MICROSCOPIA OTTICA IN BIOLOGIA CELLULARE [675SM]

MICROSCOPY IN CELL BIOLOGY –

aa 2022/2023, 2nd semester

Aula 5A, edificio H2bis ~~14-17~~ 15-18

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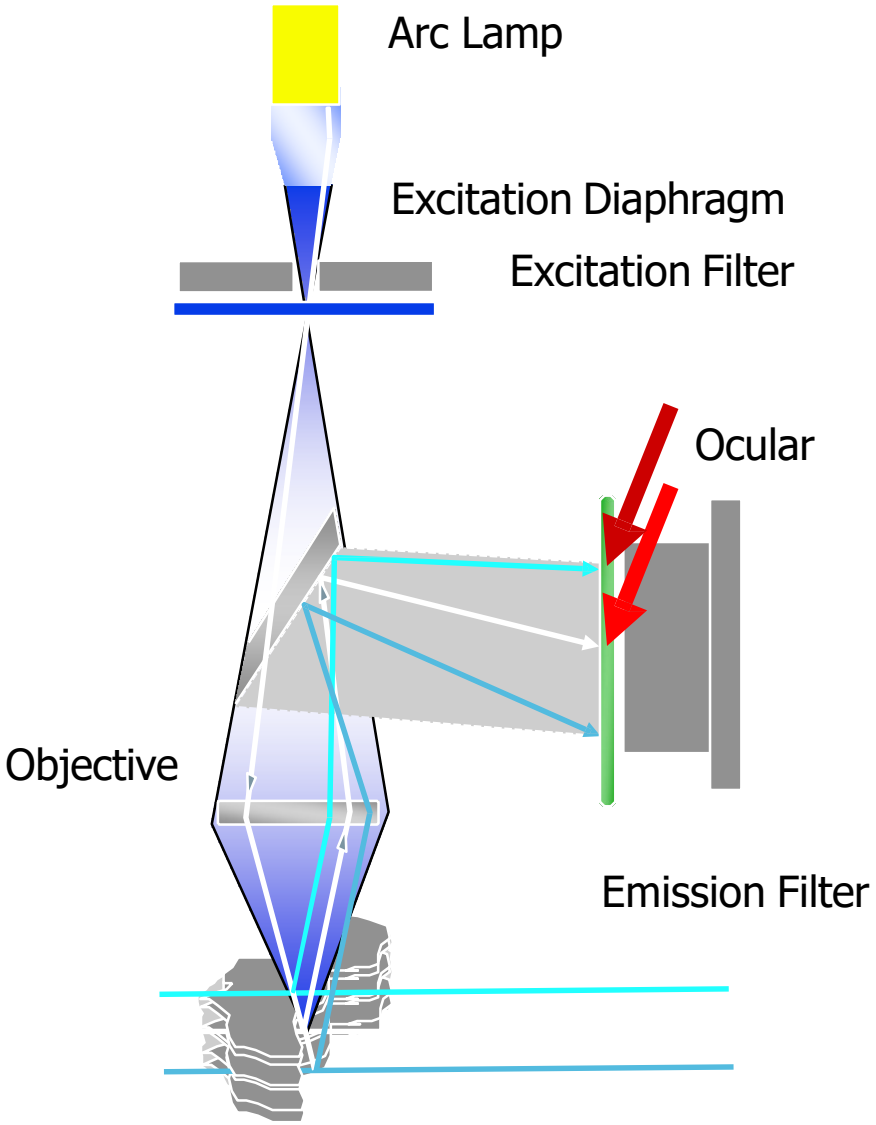
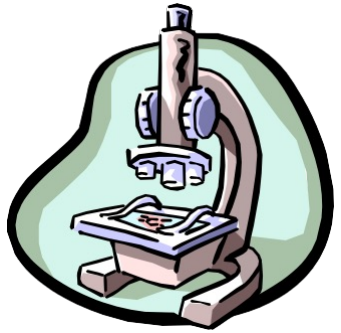
UNIVERSITÀ DEGLI STUDI DI TRIESTE
Dipartimento di Scienze della Vita

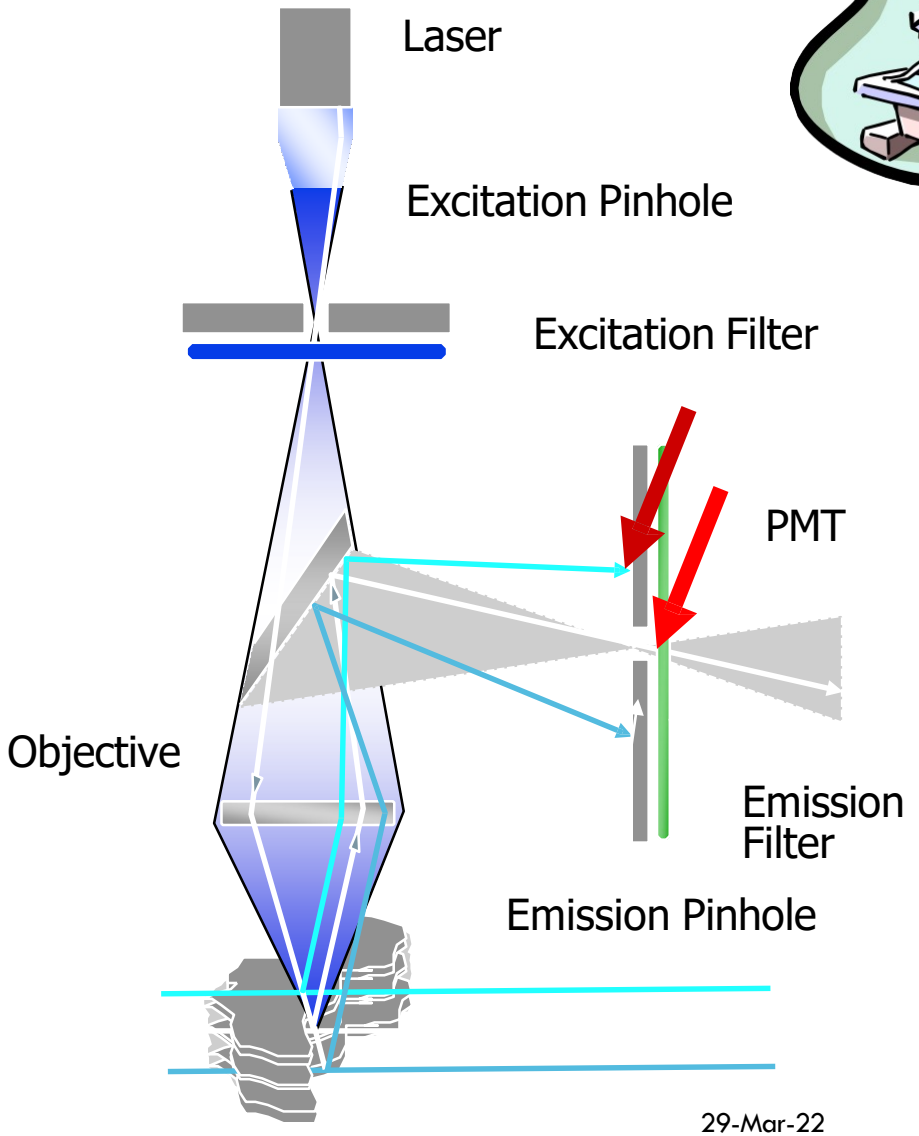
date	lesson/lab	aula	time
01/03/23	intro	aula5A, H2bis	14-15 (GF and BT), 16-17 (NS)
08/03/23	lesson1	aula5A, H2bis	15-18
15/03/23	lab1	sala microscopia F2, C1	15-18
22/03/23	lesson2	aula5A, H2bis	15-18
29/03/23	lab2	sala microscopia F2, C1	15-18
05/04/23	lesson3	aula5A, H2bis	15-18
19/04/23	lesson4	aula5A, H2bis	15-18
26/4/23	lesson5	aula5A, H2bis	15-18
3/5/23	lab3	CIMA center	15-18
10/5/23	lab4	aula5A, H2bis	15-18

Fluorescence Microscope

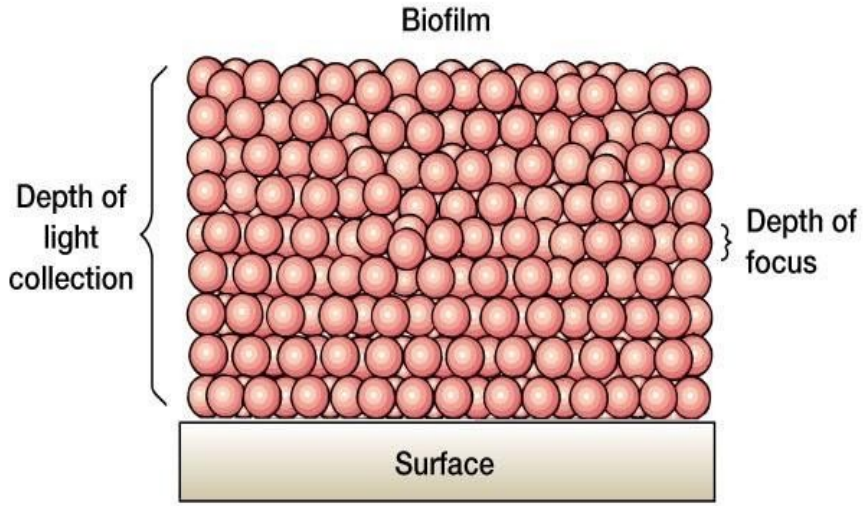


Confocal Fluorescence Microscope





Conventional light microscope



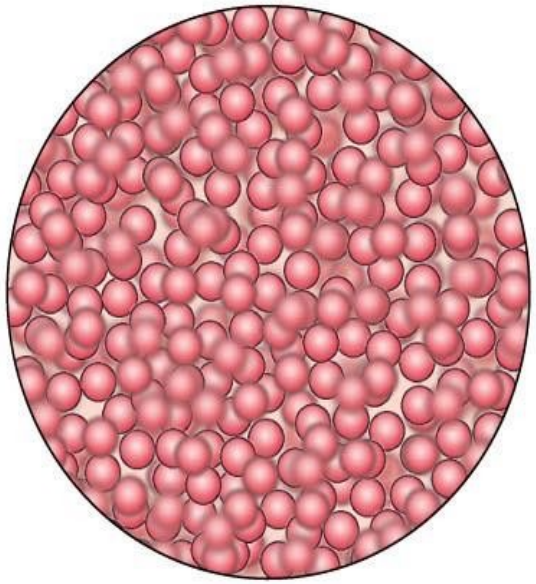
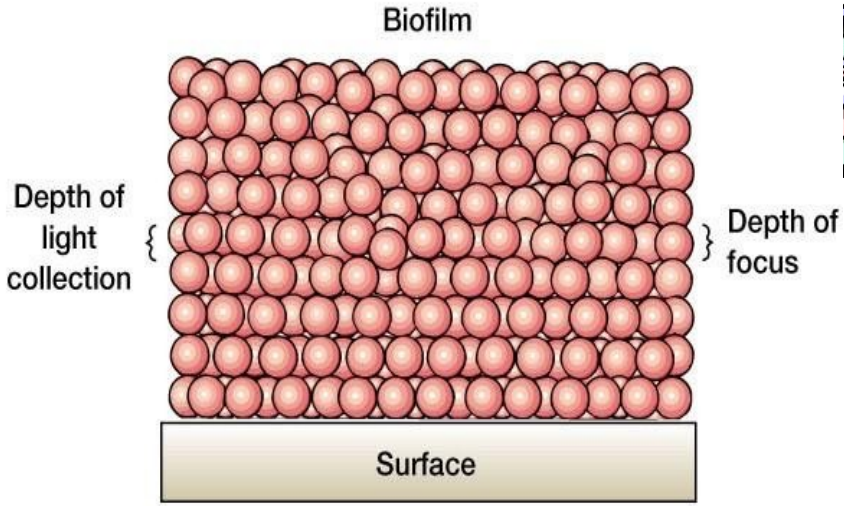


Image in field of view

(a)

Confocal scanning laser microscope



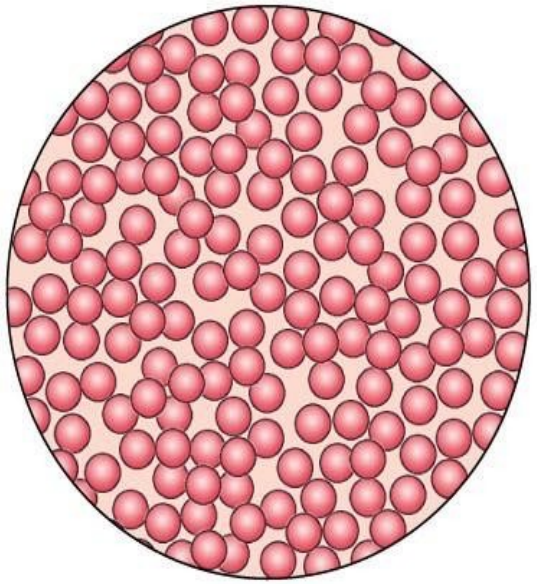
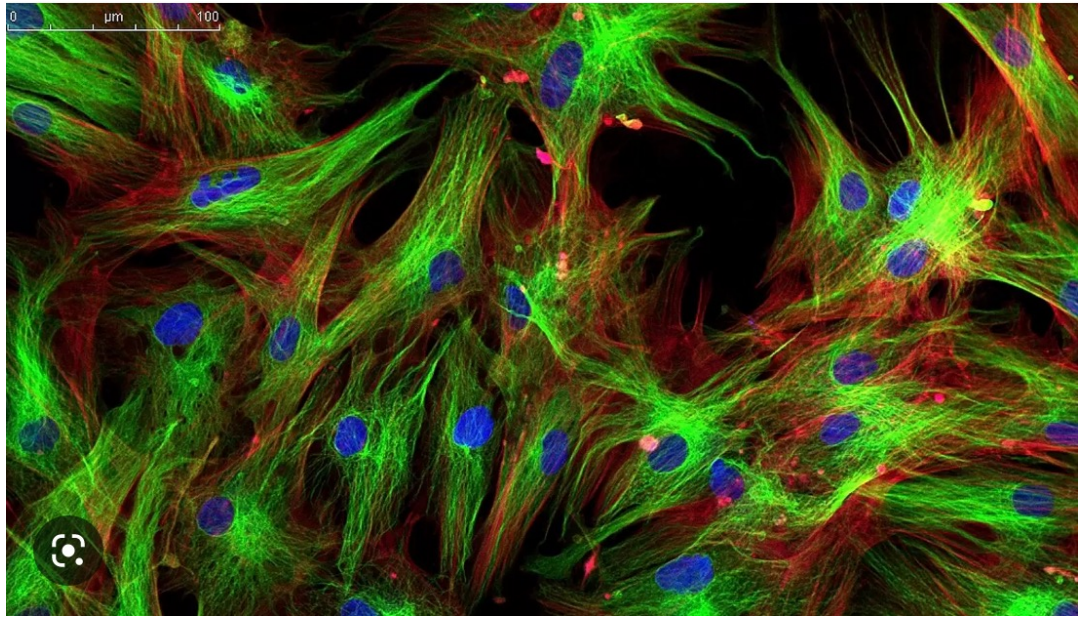
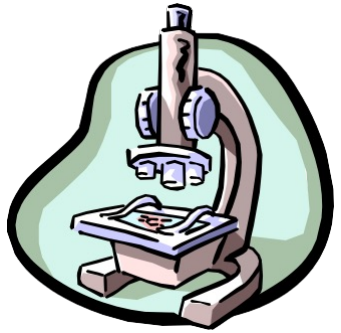
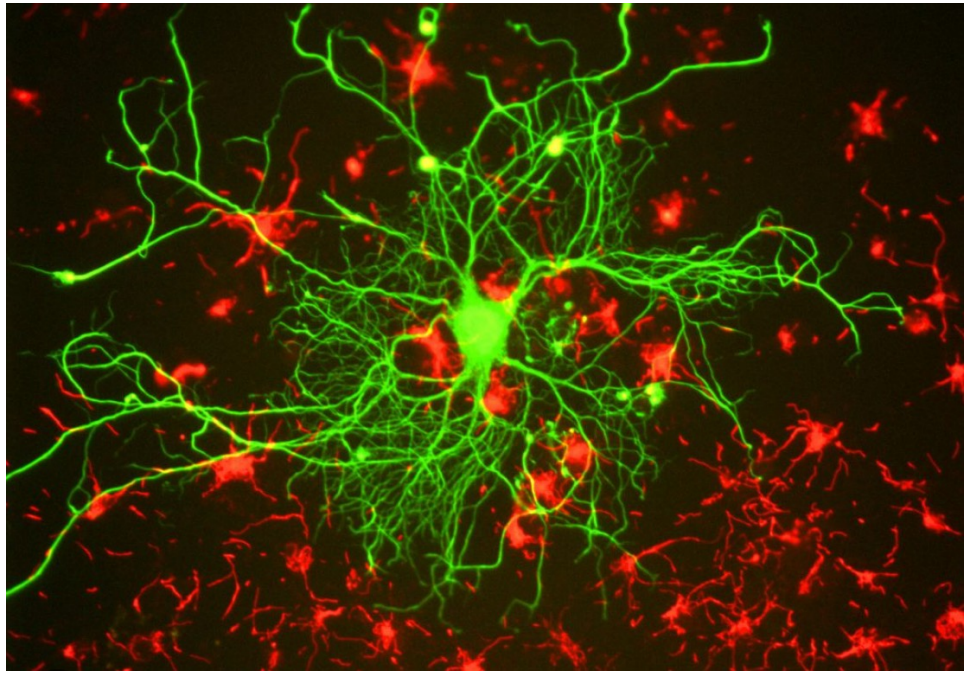


Image in field of view

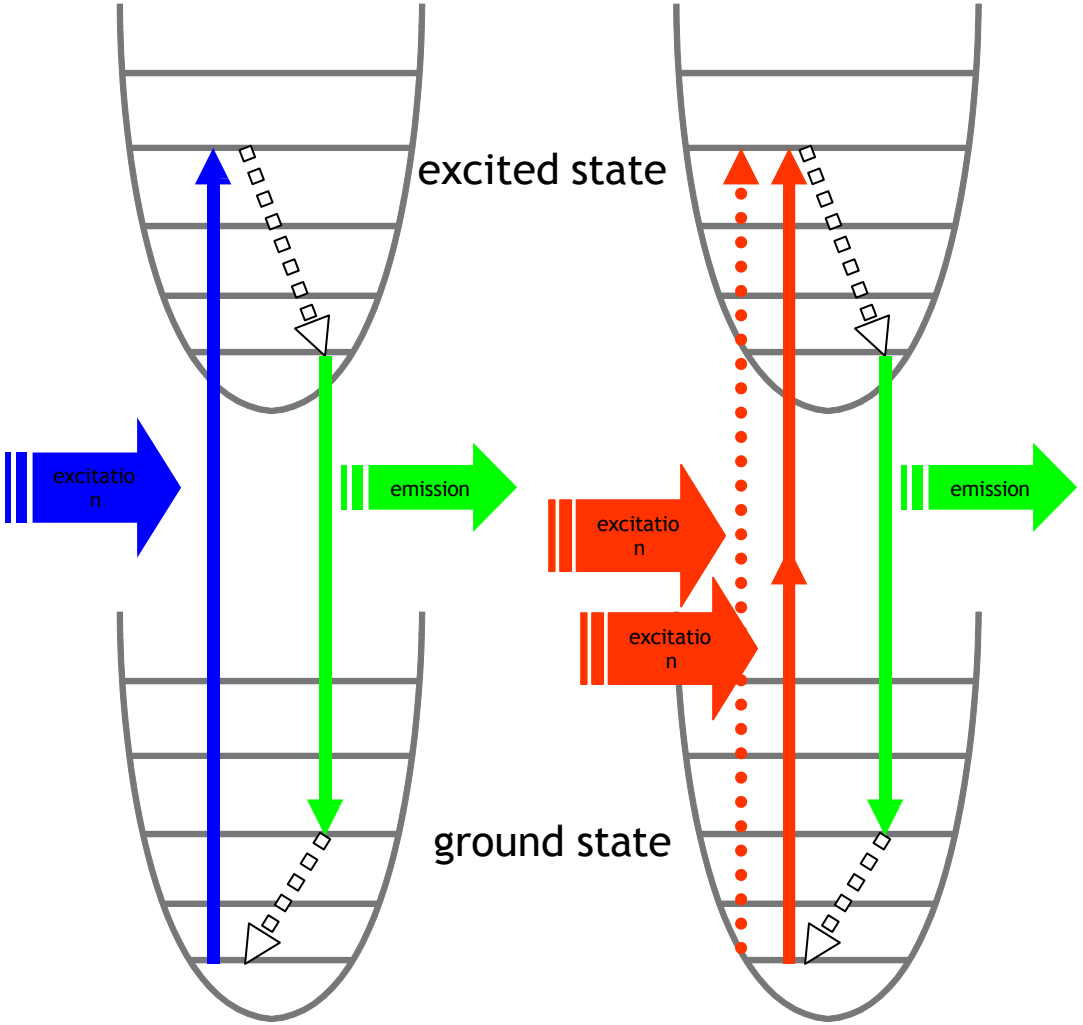
(b)

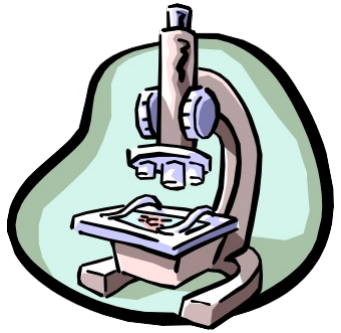

0
µm
100





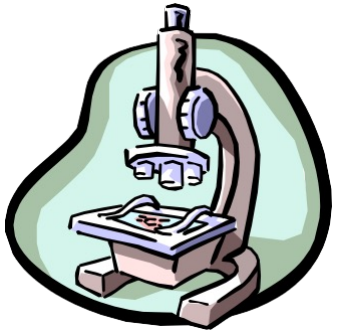
2-PHOTON EXCITATION

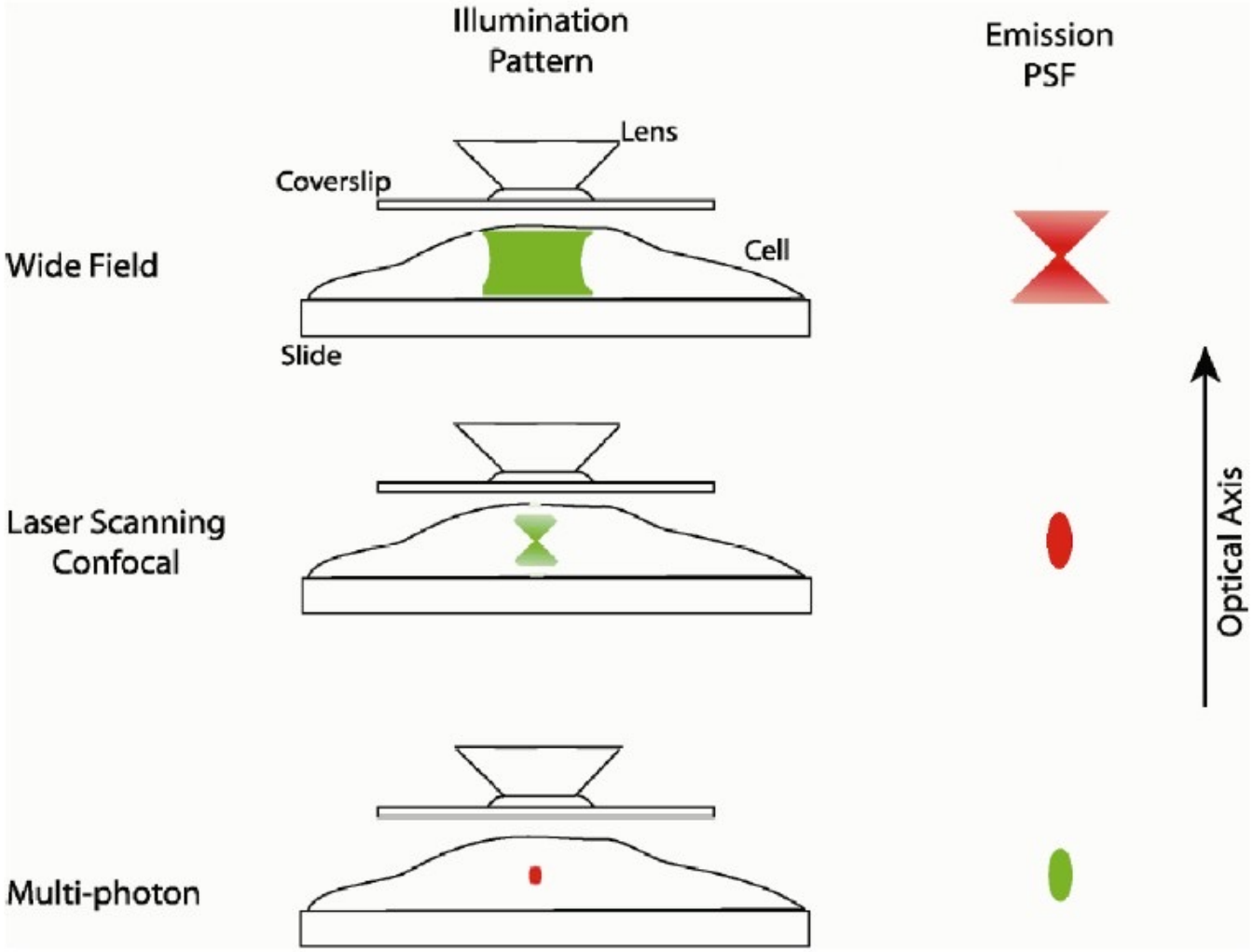




Two-photon excitation occurs through the absorption of two lower energy

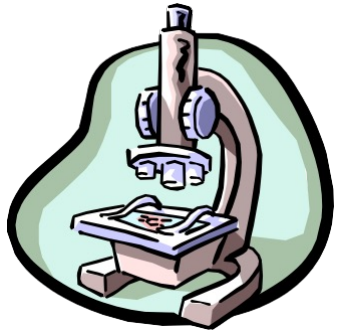
WIDE-FIELD VS. CONFOCAL VS. 2-PHOTON



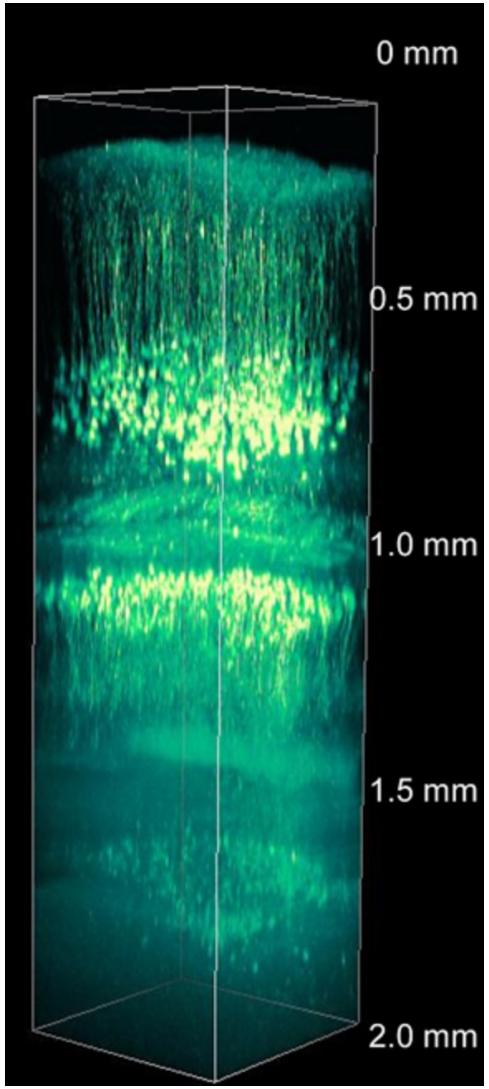


Drawing by P. D. Andrews, I. S. Harper and J. R. Swedlow

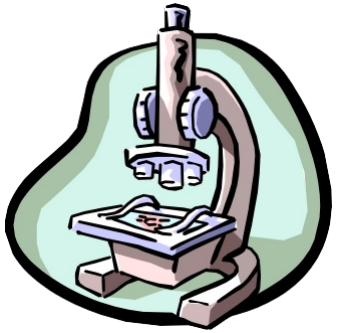
FAR FIELD: TWO-PHOTON

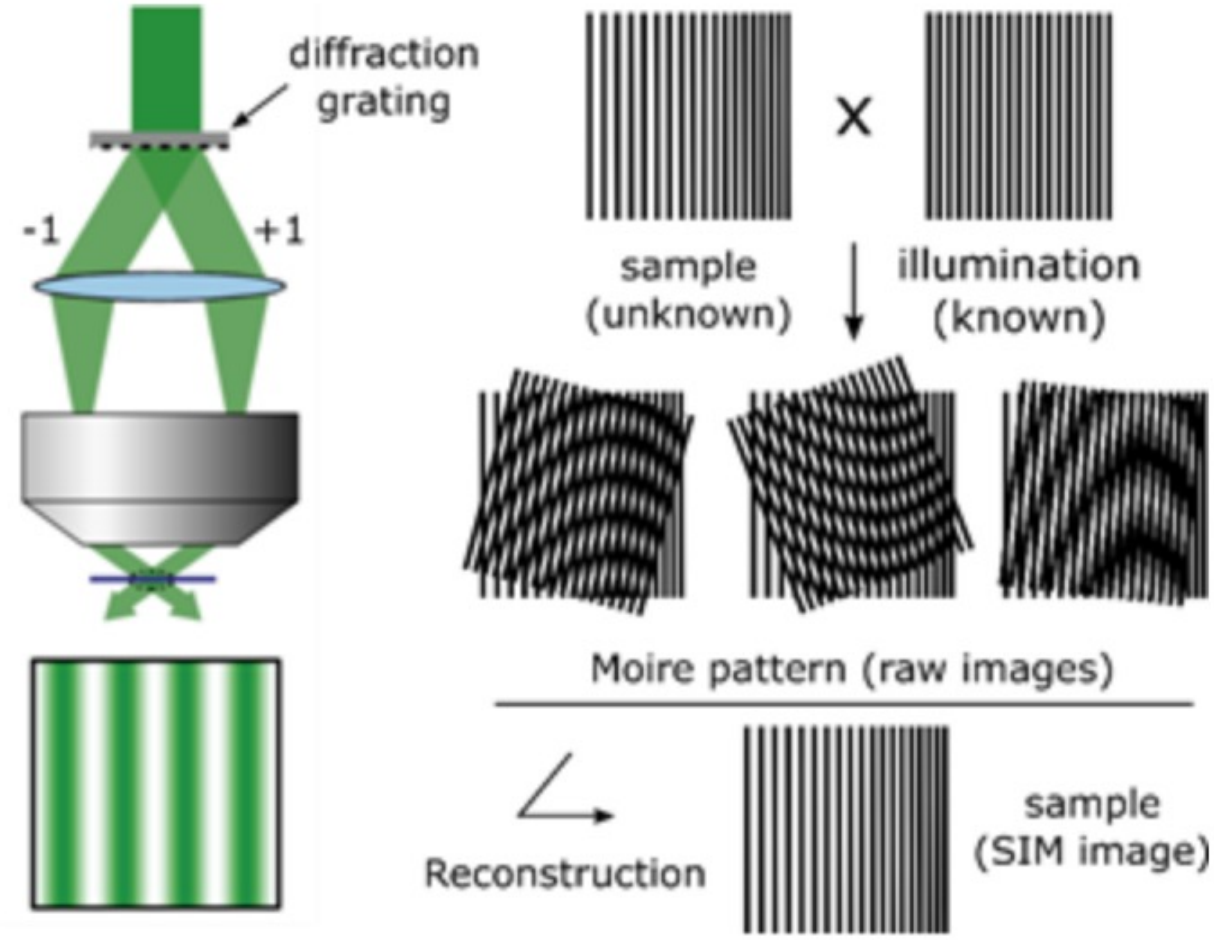


- Non-linear 2-photon excitation and pinhole detection decrease SPF beyond classical limits
- $2^{1/2}$ improvement in resolution
- High penetration depth (IR wavelengths for stimulation)



STRUCTURED-ILLUMINATION MICROPSCOPY (SIM)

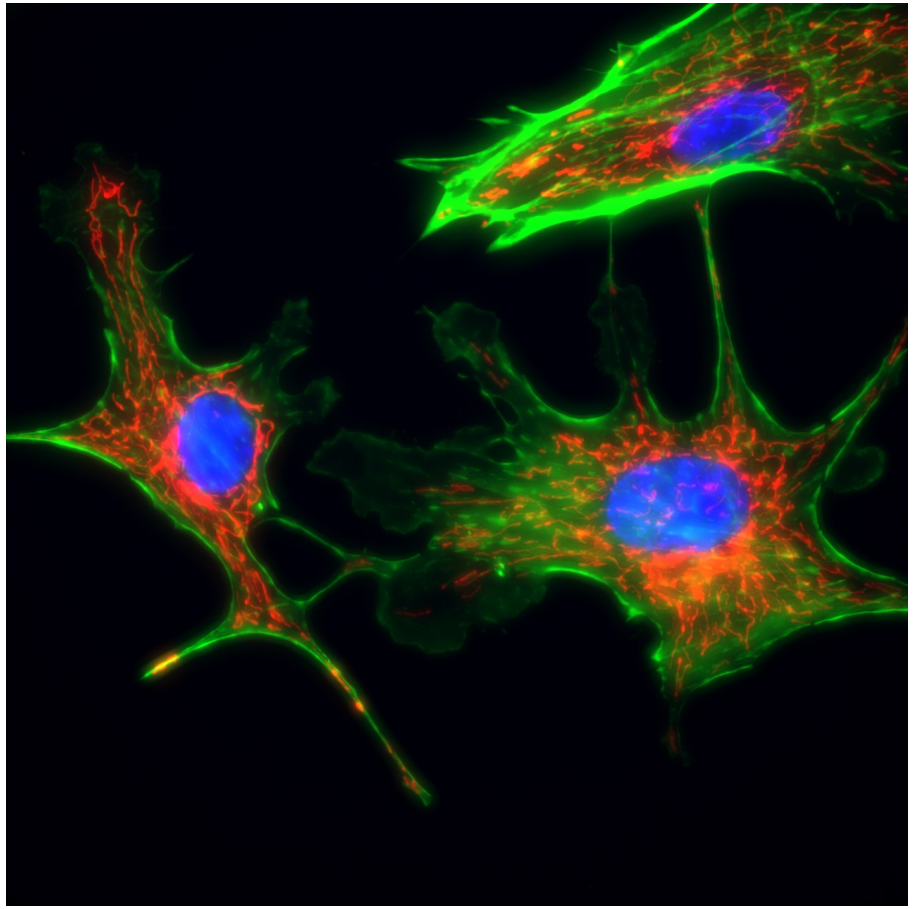


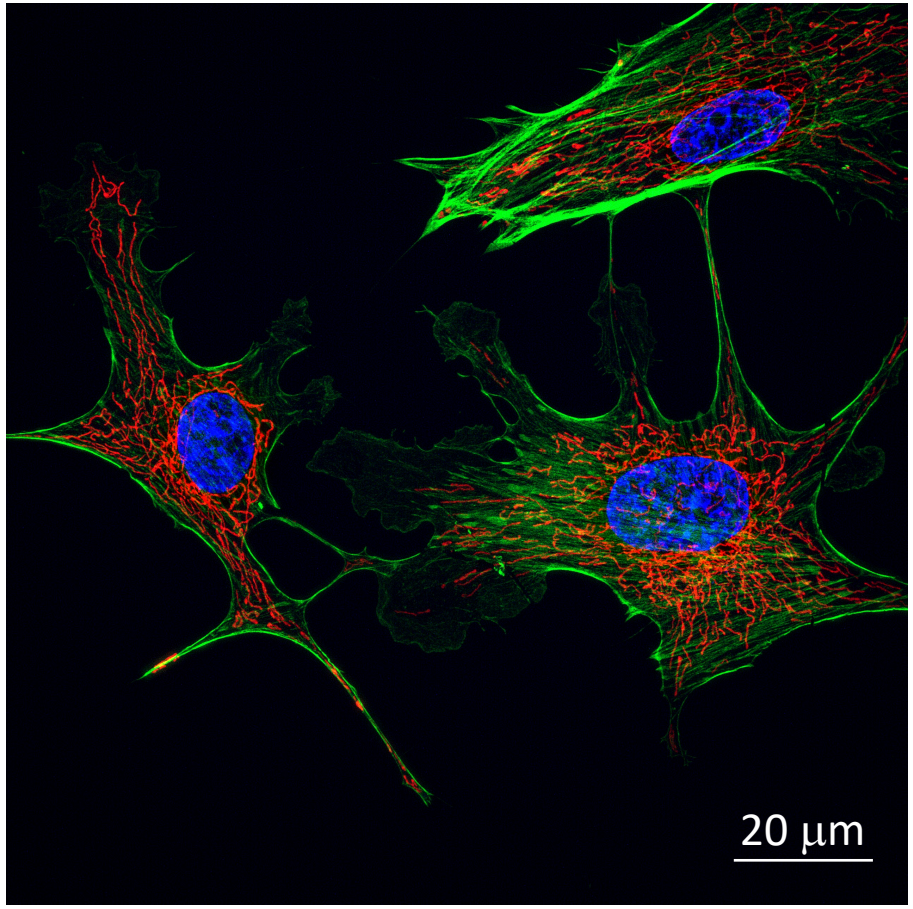


- Structural illumination for enhancing spatial resolution
- 100 nm resolution possible
- Software analysis of Moiré fringe effects

Advanced microscopy techniques

SIM-Structural illumination microscopy





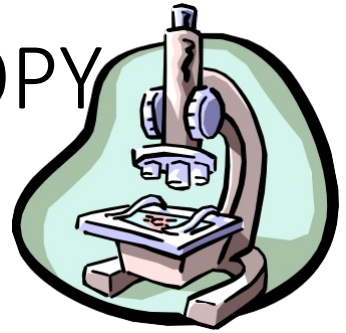
Advanced microscopy techniques

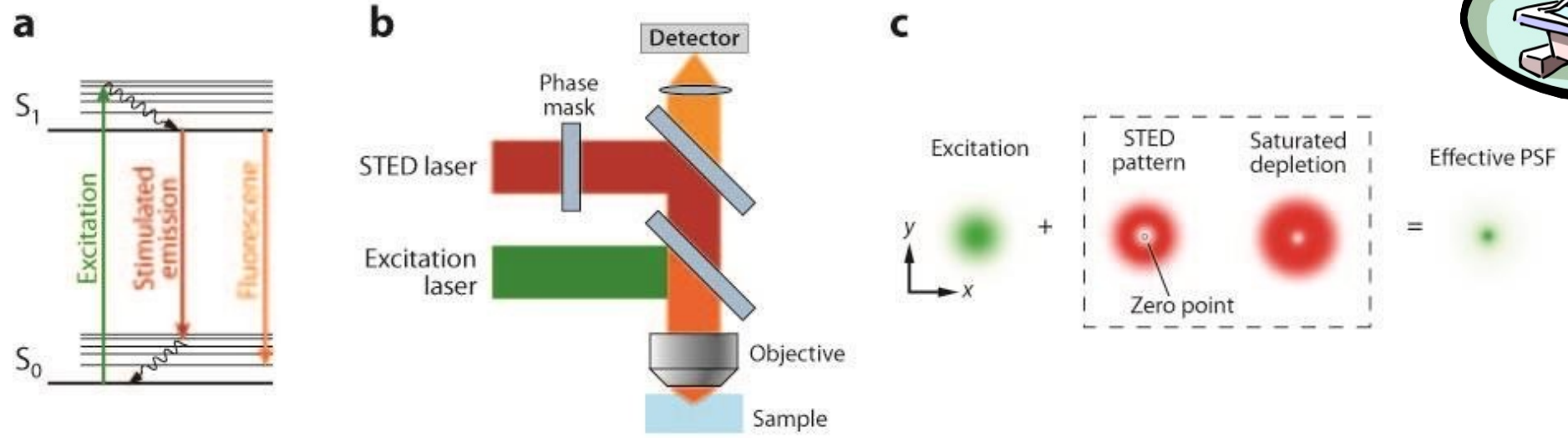
CIMA SIM superresolution vs MP vs confocal

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)

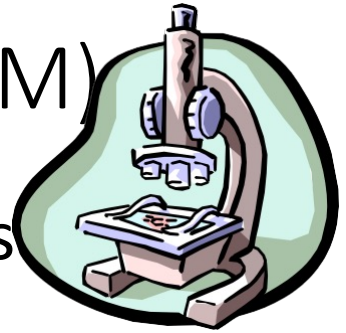
STIMULATED EMISSION DEPLETION (STED) MICROSCOPY





(*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).

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.);

STORM-Stochastic Optical Reconstruction Microscopy

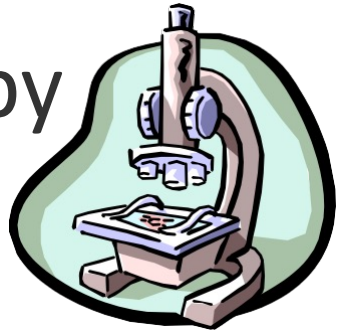
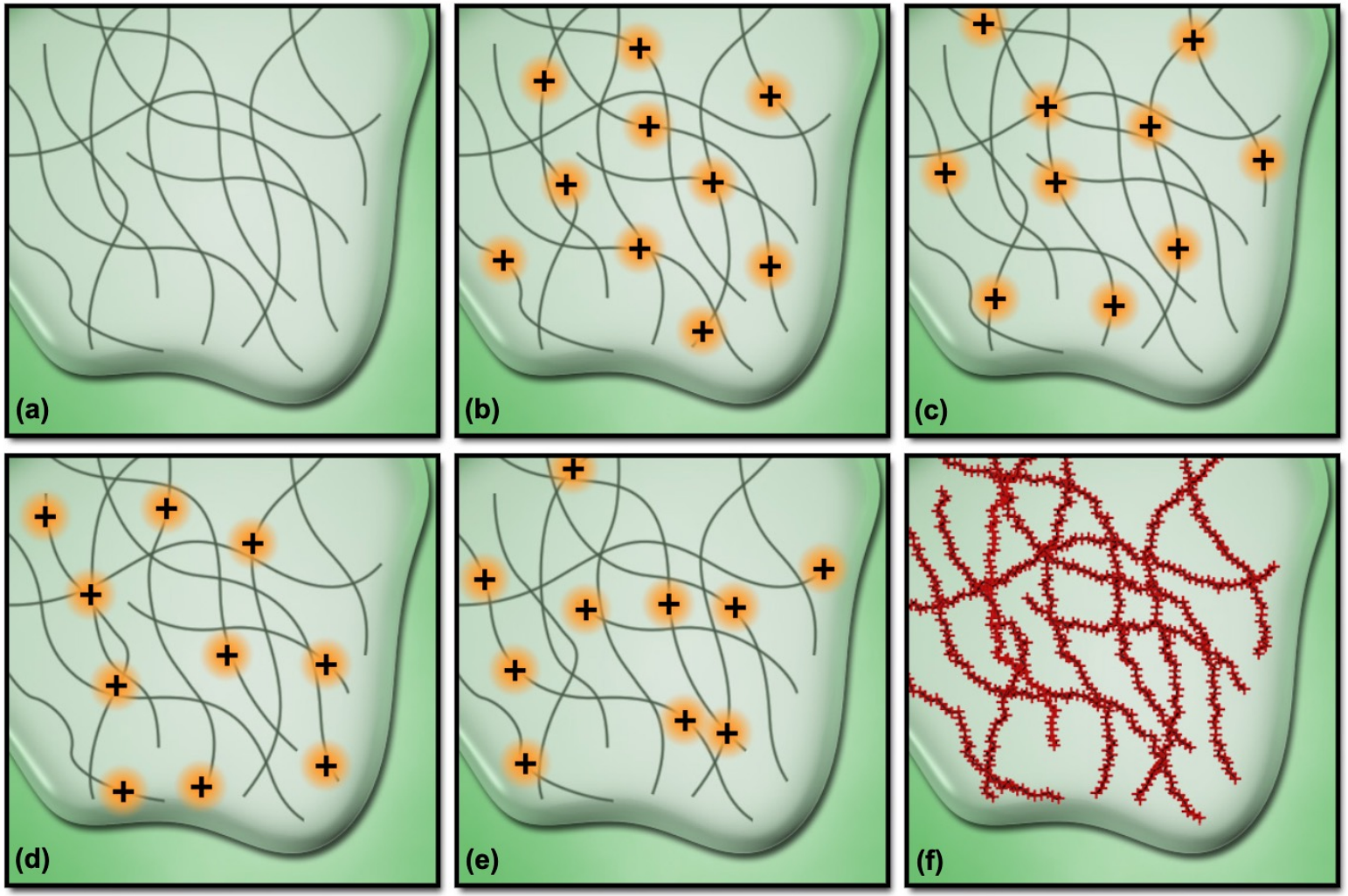
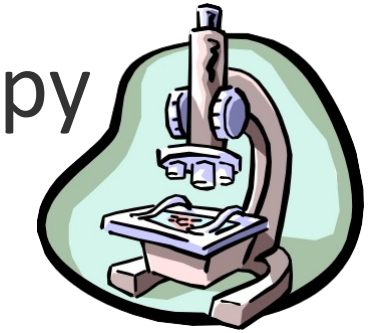


Figure 1 - Basic Principles of STORM Superresolution Imaging

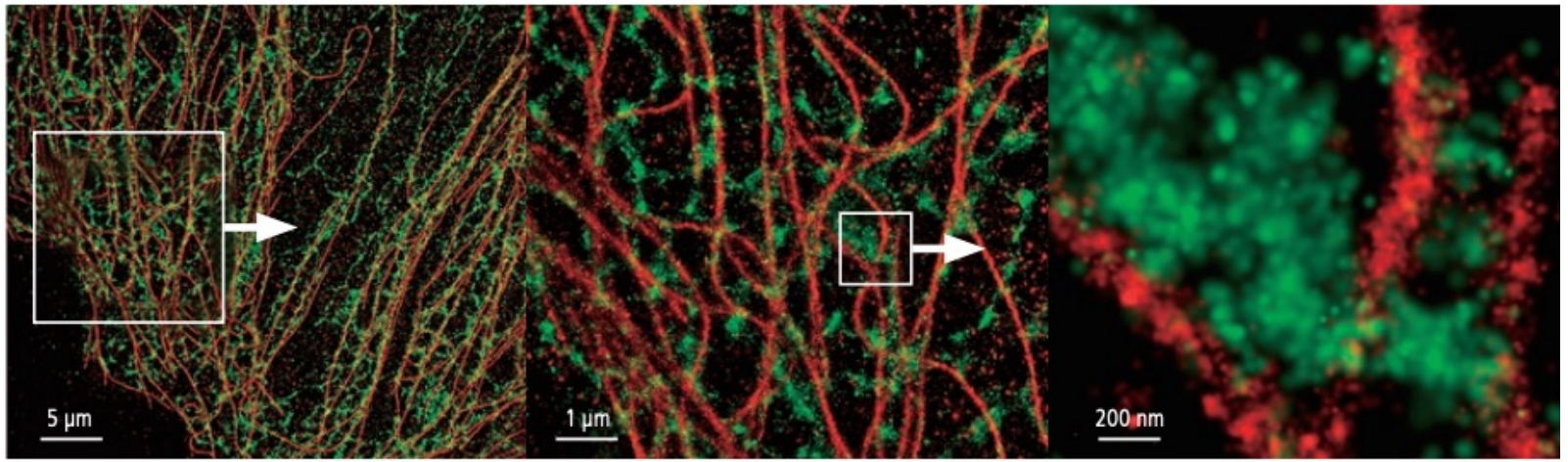


https://www.microscopyu.com/tutorials/stochastic-optical-reconstruction-microscopy-storm-imaging

STORM-Stochastic Optical Reconstruction Microscopy

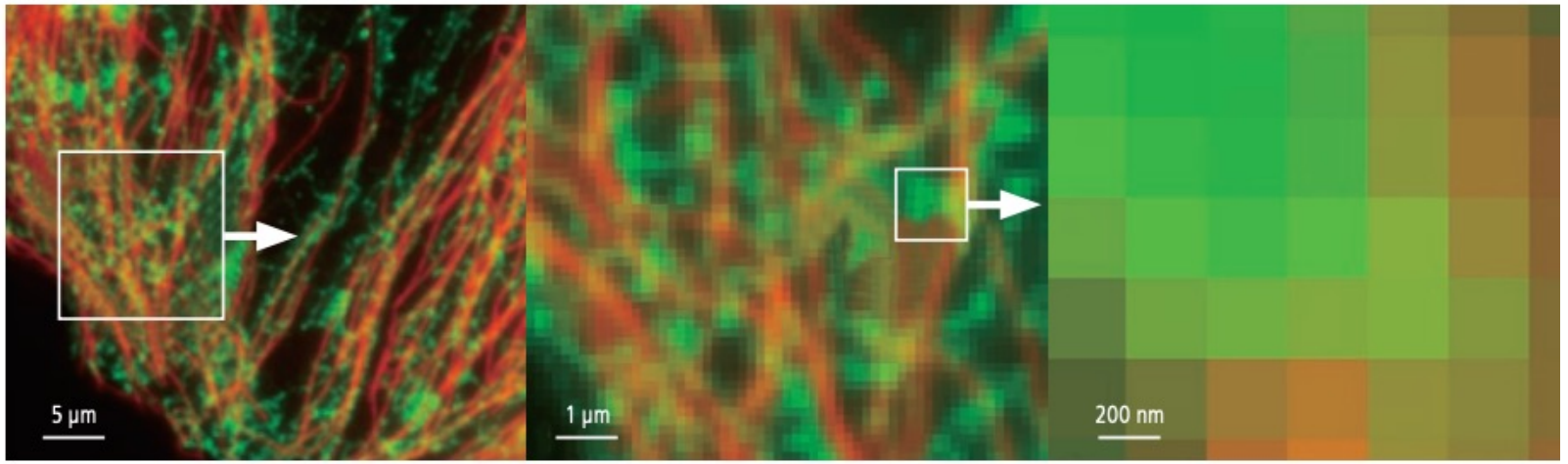


N-STORM images



50 nm resolution

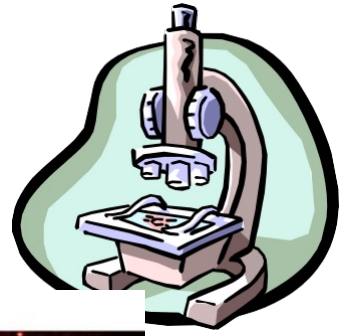
Conventional widefield images

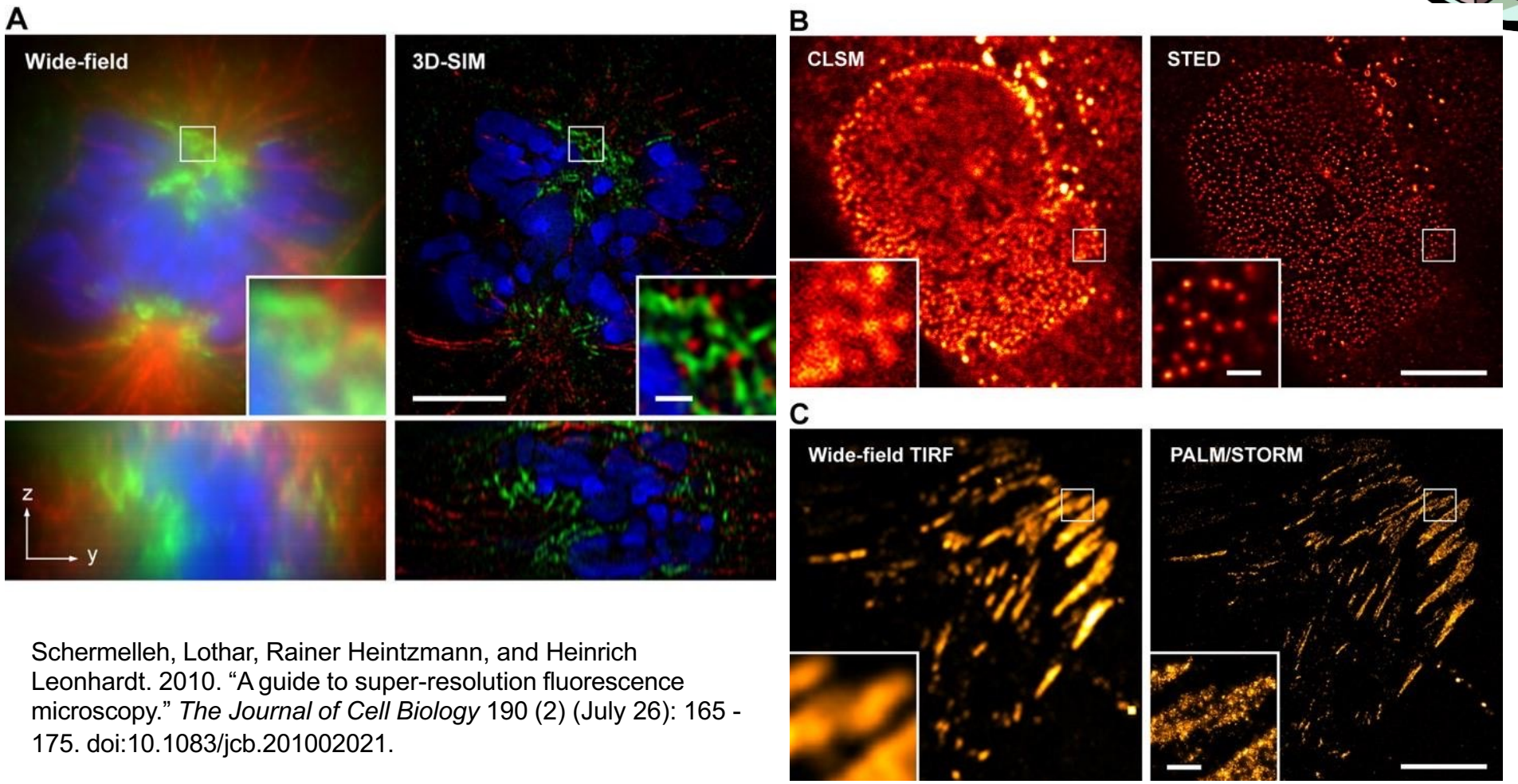


African green monkey kidney cells (BSC-1) labeled with Alexa Fluor® 647 (Tubulin) and ATTO 488 (Calreticulin)
Photos courtesy of: Dr. Michael W. Davidson, National High Magnetic Field Laboratory, Florida State University

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

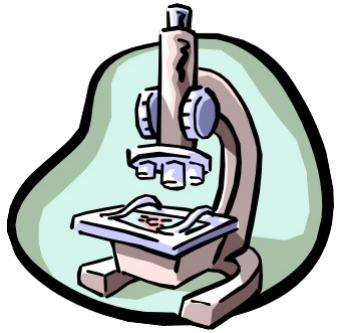
SUPER-RESOLUTION MICROSCOPY OF BIOLOGICAL SAMPLES

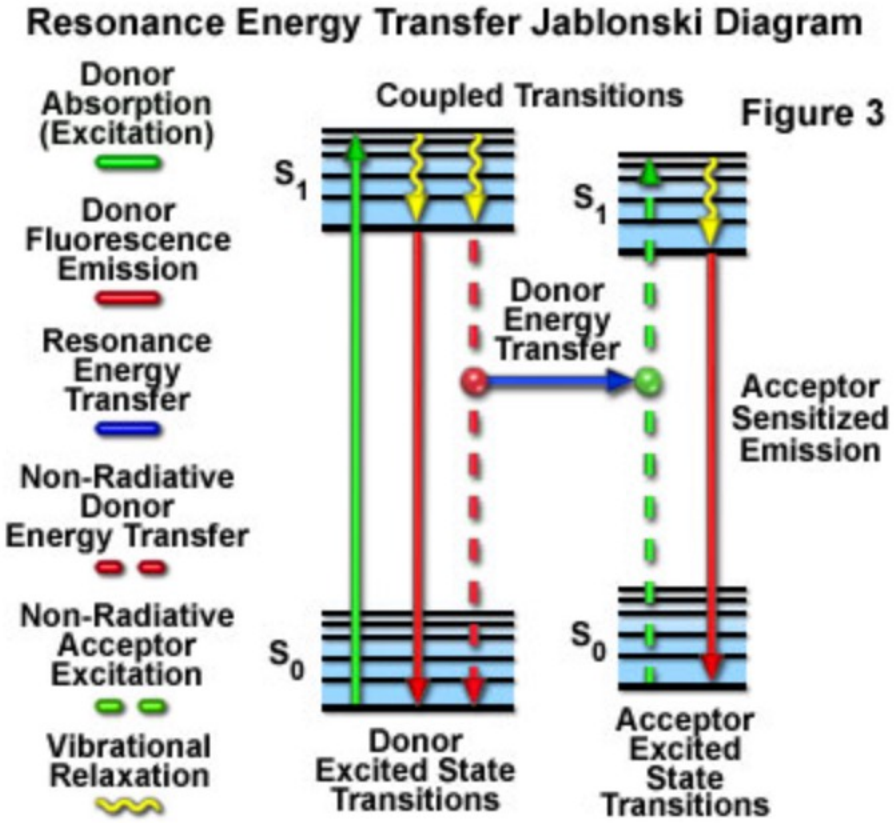


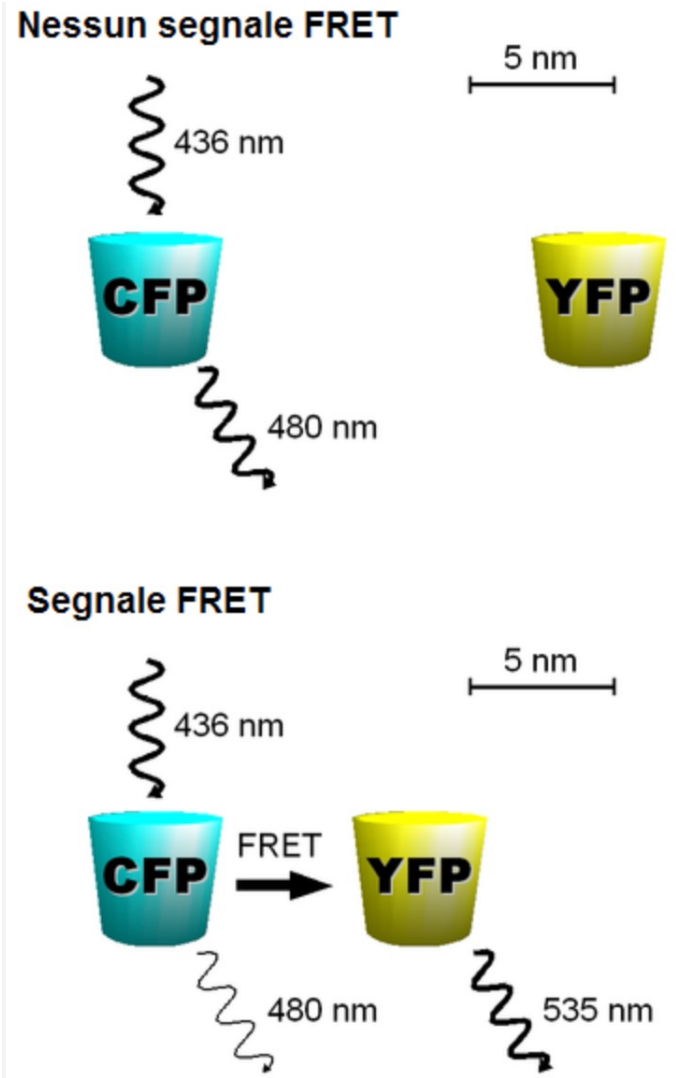


Schermelleh, Lothar, Rainer Heintzmann, and Heinrich Leonhardt. 2010. “A guide to super-resolution fluorescence microscopy.” *The Journal of Cell Biology* 190 (2) (July 26): 165 - 175. doi:10.1083/jcb.201002021.

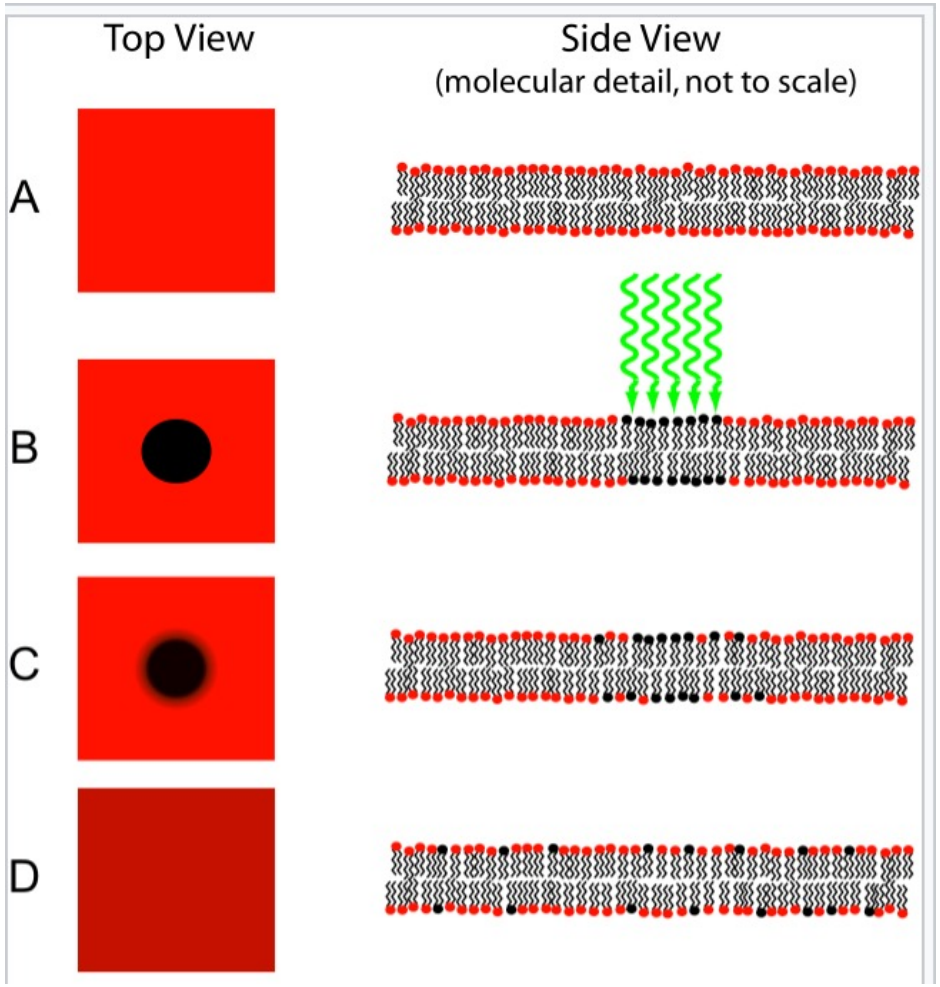
Fluorescence Resonance Energy Transfer (FRET) Microscopy

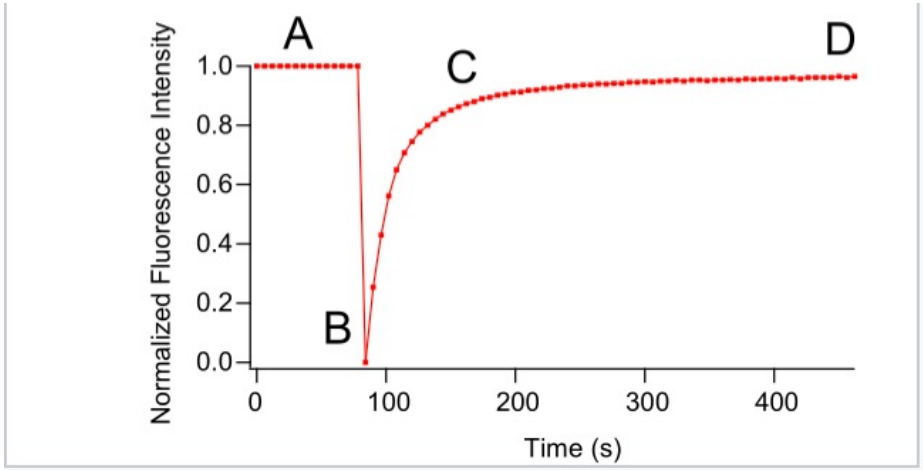




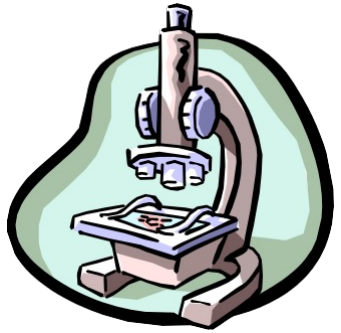


Fluorescence recovery after photobleaching

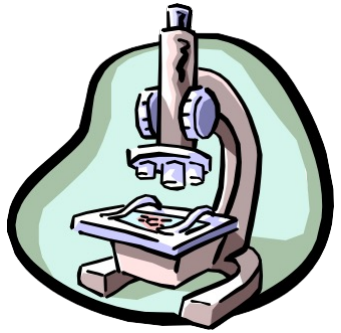




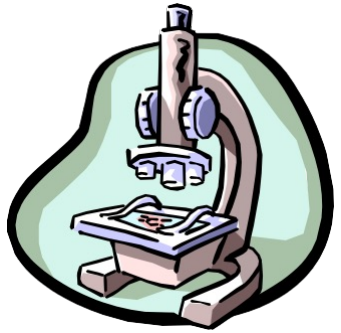
Principle of FRAP A) The bilayer is uniformly labeled with a fluorescent tag B) This label is selectively photobleached by a small (~30 micrometre) fast light pulse C) The intensity within this bleached area is monitored as the bleached dye diffuses out and new dye diffuses in D) Eventually uniform intensity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



COMPARISON

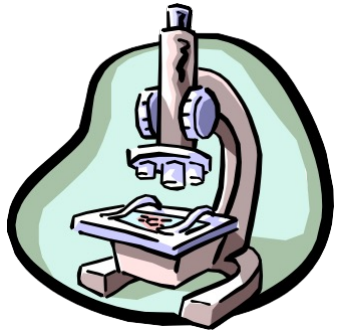


method	excitation	detection	sectioning	use
Wide field	Whole sample	Whole sample	No sectioning	Simple fluorescence samples
confocal	Whole sample	One z-plane	350-500nm	High contrast images, optical sectioning
2-Photon	One z-plane	One z-plane	500-700nm	Deep tissue imaging, optical sectioning
FRET				Protein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



Live imaging microscopy

LIVE IMAGING



- Allows study of dynamic processes in real time in stead of snap-shot of one timepoint

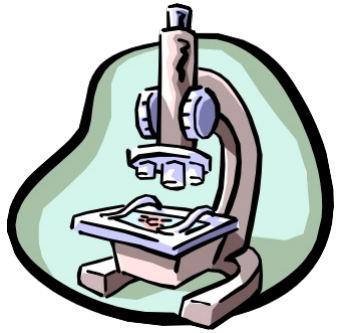
- Results in movie instead of single image

- Dynamic processes of subcellular compartments, cells, cellular networks, organs, entire animals

Examples for dynamic processes of interest

- Cell migration
- Morphological changes
- Physiological parameters (ion concentrations, vesicle dynamics)

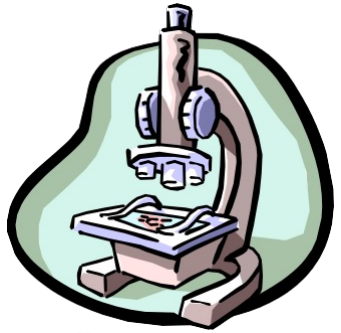
LIVE IMAGING - CHALLENGES



Keeping cells alive

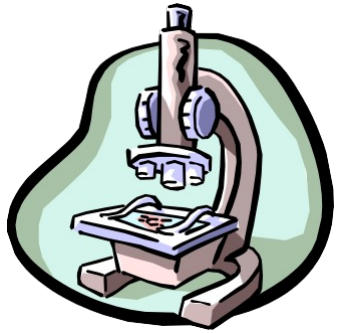
- physiological environment
    - Temperature
    - Ionic and osmotic environment
    - Oxigenation
    - pH (ev. CO_2 concentration for carbonate buffer)
- Mechanical stress
- Phototoxicity
- Chemical toxicity of dyes

LIVE IMAGING - INCUBATION

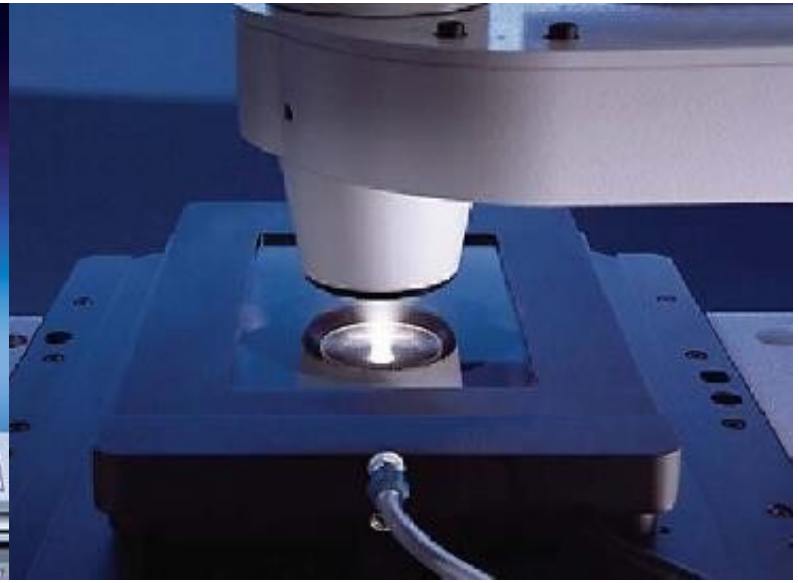


Cell Health Parameter	Short-Term Study	Longer-Term Study
pH	HEPES Buffer	CO_2 Incubator
Temperature	Stage Warmers	Objective Lens Warmers Cell chambers with integrated heating elements Enclose microscope within a heated box
Humidity	Open cell chambers	Tightly sealed cell chambers
Oxygenation	Large volume of media	Media changes during study Large media volume
Osmolarity	Sealed chamber	Enclose system within a humidified chamber

LIVE IMAGING - INCUBATION



Incubation chambers can surround the microscope stand or just the cell dish. Both can maintain cells at the correct temperature for long periods of time.



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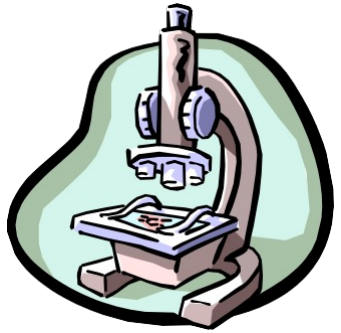

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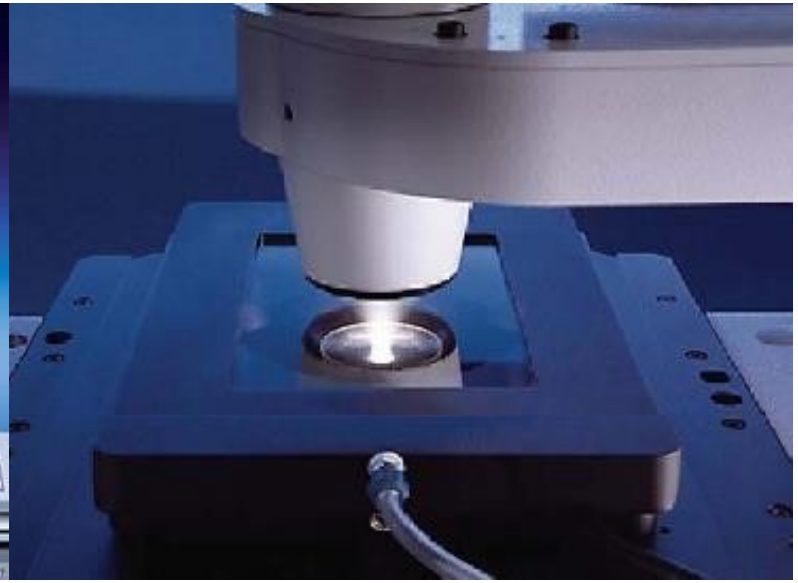
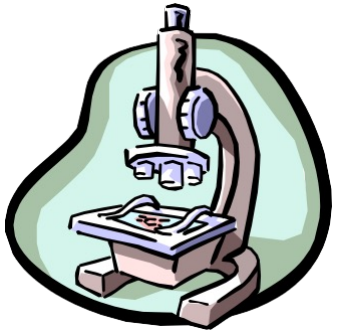


INCUBATOR-MICROSCOPE

LCV110 Incubator Fluorescence Microscope

OLYMPUS
Your Vision, Our Future

SMALL FORMAT CHAMBER



CONTRASTING TECHNIQUES

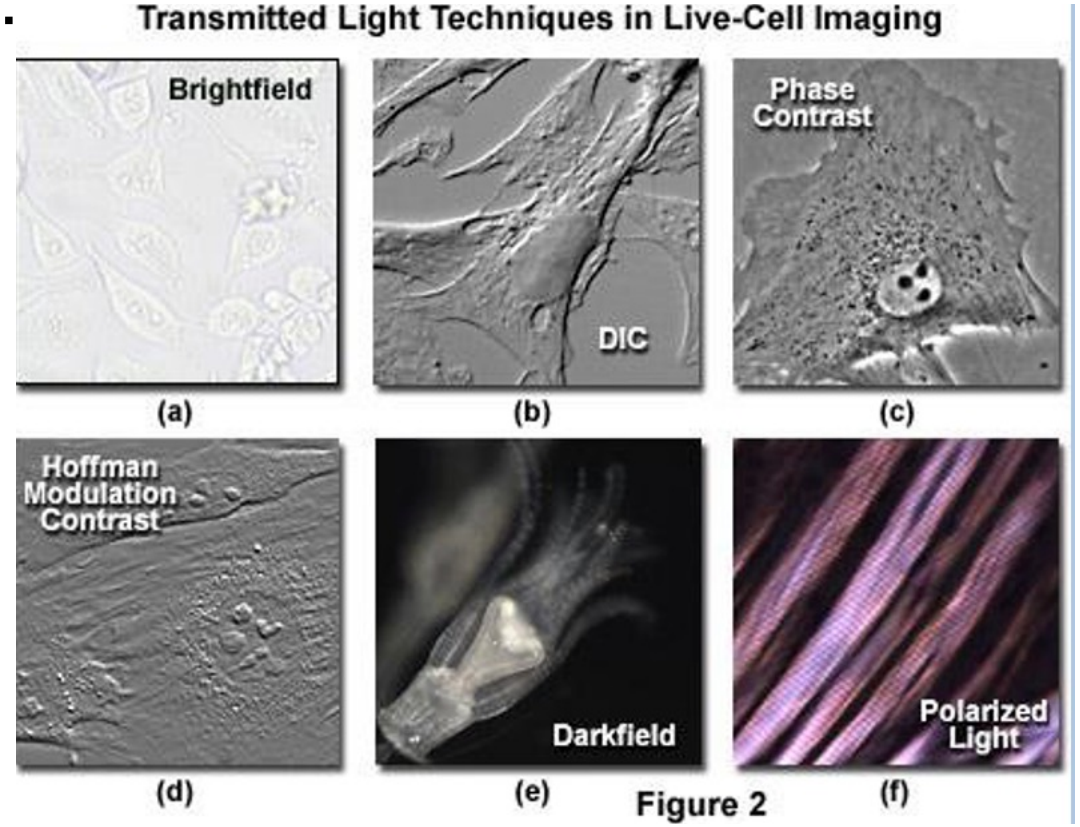
Optical Techniques to Enhance contrast:

Differential Interference Contrast (DIC)
Phase Contrast

Darkfield

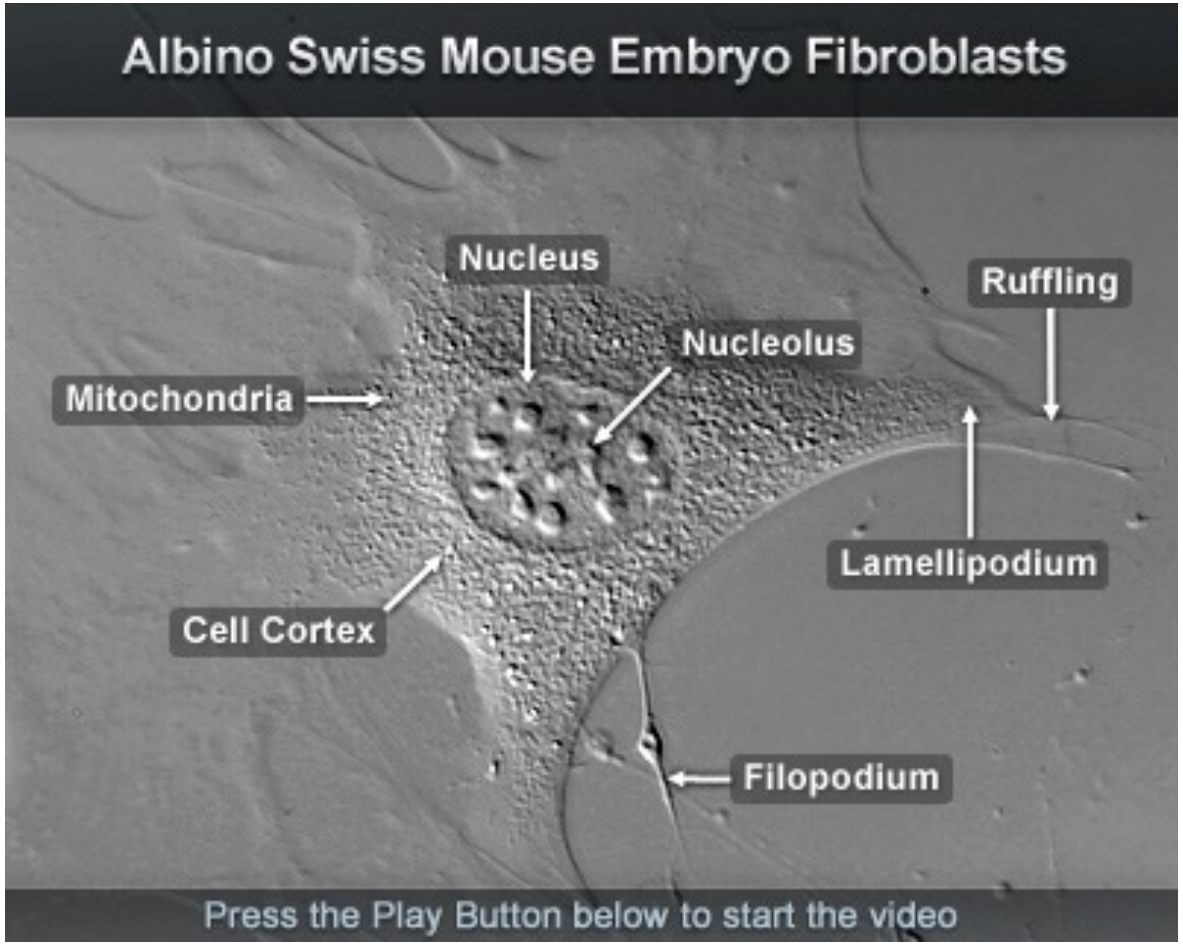
Polarized Light

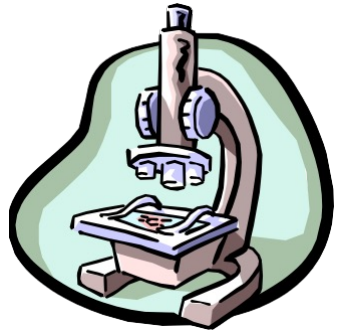
Fluorescence



CONTRASTING TECHNIQUES

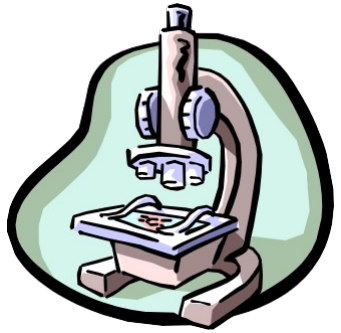
https://www.microscopyu.com/gallery-images/albino-swiss-mouse-embryo-fibroblasts-3t3-line





DIC

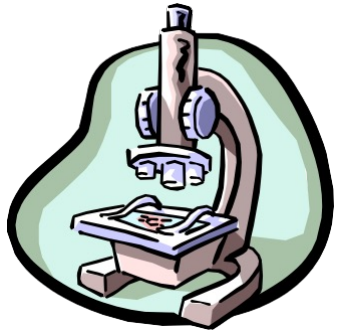
CONTRASTING TECHNIQUES



https://youtu.be/iKsUiyll2BM

Phase contrast

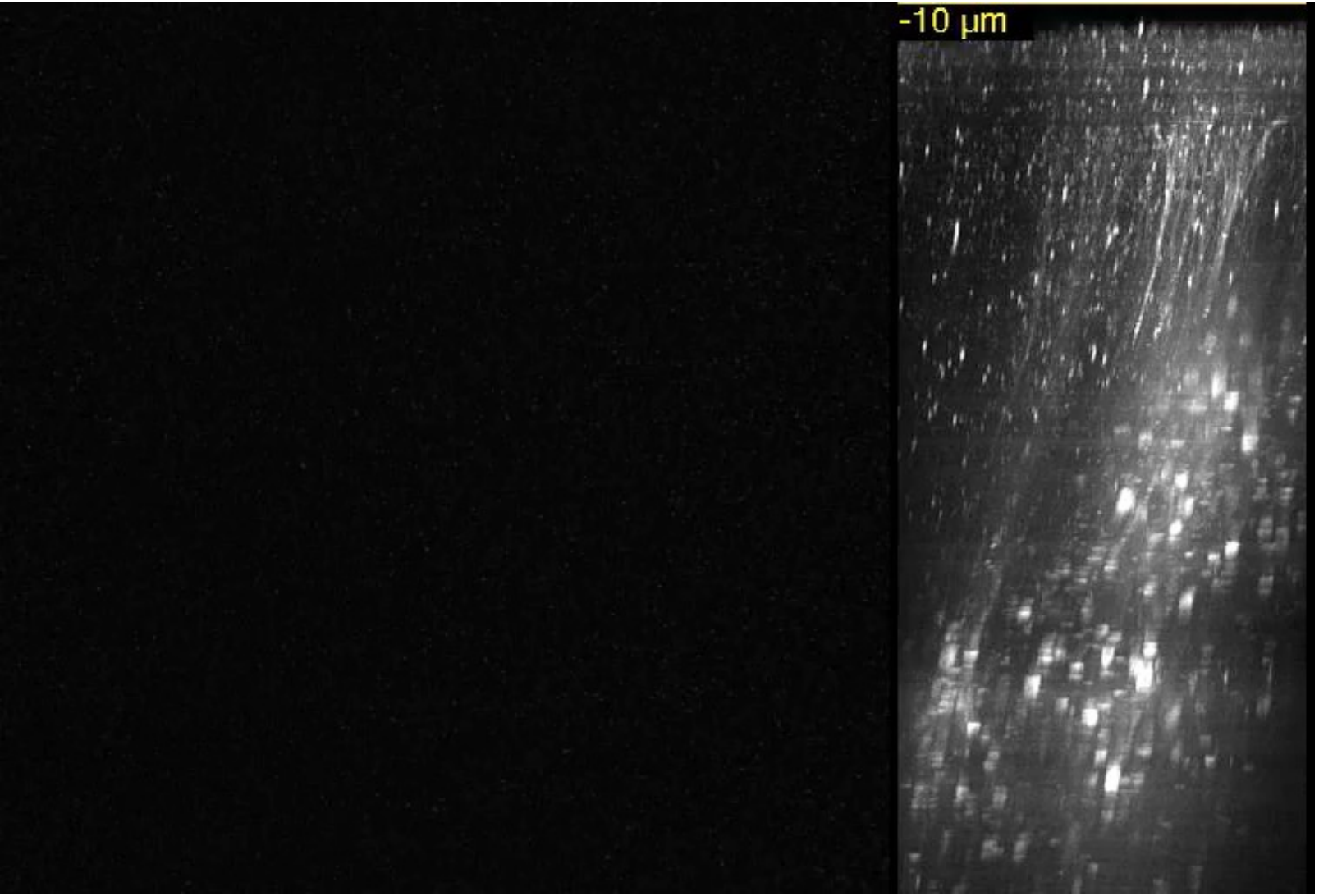
CONTRASTING TECHNIQUES

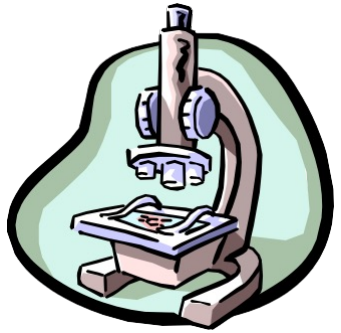


https://www.youtube.com/watch?v=7HRVcHNsaks

Phase contrast

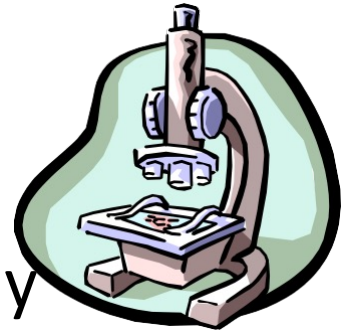
CONTRASTING TECHNIQUES





flourescence

PHOTOTOXICITY

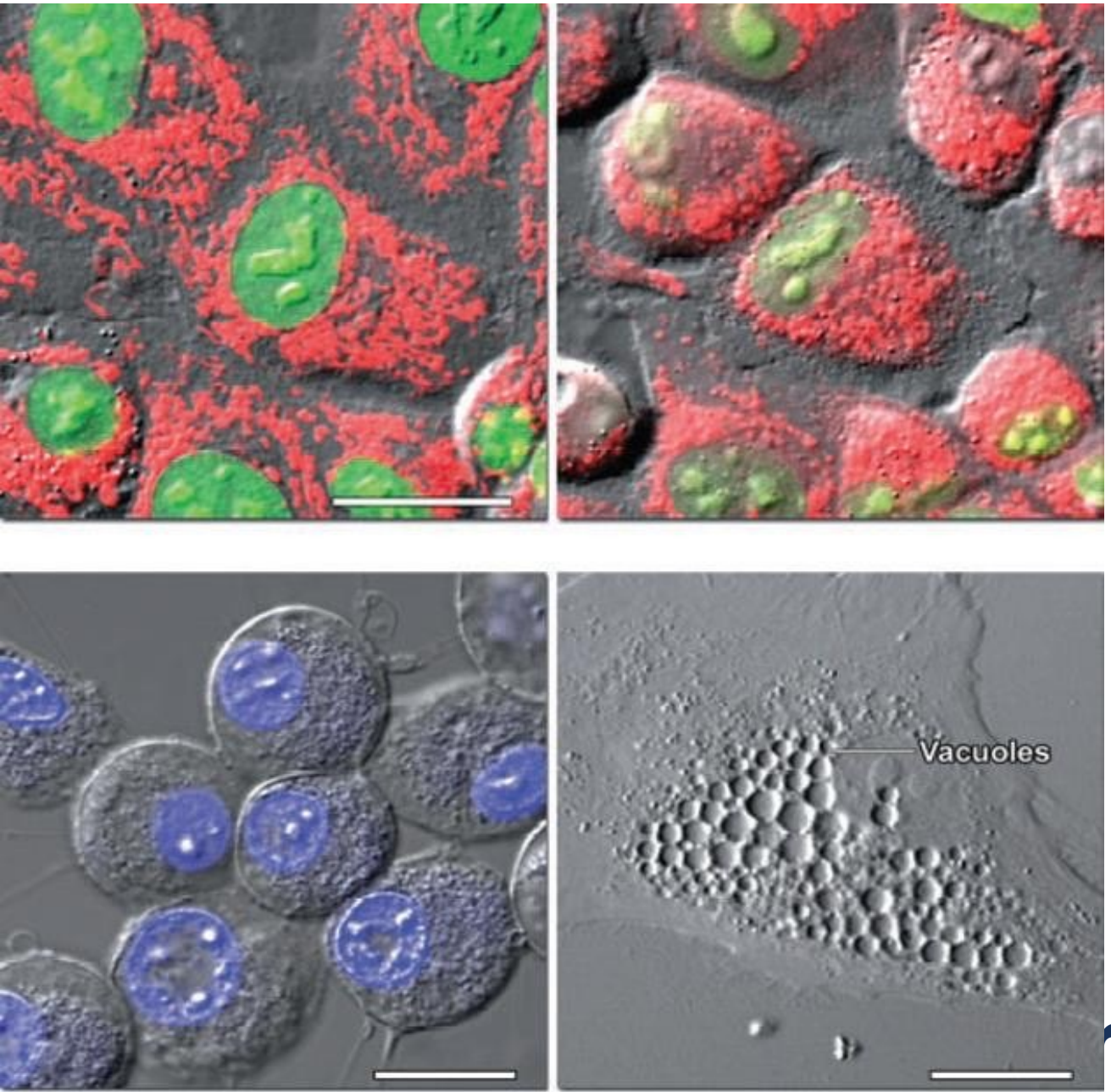


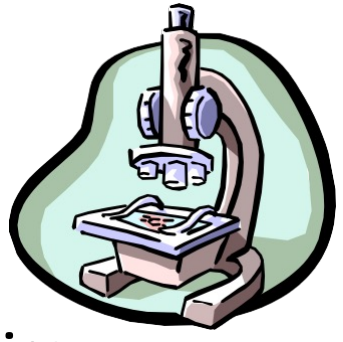
The **central** challenge in live cell imaging – Cells are inherently photosensitive!

- Formation of free radicals by fluorophores only exacerbates the problem
- Free radical formation cannot be prevented, only managed by careful tuning of imaging parameters to reduce light exposure

Live cell imaging requires “gentle imaging” - short exposures of low intensity Illumination

PHOTOTOXICITY-EXAMPLES

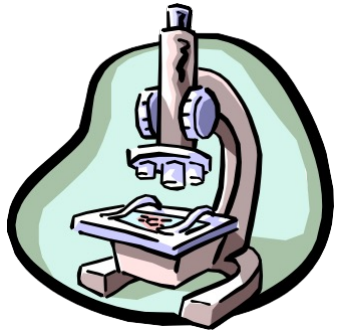




Phototoxicity during live-cell imaging.
(a)(RK13) cells expressing EYFP fused to a nuclear localization signal (green nucleus) were treated with the synthetic dye MitoTrackerTM
(b)Same view field as panel a after time-lapse imaging for 2 hours at 15 second intervals.

(c)HeLa cells labeled with Hoechst 33342 imaged for 30 minutes at 10- second intervals Cells have detached from the coverslip and are rounding.
(d)Vacuole formation in a fibroblast cell after imaging for 8 hours at 30- second intervals using tungsten halogen illumination and DIC optics.

FLOURESCENCE LABELLING OF LIVE CELLS



- Detect specific molecules (immunocytochemistry, fluorescent proteins)
- Must keep the specimen transparent
- Targeting live cell (membrane permeability)
- Eventually detect multiple molecules at the same time

FLOURESCENCE LABELLING OF LIVE CELLS

Technique	Example	Pros	Cons
Cell Permeable Dyes	MitoTracker Red CMXRos DAPI BAPTA / FURA	•Simple •In vivo labeling •Short incubation times	•Often toxic to cells, especially in higher concentrations
Fluorescent Proteins	EGFP	•Simple; in vivo •Well developed •Very specific labeling	•Large tag •Oligomerization •Overexpression of exogenous protein
Direct labeling of Protein	FITC labeled actin	•Very specific labeling	•Highly purified protein required •Delivery into cell
Auto-labeling of protein fusions	HaloTag™ SnapTag™	•In vivo labeling	•Large tag •Limited colors
Labeling peptide tags	FlAsH and ReAsH	•Specific labeling •Small label •In vivo labeling	•Limited colors

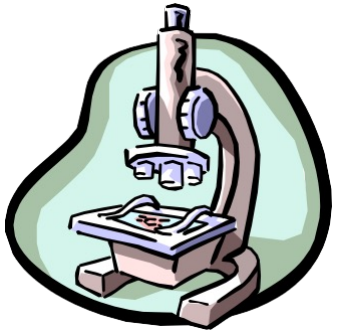
FLOURESCENCE LABELLING OF LIVE CELLS

➢For live cell experiments, choose FPs with high photostability

Protein (Acronym)	Ex (nm)	Em (nm)	EC (x 10^{-3})	QY	Photostability	Quaternary Structure	Brightness (% of EGFP)
Green Fluorescent Proteins							
EGFP	488	507	56.0	0.60	++++	Monomer*	100
Emerald	487	509	57.5	0.68	+++	Monomer*	116

Protein (Acronym)	Ex (nm)	Em (nm)	EC (x 10^{-3})	QY	Photostability	Quaternary Structure	Brightness (% of EGFP)
mApple	568	592	75.0	0.49	+++	Monomer	109
mStrawberry	574	596	90.0	0.29	+	Monomer	78
AsRed2	576	592	56.0	0.05	+	Tetramer	8
mRFP1	584	607	50.0	0.25	++	Monomer	37
JRed	584	610	44.0	0.20	++	Dimer	26
mCherry	587	610	72.0	0.22	+++	Monomer	47

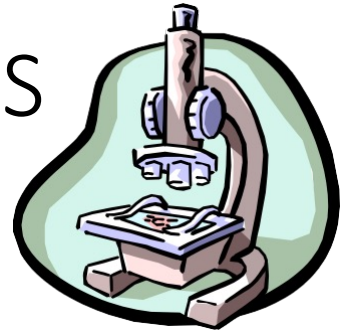
CHANGES IN FLOURESCENCE



- Changes of flourescence intesity
  - Examples: calcium imaging, vesicle release

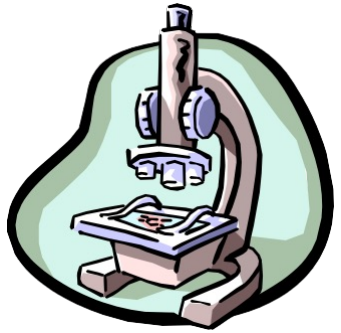
- Changes of flouresence localisation
  - Examples: Mitochondrial dynamics

MINIMIZING DAMAGE WHILE GETTING GOOG IMAGES

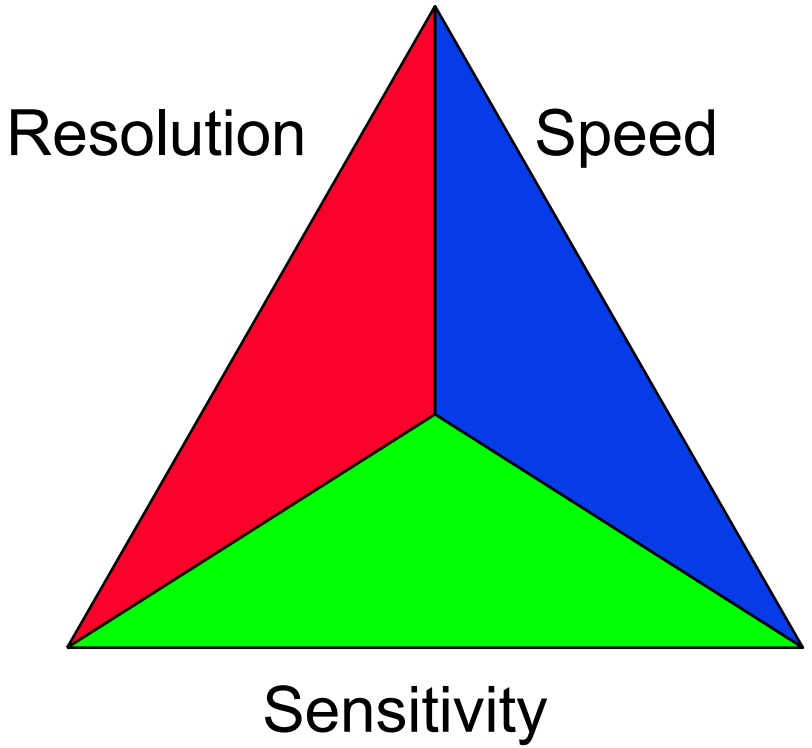


- Resolution vs Speed vs Sensitivity
- Light Gathering Power and Focus Maintenance
- Which immersion media
- Correct coverslip thickness
- 3D Imaging

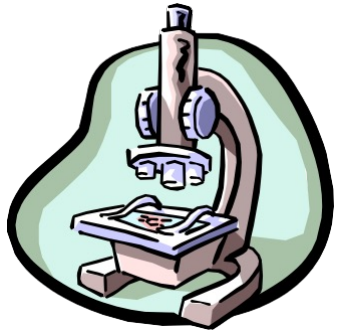
RESOLUTION-SPEED-SENSITIVITY



- Spatial Resolution
  - Depends on application optical magnification and lens apperture
  - Smaller and more pixels = higher resolution
- Sensitivity
  - Larger pixels = higher sensitivity
  - Higher sensitivity = shorter exposure time
  - Shorter exposure time = higher frame rate
  - Larger pixels = lower resolution
- Speed
  - Less pixels = faster readout
  - Faster Readout = higher framerate
  - Less pixels = lower resolution
  - requires fast readout and high sensitivity



CORRECTION IMMERSION MEDIA

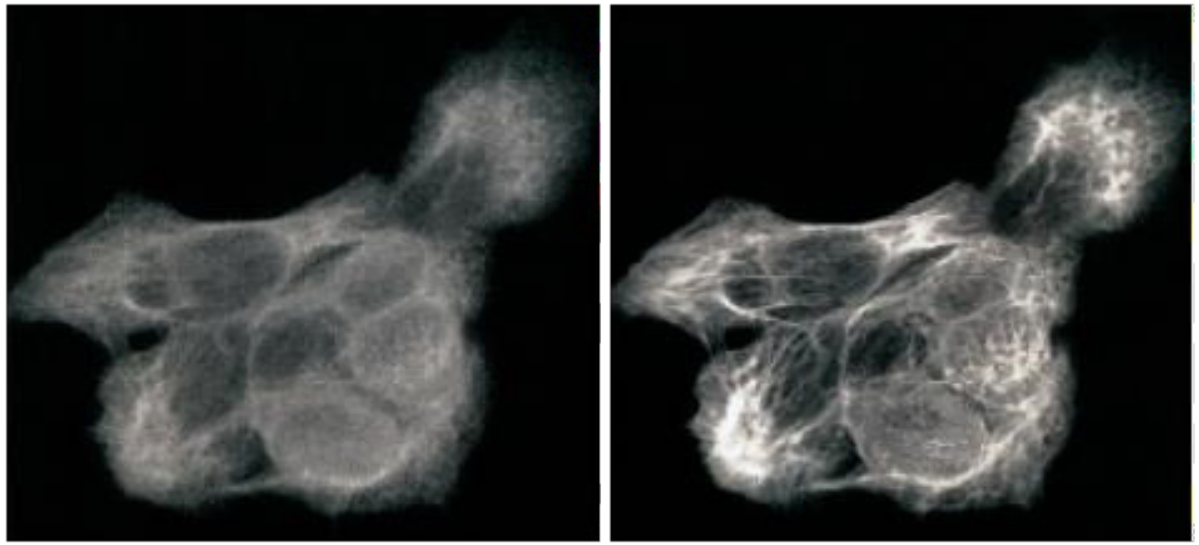


oil immersion objective

NA=1.4

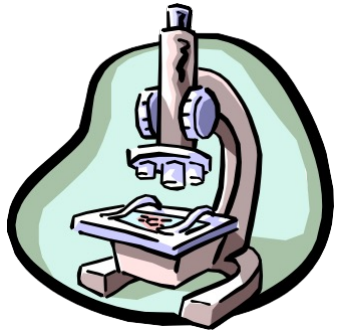
water immersion objective

NA=1.2

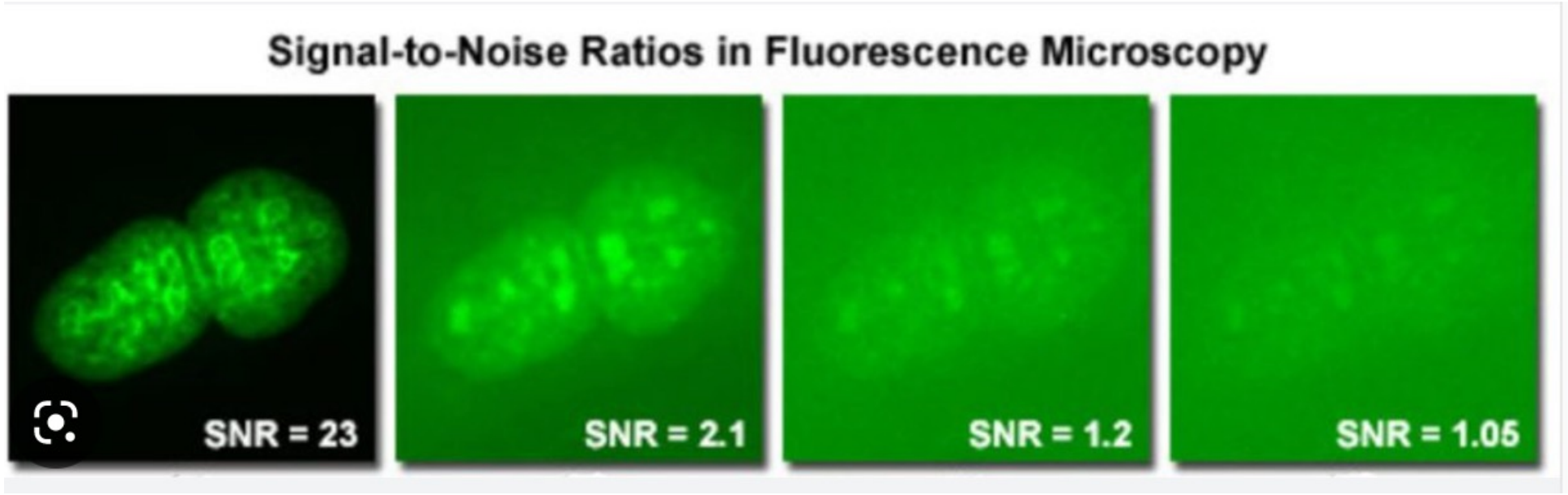


Using water immersion objectives for samples in liquid media captures more signal and preserves resolution better than refractive index mismatched oil immersion objectives.

SIGNAL-TO-NOISE

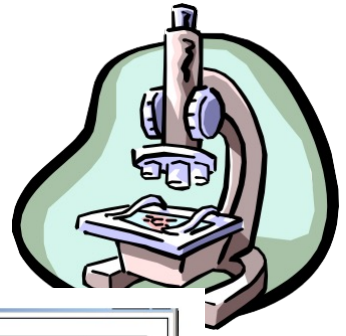


- Camera Sensitivity -
  - Signal needs to be 2-3x brighter than background noise to be detected.

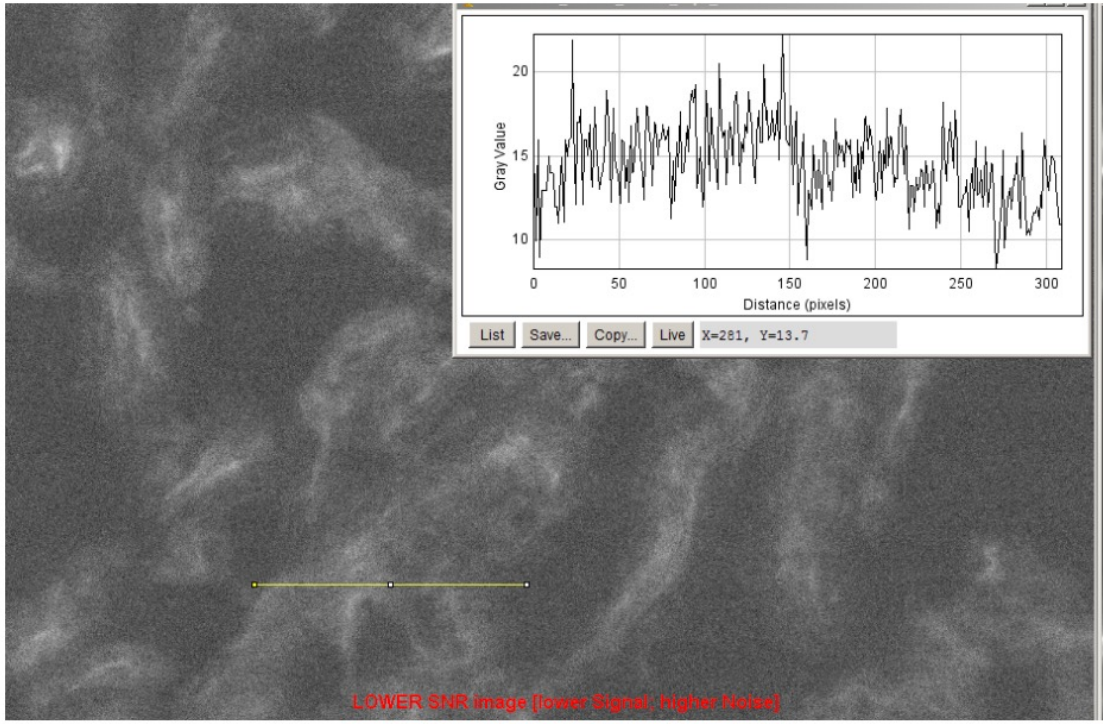


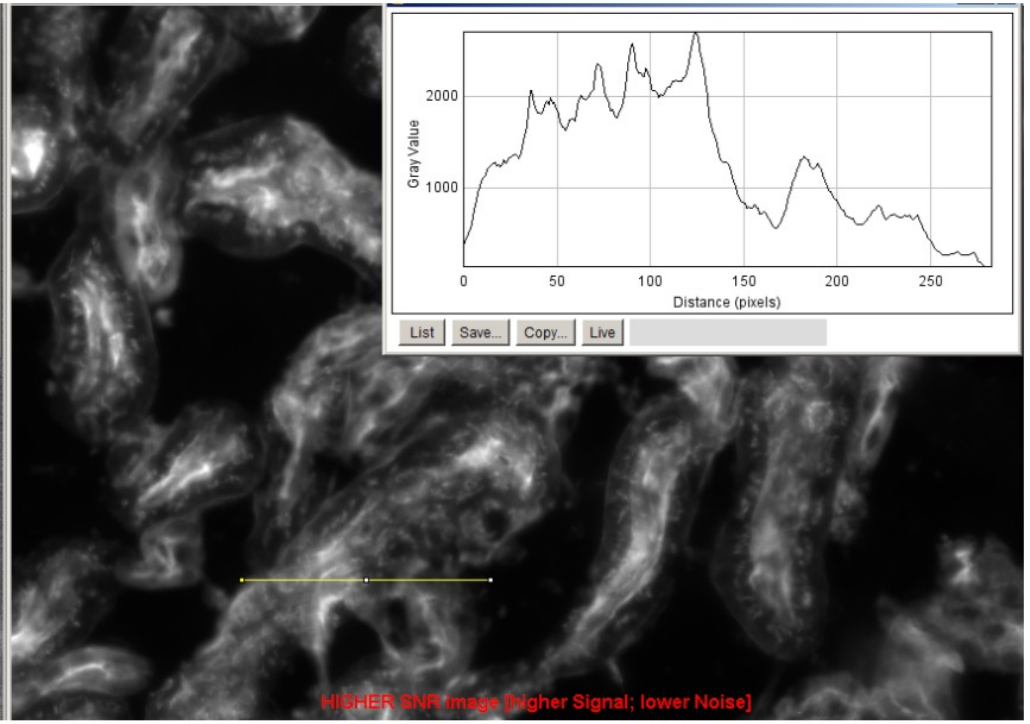
  - Increasing the exposure time (integration time) or increasing the illumination intensity produces more signal.

SIGNAL-TO-NOISE



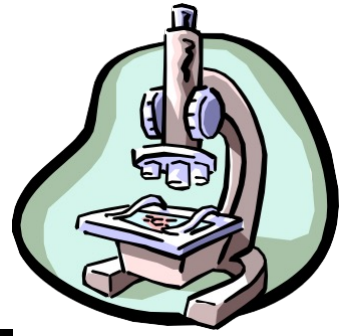
https://scientificimaging.com/knowledge-base/signal-and-noise-quantitative-explanation/

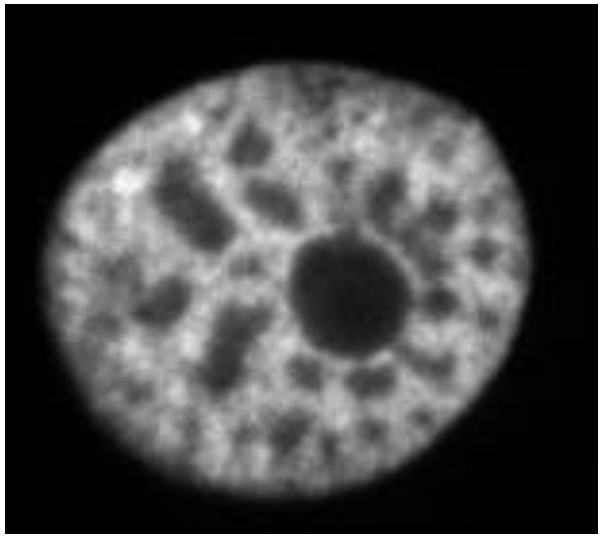




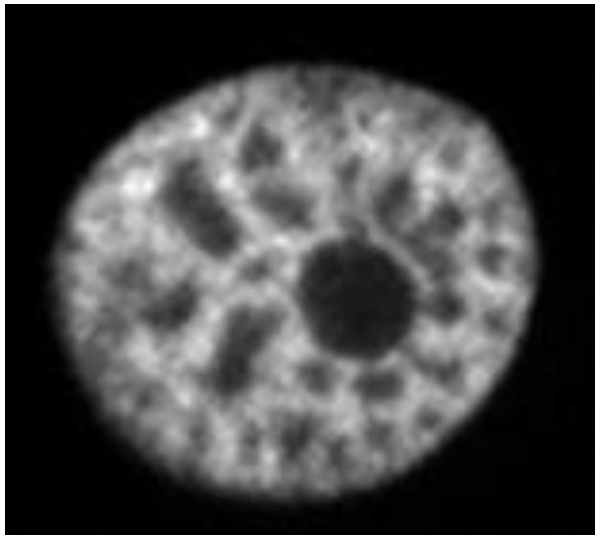
A fluorescence slide was imaged with the same excitation & filters, but with different camera exposures. The LOWER SNR image was obtained with a short=2.5ms exposure and the HIGHER SNR image was obtained with a long=1sec exposure.

RESOLUTION VS SPEED

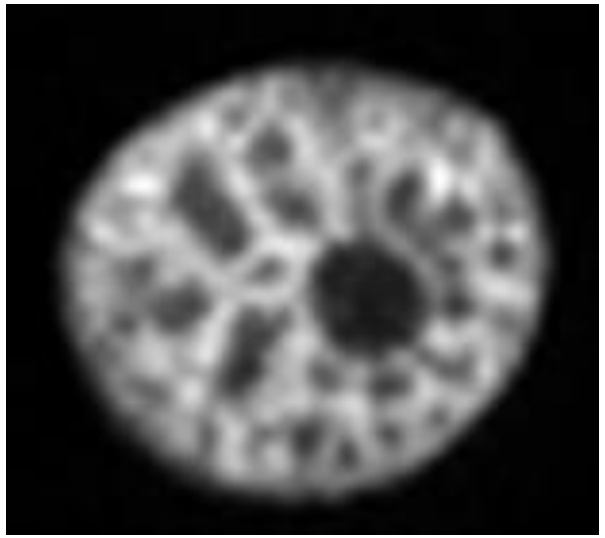




512ms
1x1 binning



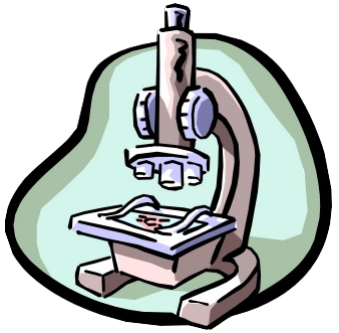
117ms
2x2 binning



28ms
4x4 binning

Using binning shortens exposure times reducing cell phototoxicity and fluorophore photobleaching

CHANGES IN FLOURESCENCE



- Changes of flourescence intesity
  - Examples: calcium imaging, vesicle release

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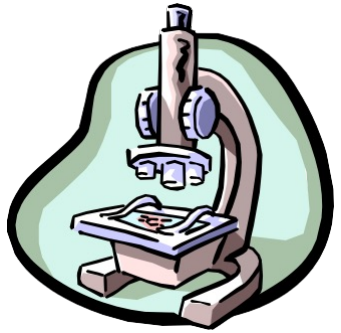
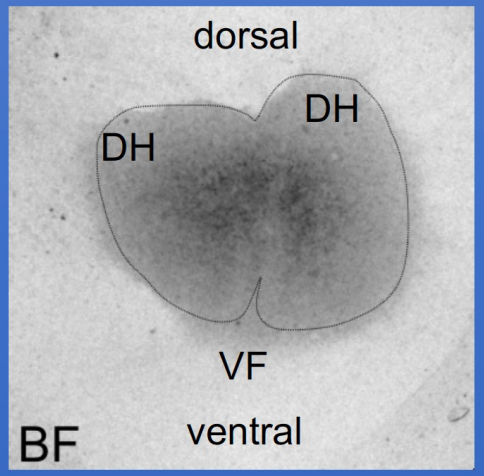
  - Examples: Mitochondrial dynamics

IMAGING CHANGES IN ION CONCENTRATIONS

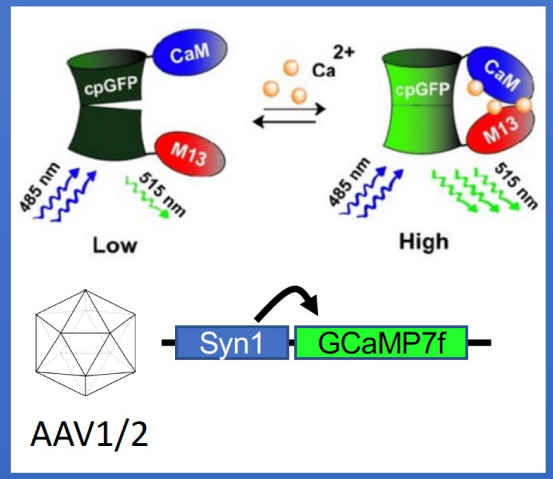


A commonly performed live flourescence imaging method is ion imaging (calcium, chloride, magnesium, etc) using either fluorescent dyes or proteins especially designed to change their emission behavior upon binding of the respective ion.

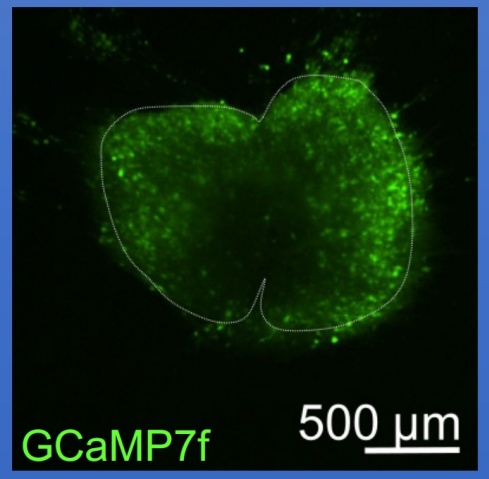
IMAGING CHANGES IN ION CONCENTRATIONS

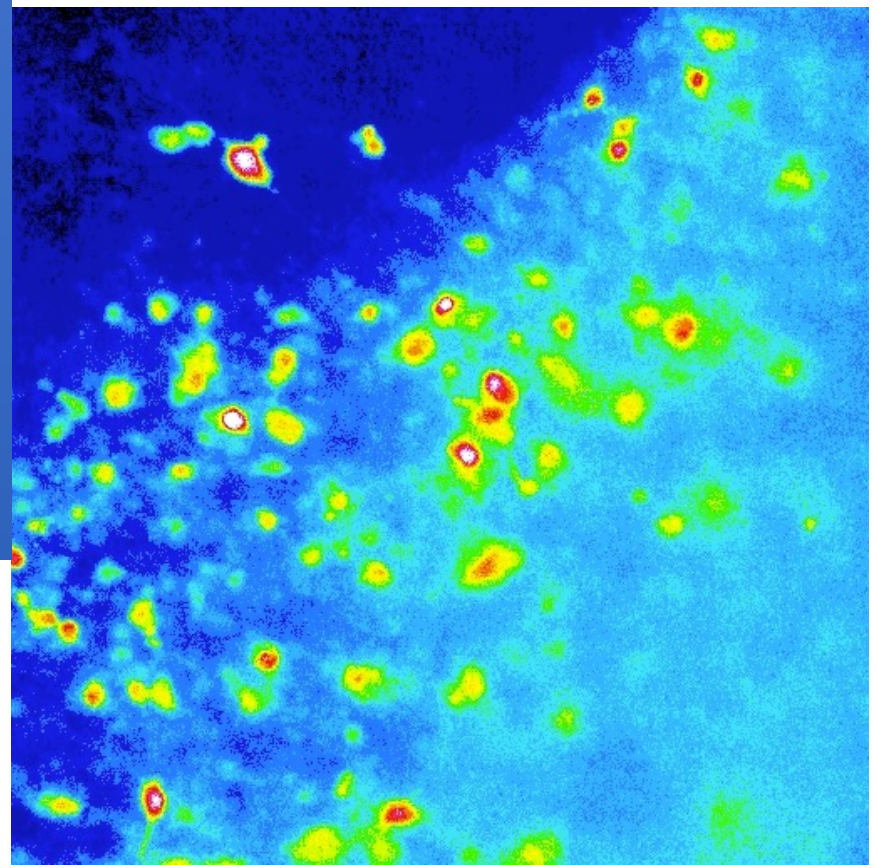


Organotypic spinal cord culture at DIV 18. DH, dorsal horn; VF, ventral fissure.



Organotypic spinal cord cultures were infected with AAV1/2 at DIV13, expressed calcium indicator GCaMP7f under control of the neuron-specific promotor Syn1 for 4-6 days. Image of GCaMP7f fluorescence.

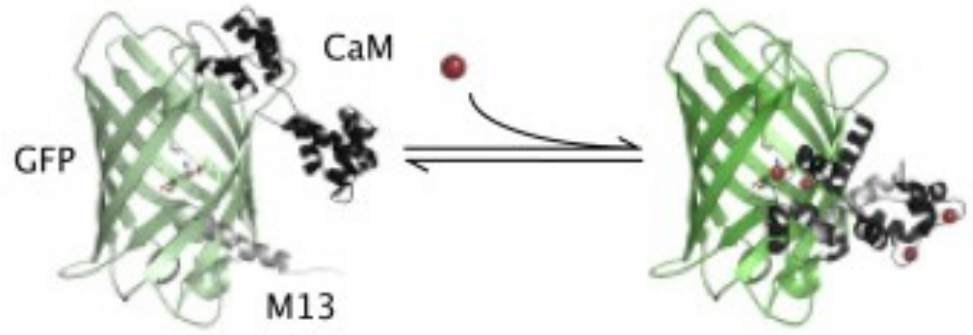


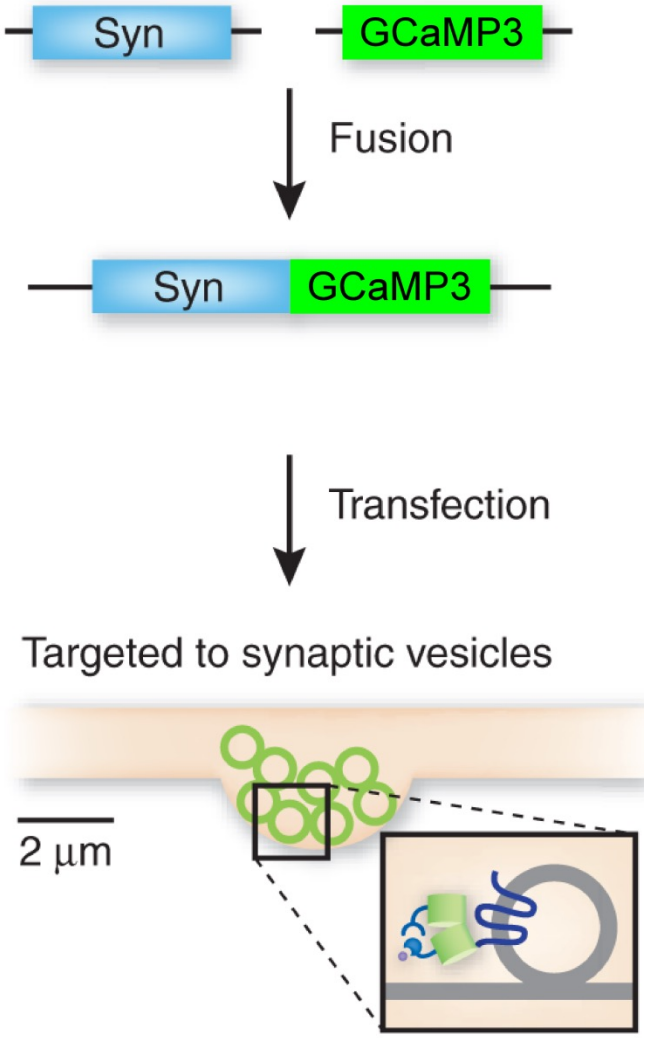


Thalhammer et al, 2022

SYNAPTIC TARGETING OF GCaMPs

GCaMP

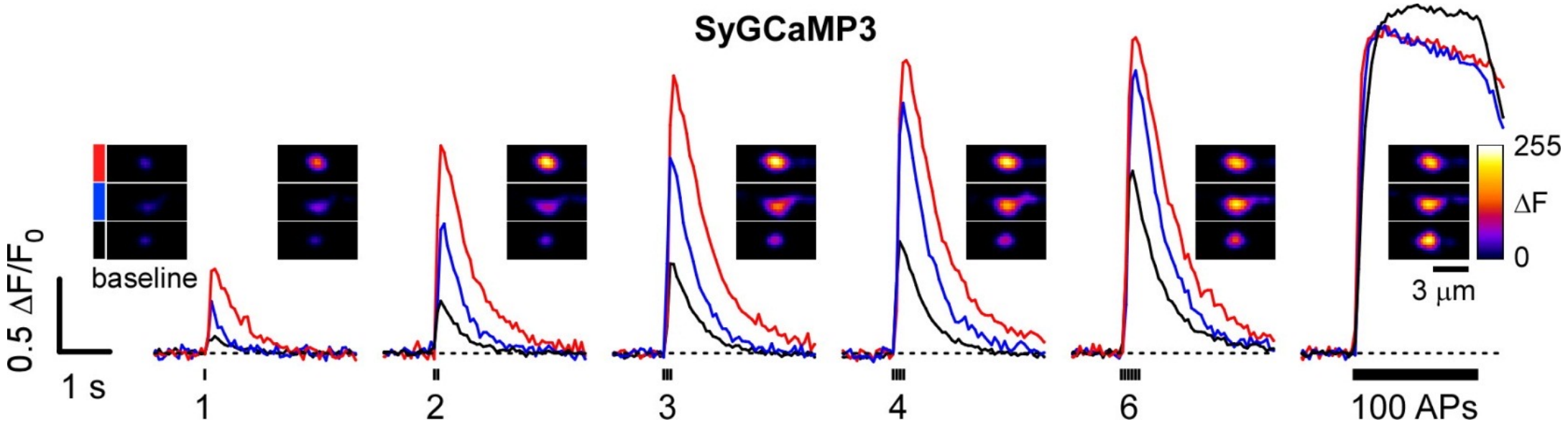




SyGCaMP3 CAN DETECT SINGLE ACTION POTENTIALS

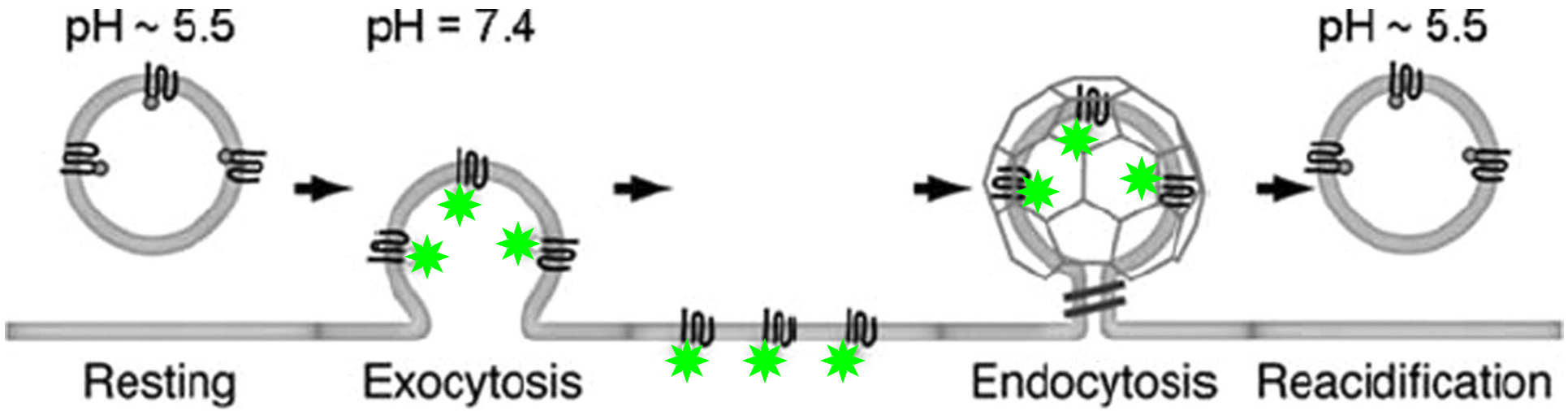
Speed: 5x

OVEREXPRESSION OF P/Q-TYPE CALCIUM CHANNELS BOOSTS PRESYNAPTIC CALCIUM TRANSIENTS

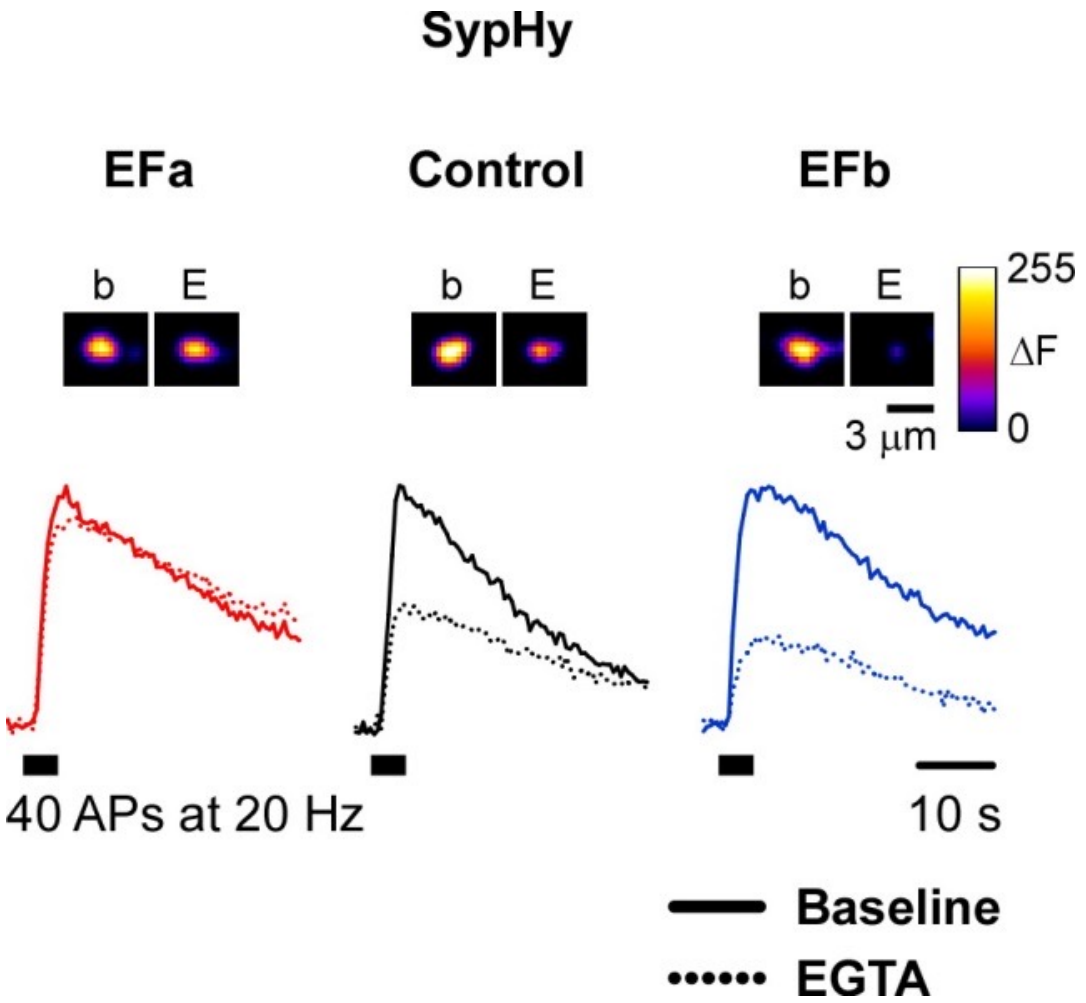


Thalhammer et al, 2017

SYNAPTOPHYSIN-PHLUORIN (SYPHY) – IMAGING OF VESICLE RELEASE

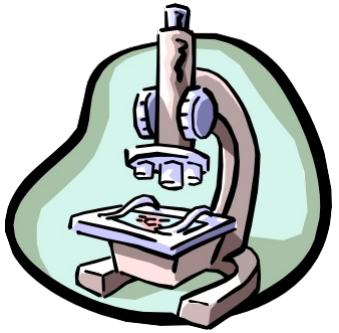


$CA_V2.1$[EFA/B] ARE DIFFERENTIALLY COUPLED TO THE NEUROTRANSMITTER RELEASE MACHINERY



Thalhammer et al, 2017

CHANGES IN FLOURESCENCE



- Changes of flourescence intesity
  - Examples: calcium imaging, vesicle release

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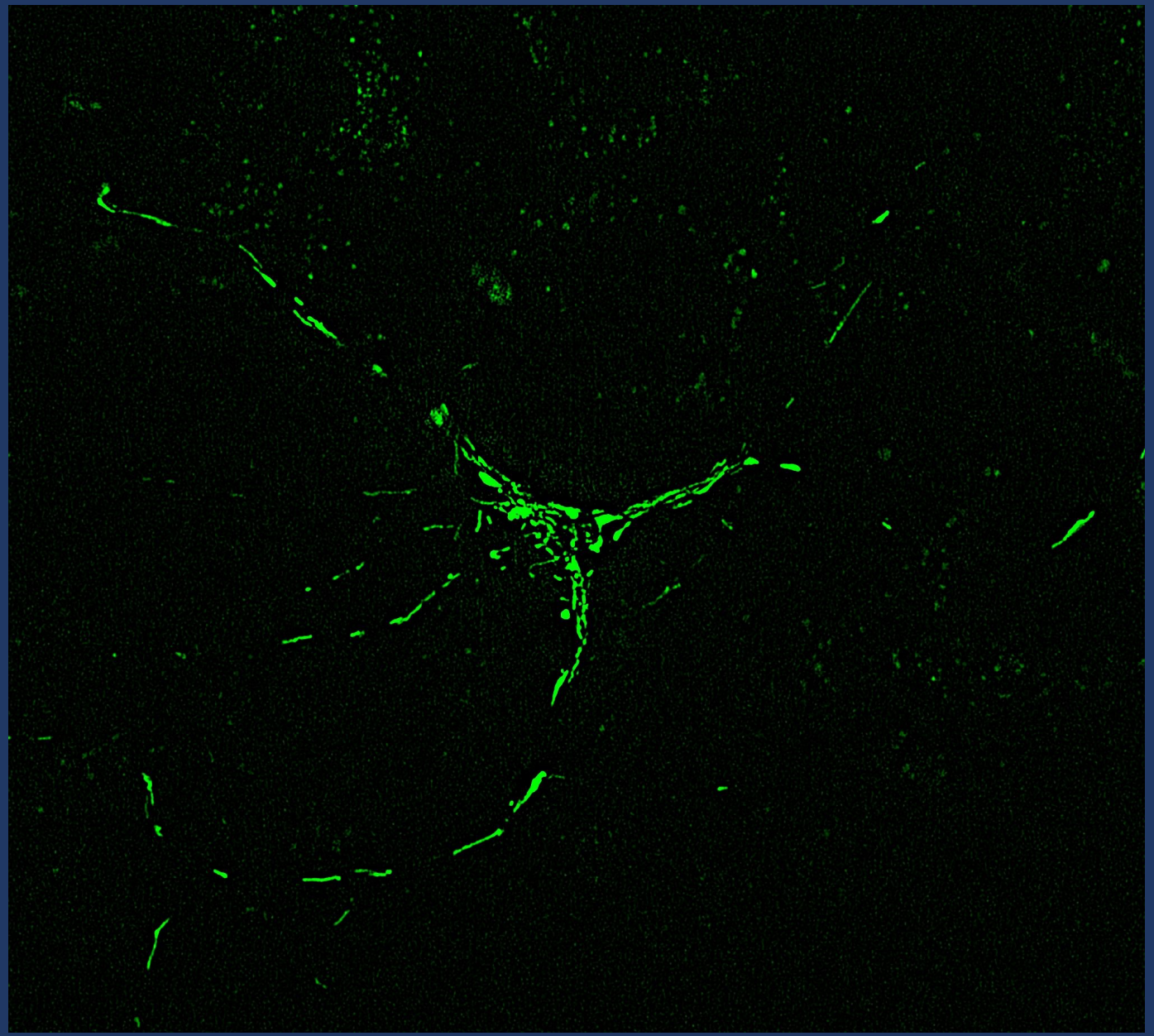
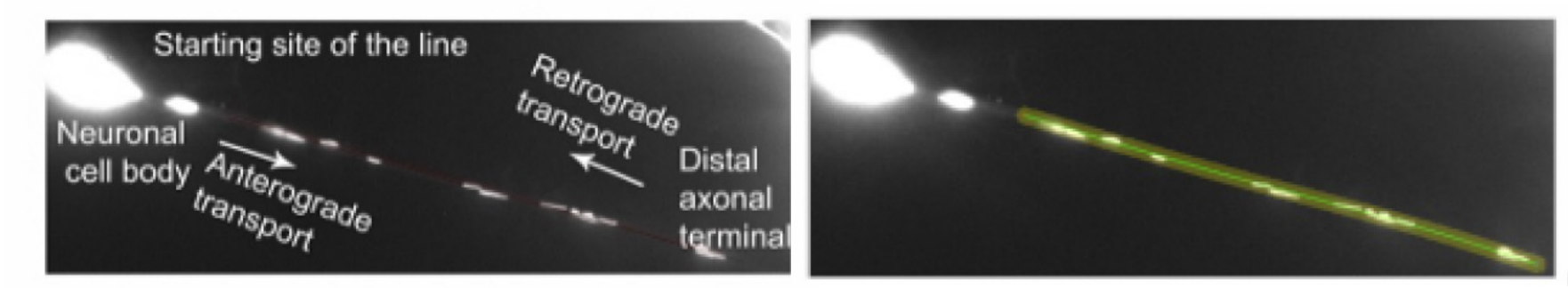
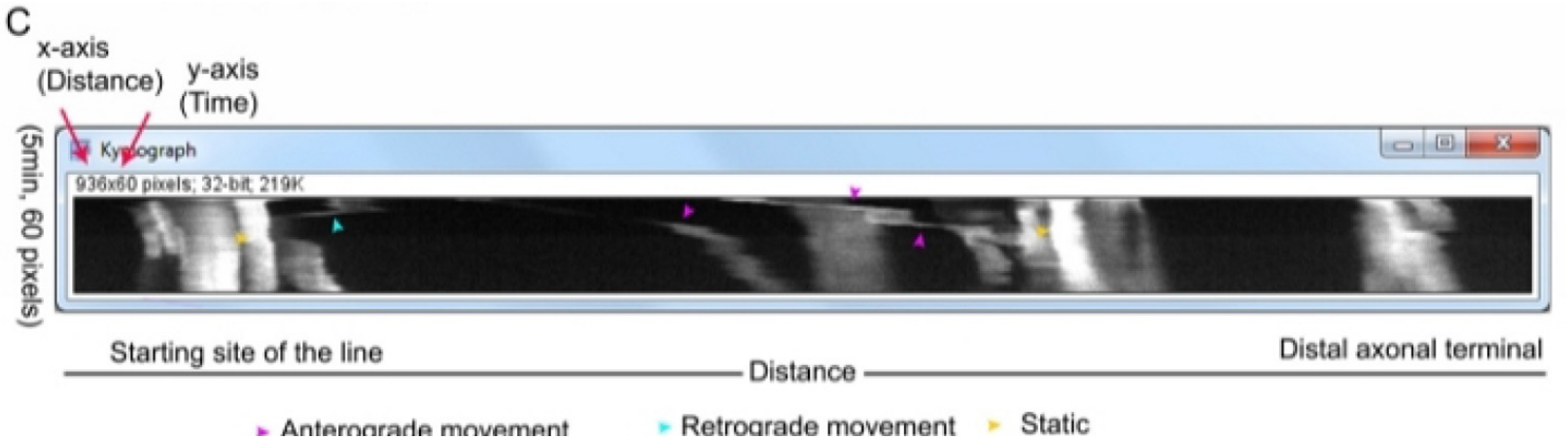
MITOCHONDRIAL PARTICLE DYNAMICS



MITOCHONDRIAL PARTICLE DYNAMICS

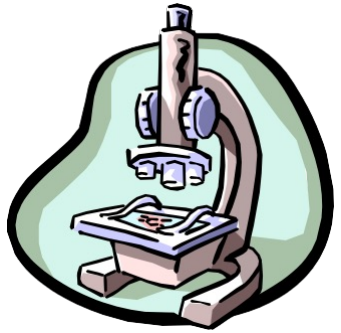




Mou et al, 2020

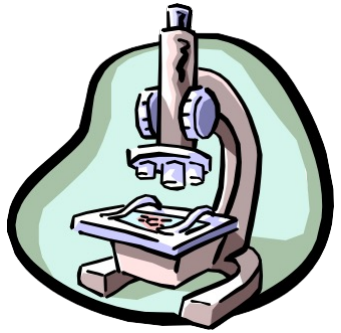
MITOCHONDRIAL PARTICLE DYNAMICS

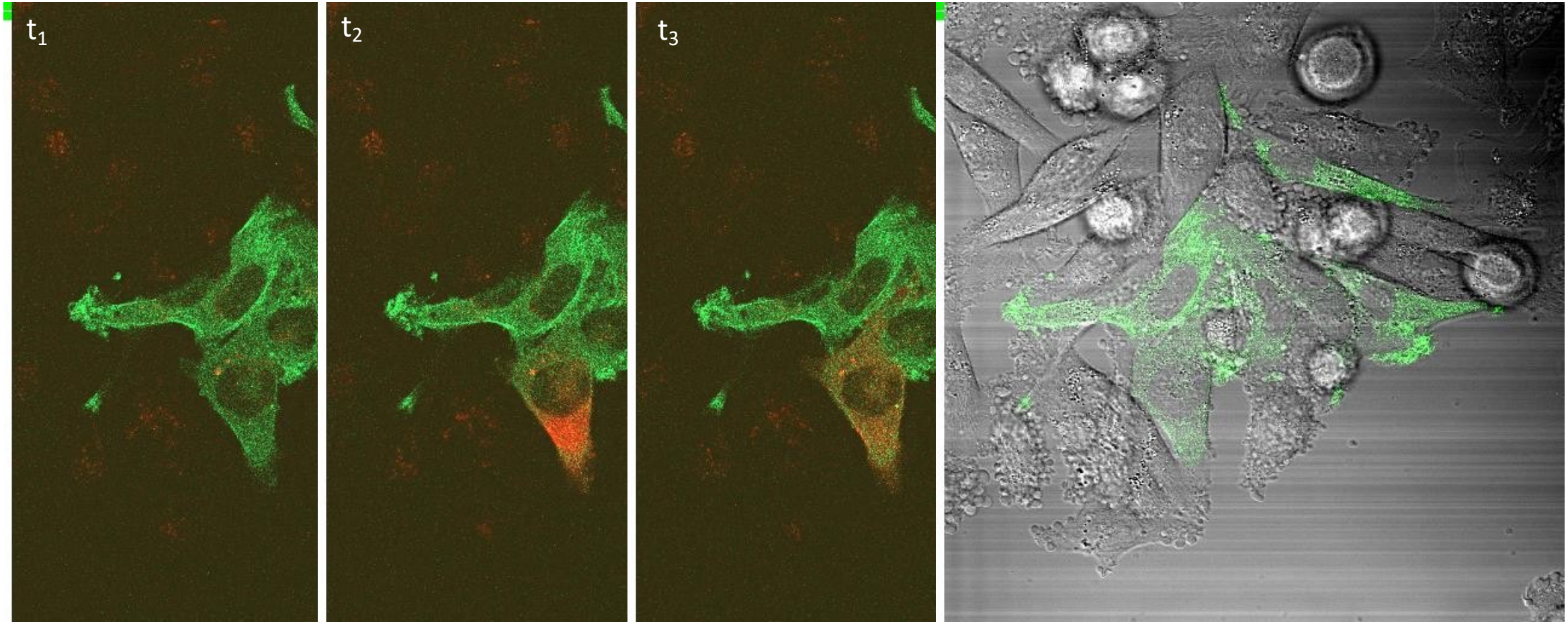
OTHER LIVE IMAGING TECHNIQUES



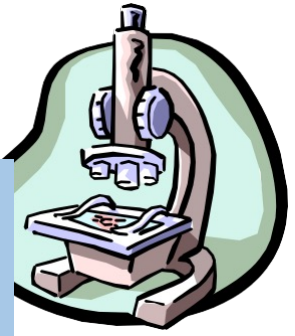
- Vesicle fusion/release (TIRF or pH-sensitive GFP)
- FRAP
- FRET
- Photoactivation/Photoconversion

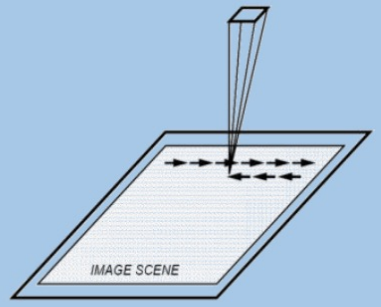
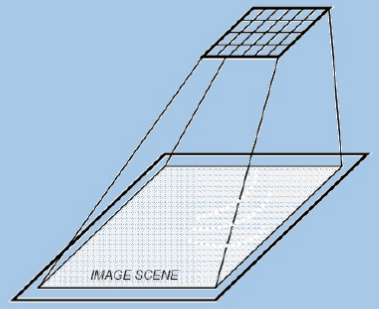
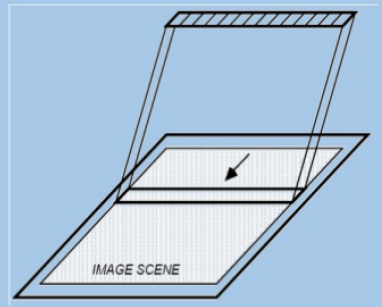
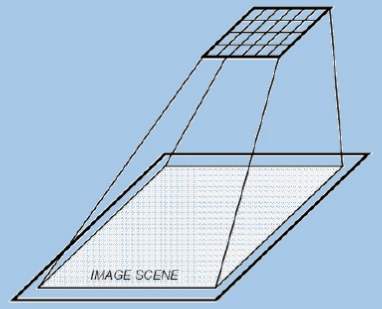
OTHER LIVE IMAGING TECHNIQUES



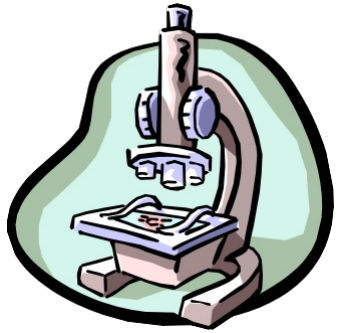


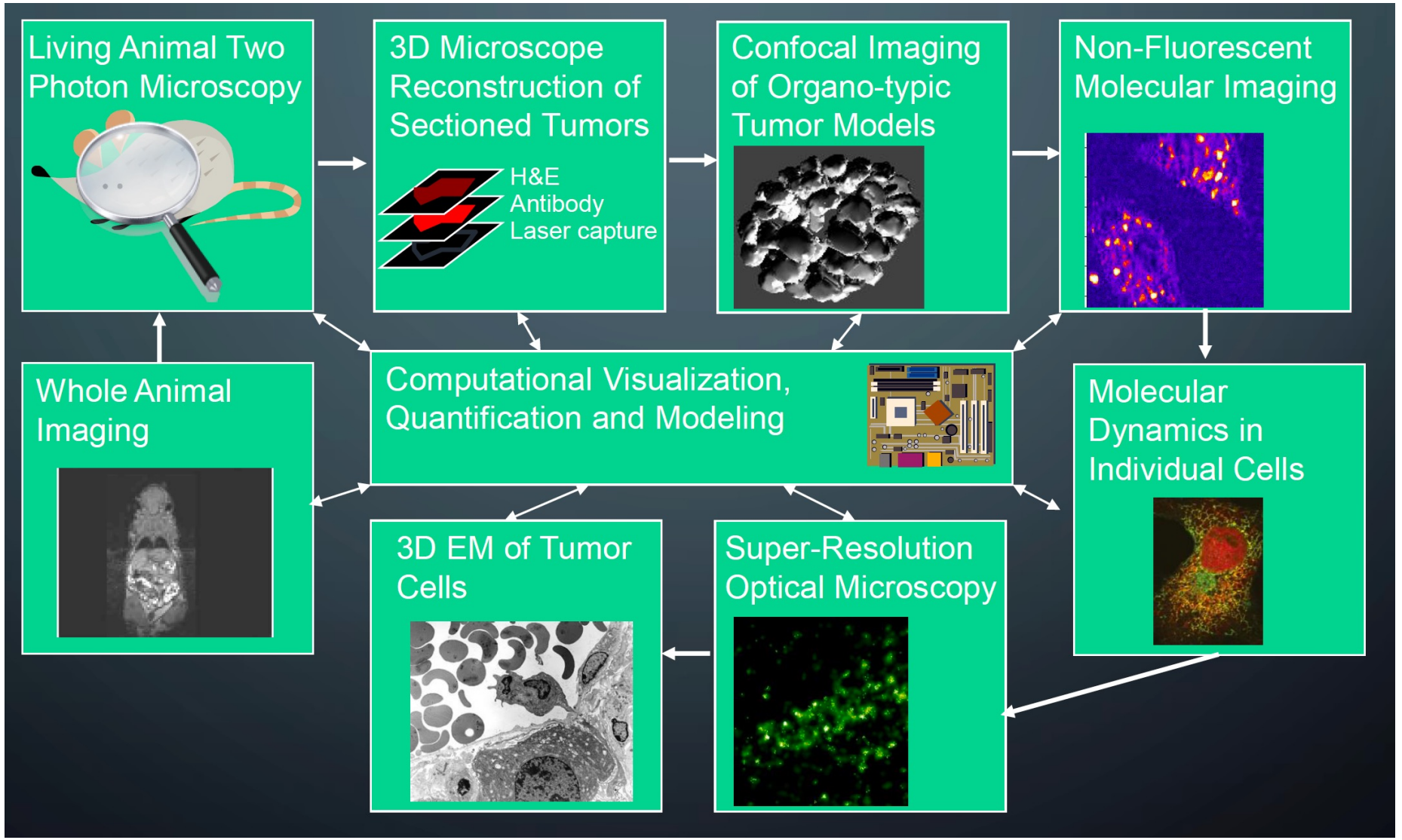
DETECTION



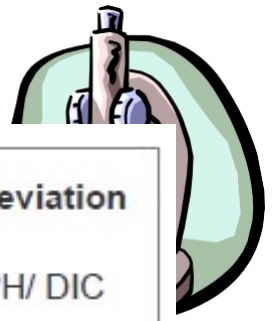
Point Scanning	Multi Point Scanner	Line Scanning	Area Scanning
			
Point Detector	**Area Detector**	**Line Detector**	**Area Detector**
Photomultiplier	CCD camera	Photomultiplier Array	CCD camera
Avalanche Photodiode	CMOS camera	CCD Line Array	CMOS camera
GaAsP			

COMBINING TECHNIQUES



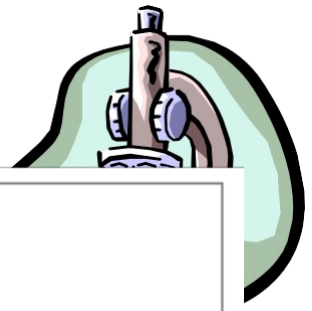


SUMMARY



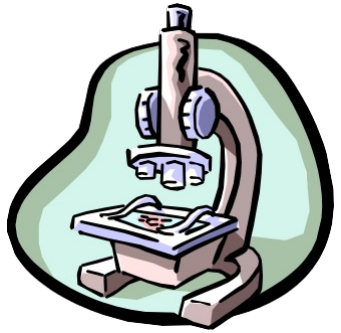
Biological application	Microscopy Approach	Abbreviation
Cell shape – cell migration – organelle kinetics	Transmission Microscopy : Bright Field, Dark Field, Phase Contrast, Differential Interference Contrast	BF/ PH/ DIC
Protein colocalization - dynamics, organisation and structure of cells , organelles, proteins	Epifluorescence / Confocal / Spinning disk	
3D imaging of live cells	Confocal/ Spinning disk	
3D imaging of tissues, model organisms, small animals – study structure and development	Multiphoton microscopy/ light sheet microscopy	
Quantification of ion concentrations (calcium, magnesium)	ion imaging	
Biosensing and protein-protein interactions	Forster resonance energy transfer	FRET
Biosensing and protein-protein interactions without light excitation	Bioluminescence resonance energy transfer	BRET
Protein diffusion and kinetics	Fluorescence recovery after photo-bleaching	FRAP
Protein diffusion with high spatio-temporal accuracy (µs-s and 300-400nm)	Fluorescence correlation spectroscopy	FCS
Protein diffusion with single molecule mapping and high spatio-temporal resolution	Single particle tracking	SPT
Protein diffusion with single molecule mapping and high spatio-temporal resolution in dense samples	SPT photoactivated localisation microscopy	sptPALM

SUMMARY



Type	Common Application	Pro	Con
Fluorescent Proteins (FP)	Targeting probe to specific cellular compartments or to interact with particular proteins FRET	Direct in situ labeling Can be attached to a target genetically Very specific Low toxicity	Large Size Beta barrel shape may hinder molecules under study Photobleach Low signal intensity
Fluorescent Peptides	Similar applications as FP but utilized when size of FP may hinder study	Small size Low Toxicity	Targets Specific Interactions Often requires coupling to molecules
Organic Fluorophores	Can target cell structures Label mRNA sequences	Small Good for multiplexing Limited photostability Wide range of colors	Require coupling to another molecule Photobleach Hydrophobic Somewhat toxic Lower specificity
Indicator Probes	Indicate presence of ions and metals	Very specific	Often requires coupling to molecules
Quantum Dots	Long term imaging studies Real-time tracking over extended periods	Very photostable Good for multiplexing Intense signal Enable very sensitive detection	Coupling to another molecule necessary Interference

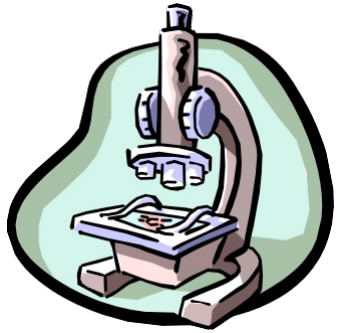
SUMMARY



Platform	To improve efficiency	To improve sensitivity and S/N	To minimize light exposure
All	Hard-coated filters (see Box 1). Dye-specific filters. Avoid excess optical elements (lenses, DIC components). 100% light to detector port. High NA objectives. Spectral un-mixing. Avoid phase objectives.	**General:** Phenol-red-free medium. Restorative deconvolution. **Camera-based systems:** Avoid color cameras. Bin high-resolution cameras 2×2 (exposure times of 200-500 ms). Slow camera read times. Use EM-CCD for high speed (exposure times of <100 ms).	Keep excitation light levels low. Avoid blue dyes. Minimum resolution (60×, 1.4 NA): x,y ~0.1-0.2 µm z ~0.3-0.4 µm t=2.3× timescale of events. Minimum number of probes. Use oxygen-radical scavengers.
WFM	Remove DIC prism and analyzer when imaging fluorescence.	Perform post-acquisition restorative deconvolution (Fig. 3C,D).	Find cells with transmitted light. Use ND filters (<10% lamp power). Use UV- and IR-blocking filters. Use halogen lamps if possible.

Wide-field microscopy

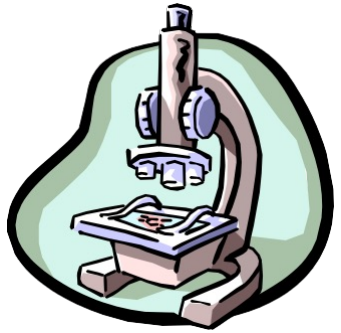
SUMMARY



Platform	To improve efficiency	To improve sensitivity and S/N	To minimize light exposure
CLSM	Use long-pass or wide band-pass filters and un-mix post acquisition. Open the pinhole ≥2 Airy units. Remove DIC prism.	High PMT voltage (≥800 V). Restorative deconvolution. Avoid spectral-array detectors.	Low laser power. AOTF laser blanking. No line or frame averaging. Fast scan speed (≥8 µs/pixel). Image regions of interest when possible. Recommended laser powers: 405 nm Avoid 488 nm <2% (30 mW) 514 nm <2% (30 mW) 543 nm <50% (1 mW) 633 nm <5% (5 mW)

Confocal laser scanning microscopy

RESOLUTION-SPEED-SENSITIVITY



- Spatial Resolution
  - Depends on application optical magnification and lens apperture
  - Smaller and more pixels = higher resolution
- Sensitivity
  - Larger pixels = higher sensitivity
  - Higher sensitivity = shorter exposure time
  - Shorter exposure time = higher frame rate
  - Larger pixels = lower resolution
- Speed
  - Less pixels = faster readout
  - Faster Readout = higher framerate
  - Less pixels = lower resolution
  - requires fast readout and high sensitivity