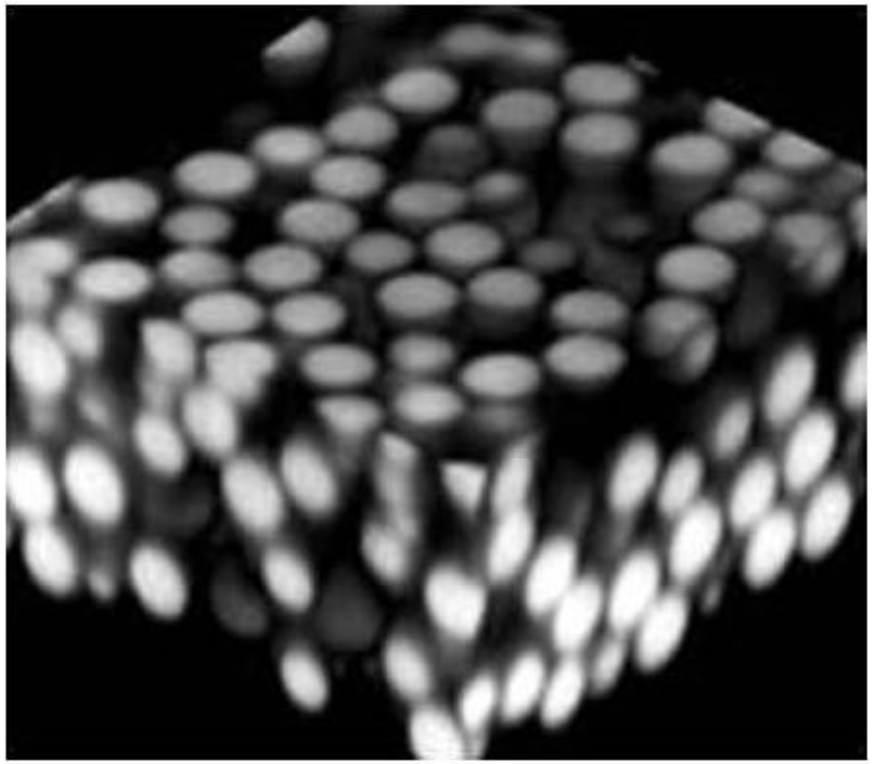
Fast imaging via moveable mirrors



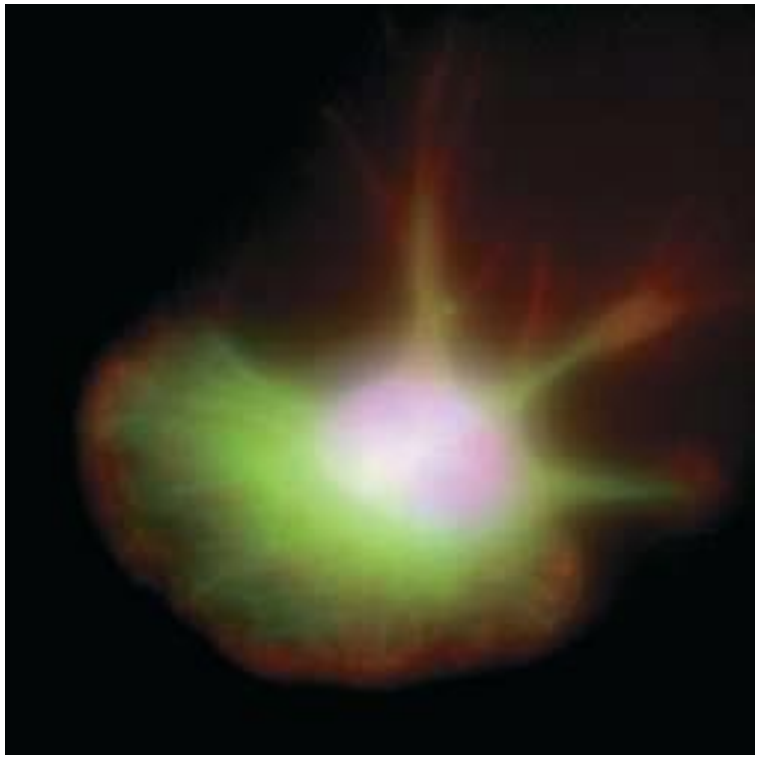


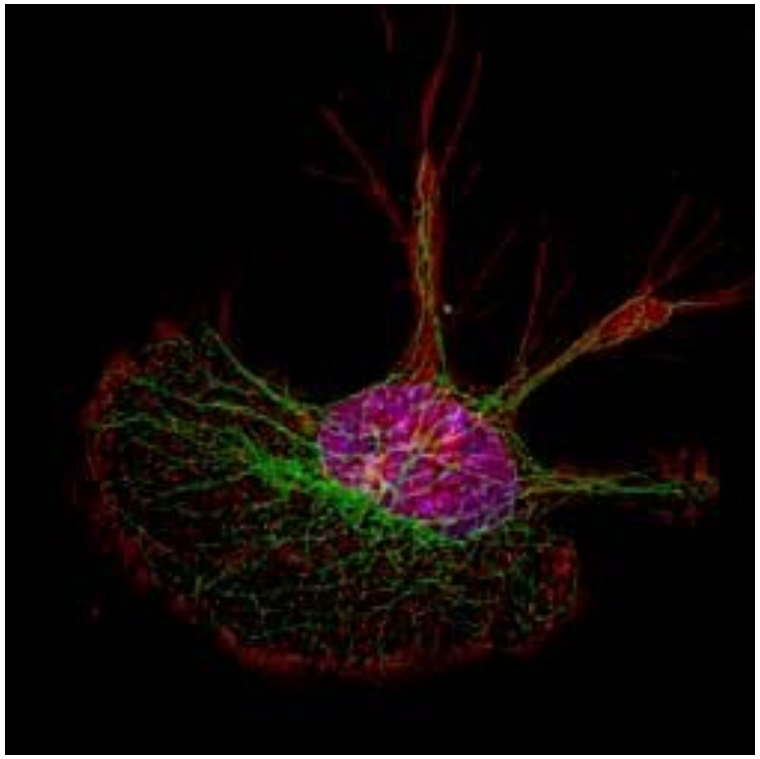
3-D SECTIONING WITH CONFOCAL

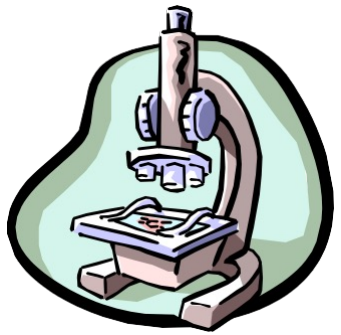




3D-dimensional reconstruction of a series of 2D images of PMMA spheres

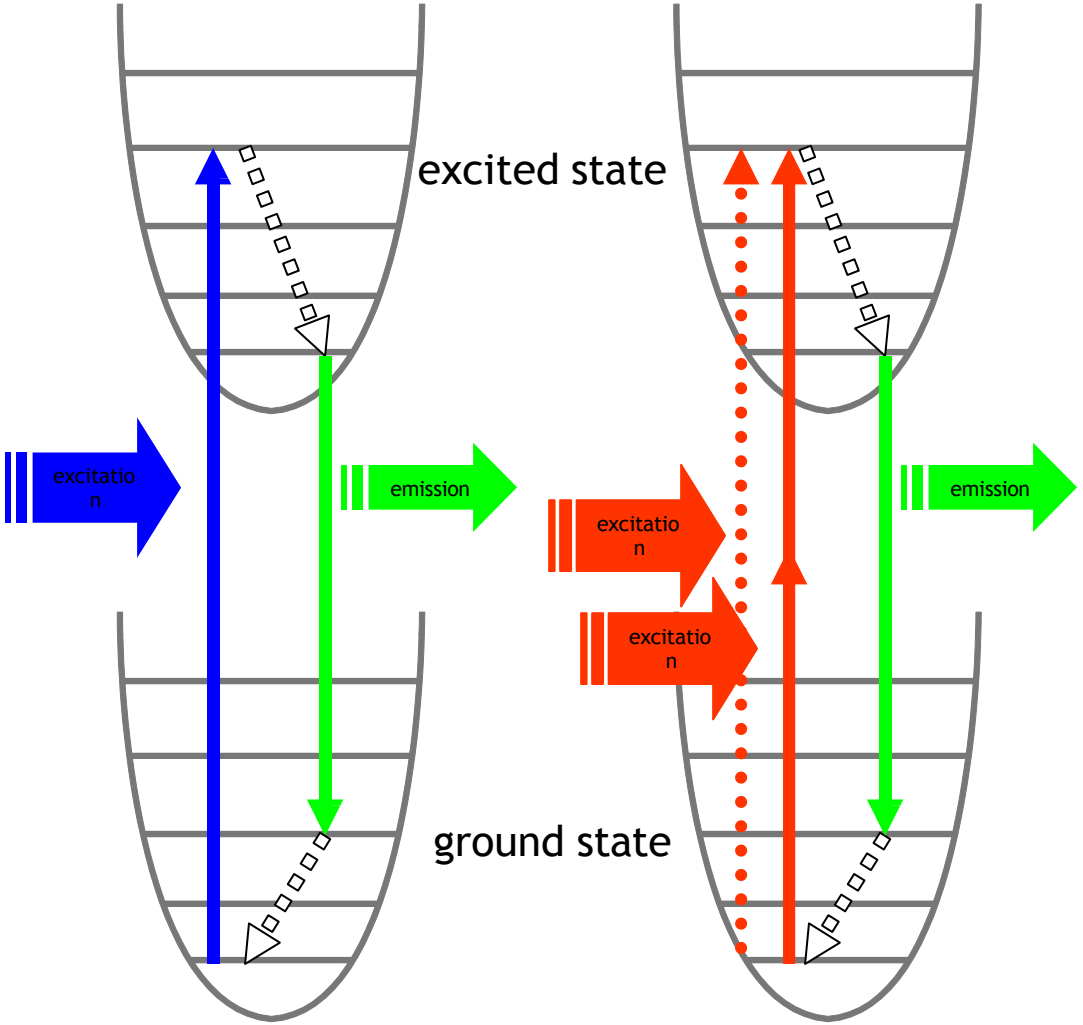


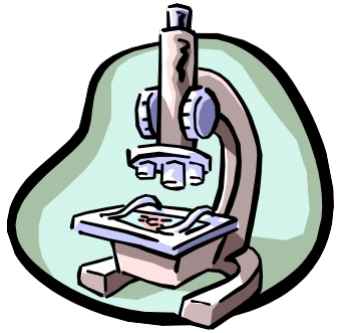




A macrophage cell was stained with fluorochrome-labeled reagents specific for DNA (blue), microtubules (green), and actin microfilaments (red). The series of fluorescent images obtained at consecutive focal planes (optical sections) through the cell were recombined in three dimensions.
(a) In this three-dimensional reconstruction of the raw images, the DNA, microtubules, and actin appear as diffuse zones in the cell. (b) After application of the deconvolution algorithm to the
images, the fibrillar organization of microtubules and the localization of actin to adhesions become readily visible in the reconstruction.

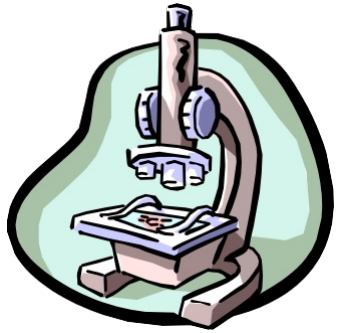
2-PHOTON EXCITATION

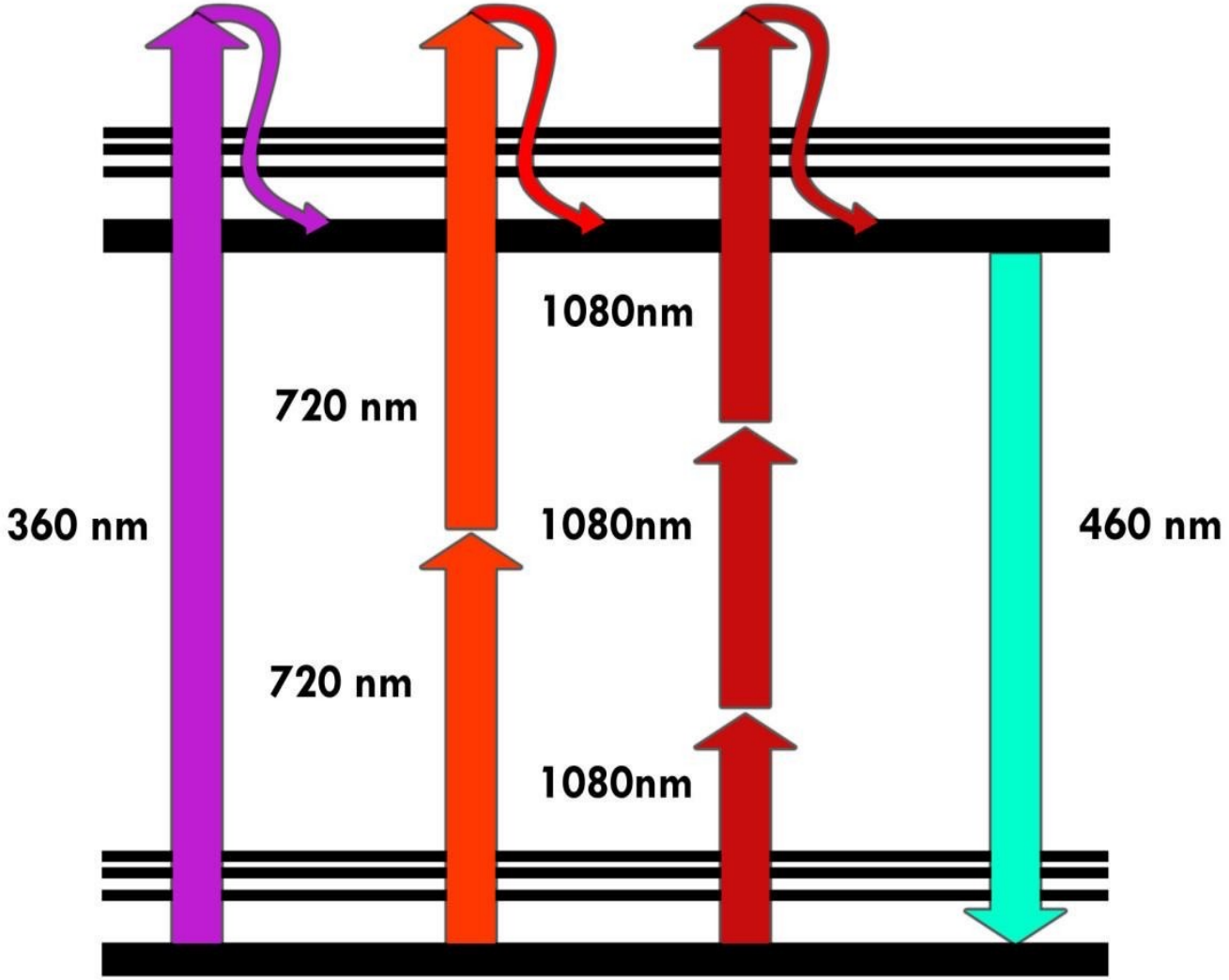




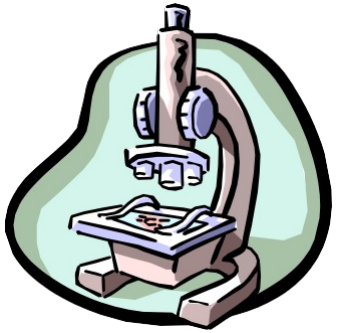
Two-photon excitation occurs through the absorption of two lower energy

FROM 2-PHOTON TO MULTIPHOTON…





SINGLE- vs TWO-PHOTON EXCITATION



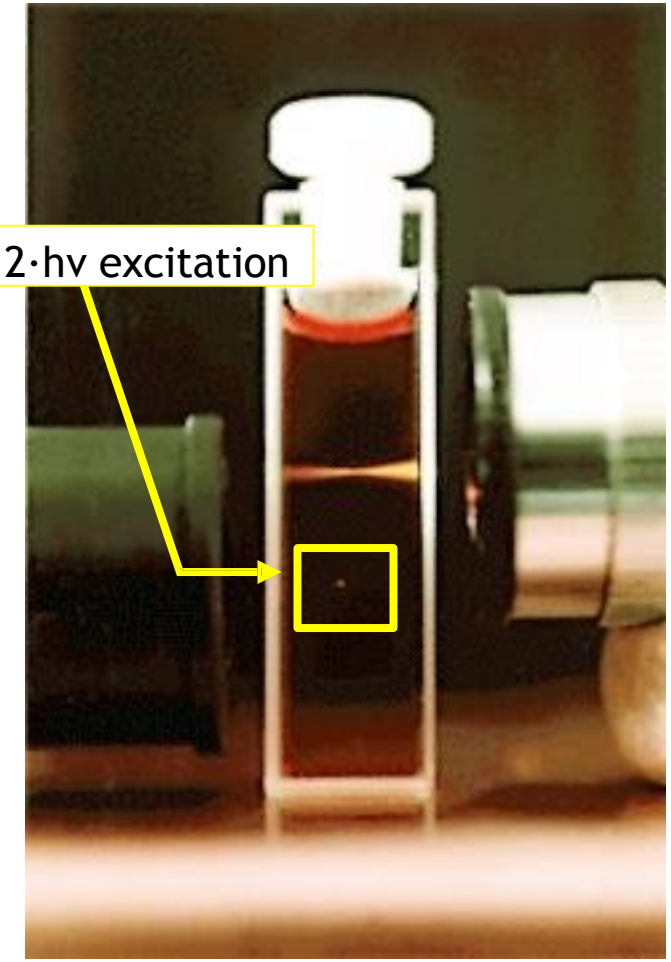
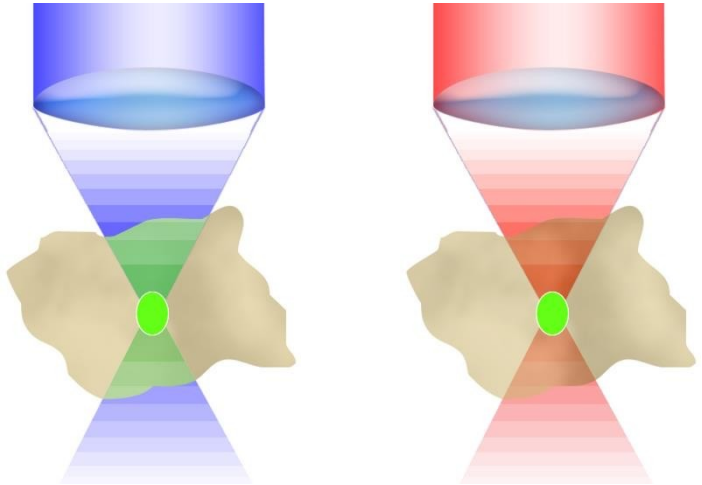


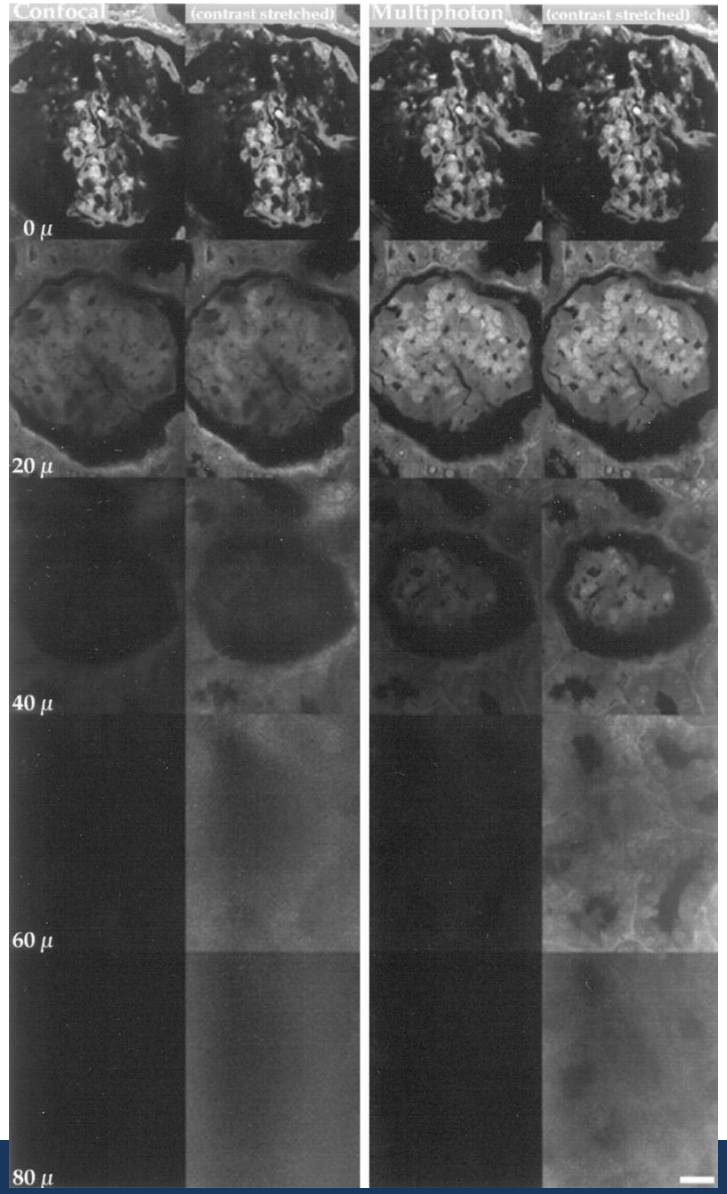
Image source: Current Protocols in Cytometry Online Copyright © 1999 John Wiley & Sons, Inc. All rights reserved.

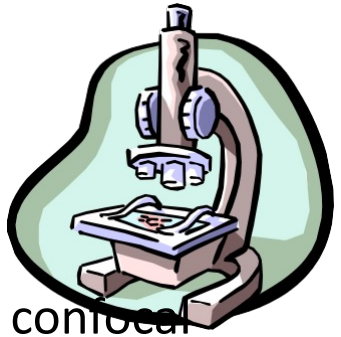
The cuvette is filled with a solution of a dye, safranin O, which normally requires green light for excitation. **Green light (543 nm)** from a continuous-wave helium-neon laser is focused into the cuvette by the lens at upper right. It shows the expected pattern of a continuous cone, brightest near the focus and attenuated to the left. The lens at the lower left focuses an invisible **1046-nm infrared beam** from a mode-locked Nd-doped yttrium lanthanum fluoride laser into the cuvette. Because of the two-photon absorption, excitation is confined to a tiny bright spot in the middle of the cuvette.



Slide credit: Brad Amos, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

PENETRATION DEPTH

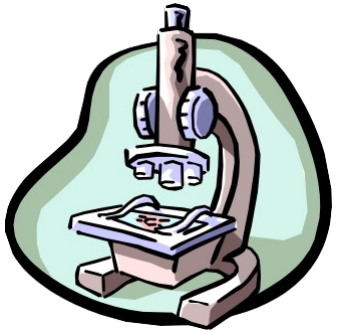


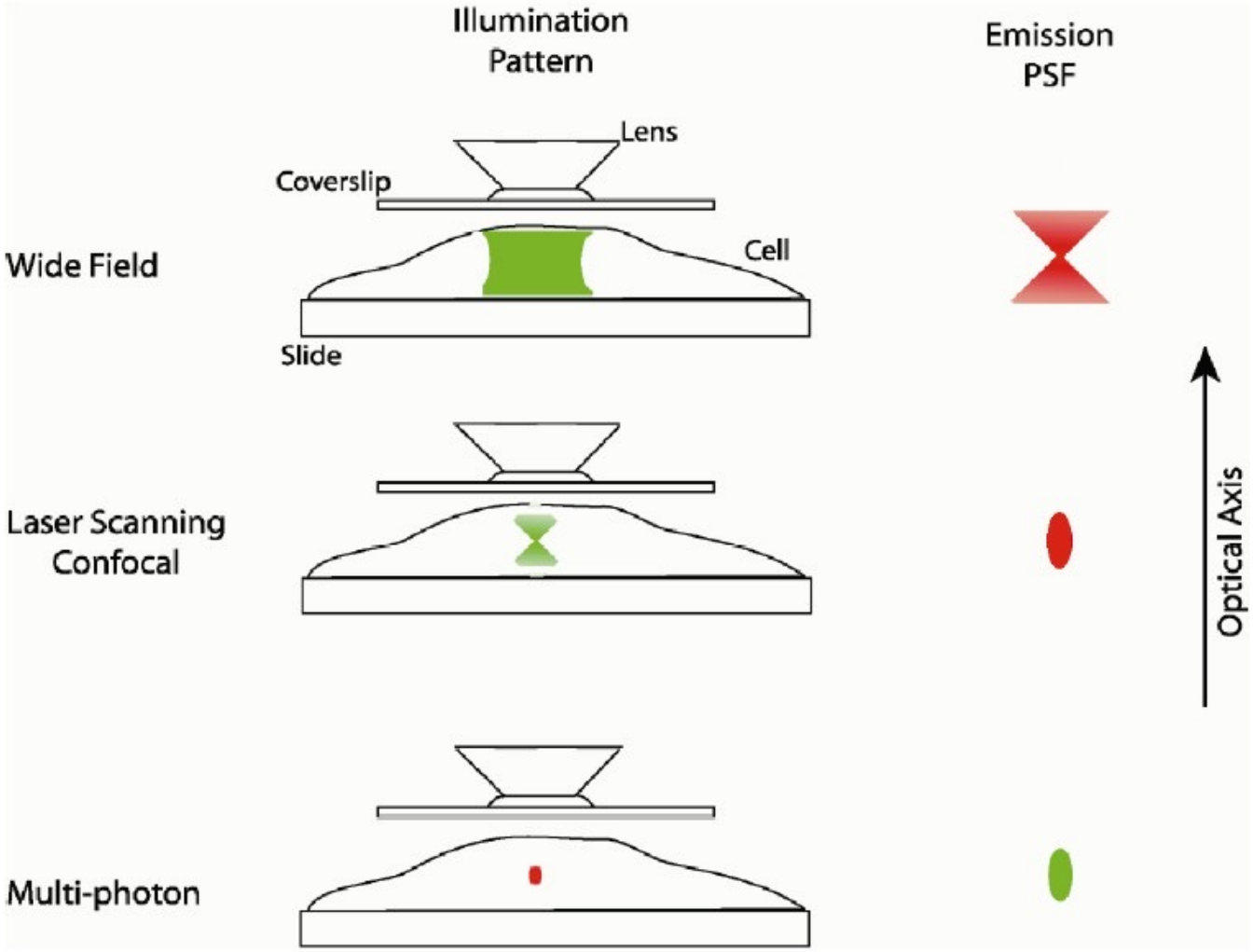


Comparison of imaging penetration depth between confocal and multiphoton microscopy. Optical sections through a glomerulus from an acid-fucsin- stained monkey kidney pathology sample imaged by confocal microscopy with 2 µW of 532-nm light (*left, columns 1 and 2*) and multiphoton microscopy with 4.3 mW of 1047-nm light (descanned; *right, columns 3 and 4*) were compared. At the surface, the image quality and signal intensity are similar; however, at increasing depth into the sample, signal intensity and quality of the confocal image falls off more rapidly than the multiphoton image. Images were collected at a pixel resolution of 0.27 µm with a Kalman
3 collection filter. Scale bar, 20 µm.

Centonze VE, White JG. Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. Biophys J. 1998 Oct;75(4):2015-24.

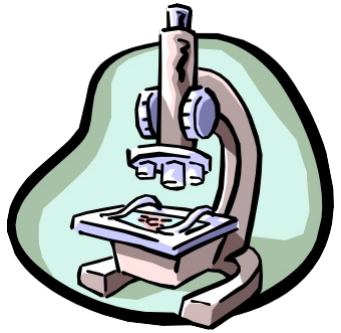
WIDE-FIELD VS. CONFOCAL VS. 2-PHOTON



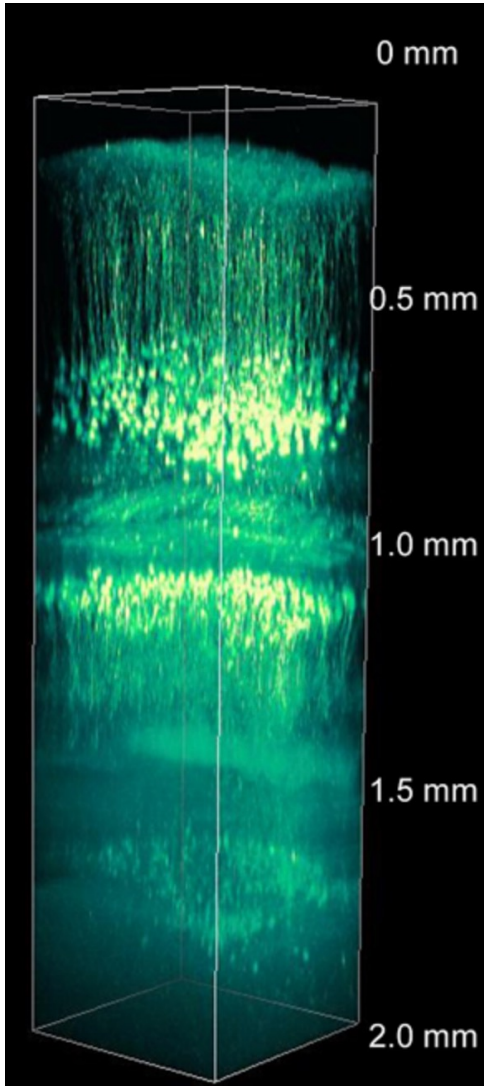


Drawing by P. D. Andrews, I. S. Harper and J. R. Swedlow

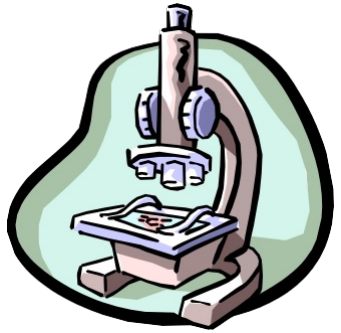
FAR FIELD: TWO-PHOTON



- Non-linear 2-photon excitation and pinhole detection decrease SPF beyond classical limits
- $2^{1/2}$ improvement in resolution
- High penetration depth (IR wavelengths for stimulation)

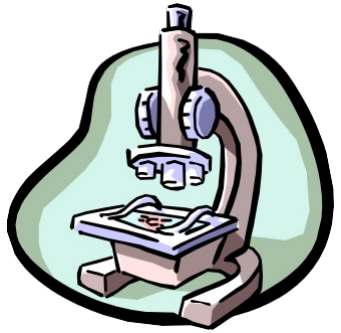


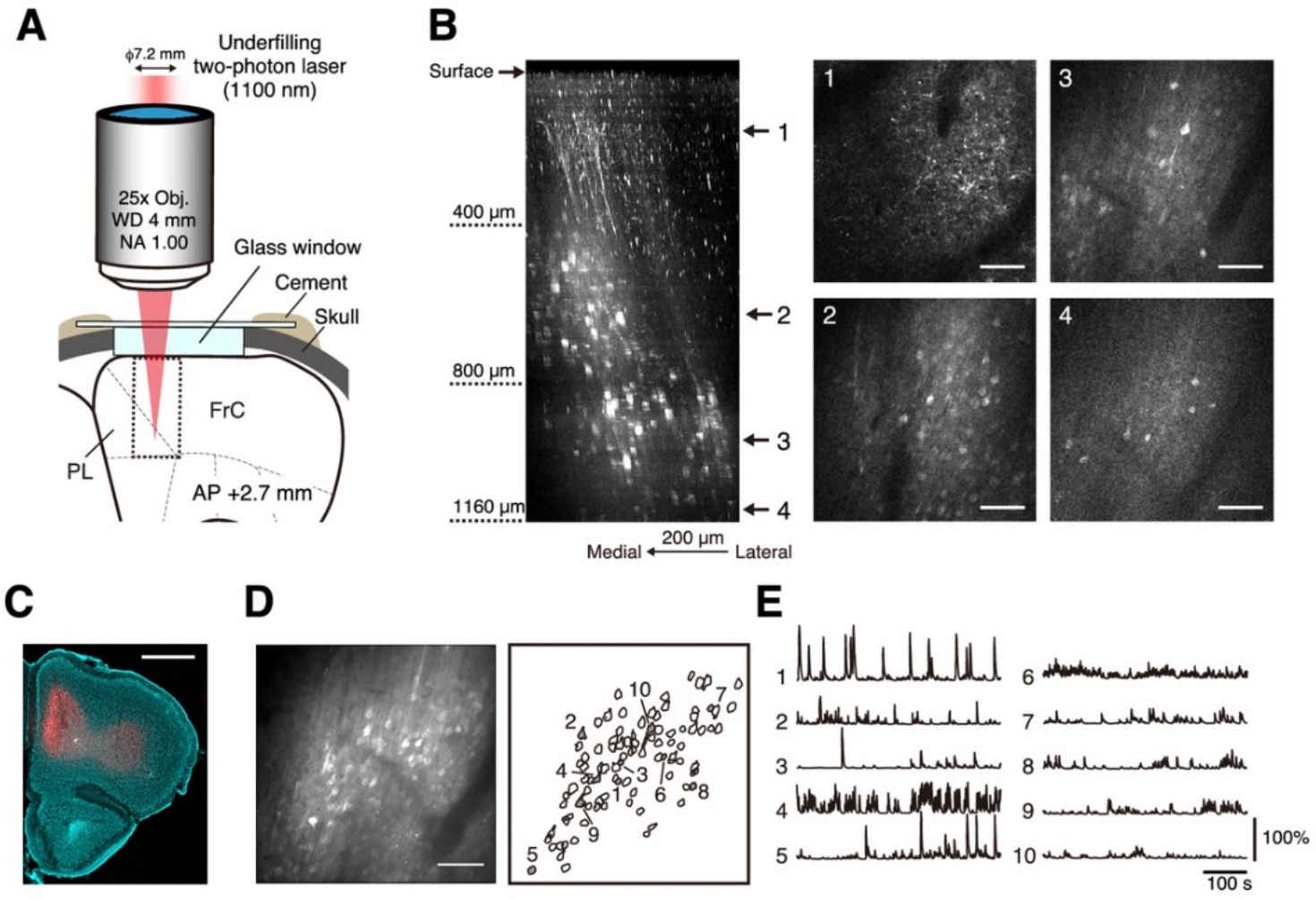
TWO-PHOTON MICROSCOPY – DYE examples



	Alexa Fluor® 350	Alexa Fluor® 488	Alexa Fluor® 546	Alexa Fluor® 555	Alexa Fluor® 568	Alexa Fluor® 594	Alexa Fluor® 647
Target	label/conjugate	label/conjugate	label/conjugate	label/conjugate	label/conjugate	label/conjugate	label/conjugate
Bibliography	Citations	Citations	Citations	Citations	Citations	Citations	Citations
TPE excitation (nm)	720	720, 830	810	810	770	810	800
Laser line (nm)	350/405	488	488	488	561	594	594/633
Standard filter set	DAPI	FITC	TRITC	TRITC	RFP	Texas Red®	Cy®5
Ex/Em (nm)	346/442	490/525	556/573	555/580	578/603	590/617	650/665

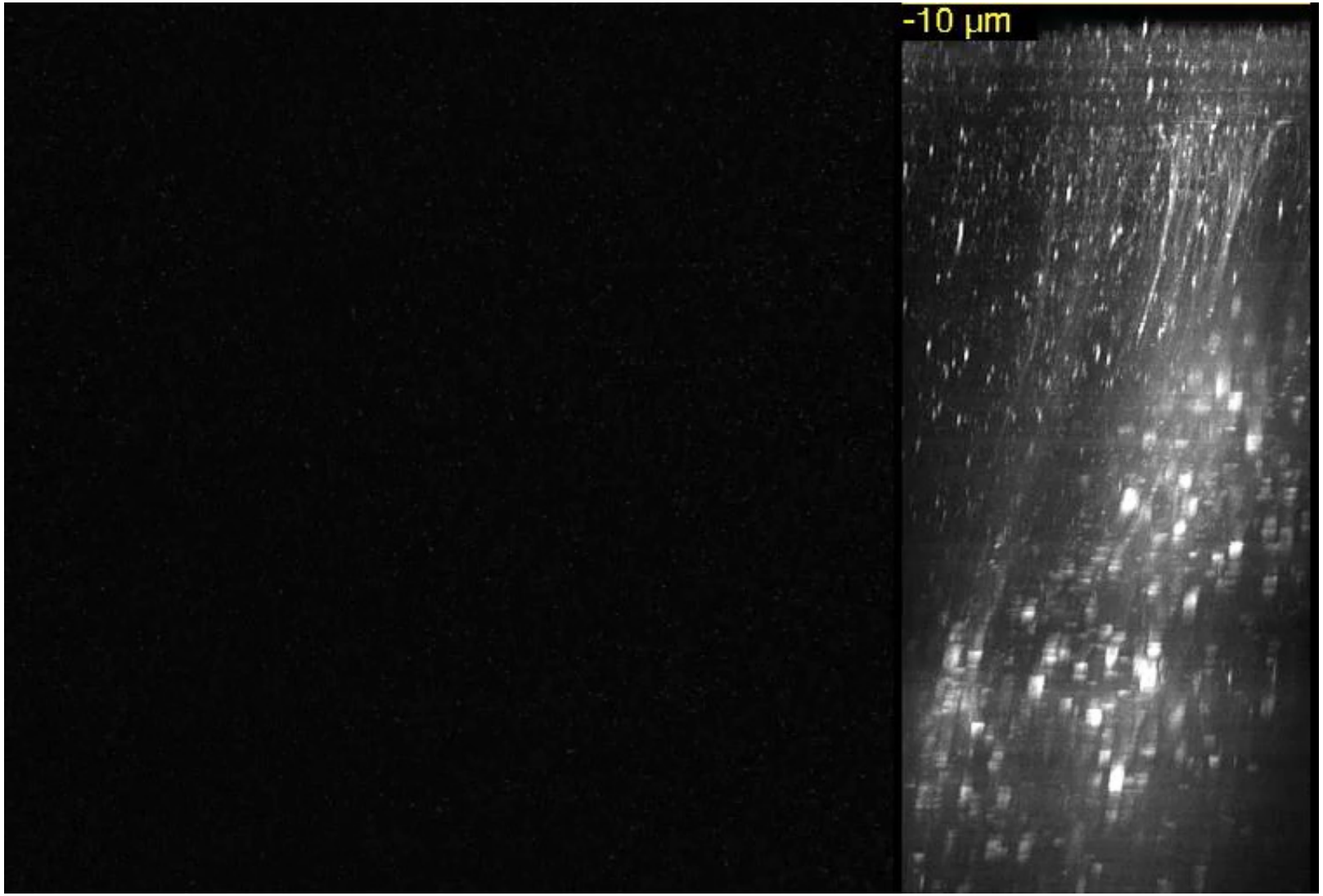
TWO-PHOTON MICROSCOPY – DYE examples

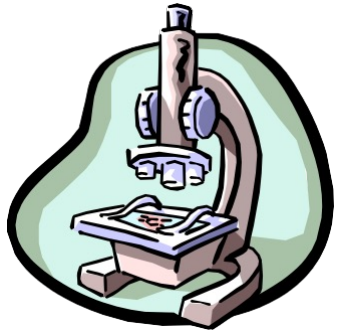




Kondo et al, 2017

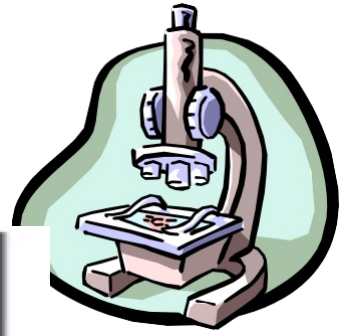
TWO-PHOTON MICROSCOPY – DYE examples

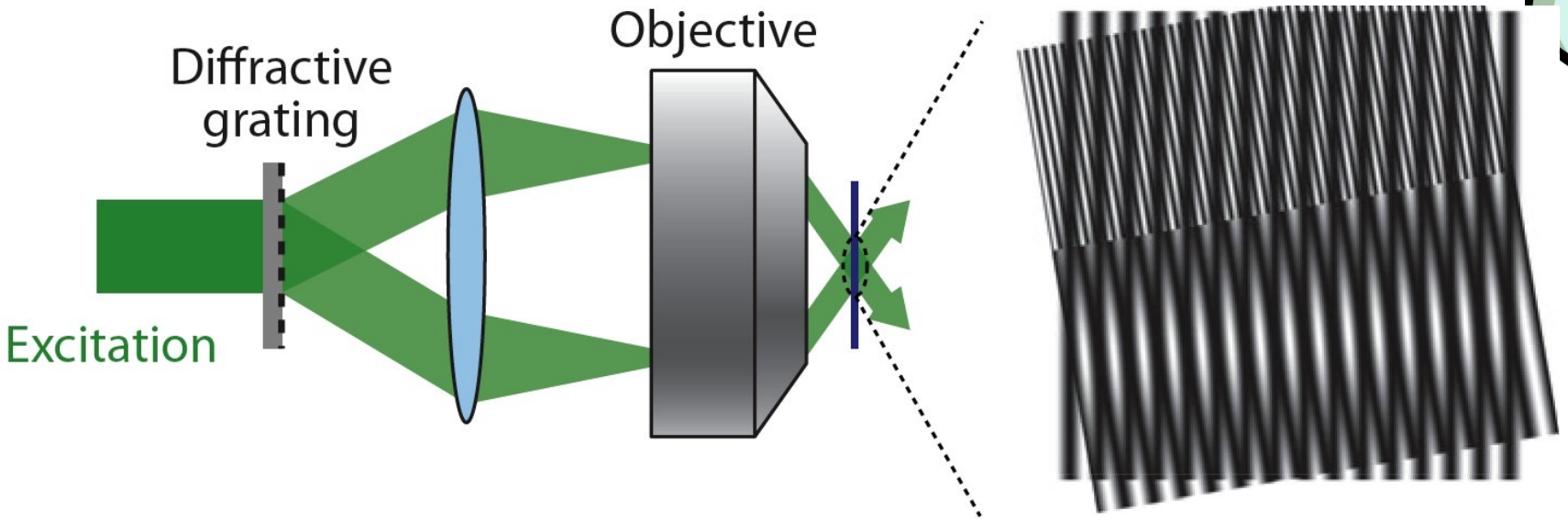




Kondo et al, 2017

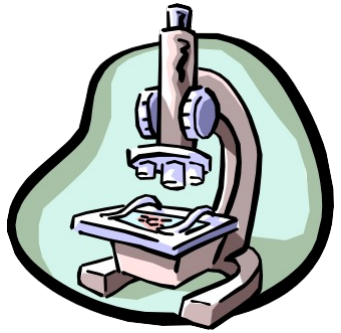
STRUCTURED-ILLUMINATION MICROPSCOPY (SIM)





100 nm resolution
possible

STRUCTURED ILLUMINATION – HISTORY



Optischen Abbildung unter Überschreitung der beugungsbedingten Auflösungsgrenze

von W. LUKOSZ und M. MARCHAND
Physikalisches Institut, Technische Hochschule, Braunschweig, Germany

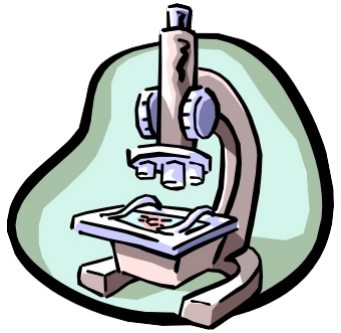
(*Received* 5 *February* 1963, *and in revised form* 1 *July* 1963)

Bekanntlich setzt die Beugung dem mit einem optischen System erreichbaren Auflösungsvermögen (präziser formuliert : der Bandbreite des vom System durchgelassenen Orts-Frequenzbandes) eine prinzipielle Grenze.

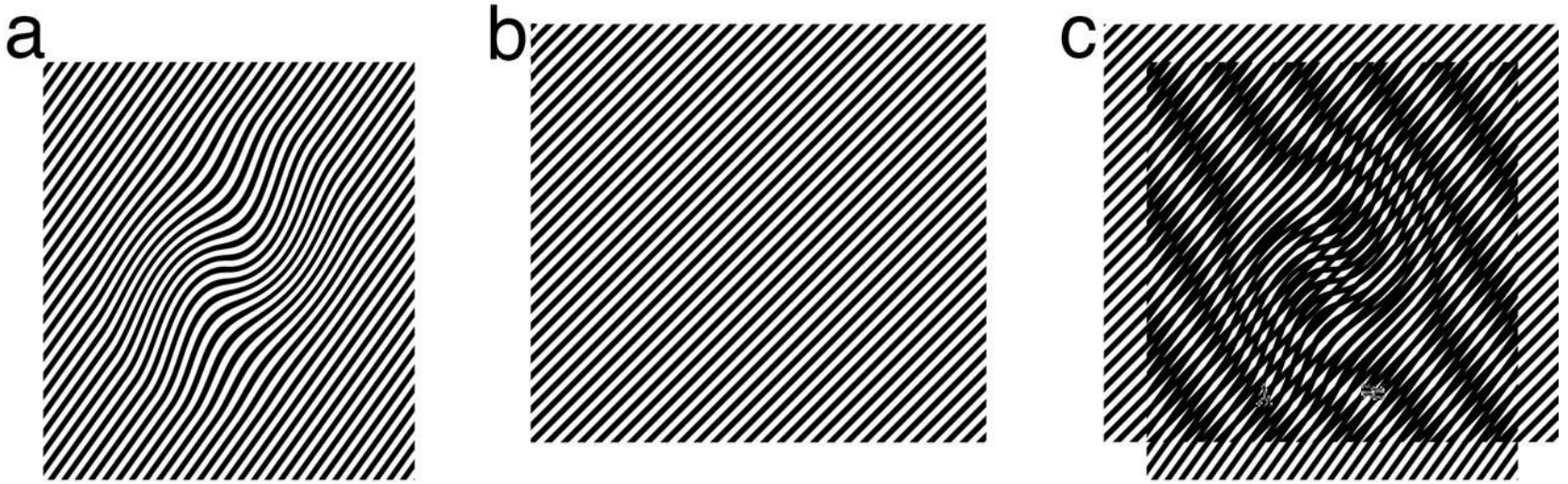
In der vorliegenden Arbeit wird ein neues Verfahren zur optischen Abbildung mit einem über die beugungsbedingten Grenzen hinausgehenden Auflüsungsvermögen erläutert : Das optische System selbst wird unverändert benutzt. In (bzw. in der Nähe) der Objektebene wird aber eine Maske mit örtlich variabler Transmission (z.B. ein Gitter) angebracht oder

- Lukosz and Marchand suggested in 1963 that lateral light patterns could be used to enhance resolution
- Practical implementation was reported by T. Wilson et al. in 1997. (Neil, M. A. A., Wilson, T. & Juskaitis, R. (1997) *Opt. Lett.* **22**, 1905–1907.)

RESOLUTION EXTENSION THROUGH THE MOIRÉ EFFECT



The word moiré is French (from the past participle of the verb moirer, meaning to water).

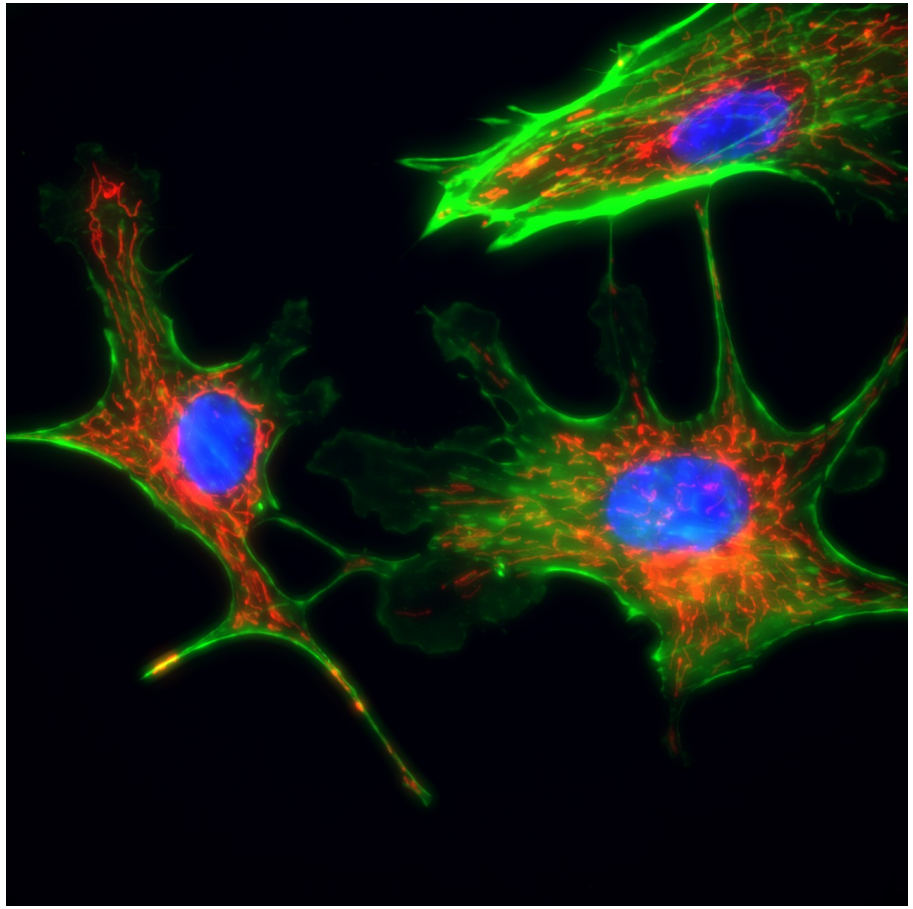


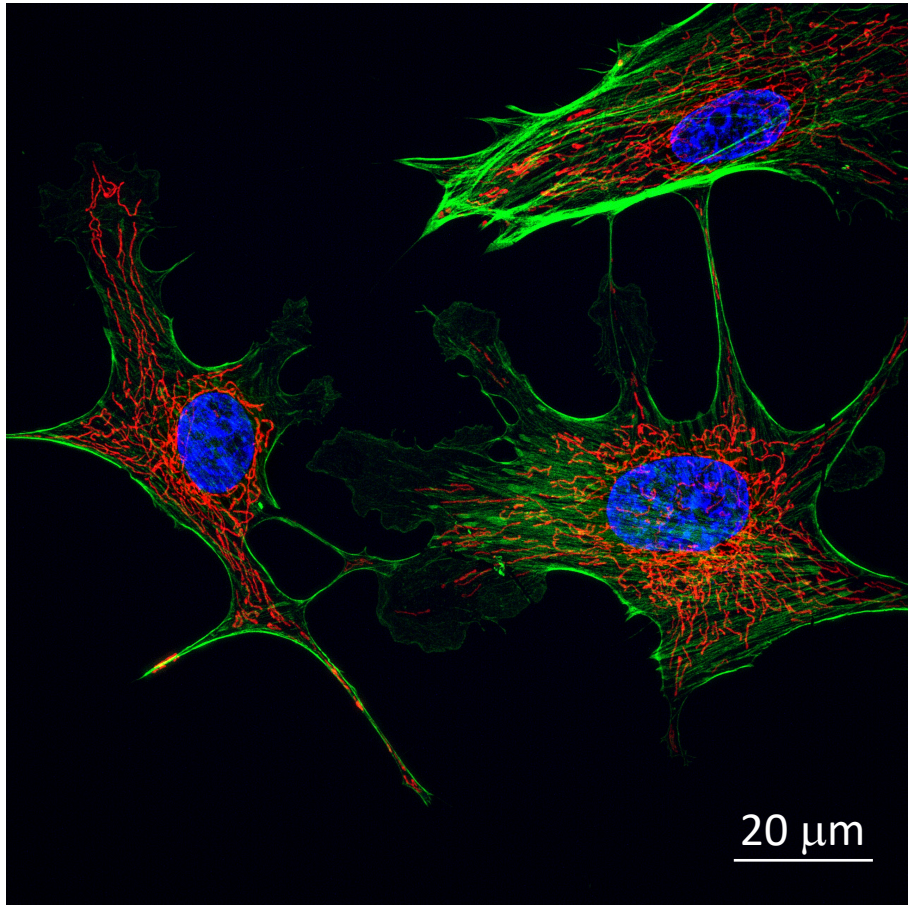
If an unknown sample structure (*a*) is multiplied by a known regular illumination pattern (*b*), moiré fringes will appear (*c*). The Moiré fringes occur at the spatial difference frequencies between the pattern frequency and each spatial frequency component of the sample structure and can be coarse enough to observe through the microscope even if the original unknown pattern is unresolvable. Otherwise-unobservable sample information can be deduced from the fringes and computationally restored.

Gustafsson, M.G.L. (2005) Proc. Natl. Acad. Sci. USA 102, 13081-13086

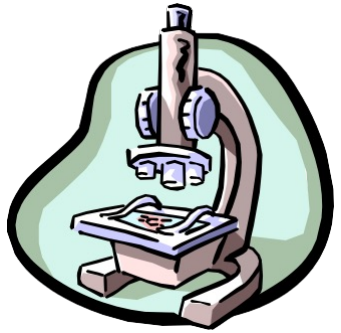
Advanced microscopy techniques

SIM-Structural illumination microscopy





CONCLUSIONS



- methods (TIRF, cofocal, SIM and 2 photon) use common dyes (good)
- confocal is easiest, most widely used

- best resolution obtainable only 100 nm (SIM)
- single molecule is problematic
- Consider sample thickness!

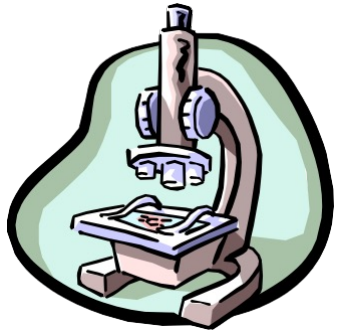
Advanced microscopy techniques

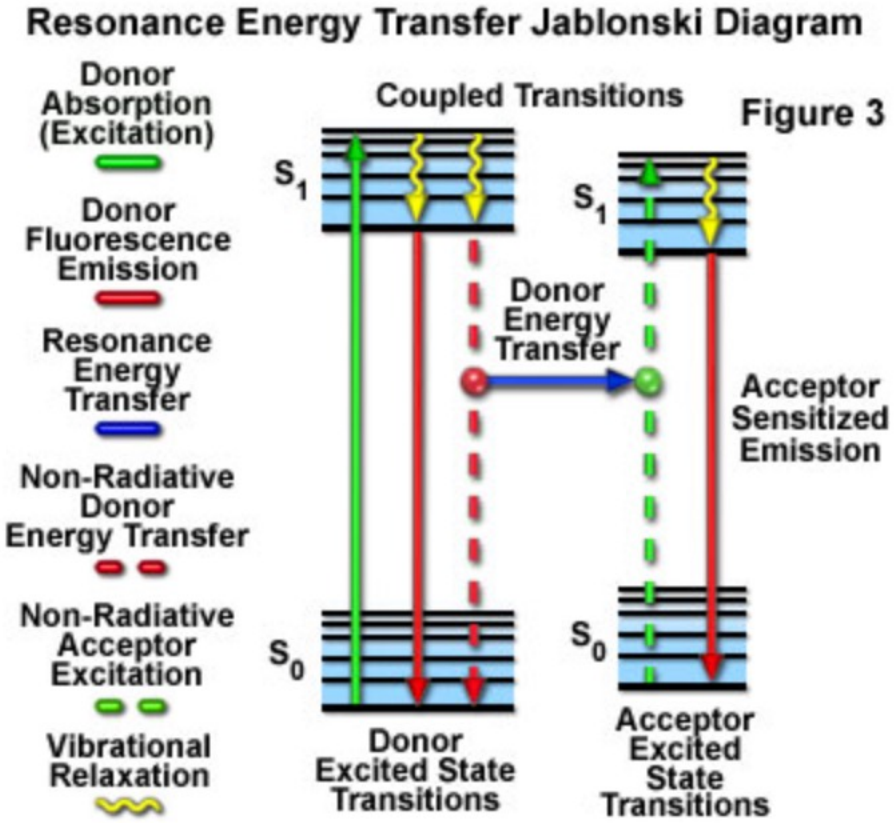
CIMA SIM superresolution vs MP vs confocal

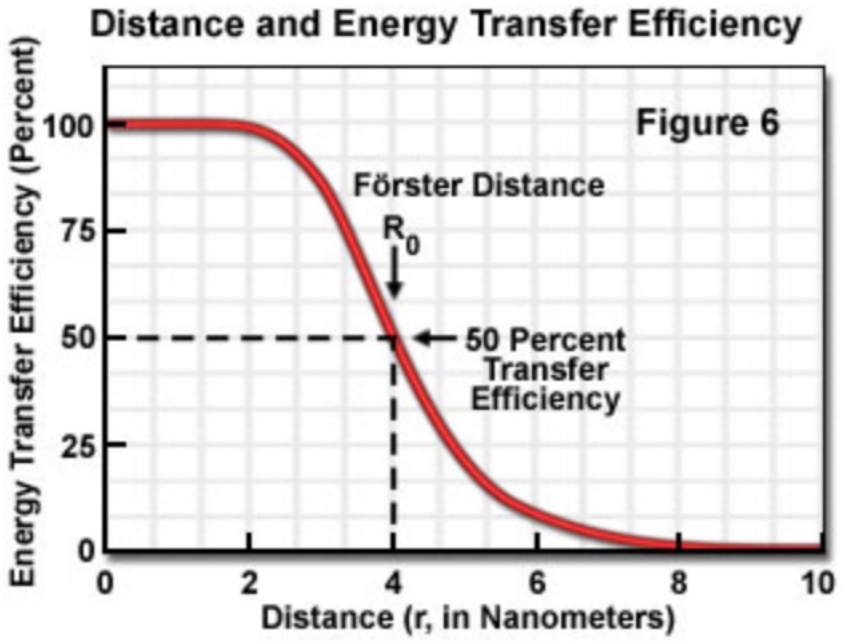
cima.services@units.it

	confocal	SIM	MP
Fixed sample	yes	yes	yes
Live sample	not ideal	yes dedicated incubation chamber with temperature, CO_2 and humidity regulation	yes to be set up customized to needs
Sample thickness	tens of μm	tens of μm preferably monolayer of cells	up to 2 mm
Wavelengths (nm)	405/488/561/640	405/488/561/640	tuneable, range 700-1000 nm
strength	Multichannel imaging for colocalisation	Structural resolution and time series (1 fps)	Penetration depth, time series (> 1 fps)

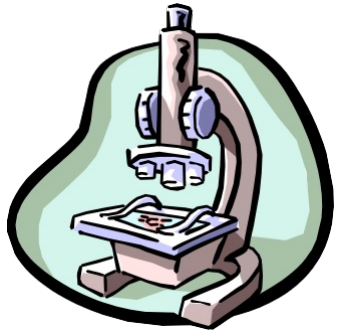
Fluorescence Resonance Energy Transfer (FRET) Microscopy

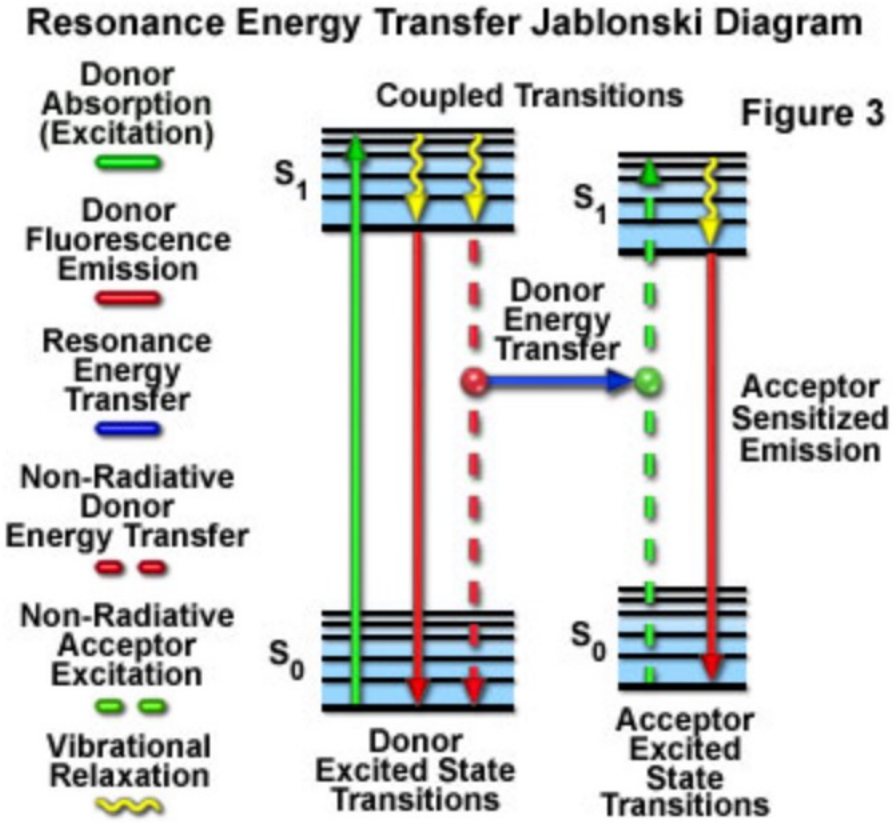


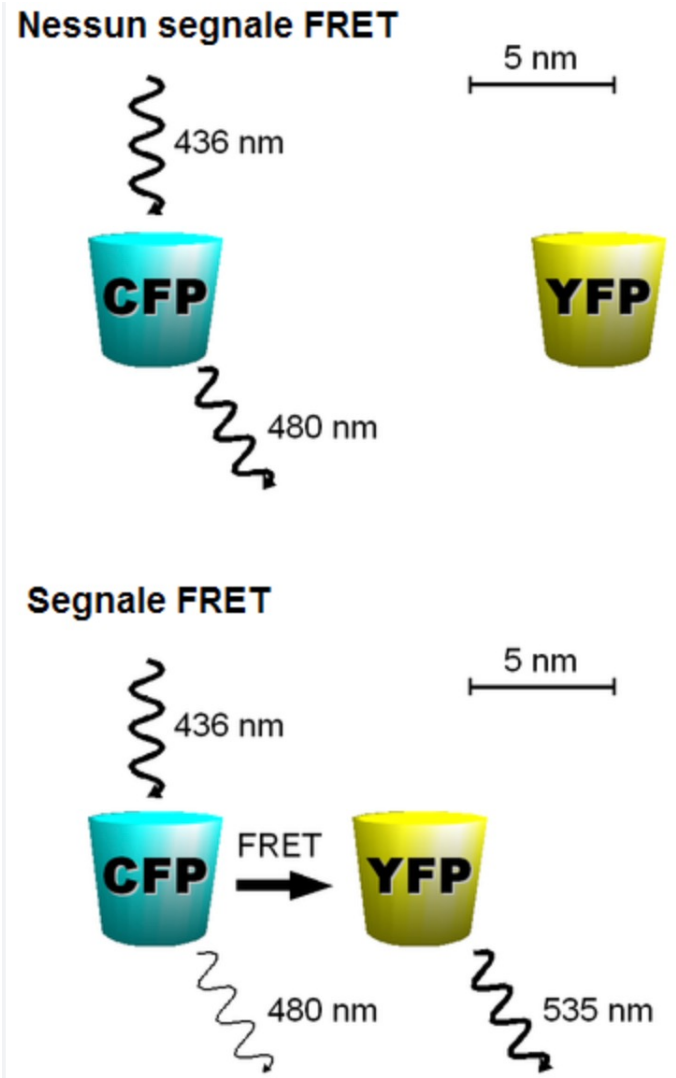




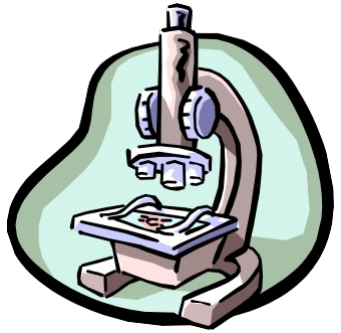
Fluorescence Resonance Energy Transfer (FRET) Microscopy

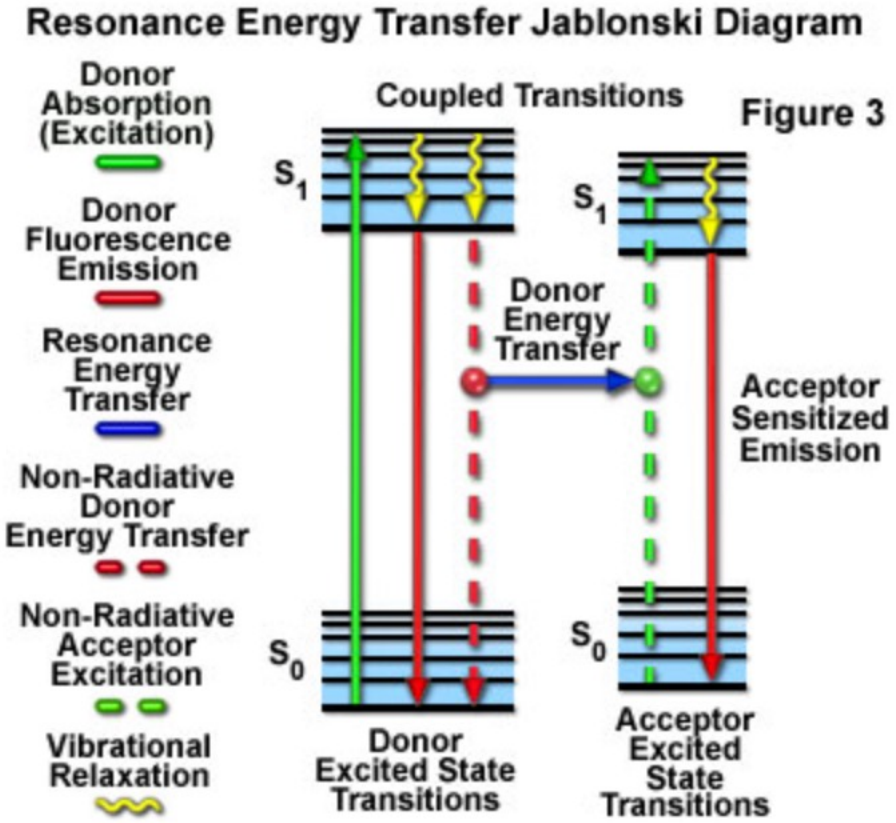


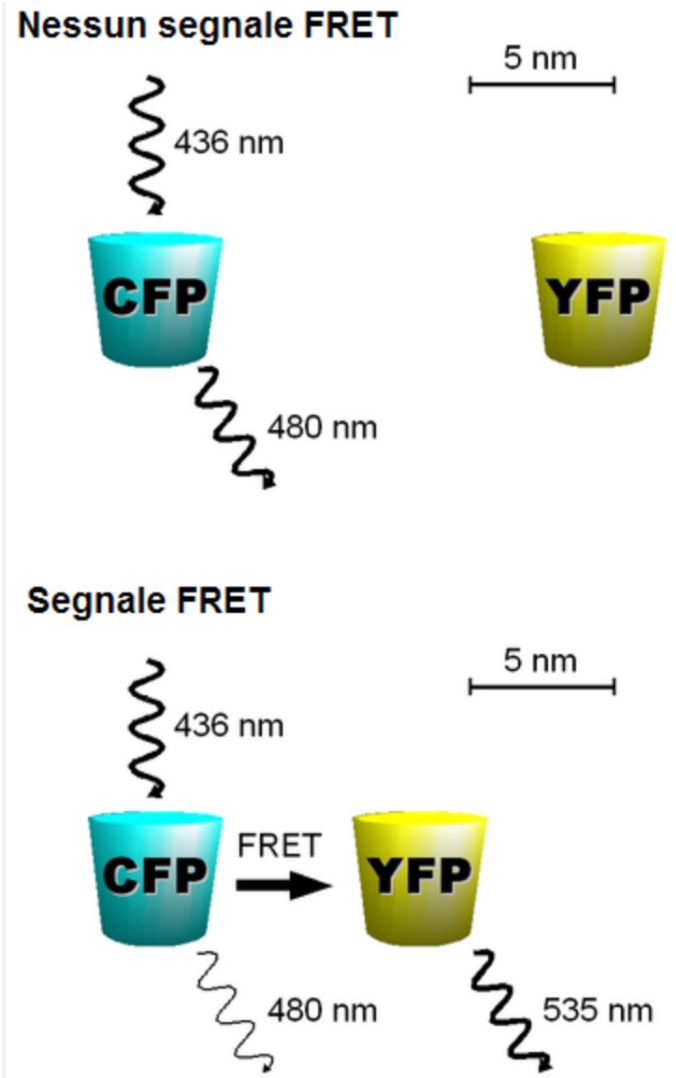




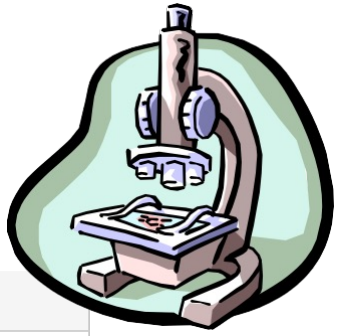
Fluorescence Resonance Energy Transfer (FRET) Microscopy

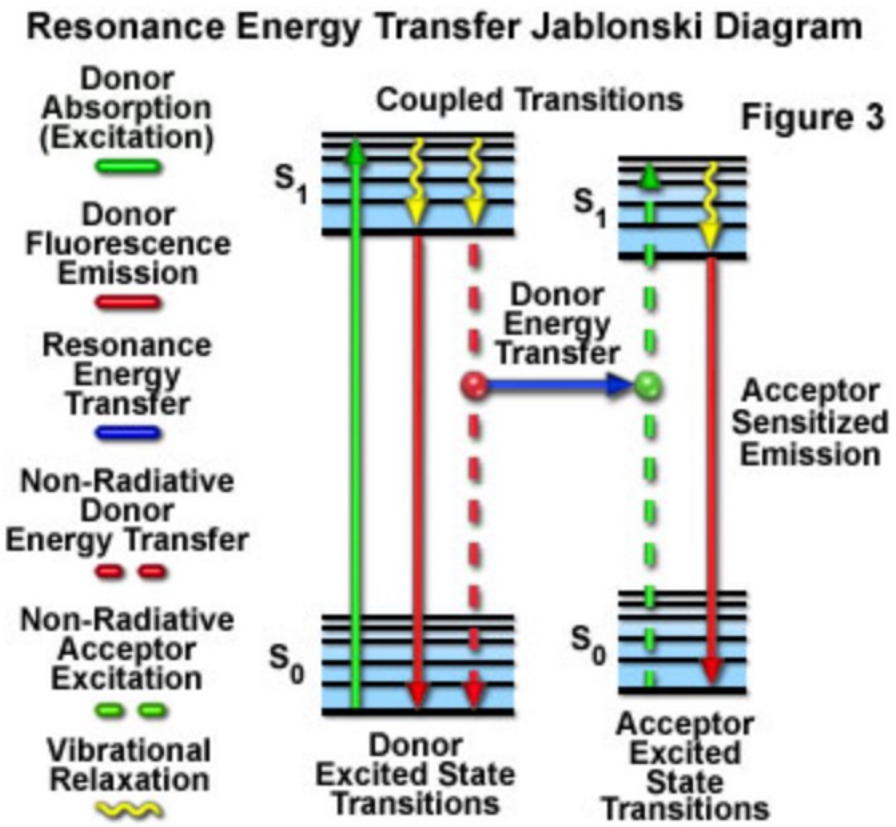






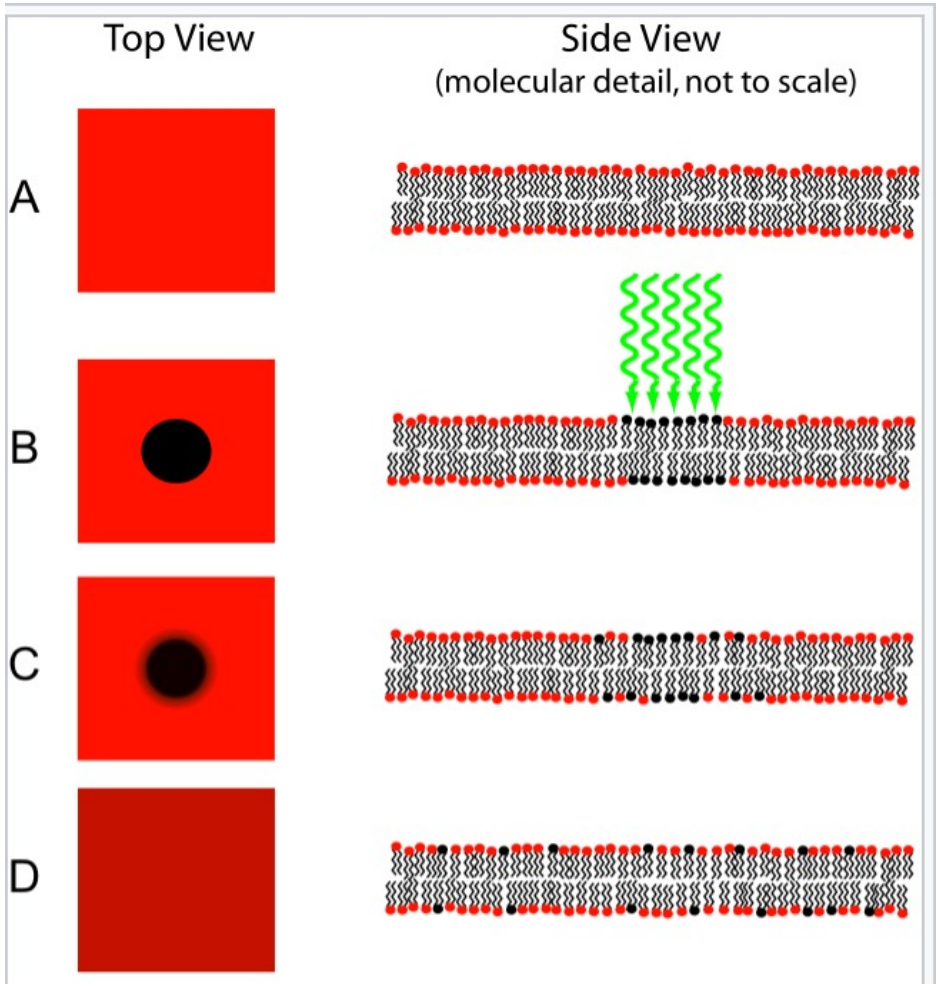
Fluorescence Resonance Energy Transfer (FRET) Microscopy

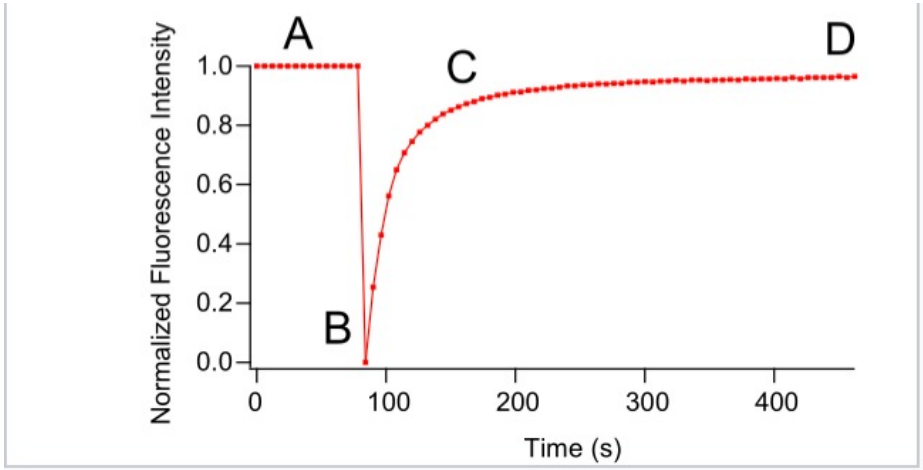




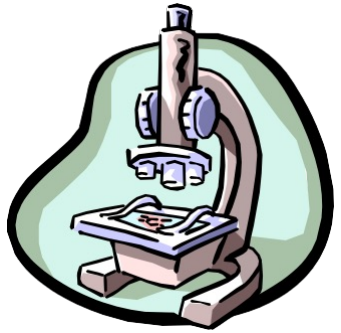
Donor	Acceptor
Tryptophan	Dansyl
IAEDANS (1)	DDPM (2)
BFP	DsRFP
Dansyl	FITC
	Octadecylrhodamine
CFP	GFP
CF (3)	Texas Red
Fluorescein	Tetramethylrhodamine
Cy3	Cy5
GFP	YFP
BODIPY FL (4)	BODIPY FL (4)
Rhodamine 6G	Malachite Green
FITC	Eosin Thiosemicarbazide
B-Phycoerythrin	Cy5
Cy5	Cy5.5

Fluorescence recovery after photobleaching

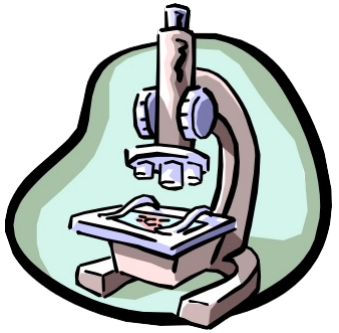


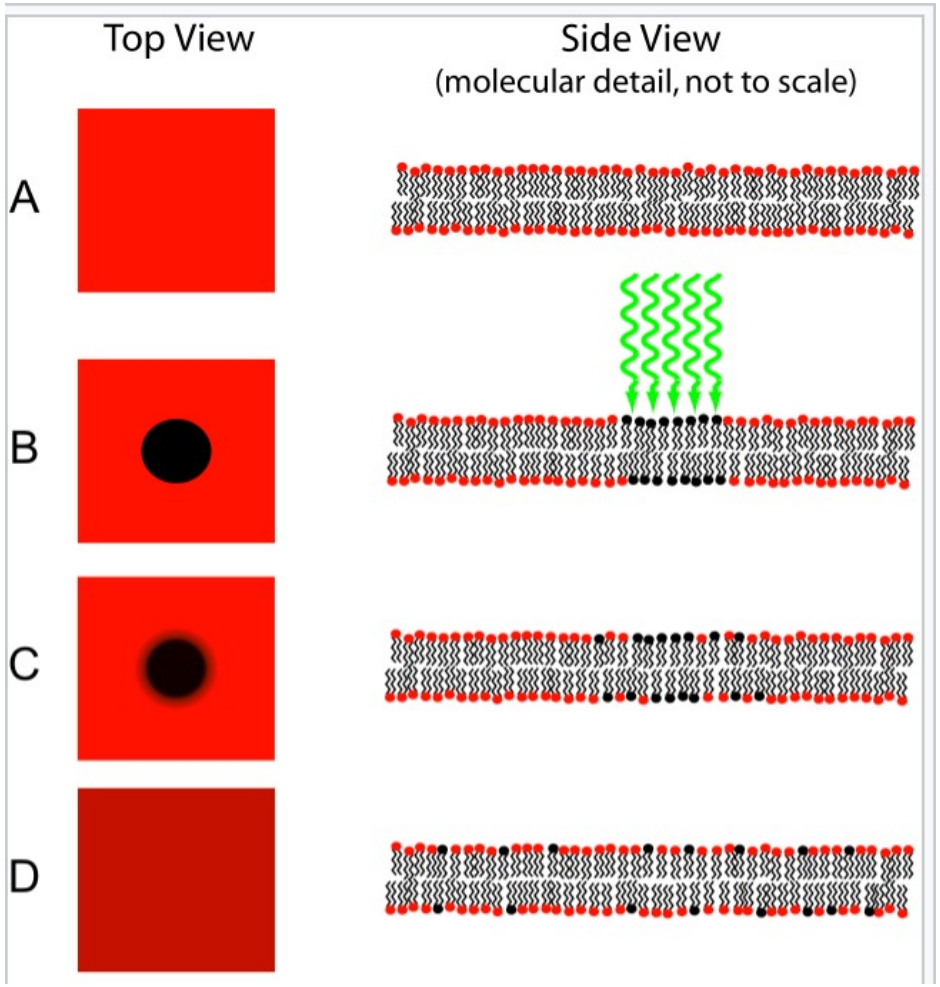


Principle of FRAP A) The bilayer is uniformly labeled with a fluorescent tag B) This label is selectively photobleached by a small (~30 micrometre) fast light pulse C) The intensity within this bleached area is monitored as the bleached dye diffuses out and new dye diffuses in D) Eventually uniform intensity is restored



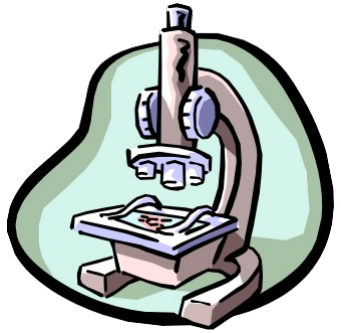
Fluorescence recovery after photobleaching





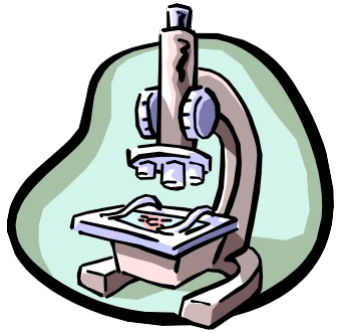
- Laser for photobleaching can be used on an epifluorescence or confocal microscope, usually with CCD camera, not PMT
- GFP dyes or photoconvertable dyes used

COMPARISON



method	excitation	detection	sectioning	use
Wide field	Whole sample	Whole sample	No sectioning	Simple fluorescence samples
confocal	Whole sample	One z-plane	350-500nm	High contrast images, optical sectioning
2-Photon	One z-plane	One z-plane	500-700nm	Deep tissue imaging, optical sectioning
FRET				Protein interactions
FRAP + photoactivation	405 laser (UV)			dynamics/mobility
TIRF	Only bottom plane	Only bottom plane	150-200nm	Membrane dynamics

SUPER-RESOLUTION MICROSCOPY



Goal: obtain sub-100 nm resolution

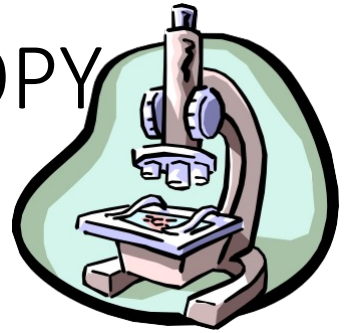
pioneered by Stefan Hell in mid-1990s
Max Plank Institute (Germany)

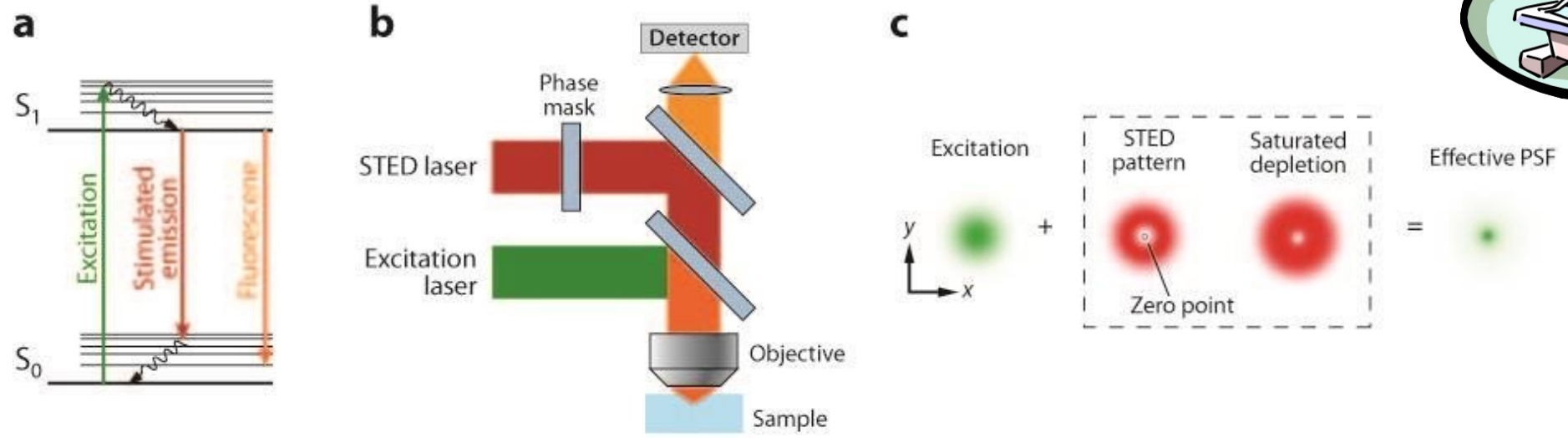
two methods:

(i) Spatially Patterned Excitation
STED, SIM

(ii) Localization Methods
STORM, PALM

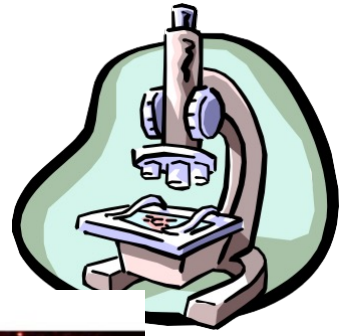
STIMULATED EMISSION DEPLETION (STED) MICROSCOPY

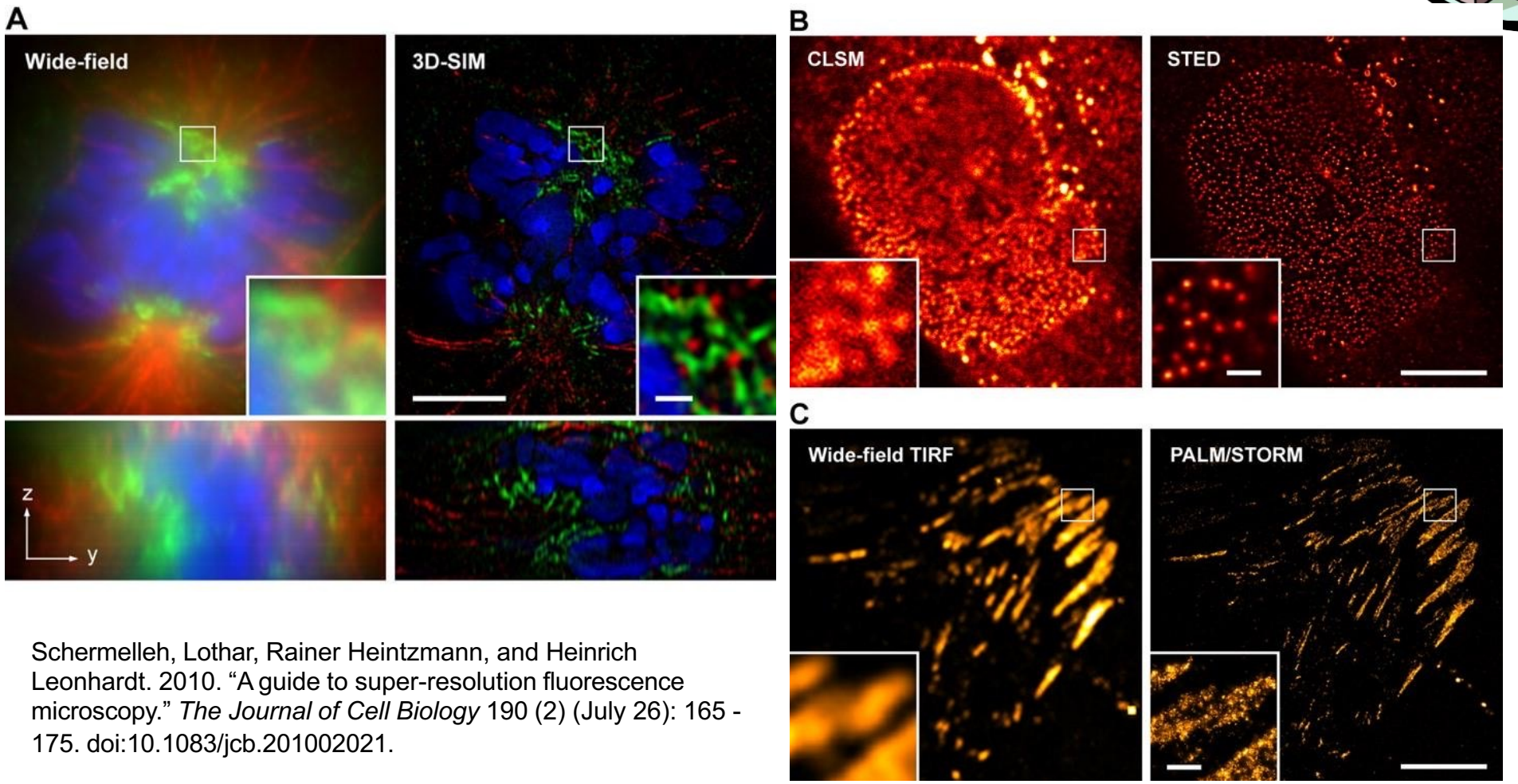




(*a*) The process of stimulated emission. A ground state (S_0) fluorophore can absorb a photon from the excitation light and jump to the excited state (S_1). Spontaneous fluorescence emission brings the fluorophore back to the ground state. Stimulated emission happens when the excited-state fluorophore encounters another photon with a wavelength comparable to the energy difference between the ground and excited state. (*b*) The excitation laser and STED laser are combined and focused into the sample through the objective. A phase mask is placed in the light path of the STED laser to create a specific pattern at the objective focal point. (c) In the *xy* mode, a donut-shaped STED laser is applied with the zero point overlapped with the maximum of the excitation laser focus. With saturated depletion, fluorescence from regions near the zero point is suppressed, leading to a decreased size of the effective point spread function (PSF).

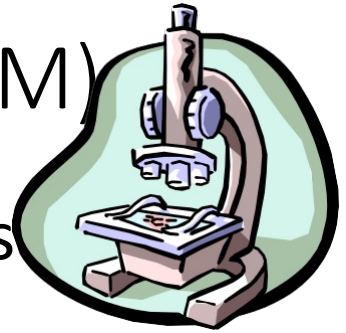
SUPER-RESOLUTION MICROSCOPY OF BIOLOGICAL SAMPLES.





Schermelleh, Lothar, Rainer Heintzmann, and Heinrich Leonhardt. 2010. “A guide to super-resolution fluorescence microscopy.” *The Journal of Cell Biology* 190 (2) (July 26): 165 - 175. doi:10.1083/jcb.201002021.

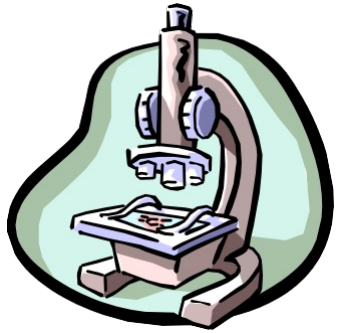
SINGLE MOLECULE LOCALISATION MICROSCOPY (SMLM)



- Induction of fluorescence emission of only a subset of molecules at a given time in order to localize each of them individually. By repeating this process, you can accumulate enough localizations to reconstruct a final super-resolved image.

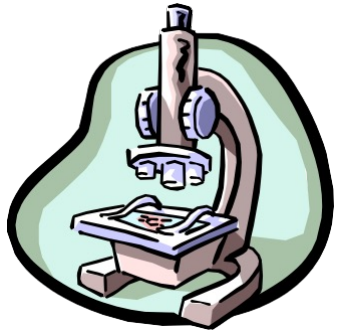
STORM and PALM are based on SMLM principle. The only difference is the way to induce the stochastic emission of the fluorophores:
•In STORM, fluorescent organic dyes (cyanines, rhodamines, …etc.) together with a specific imaging buffer (abbelight's buffer) are used to allow blinking of the fluorescent molecules;
•In PALM, photoactivatable, photoconvertible or photoswitchable proteins are used (ex: PA-GFP, PA-mCherry, mEOS, mMAPLE, …etc.);

PALM-Photoactivated localization microscopy



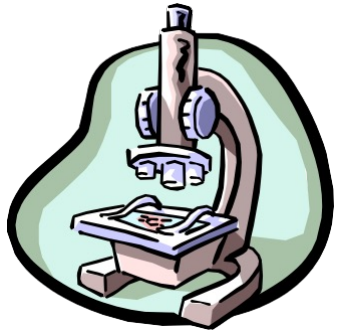
- invented by E Betzig and H Hess (2014 Nobel Prize for Chemistry)
- uses photoactivatable fluorophores to resolve spatial details of tightly packed molecules
- The laser stochastically activates fluorophores until all have emitted. Fluorophores emit for a short period but eventually bleach -> a more accurate view of positions can be obtained.

PALM-Photoactivated localization microscopy



- Signals from each fluorophore are still subject to the 300 nm diffraction of light limit. However, because each has been activated separately, the centre of mass can be calculated accurately
- The point spread function (PSF) is used to determine the location down to a resolution of 20 nm
- By mapping each of the more accurately defined points together, a complete super-resolution microscopy image can be compiled.

PALM-Photoactivated localization microscopy



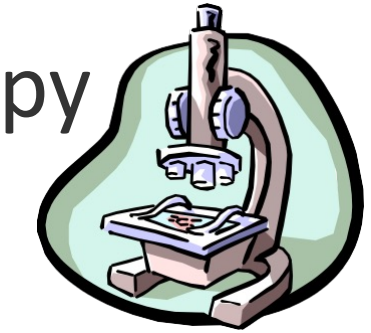
Three commonly used types of fluorophores are:

•Photoactivatable Fluorophores, e.g.: PAmCherry, PA-GFP, which emit light upon activation with UV.

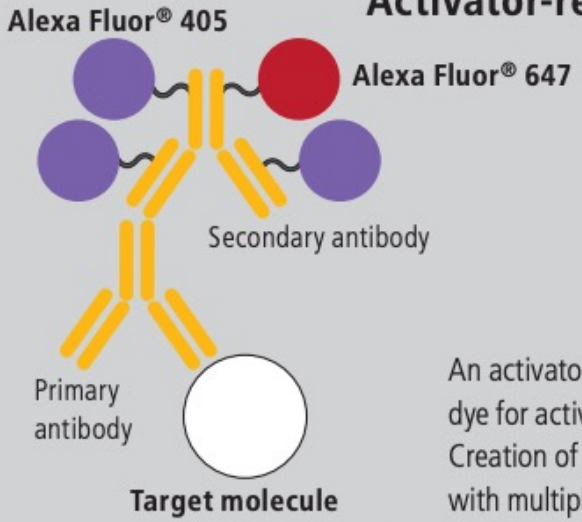
•Photoconvertible Fluorophores, which change their emission spectrum upon activation with UV light (e.g.: mEOS proteins).

•Photoswitchable Fluorophores: Typically chemical dyes (e.g. Alexa Fluor 647, DyLight555) which can switch between dark, non-fluorescent and bright, fluorescent states repeatedly.

STORM-Stochastic Optical Reconstruction Microscopy



Activator-reporter pair

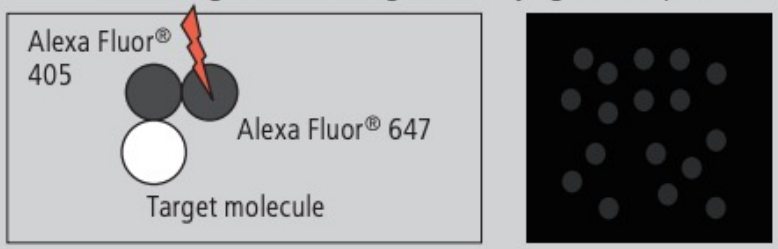


Dye for activator	Dye for reporter
Alexa Fluor® 405	Alexa Fluor® 647
Cy2	Alexa Fluor® 647
Cy3	Alexa Fluor® 647

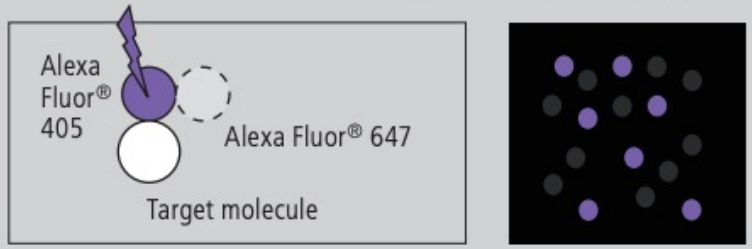
An activator-reporter pair consists of a shorter-wavelength dye for activation and a longer-wavelength dye for detection. Creation of three-color, super-resolution images is possible with multiple dye-pairs.

Sequential activation illumination with activator-reporter pair

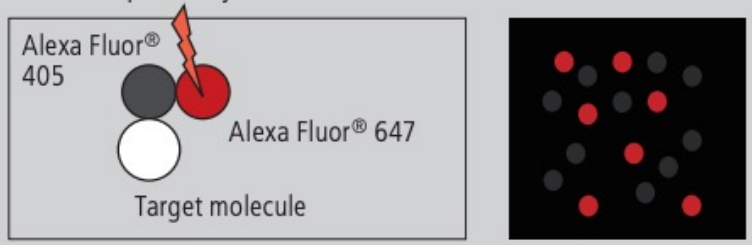
STEP 1 Most activator-reporter dye pairs are converted to a non-emissive state by combining them with high intensity light and specialized imaging buffer additives.



STEP 2 Absorption of light by an activator results in transfer of energy to a nearby reporter dye, accelerating the transition of the reporter dye from a non-emissive to a ground state. The use of spectrally distinct activator dyes allows for the use of the same reporter dye for multiple imaging channels.

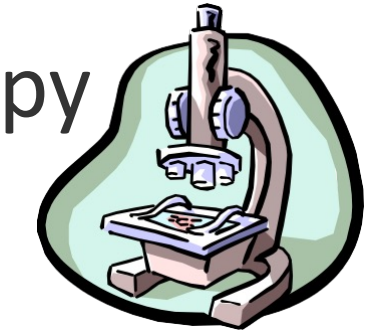


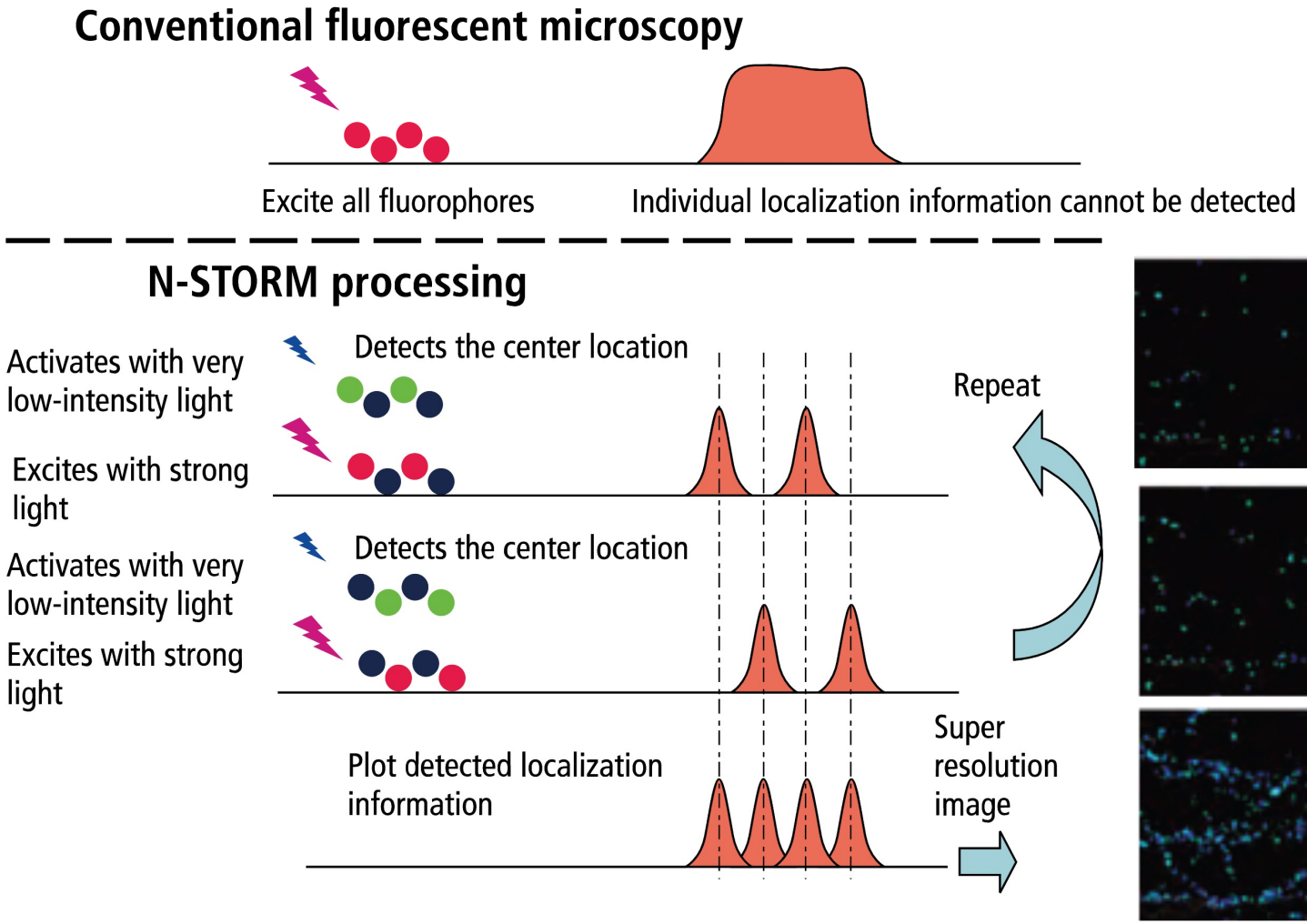
STEP 3 High intensity illumination results in fluorescence emission from the activated reporter dye.



https://downloads.microscope.healthcare.nikon.com/phase4/literature/Brochures/Super-Resolution_2CE-SCJK-3.pdf

STORM-Stochastic Optical Reconstruction Microscopy





reconstructs a super-resolution image by combining the high-accuracy localization information of individual fluorophores

https://downloads.microscope.healthcare.nikon.com/phase4/literature/Brochures/Super-Resolution_2CE-SCJK-3.pdf

STORM-Stochastic Optical Reconstruction Microscopy

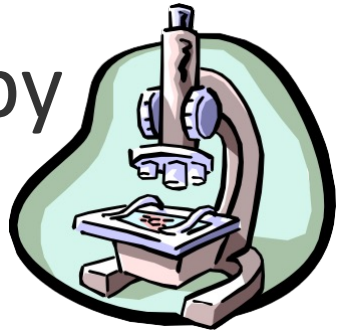
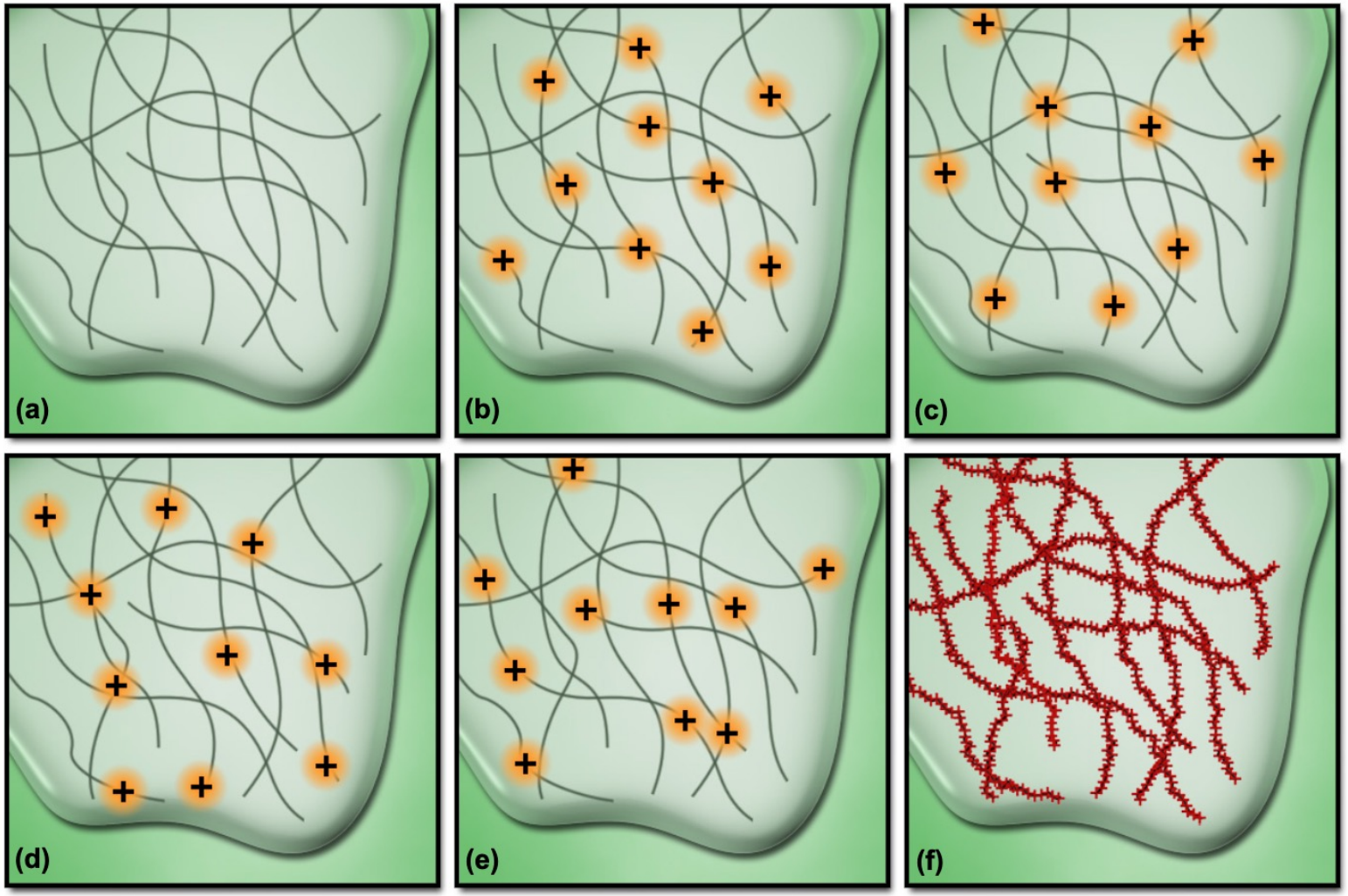
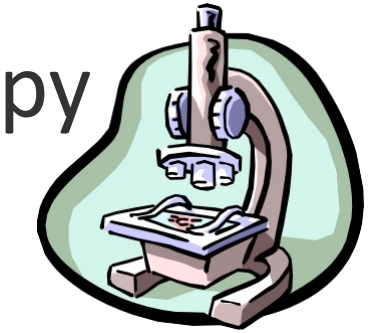


Figure 1 - Basic Principles of STORM Superresolution Imaging

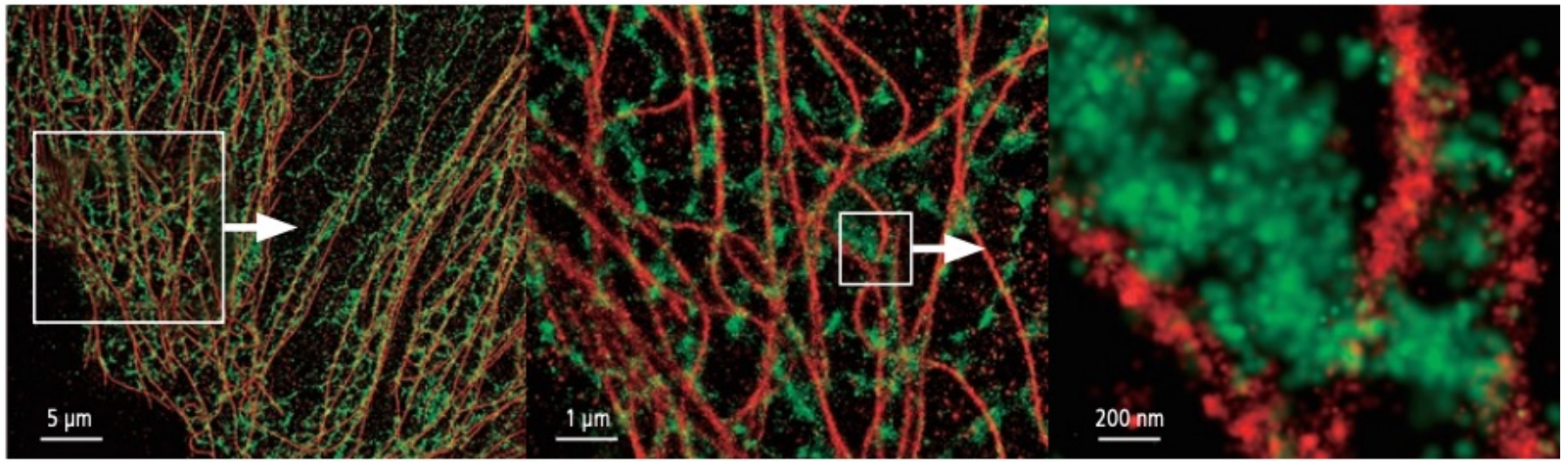


https://www.microscopyu.com/tutorials/stochastic-optical-reconstruction-microscopy-storm-imaging

STORM-Stochastic Optical Reconstruction Microscopy

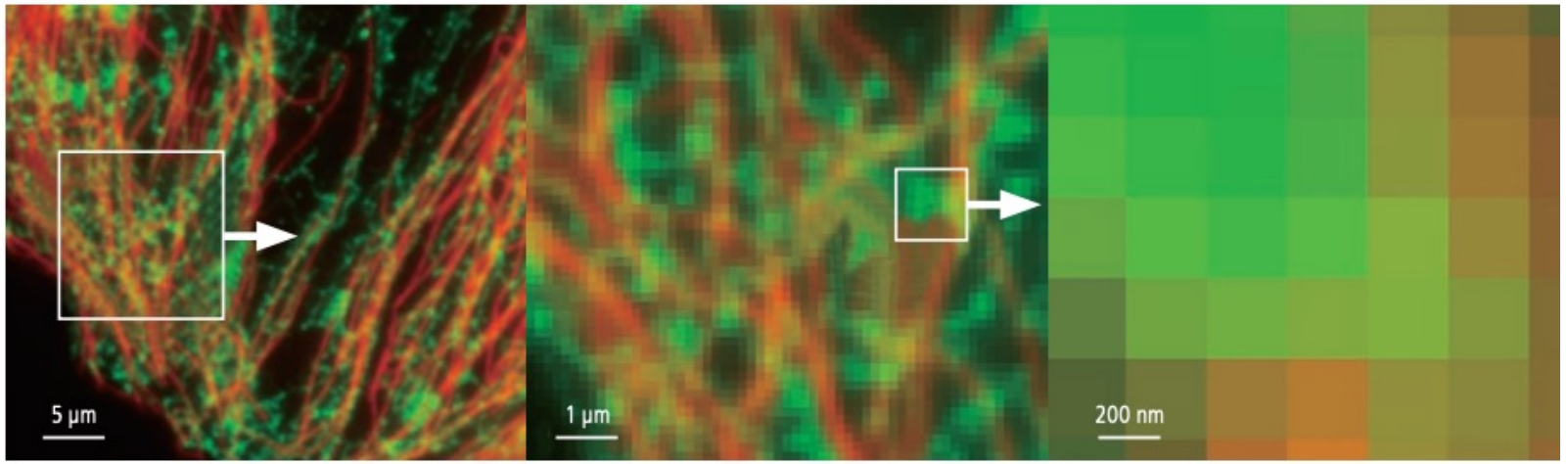


N-STORM images



50 nm resolution

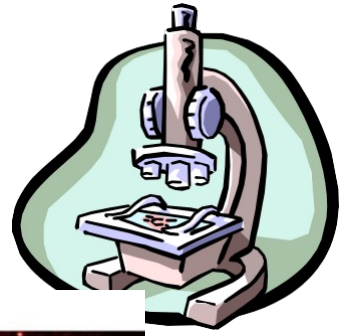
Conventional widefield images

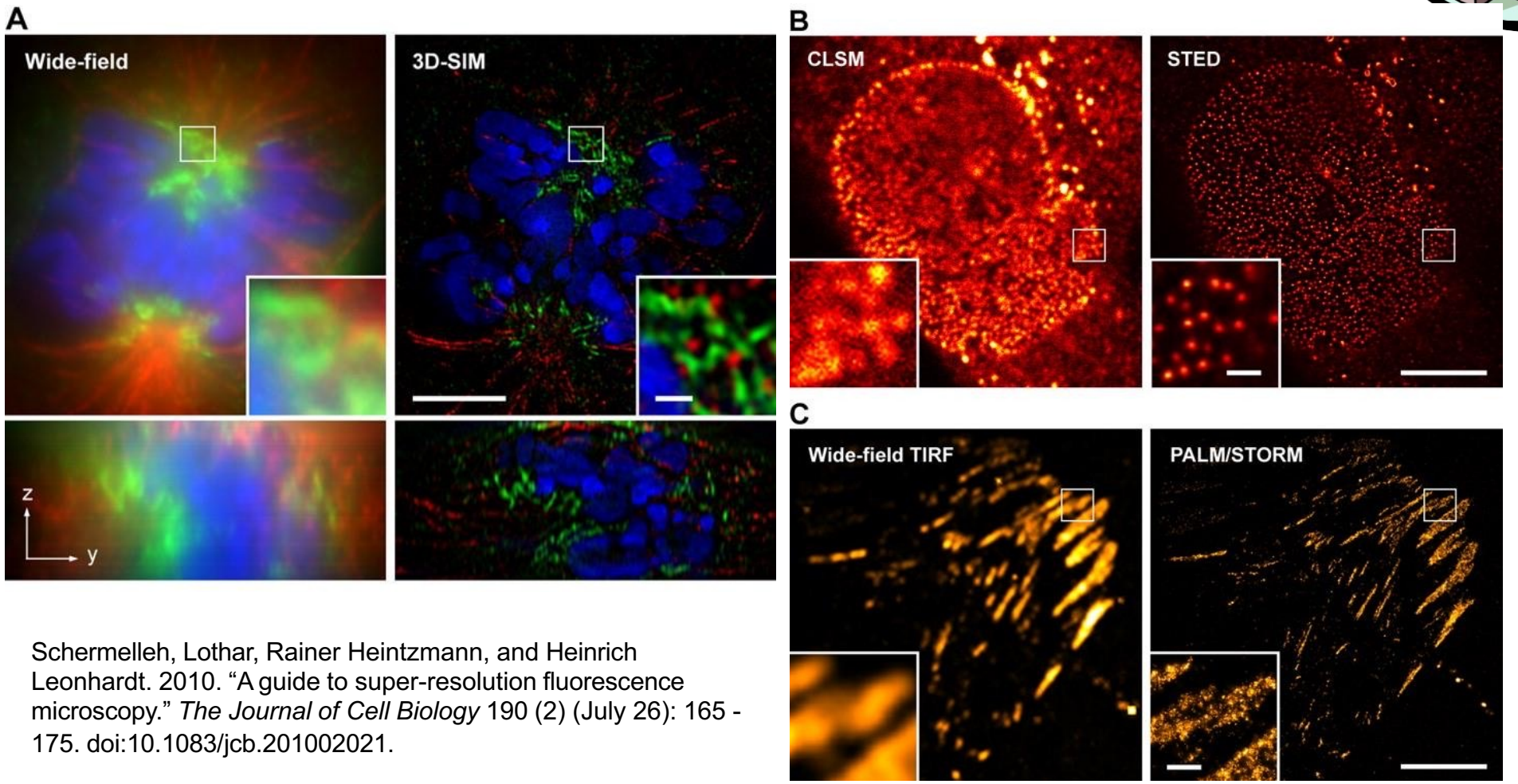


African green monkey kidney cells (BSC-1) labeled with Alexa Fluor® 647 (Tubulin) and ATTO 488 (Calreticulin)
Photos courtesy of: Dr. Michael W. Davidson, National High Magnetic Field Laboratory, Florida State University

https://downloads.microscope.healthcare.nikon.com/phase4/literature/Brochures/Super-Resolution_2CE-SCJK-3.pdf

SUPER-RESOLUTION MICROSCOPY OF BIOLOGICAL SAMPLES





Schermelleh, Lothar, Rainer Heintzmann, and Heinrich Leonhardt. 2010. “A guide to super-resolution fluorescence microscopy.” *The Journal of Cell Biology* 190 (2) (July 26): 165 - 175. doi:10.1083/jcb.201002021.