Identification and Localization of Proteins in a Single Cell

Molecular Medicine Course 2012

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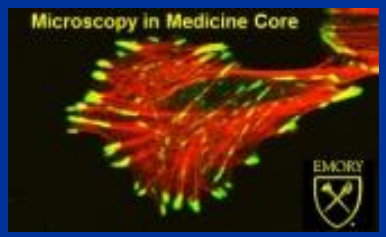
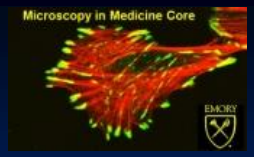
January 31, 2013

http://medicine.emory.edu/MIMCore

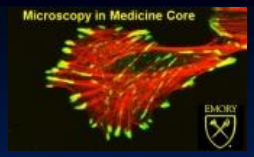


EMORY



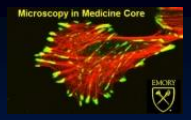
OUTLINE

1. Rationale for microscopic imaging of proteins: Why bother?
2. How to visualize proteins: Choosing among visible markers
3. Examples of immunofluorescence labeling
4. What to visualize proteins with: Choosing among microscopes
5. Recent advances in imaging
6. Future challenges in microscopic imaging



Rationale for microscopic imaging of proteins:

Why bother?



RATIONALE for Visualizing Proteins within the Cell: WHY BOTHER?

- Human genome contains around 20,300 human protein-coding genes
- What do these proteins do?
- Key to function may lie in LOCATION of protein within the cell
- Compartmentalization is often a key to function
- What does the protein interact with?

RATIONALE for Visualizing Proteins within the Cell

- The cell is highly compartmentalized within the three-dimensional architecture that cannot be discerned through standard biochemical approaches, which lack the necessary **spatial and temporal resolution.**

- Biochemical methodologies have been most powerful in characterizing molecular events and interactions, but also **important** is

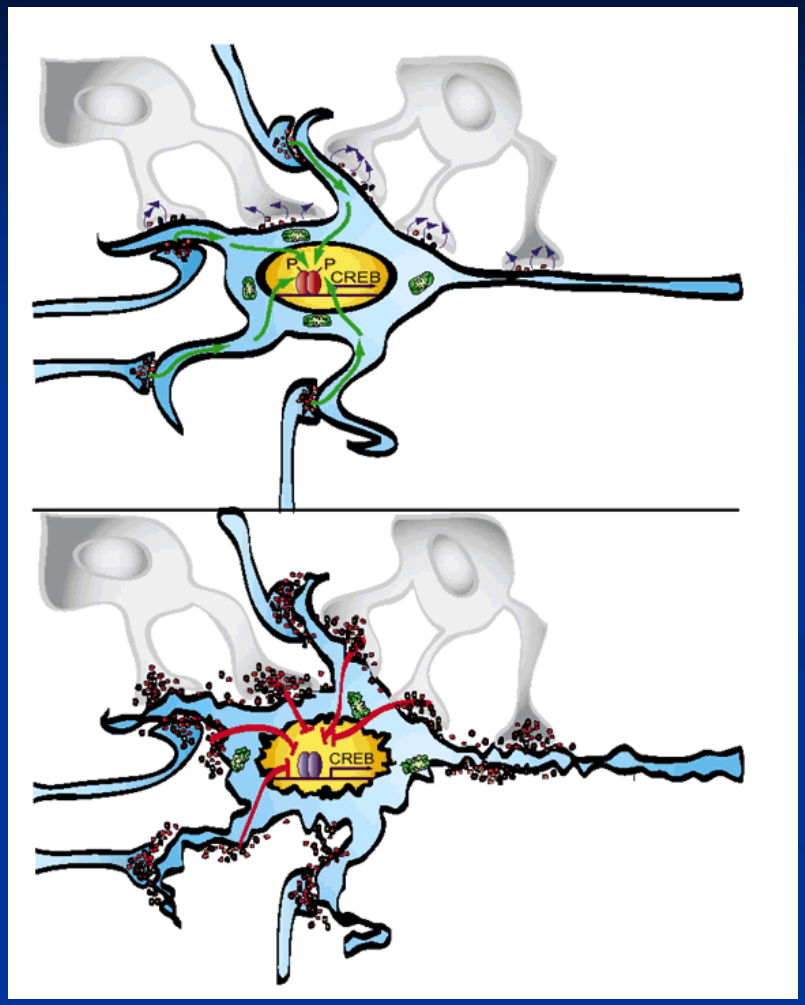
LOCATION, LOCATION, LOCATION!

- **LABEL** proteins with visible markers and **OBSERVE** location/dynamics

- Advanced light microscopy techniques can visualize proteins within their intracellular context, track cellular dynamics and **corroborate biochemical/molecular data on protein-protein interactions.**

Geiger B. 2001. EMBO reports 21:882-884.

Protein Localization: A Matter of Life or Death



Survival

Cell death

Synaptic NMDA receptor activation (top) promotes efficient signaling to the nucleus and phosphorylation of CREB transcription factor, leading to neuronal survival

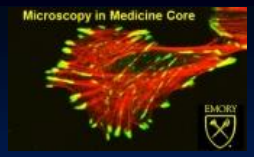
Extrasynaptic NMDA receptor activation triggers a "shut-off" pathway promoting dephosphorylation of CREB, leading to neuronal cell death

From: Riccio and Ginty. Nature Neuroscience 2002;5:389.

Aberrant Trafficking of Transmembrane Domain (TMD) Proteins in Human Disease

Human disease	Clinical features	TMD protein	Trafficking defect
Brugada syndrome	Cardiac disease	α subunit of cardiac sodium channel	ER retention of sodium channel subunits and defective cell surface sodium transport
Congenital Long QT syndrome	Heart disease	Voltage-gated K+ channels	ER retention and degradation
Familial hypercholesterolemia	Vascular disease	LDL receptor	Increased cholesterol caused by ER retention and degradation

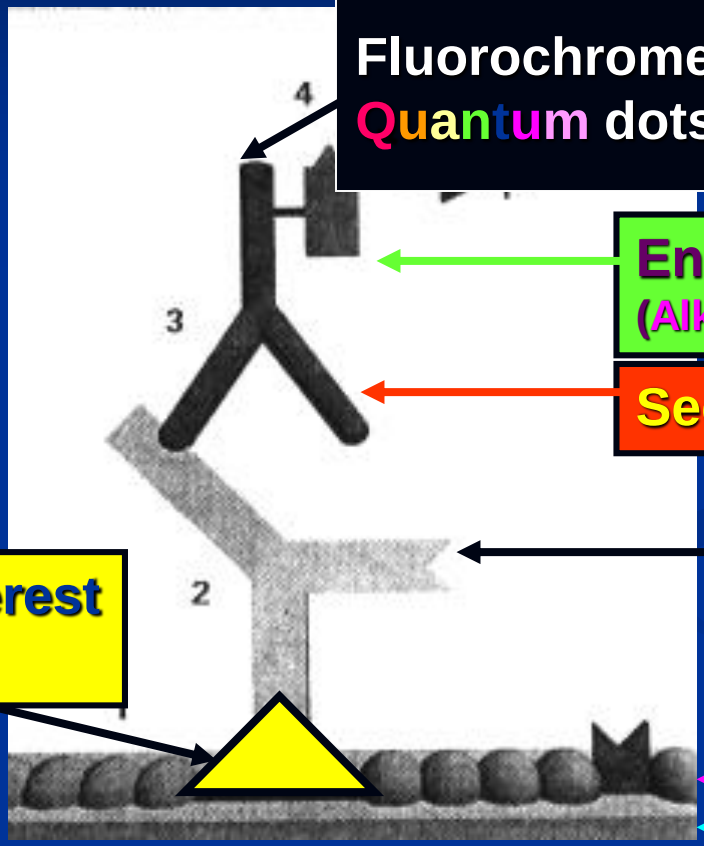
From: Cobbold et al. Trends Cell Biol 2003;13 (Supplementary Table)



How to visualize proteins:

Choosing among visible markers

Immunostaining: Use of Immunoglobulin (IgG) with Microscopically Visible Marker to Antigen



Fluorochrome (Alexa 488, Alexa 568, Cy5)
Quantum dots

Enzyme activity linked to 2° Ab
(Alkaline phosphatase, DAB)

Secondary Antibody

Primary Antibody

Protein of interest
(Antigen)

Blocker

Membrane

Courtesy of Dr. Janet Klein
Emory Renal Division

Immunostaining:
Visible Marker to Antigen is Chromogenic***

Immunolocalization using enzyme detection: enzymes conjugated to secondary Abs

- **Peroxidase**: detected using DAB as electron acceptor with H_2O_2 as substrate

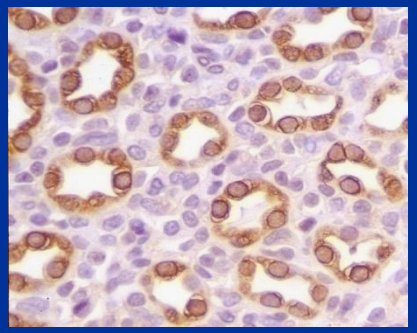
BROWN precipitate at site of activity

- **Alkaline phosphatase**: BCIP/NBT as substrate

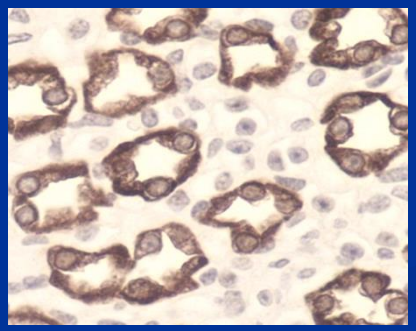
BLACK/PURPLE precipitate at site of activity

VISIBLE MARKER IS CHROMOGENIC: USE BRIGHTFIELD MICROSCOPY

Rat IMCD
UT-A C-terminal Ab



Mouse IMCD
UT-A N-terminal Ab



Urea transporter protein visualized with avidin/biotinylated enzyme complex

Courtesy of J. Klein, Renal Division, Emory SOM

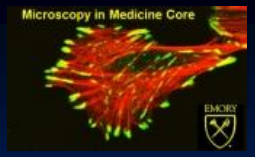
Two Revolutions for Protein Localization in Cells

1. Revolution in Labeling Proteins

   - Monoclonal antibodies
   - GFP technology

2. Revolution in Imaging Modalities

   - Fluorescence microscopy
   - Live cell imaging
   - Super resolution microscopy
     (down to 30 nm resolution)



Immunostaining:
Visible Marker to Antigen is Fluorescent

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Immunofluorescence

Direct: Primary Ab directly coupled to fluorochrome

Rarely used due to

Limited # of fluorochromes that can be attached to Ab

Indirect: Fluorescent label on secondary Abs

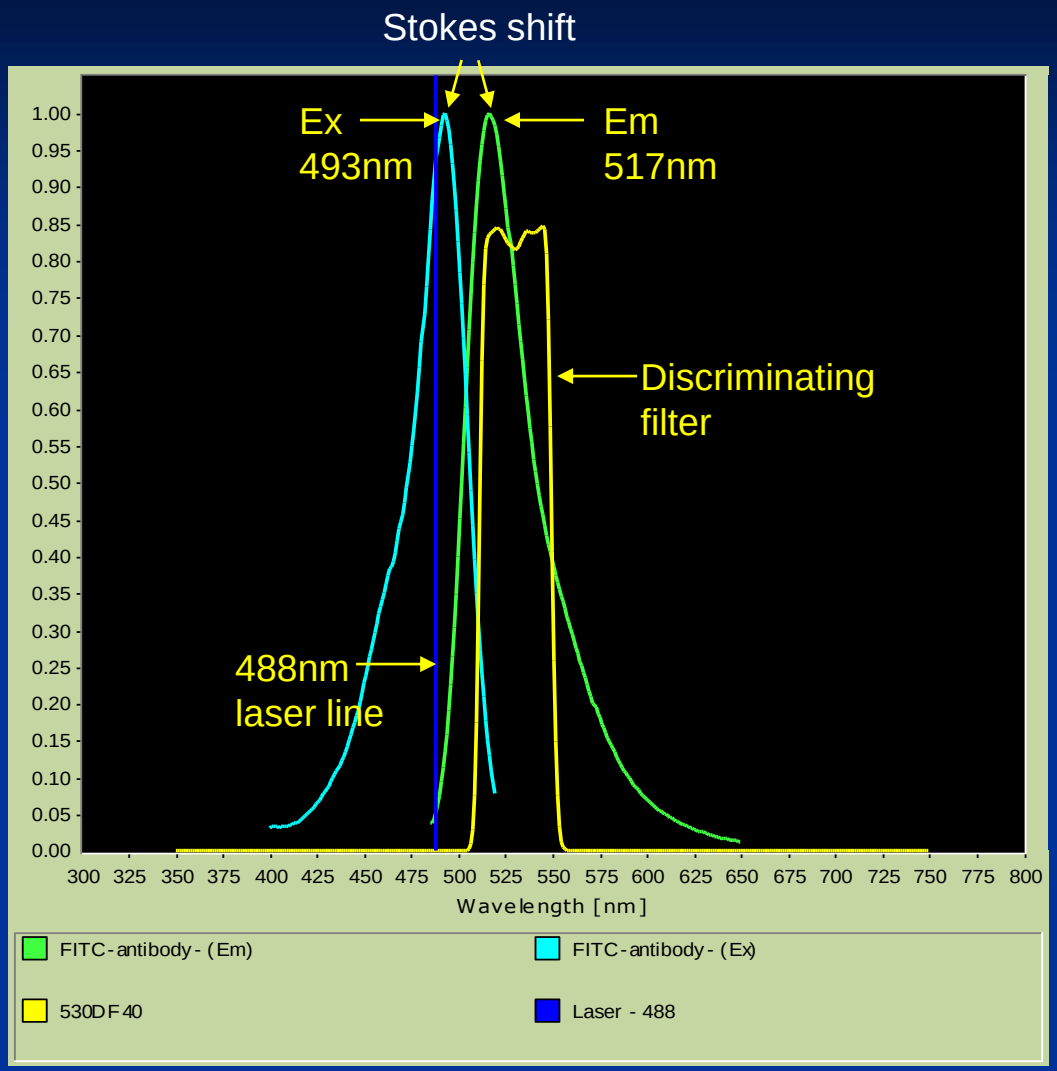
VISIBLE MARKER IS FLUORESCENT: USE FLUORESCENCE MICROSCOPY

WIDE-FIELD (Conventional)

CONFOCAL MICROSCOPY

MULTIPHOTON (GTECH OR NEURO)

Excitation and Emission Spectra of FITC (fluorescein) Ex/Em=493/517 nm



488 nm line of argon laser excites near peak maximum of Ex:493 nm

Fluorescein as fluorochrome:

- high probability of absorbing photon (high extinction coefficient)
- high probability of emitting photon (high fluorescence quantum yield)
- Stokes shift=30 nm

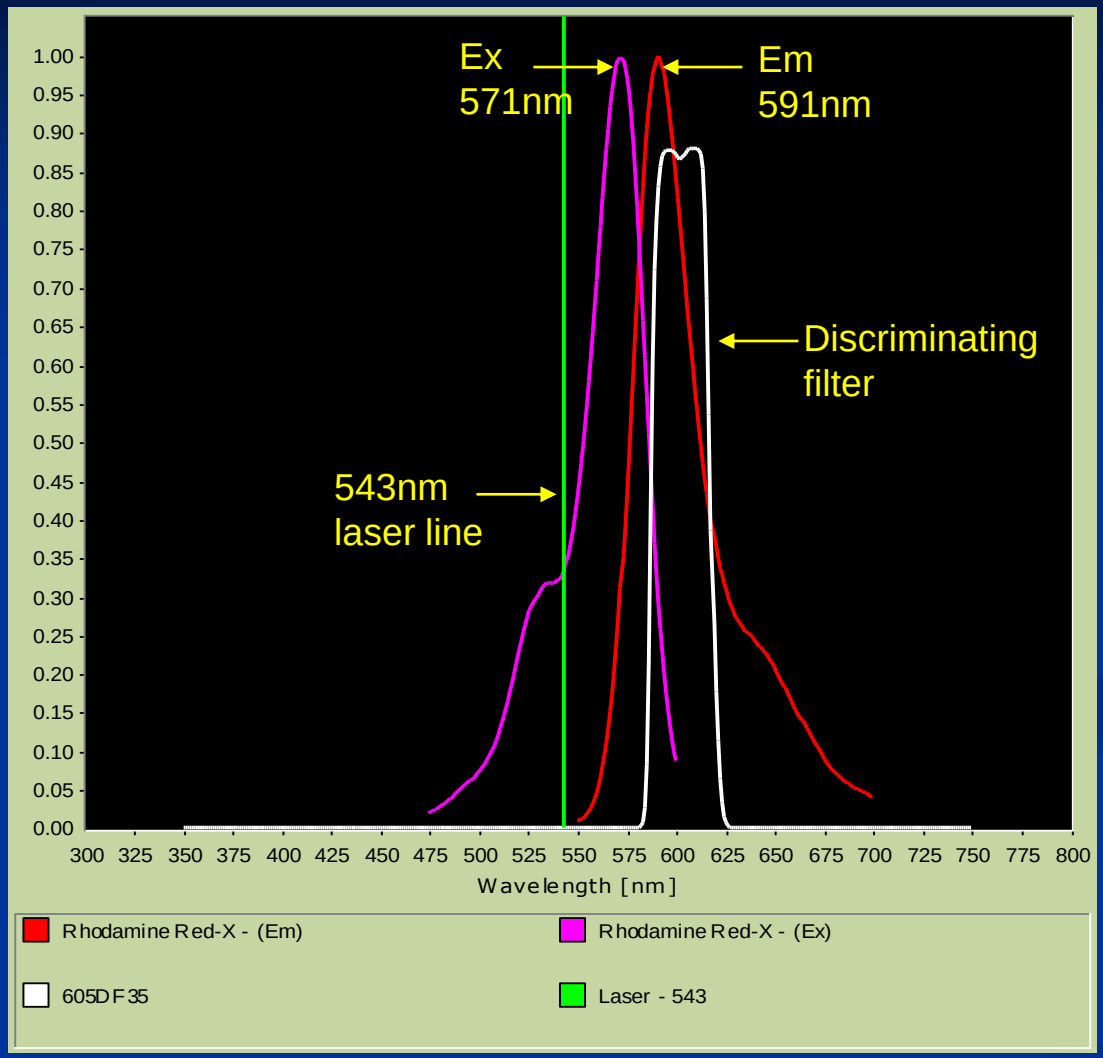
Disadvantages of **fluorescein**:

- Rapid **photobleaching** (irreversible photochemical destruction of dye)
- **pH sensitive**

EMORY

http://fluorescence.nexus-solutions.net/

Excitation and Emission Spectra of Rhodamine Red X Ex/Em=571/591 nm



543 nm line of argon laser does not excite near excitation maximum, but still excites molecule

RRX:

- More photostable than fluorescein
- Not pH sensitive

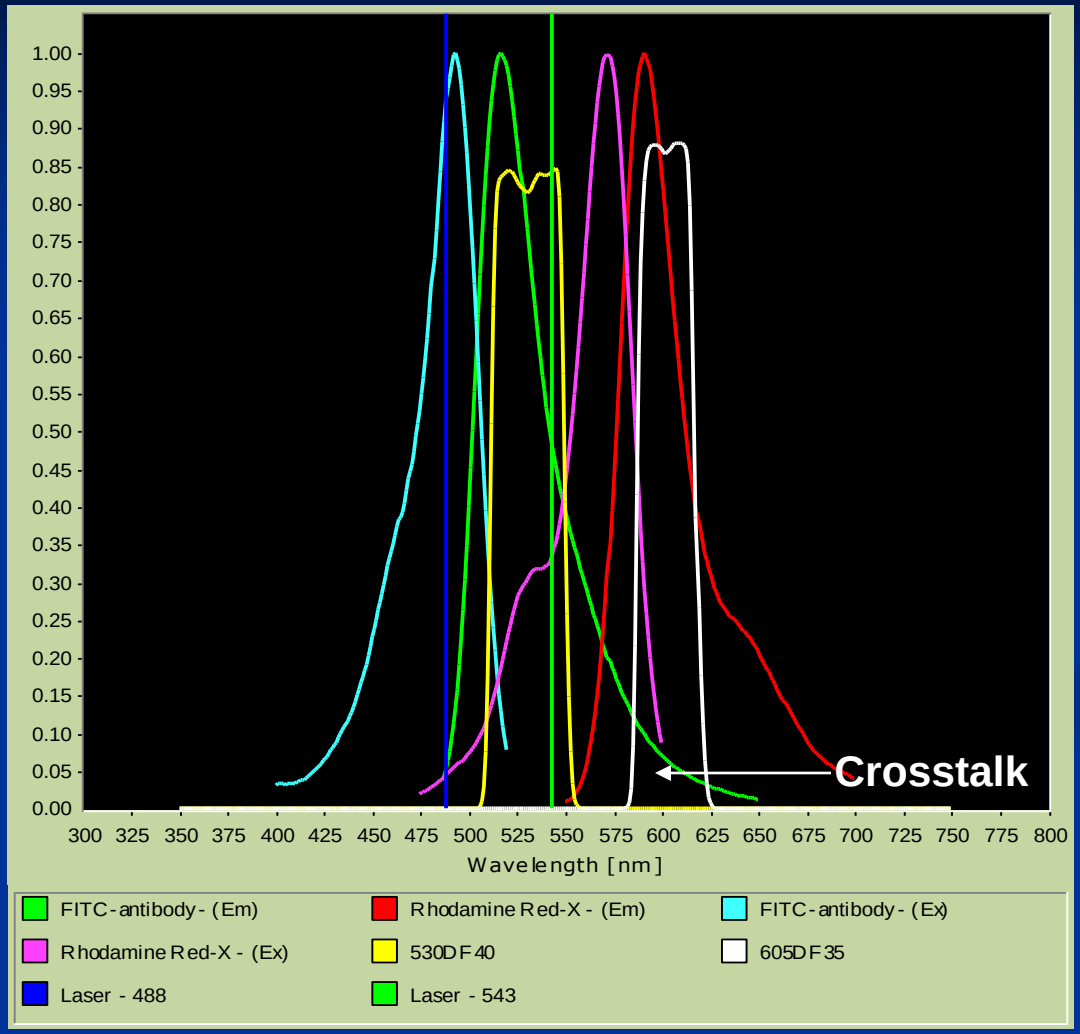
Alexa Fluor dyes (Molecular Probes) may be preferable to **FITC** or **Rhodamine**:

- Brighter
- More photostable
- Not pH sensitive
- Narrow emission spectra

EMORY

http://fluorescence.nexus-solutions.net/

Dual Labeling with Fluorescein and RRX



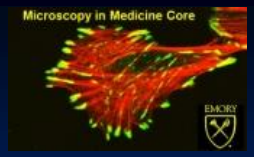
Simultaneous imaging may result in crosstalk (bleed-through) where "green channel" detects signal from red label and "red channel" detects signal from green label

One solution:

Collect individual channels separately (**sequentially**), then merge images in software or Photoshop

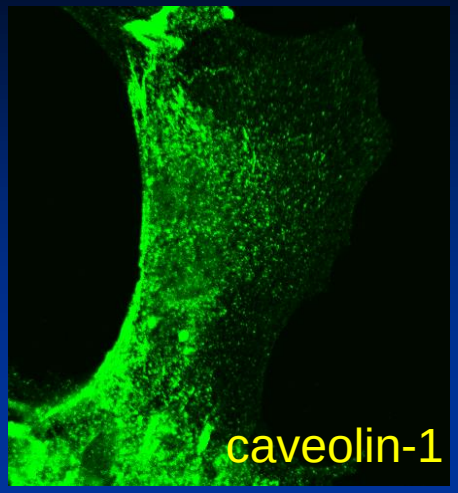
EMORY

http://fluorescence.nexus-solutions.net/

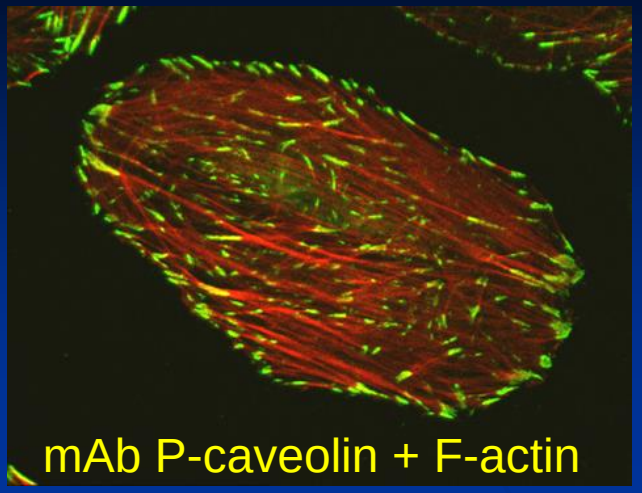


Examples of immunofluorescence labeling

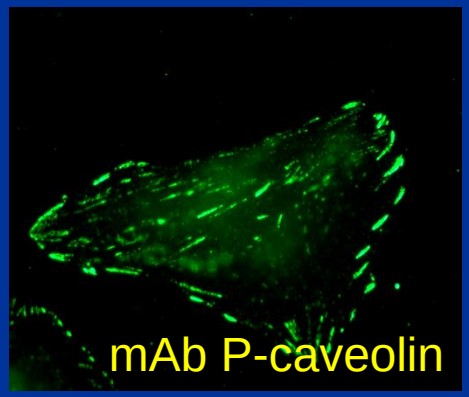
Characterization of p-caveolin Antibody Labeling

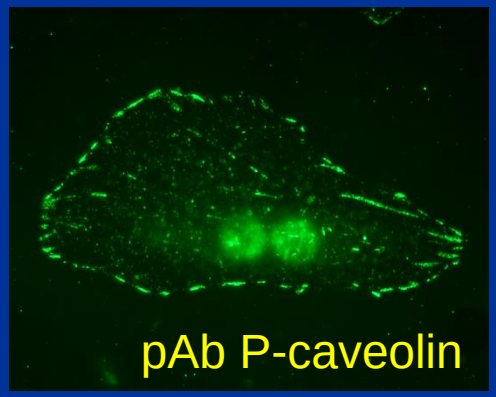


Labeling **PATTERN** similar to focal adhesions

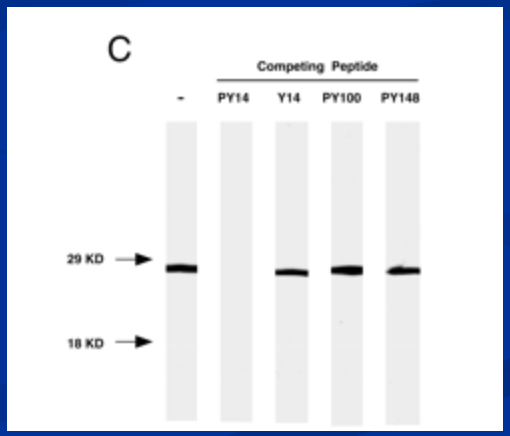


LOCATION at ends of actin stress fibers similar to focal adhesions





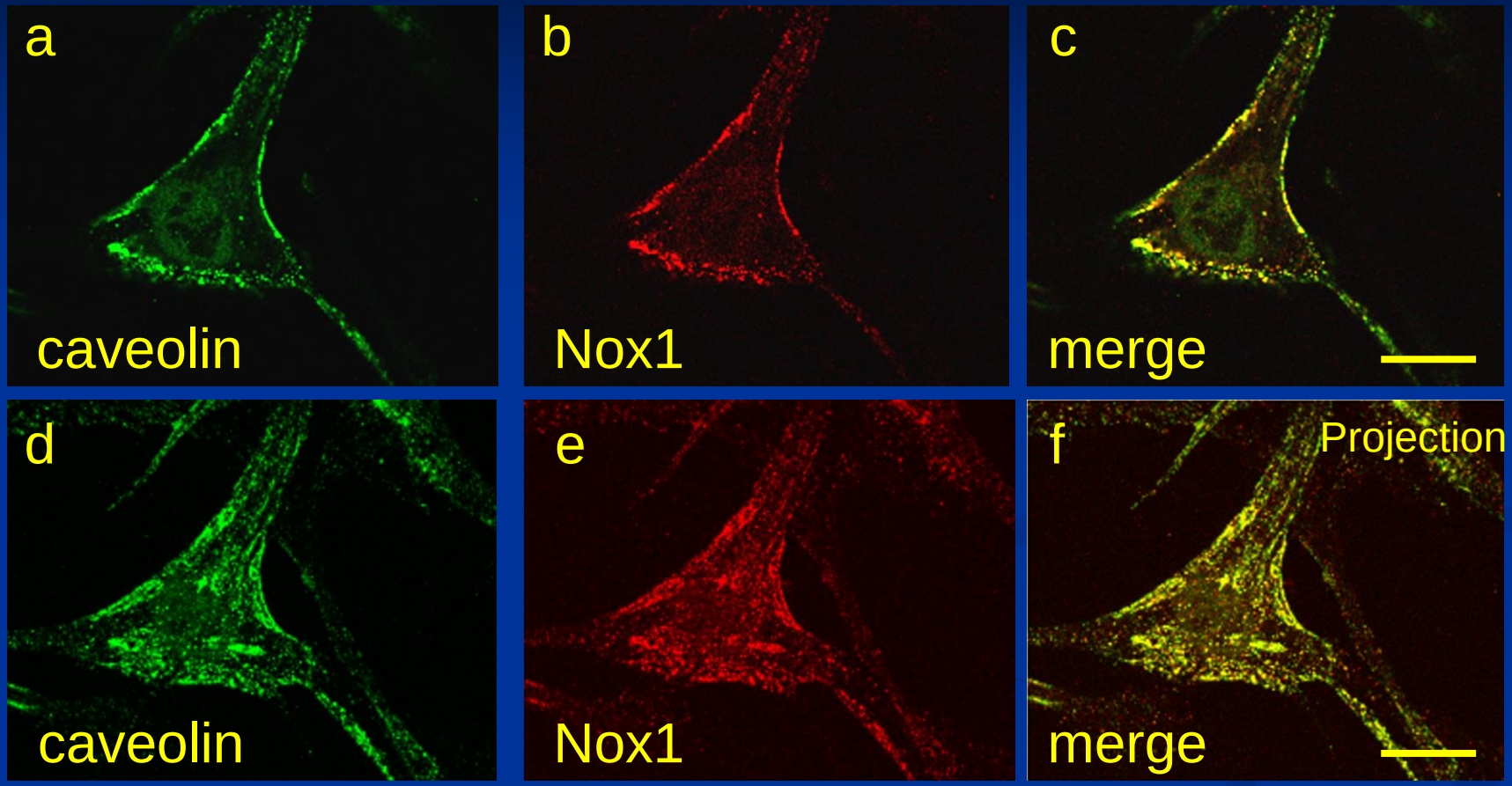
DIFFERENT ANTIBODY from different species shows similar labeling in focal adhesion pattern



Lee et al. 2000. Molec. Endocrinol. 14:1750-1775.

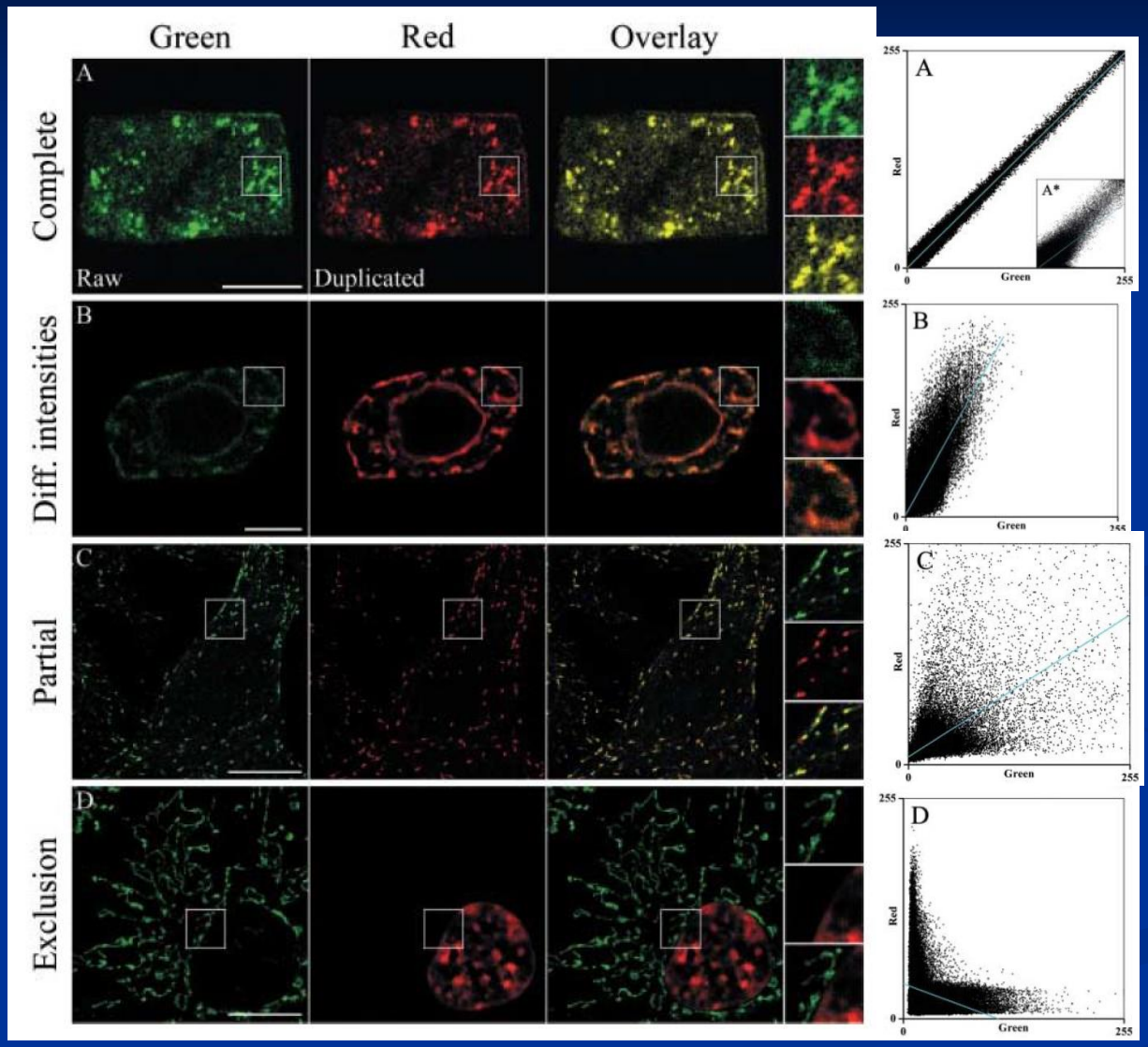
IMMUNOBLOT of mAb shows specific bands

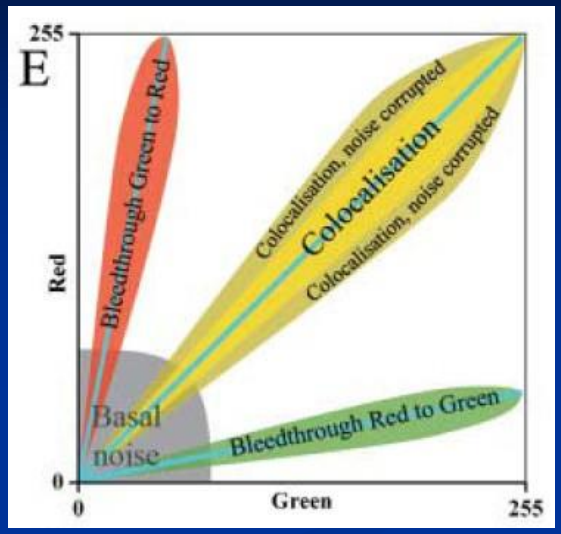
Colocalization of Nox1 with Signature Protein in Caveolae

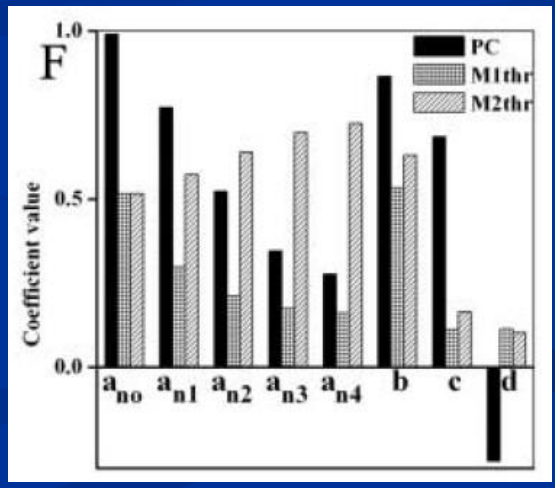


Colocalization of Nox1 with caveolin
YELLOW COLOR is not sufficient
Use coloc software quantitation

Colocalization Software

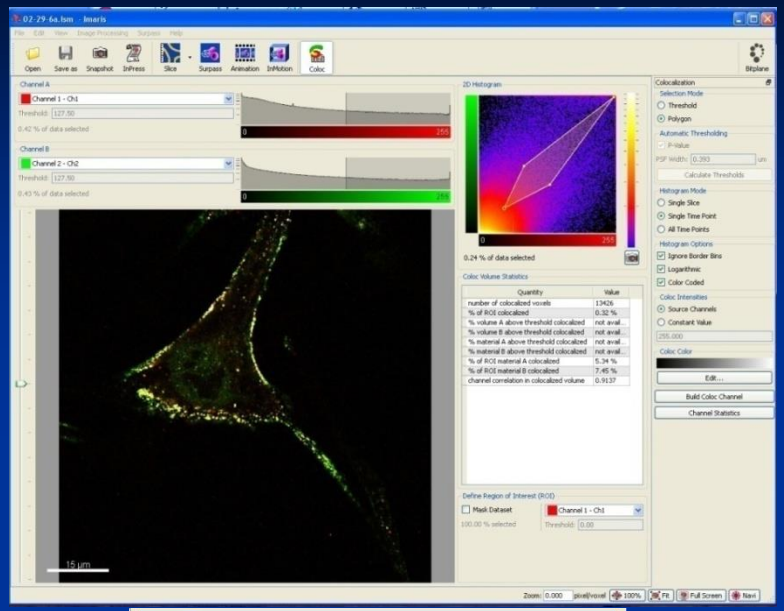


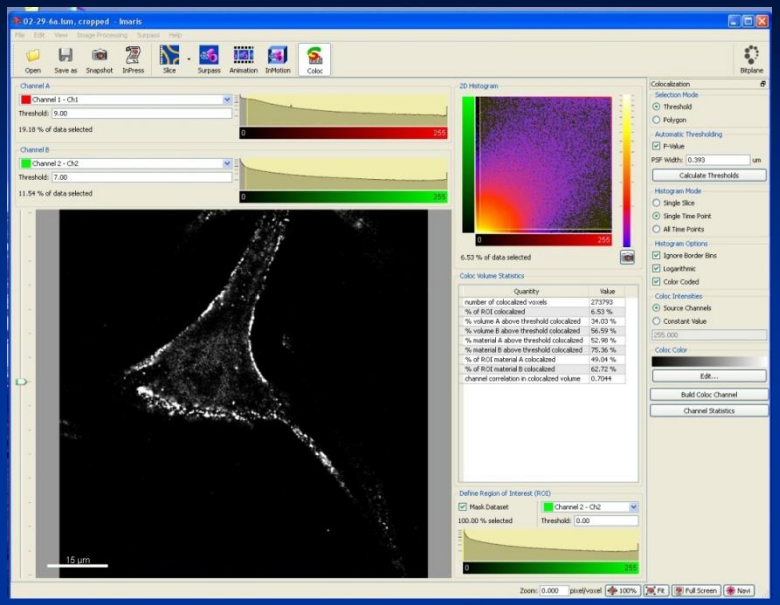


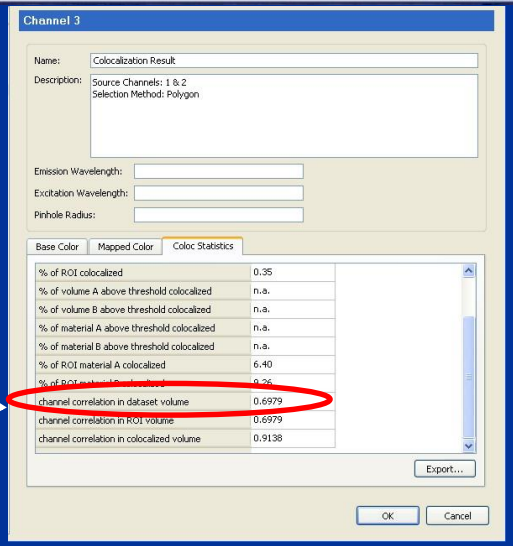


From: Bolte and Cordelieres. J Microsc 2006; 224: 213-232.

Colocalization of caveolin and Nox1 in HASMs using Imaris







Costes SV et al. Automatic and quantitative measurement of protein-protein colocalization in live cells. Biophys J 2004; 86:3993-4003.

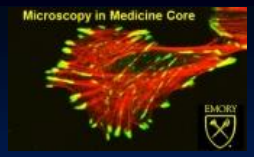
Costes et al. approach is based on PC and consists of 2 steps:

(1) Automatic threshold set to minimize noise.

(2) Statistical method based on image randomization.

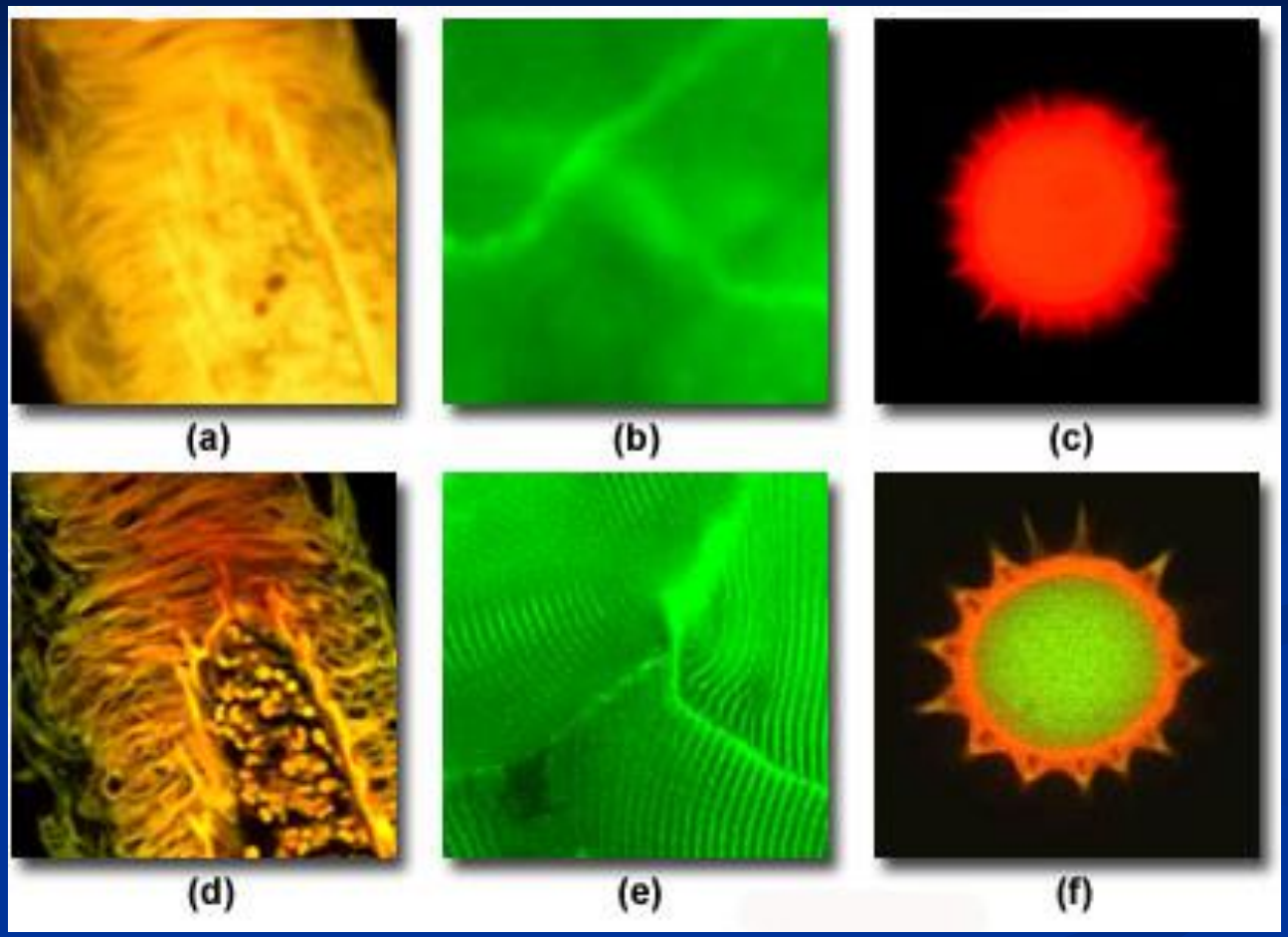
The original channel 1 image is compared to 200 "scrambled" channel 2 image blocks chosen to equal the PSF of the image. This method excludes colocalization of pixels due to chance.

From: Hilenski et al. ATVB 2004; 24:677-683.



What to visualize proteins with:
Choosing among microscopes

PROBLEM: OUT-OF-FOCUS LIGHT MAKES BLURRY IMAGES

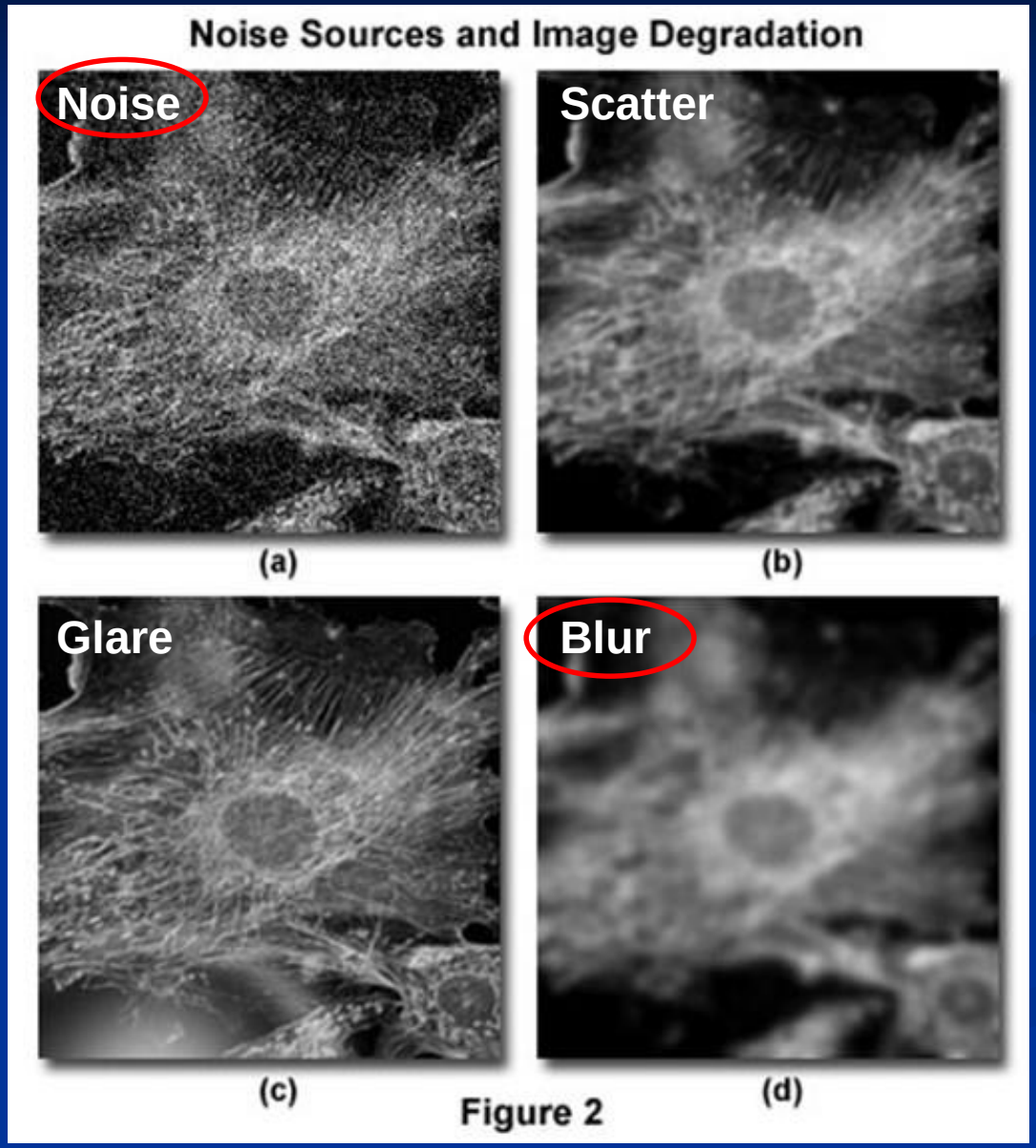


http://www.olympusconfocal.com/theory/confocalintro.html

WHICH MICROSCOPY METHOD SHOULD BE USED?

- **Wide field Deconvolution:** Computationally removes out-of-focus light.
- **Laser Scanning Confocal Microscopy:** Physically removes out-of-focus light by use of pinhole aperture. USE WHEN IMAGING THICK SPECIMENS > 5 µm thick OR WHEN YOU WANT 3-D SPATIAL INFORMATION.
- **Spinning Disk Confocal Microscopy:** Series of spinning pinholes removes out-of-focus light. USE FOR LIVE CELL IMAGING.
- **Resonance Scanning:** Scanning mirrors raster scans specimens at high speeds. USE FOR LIVE CELL IMAGING.
- **Multiphoton Microscopy:** Excitation is limited to focal plane. USE WHEN IMAGING DEEP INTO TISSUE SECTIONS (300-400 µm).
- **Super Resolution Microscopy:** Overcomes diffraction barrier. USE FOR IMAGING STRUCTURES < 200 nm (e.g., microtubules, actin filaments).

Wide field Deconvolution



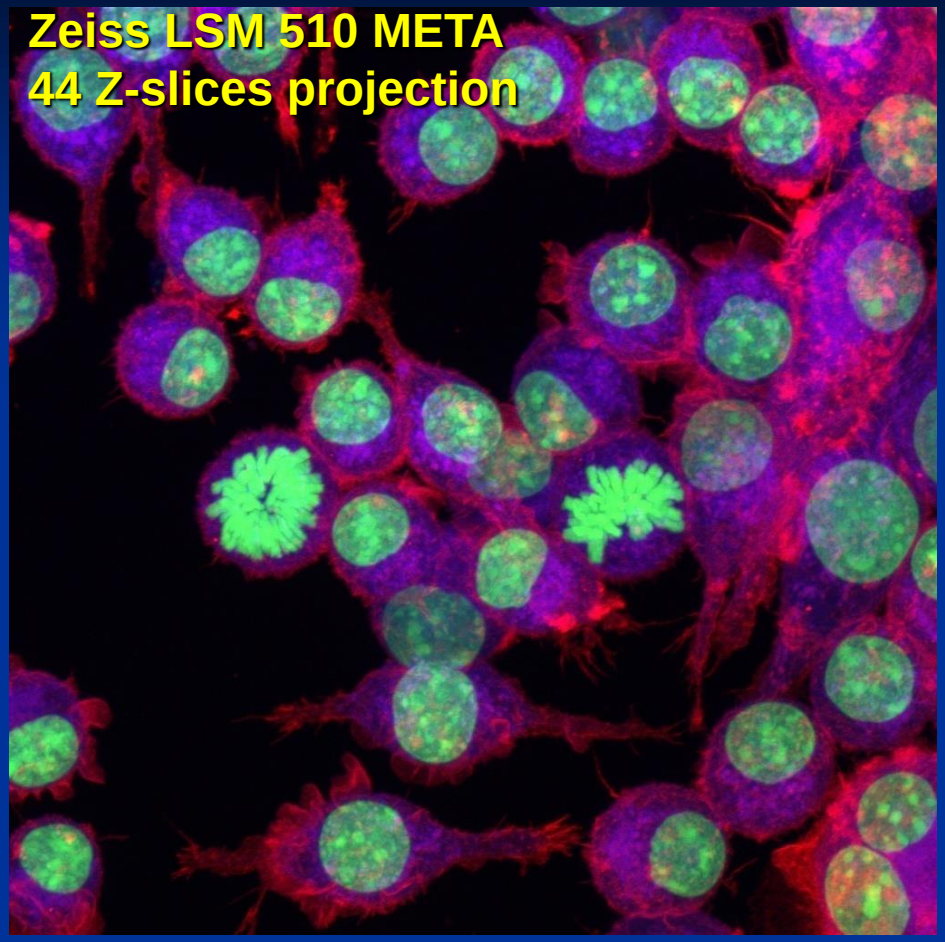
Computationally intensive deconvolution algorithms remove out of focus blur and noise

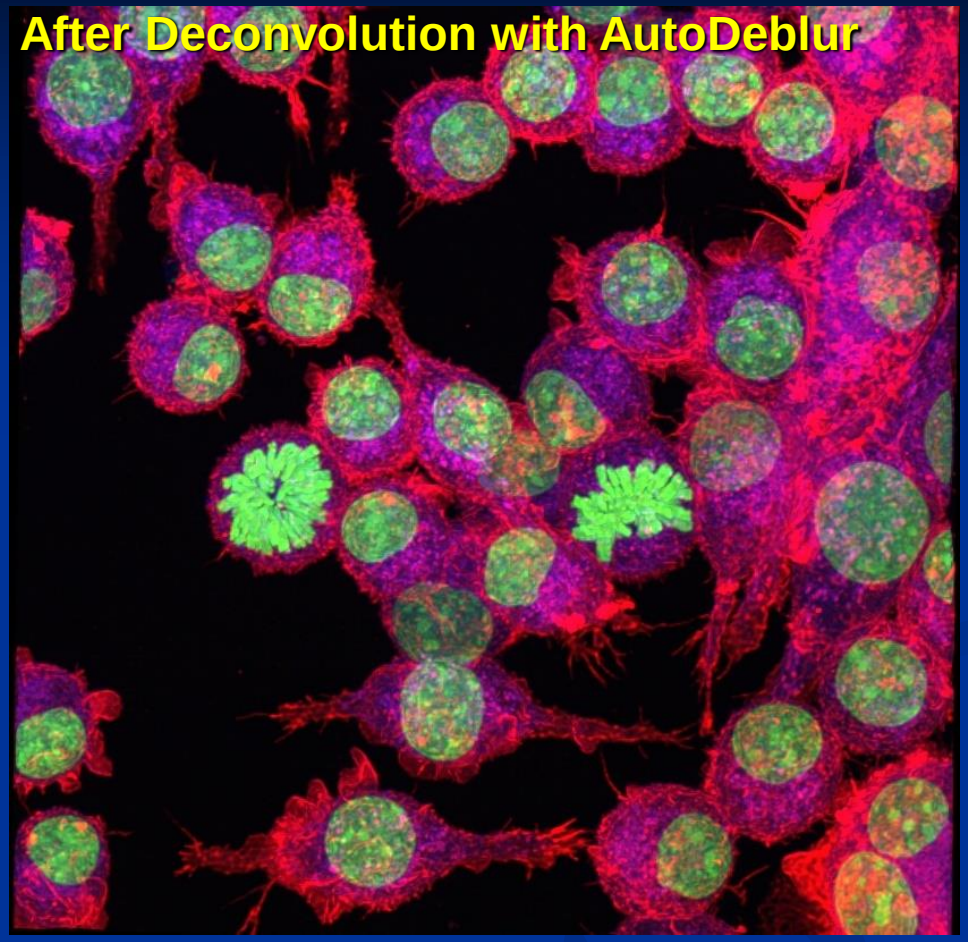
Most commonly used algorithms are deblurring (subtracts blur) and image restoration (reassigns blurred light to proper in-focus location)

Constrained iterative algorithms operate in successive cycles (thus, the term "iterative“)

http://micro.magnet.fsu.edu/primer/digitalimaging/deconvolution/deconintro.html

Effect of Deconvolution of Z stacks using AutoDeblur





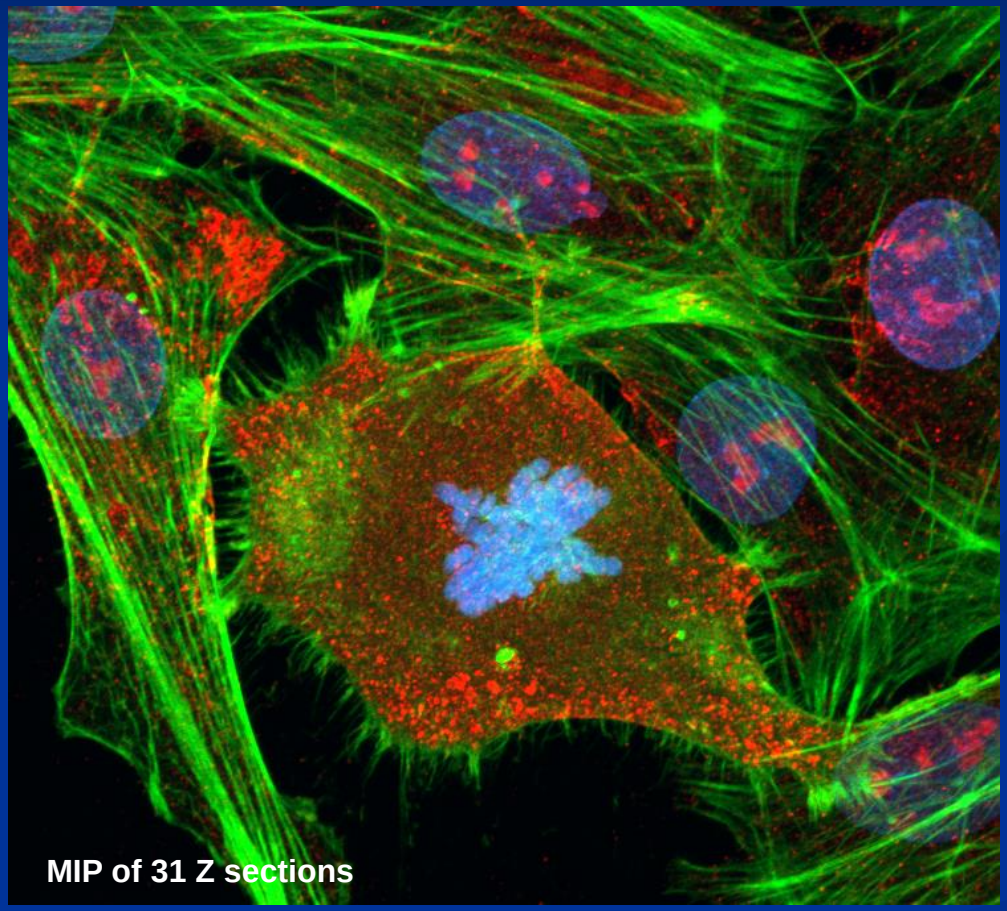
Zeiss LSM 510 META
44 Z-slices projection

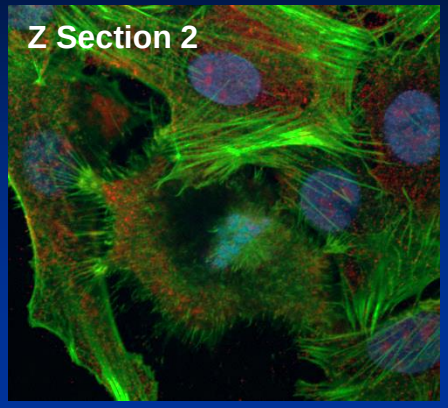
After Deconvolution with AutoDeblur

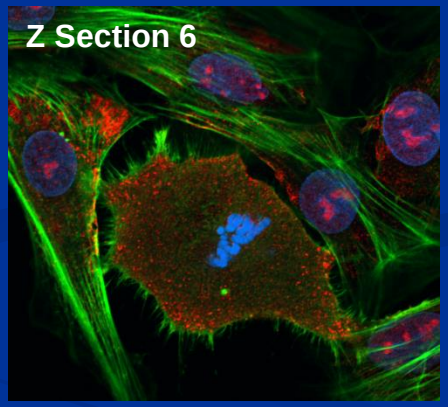
Laser Scanning Confocal: Zeiss LSM 510 META

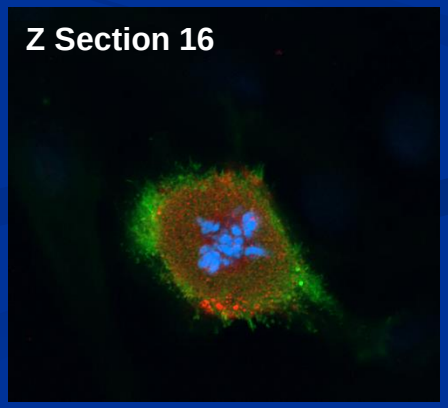
Location: WMB 303

- Out of focus fluorescence eliminated by pinhole
- Optical sectioning

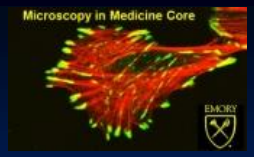






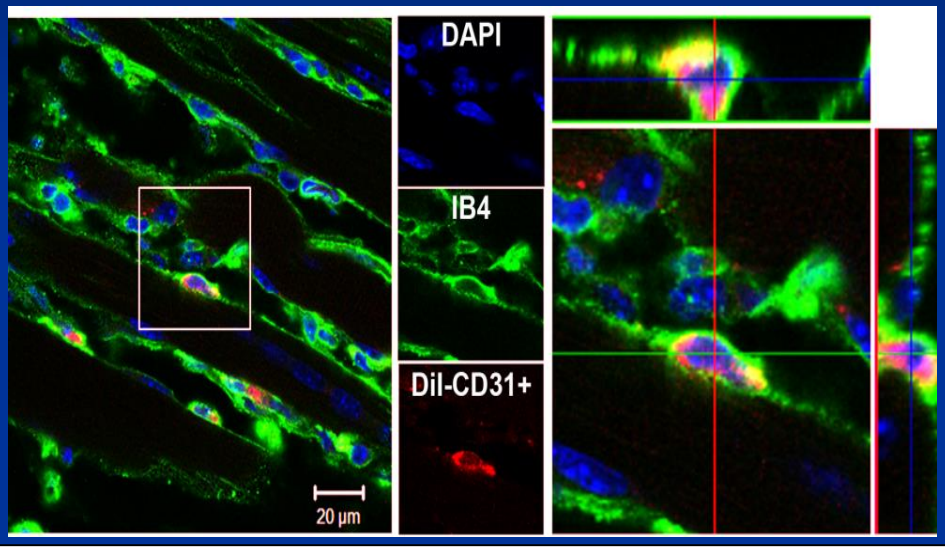


USE POINT SCANNING CONFOCAL WHEN IMAGING THICK SPECIMENS > 5 µm thick OR WHEN YOU WANT 3-D SPATIAL INFORMATION



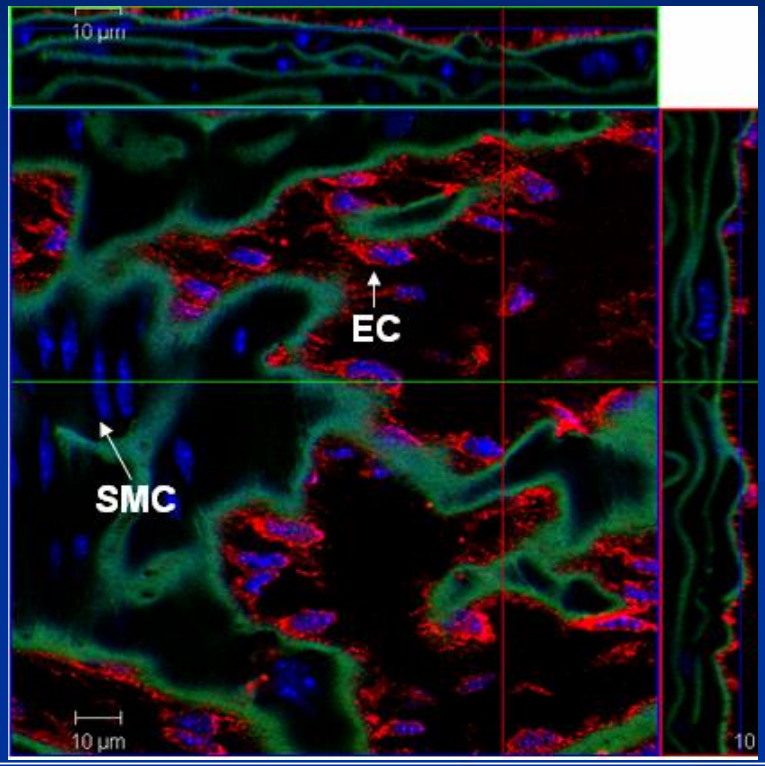
Zeiss LSM 510 META

Immunofluorescence labeling of cells in blood vessels



Vasculogenesis of mBM-CD31+ cells in a hind limb ischemia model. Injected DiI-mBM-CD31+ cells are incorporated into vessels and express an EC marker, IB4 (green fluorescence). Blue: DAPI. Image courtesy of Dr. Young-sup Yoon.

From: Kim, HB et al. Circ Res 2010;107: 602-614.



Mouse aorta en face staining
BMP4: Red
DAPI: Blue
Elastin autofluorescence: Green
BMP4 only in ECs

From: Chang et al. Circulation 2007;116: 1258-1266.

Leica TCS SP5 II

Tandem scanner (conventional and resonance) and HyD detectors

Location: WMB 1011

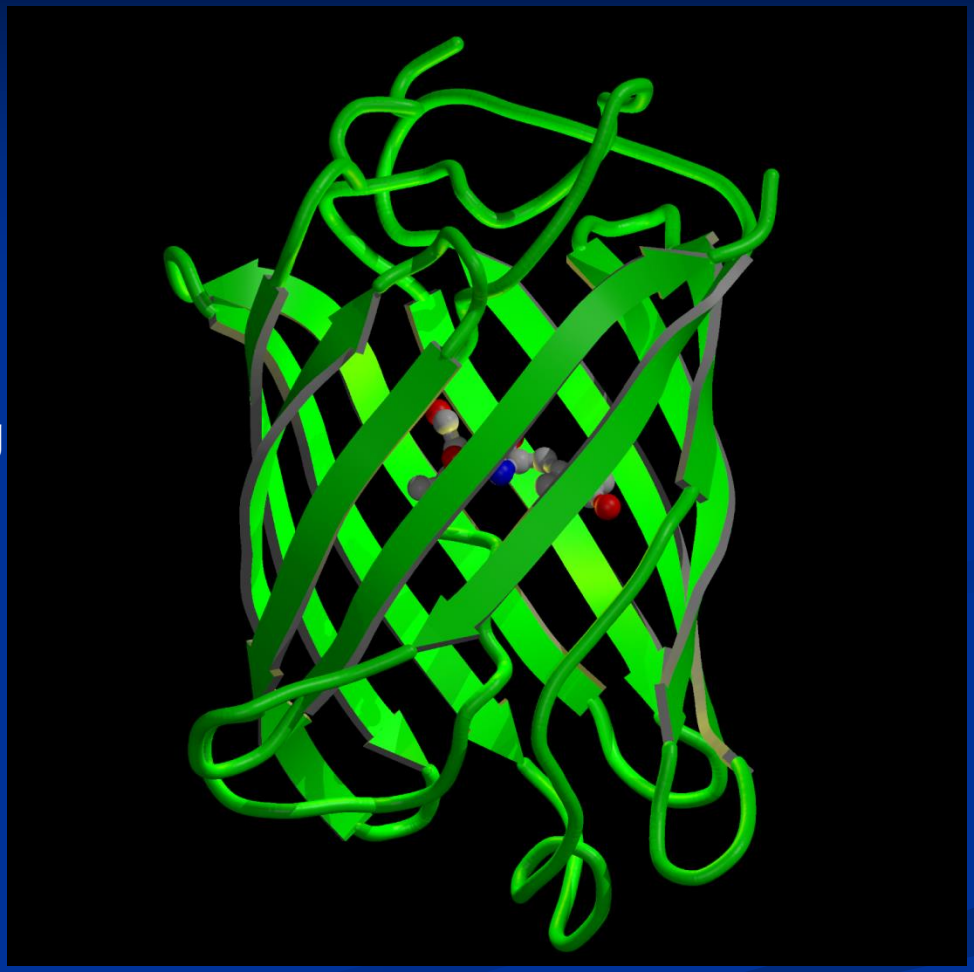
Genetically-encoded Fluorescent Indicators
Green Fluorescent Protein (GFP): Naturally fluorescent protein

Isolated from *Aequoria victoria (*jellyfish*)*

GFP used to observe patterns of gene expression by attaching promoter of gene being studied to coding region of **GFP**

Mutation has improved brightness (folding efficiency) and provided four colors: blue, cyan, green, yellow

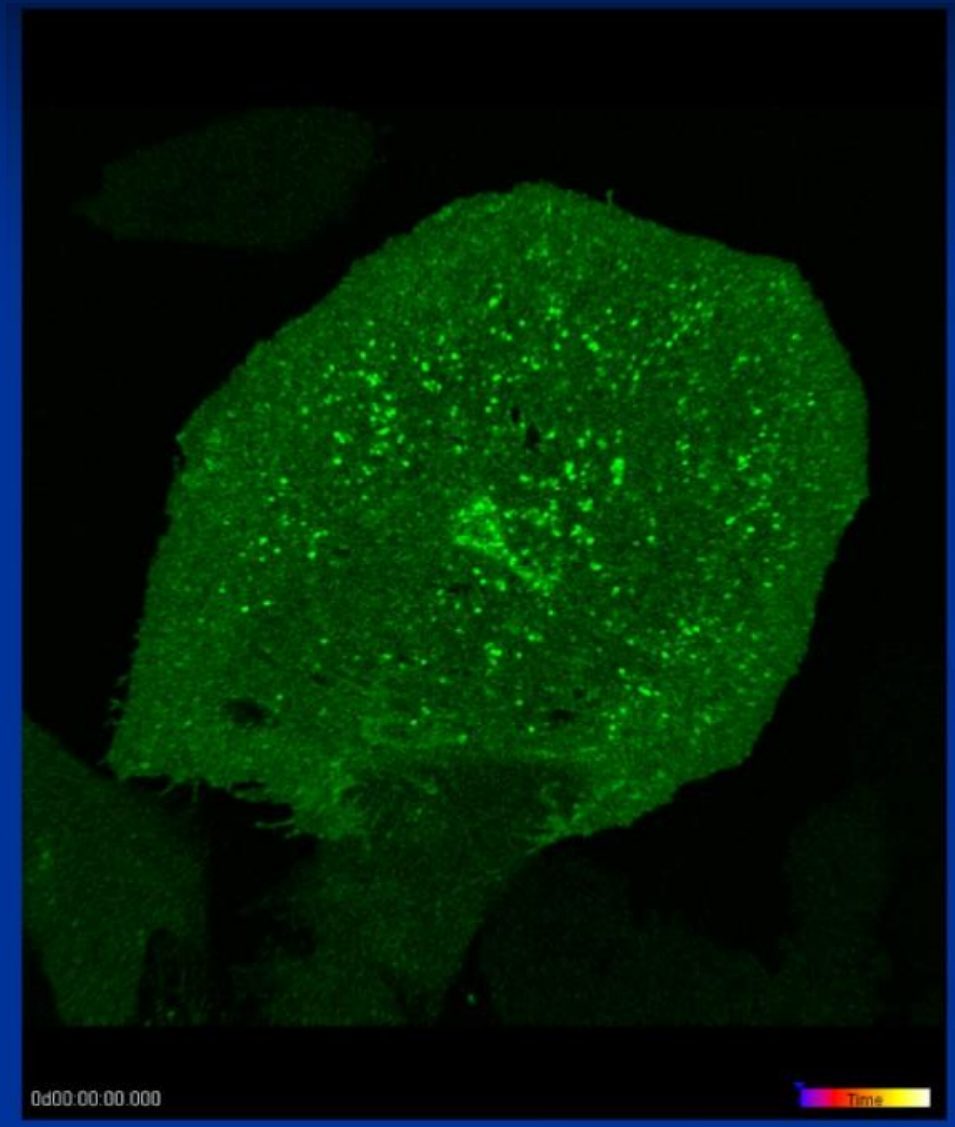
GFP variants used in FRET experiments to determine molecular proximity



GFP

Courtesy of Stephen Adams, UCSD

Leica Live Cell Imaging



Acquisition Parameters

CHO transfected with GFP-AT1R

10 Z sections (Only one shown)

Every 4 seconds

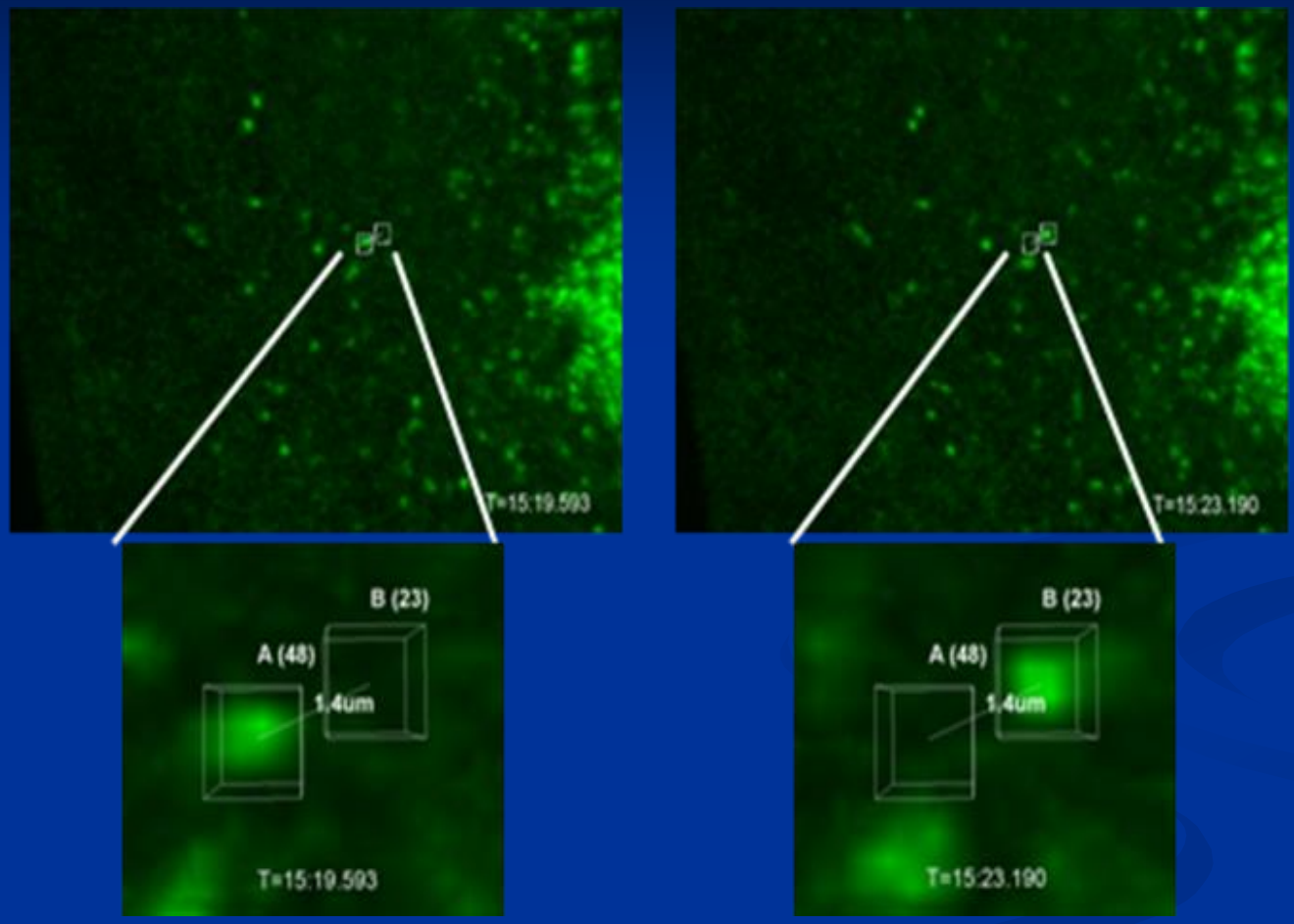
24 minutes (400 time points)

Resonance scanning

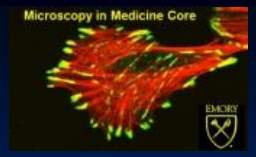
8000 Hz

40x oil NA=1.25

Imaris Tracking Measurement

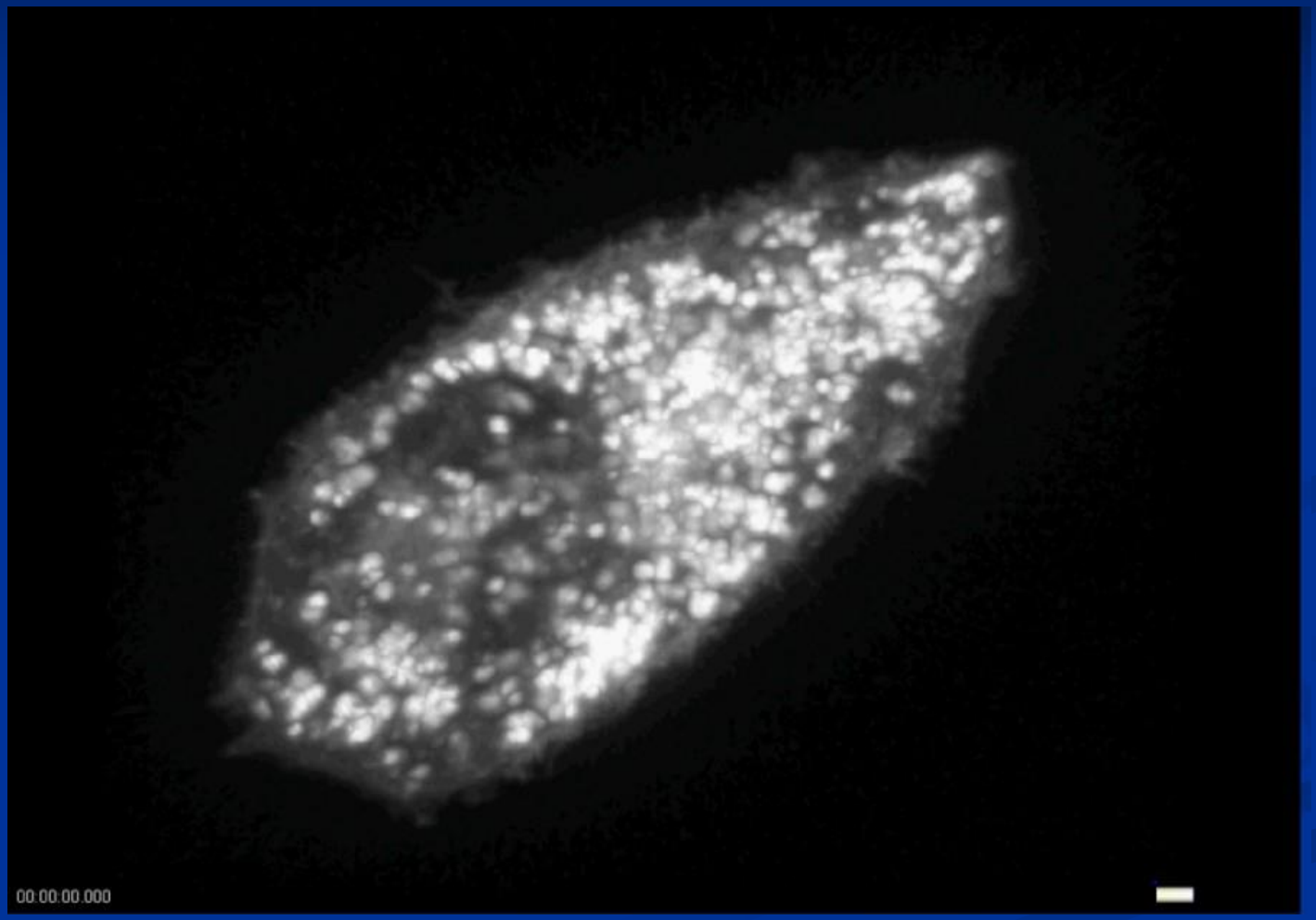


Projections of 10 Z sections of CHO cell expressing GFP-AT1R, showing 1.4 µm movement of a single puncta during a 4 second time interval.

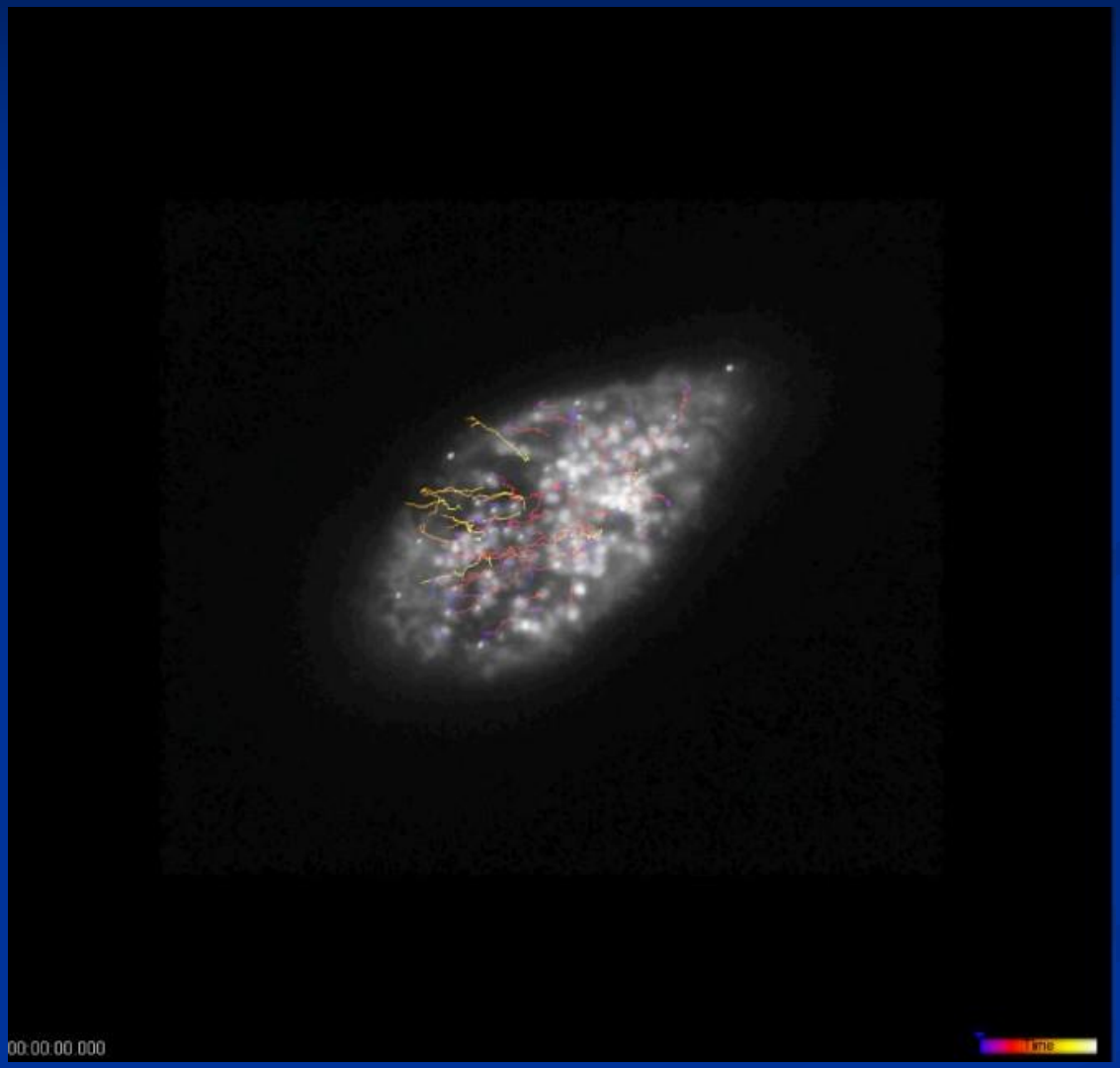


Imaris Tracking

EMORY

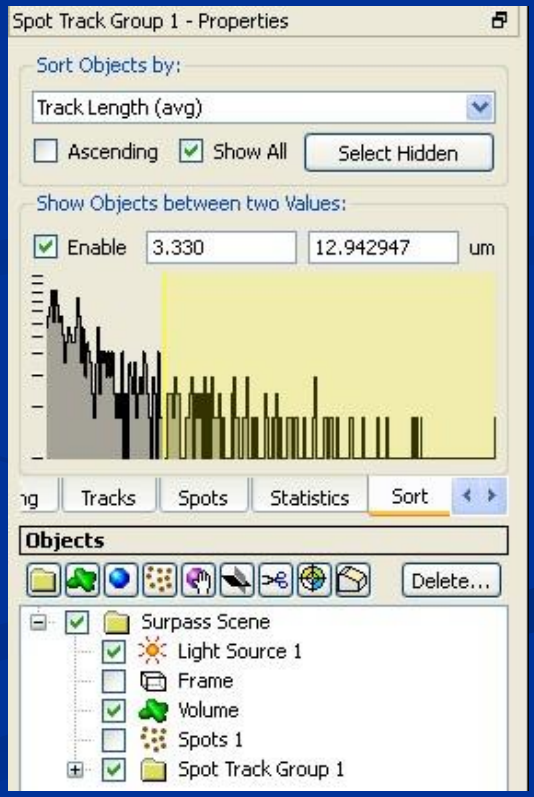


Imaris Tracking Measurement

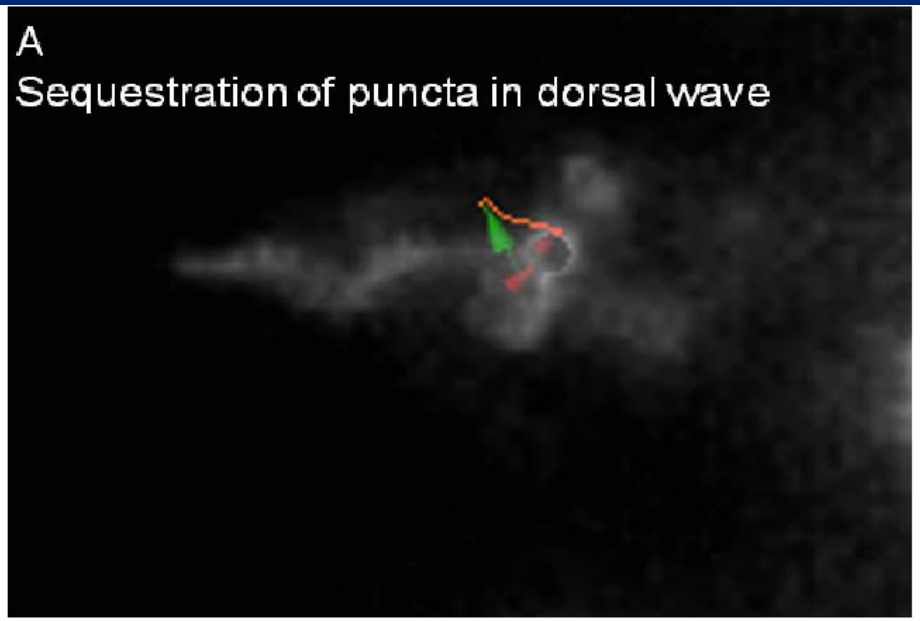


Top 4 Z-slices every 4.5 sec for 11 min (146 time points)

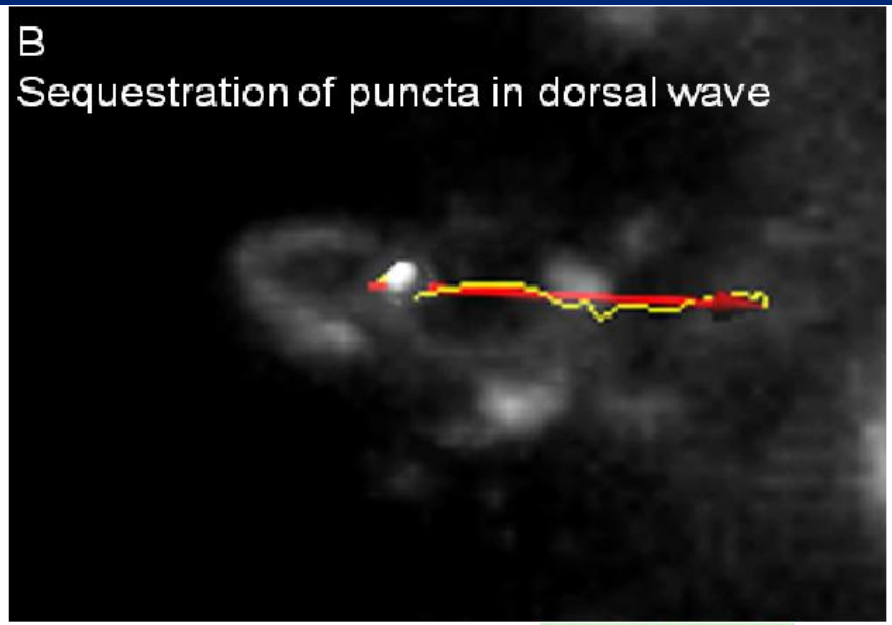
Imaris Spot, Track and Sort



Imaris Tracking Measurement

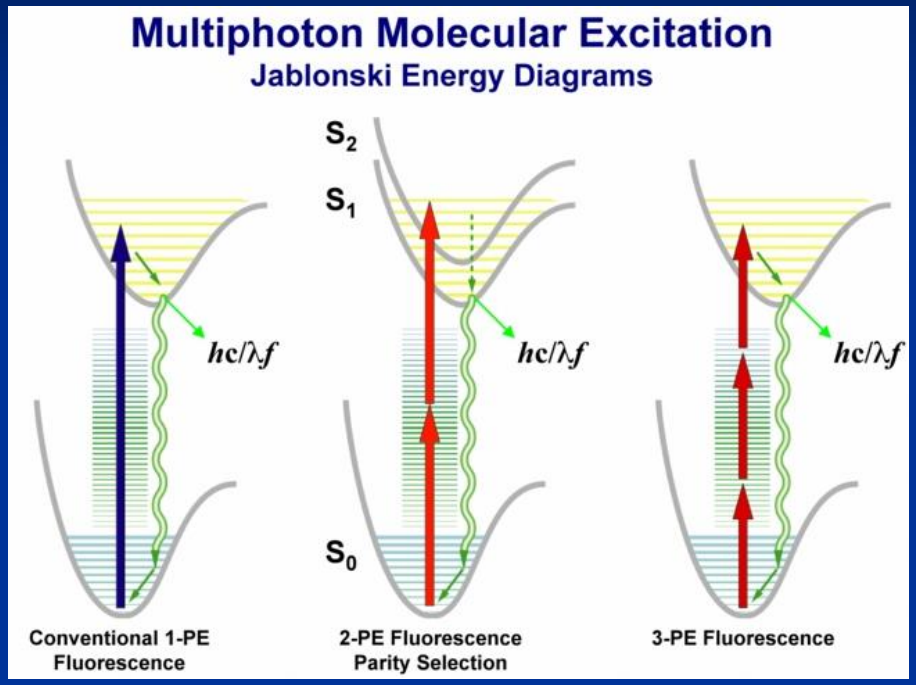


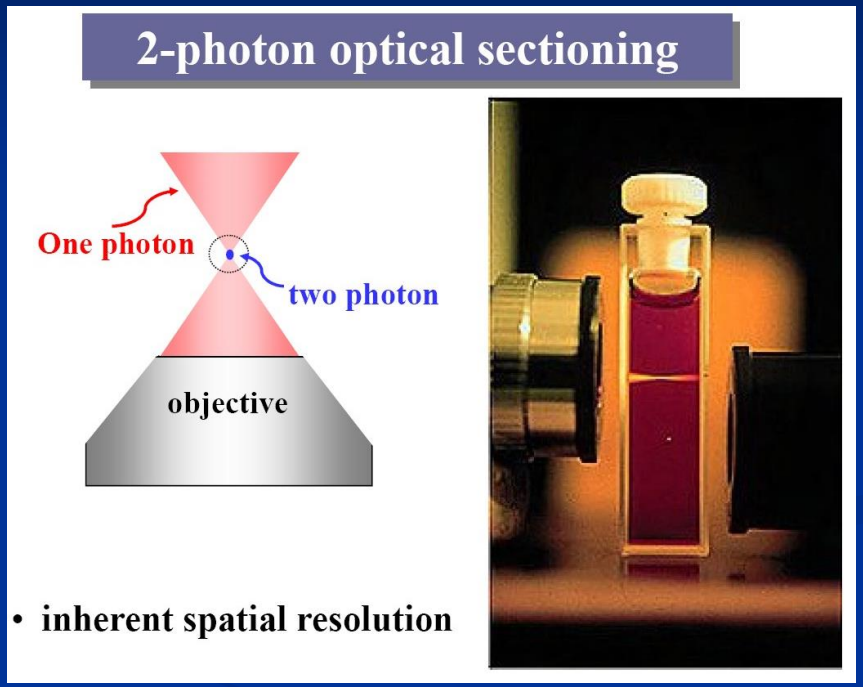
Track Displacement (avg)	1.38499999	um
Track Duration (avg)	28.03499985	s
Track Length (avg)	2.438999891	um
Track Speed Avg. (avg)	0.086999997	um/s
Track Speed Variability (avg)	0.783999979	
Track Straightness (avg)	0.568000019	



Track Displacement (avg)	6.02699995	um
Track Duration (avg)	104.1299973	s
Track Length (avg)	7.967000008	um
Track Speed Avg. (avg)	0.077	um/s
Track Speed Variability (avg)	0.529999971	
Track Straightness (avg)	0.755999982	

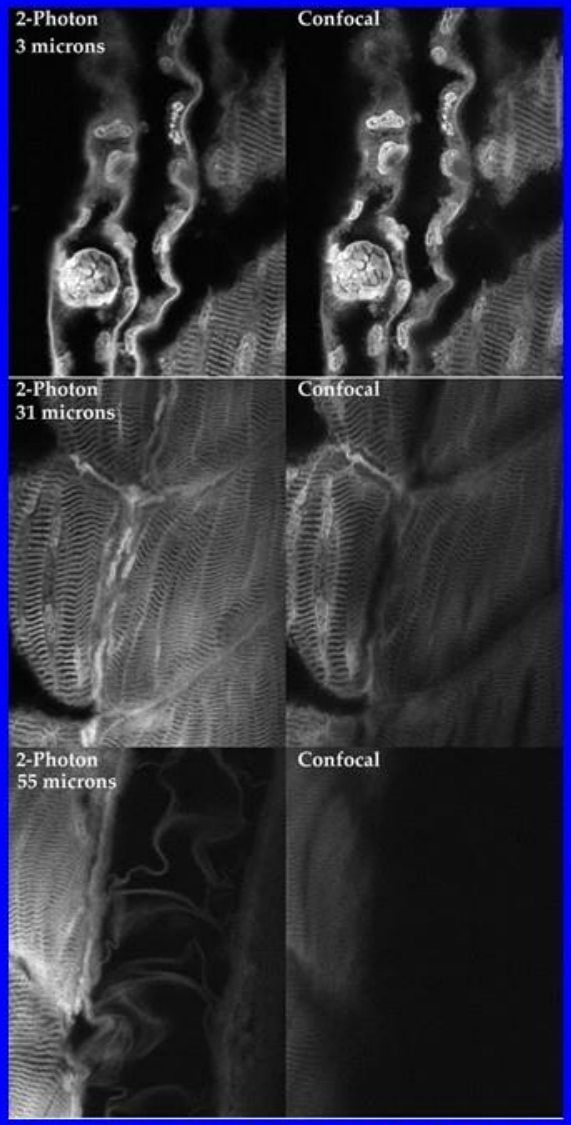
Multi-photon Imaging





- Multi-photon imaging uses ultrafast 100 femtosecond pulsed lasers
- Simultaneous absorption of 2 or more photons can occur
- Molecular excitation is equivalent to the sum of the absorbed photon energies
- Excitation is limited to the beam focus
- Photodamage is restricted to the focal plane
- 2-3 fold deeper penetration into tissue (< 500 μm)

Multi-photon Imaging

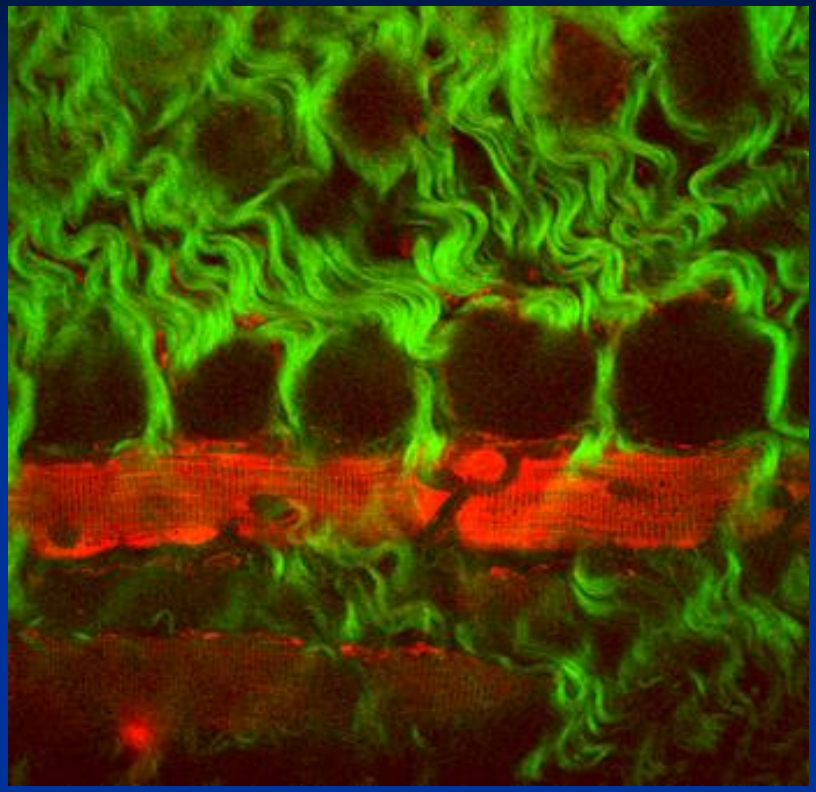


Examples of 2-p imaging

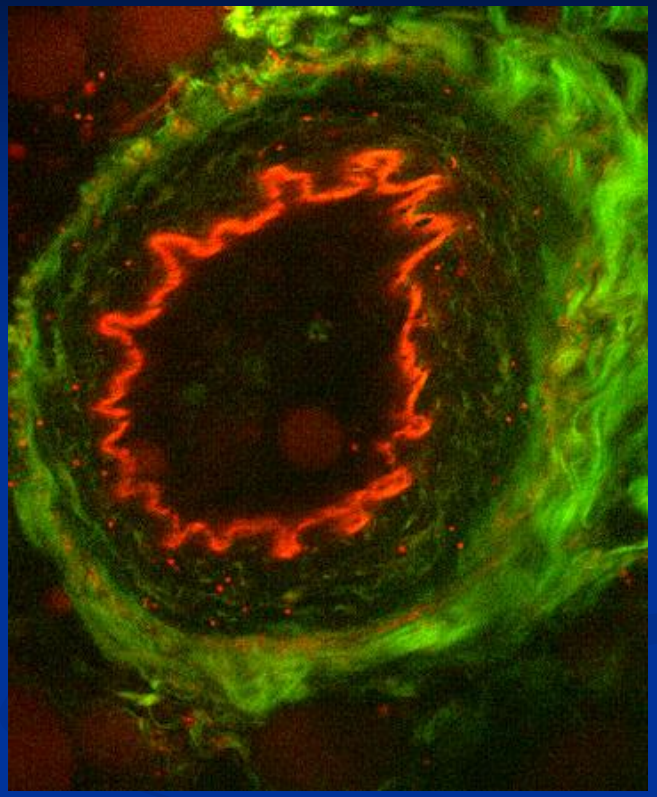
Sequence of images showing a comparison between confocal imaging (488nm excitation) and 2-photon imaging (1047nm excitation). The sample is a zebra fish that is heavily stained with safranine (the sample was prepared by B. Amos). As can clearly be seen, 2-photon imaging is able to give much better images deep into the specimen.

Images from: Multi-Photon Excitation Fluorescence Microscope Coordinator, Madison, WI

Multi-photon Imaging of Thick Tissues

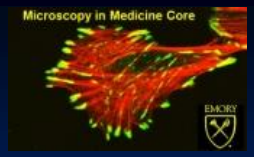


Multiphoton image of cardiac tissue from an adult mouse.
Myocytes: Visualized by NAD(P)H **RED**
Collagen: Visualized by SHG **GREEN**
http://www.drbio.cornell.edu/Infrastructure/MPM_WWW/MPM_hist/heart.htm

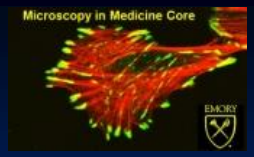


Multiphoton image of an intact, axially-oriented arteriole within a mouse lymph node.
Elastin: Pseudocolored **RED**
Collagens I and III: Pseudocolored **GREEN**
http://www.drbio.cornell.edu/Infrastructure/MPM_WWW/MPM_hist/heart.htm

LLH 2013
Emory SOM



Recent advances in imaging



Super Resolution Microscopy

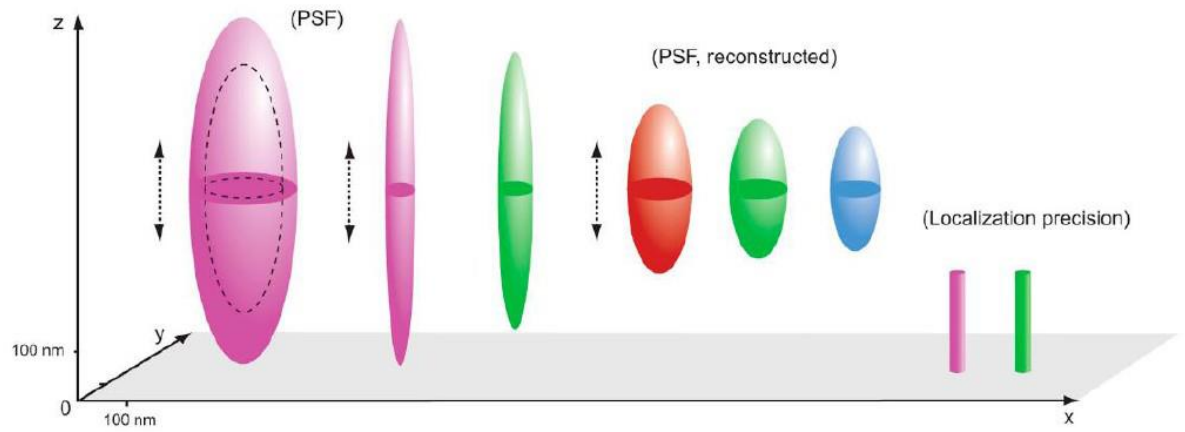
Increasing Resolution

- Localization Microscopy
  - Photoactivation Localization (PAL-M)
    - Selectively turning on a small number of photoactivatable probes, then using math to localize positions
  - Stochastic Reconstruction (STORM)
    - Stochastically switching on fluorophores, then using math to localize their positions
- Illumination Manipulation
  - Stimulated Emission Depletion (STED)
    - Uses IR laser to deplete (switch off) dyes in the neighborhood of the excitation spot
  - Structured Illumination (3D-SIM)
    - Using illumination patterns to extract information

Super Resolution Microscopy

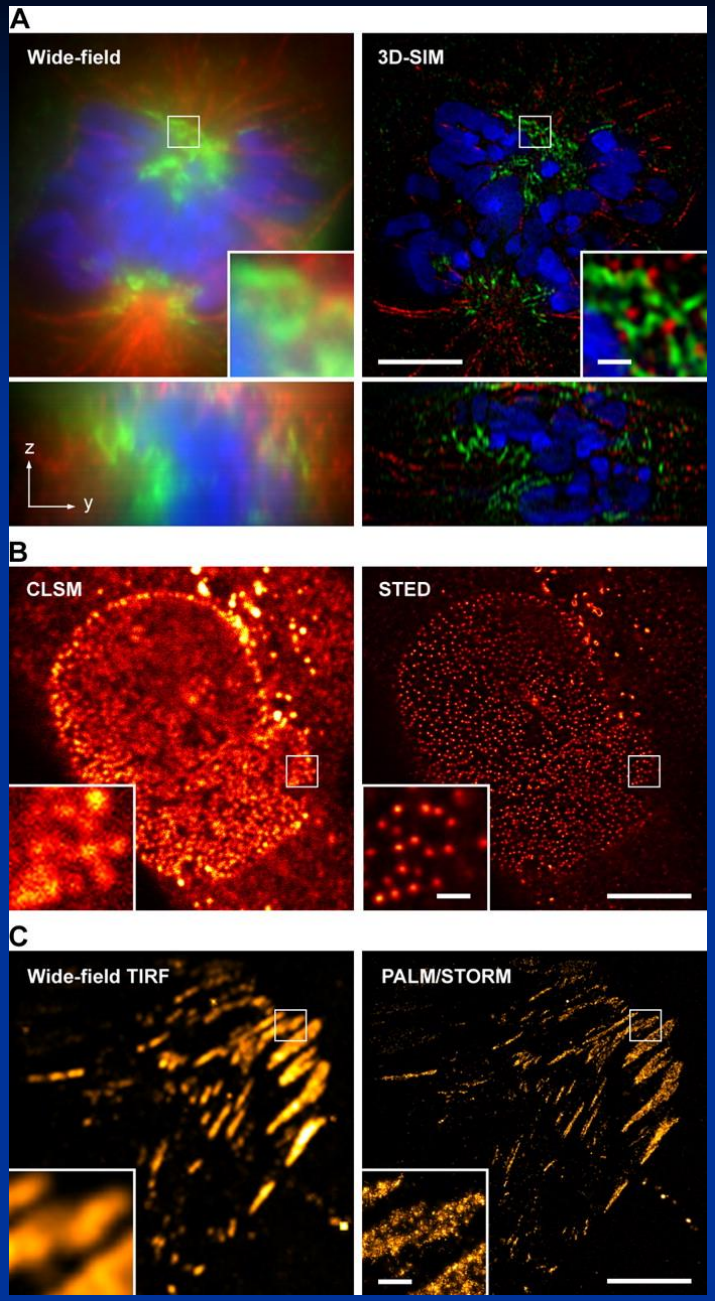
Super Resolution Comparisons

	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λem [nm]	460-670	670	520	620	520	460	670	520
~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30	
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)	
~$V_{x,y,z}$ [*10^{-3} µm³]	10-23	1.3	1.5	3.0	1.8	1.3	0.1	



J Cell Biol. 2010 Jul 26;190(2):165-75.
A guide to super-resolution fluorescence microscopy, Schermelleh L, Heintzmann R, Leonhardt H.

Super-resolution microscopy



SIM

(A) Mouse C2C12 prometaphase cell stained with primary antibodies against lamin B and tubulin, and secondary antibodies conjugated to Alexa 488 (green) and Alexa 594 (red), respectively. Nuclear chromatin was stained with DAPI (blue). 3D image stacks were acquired with a DeltaVision OMX prototype system (Applied Precision).

STED

(B) HeLa cell stained with primary antibodies against the nuclear pore complex protein Nup153 and secondary antibodies conjugated with ATTO647N. The image was acquired with a TCS STED confocal microscope (Leica).

TIRF

(C) TdEosFP-paxillin expressed in a Hep G2 cell to label adhesion complexes at the lower surface. The image was acquired on an ELYRA P.1 prototype system (Carl Zeiss, Inc.) using TIRF illumination. Single molecule positional information was projected from 10,000 frames recorded at 30 frames per second. On the left, signals were summed up to generate a TIRF image with conventional wide-field lateral resolution.

Bars: 5 µm (insets, 0.5 µm).

JCB

Schermelleh L et al. J Cell Biol 2010;190:165-175

Recent Advances: Microscopy 4-letter Words
Förster Resonance Energy Transfer (FRET)

A quantum mechanical transfer of energy between an excited fluorescent dye (the **donor**) and a dye (the **acceptor**)

- **Spatially close**
- **Appropriate orientation**
- **Sufficient spectral overlap**

Efficiency depends upon:

•How close?
2-10 nm***

•Donor and acceptor
Each pair has a characteristic R_0 (distance at which 50% FRET occurs) which depends on the **spectral overlap**

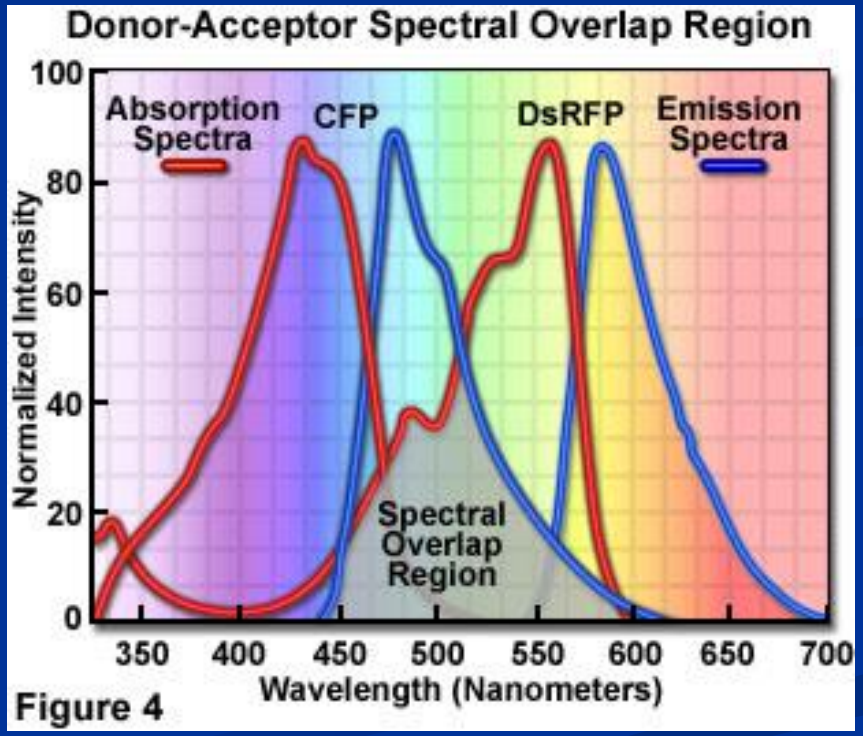
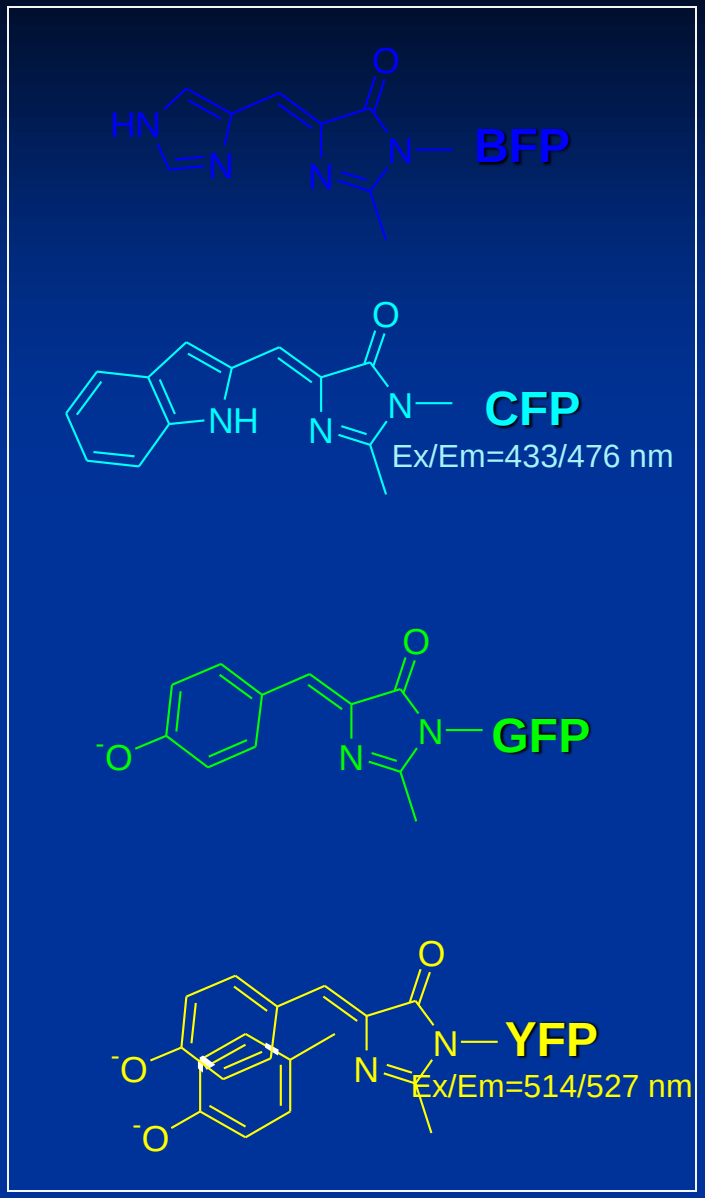


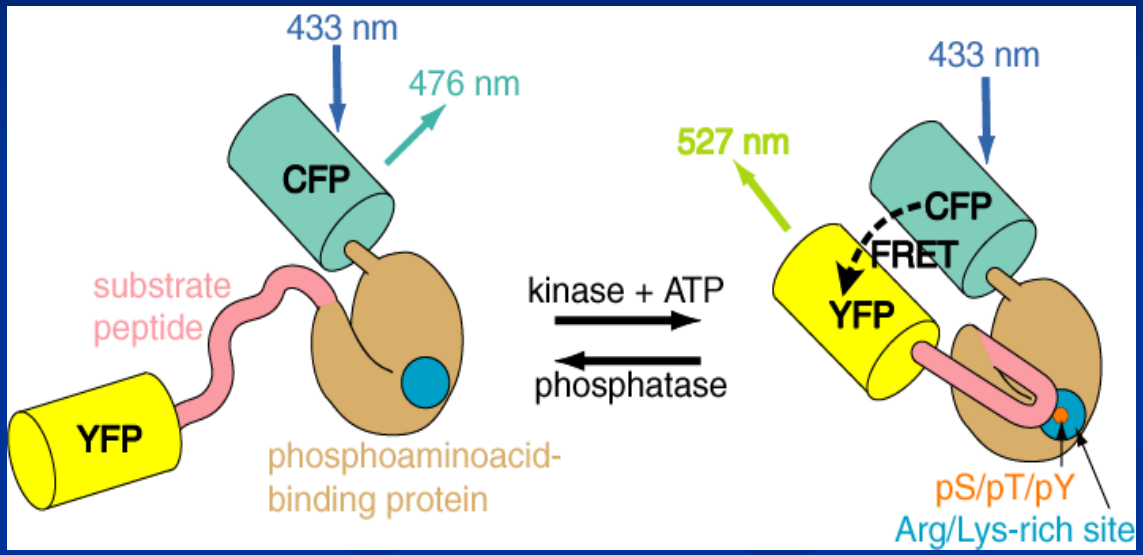
Figure 4

Courtesy of Stephen Adams, UCSD

Color mutants of GFP



Design of a Genetically-Encoded Reporter for Imaging Kinase Activities: Intramolecular FRET



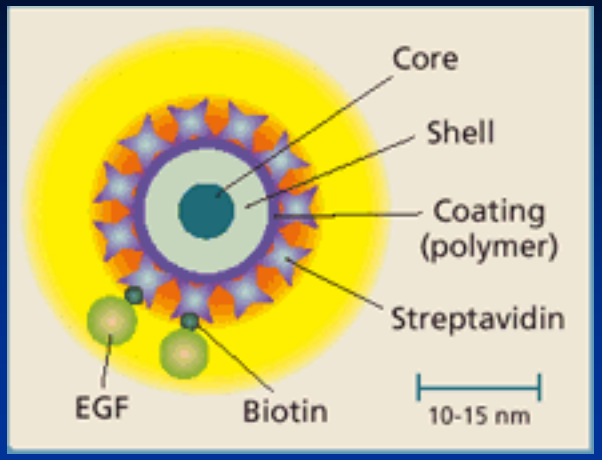
See Zhang et al. 2002 Nature Reviews/Molec Biol 3:906

Use of **FRET** allows correlation between conformational change and fluorescence

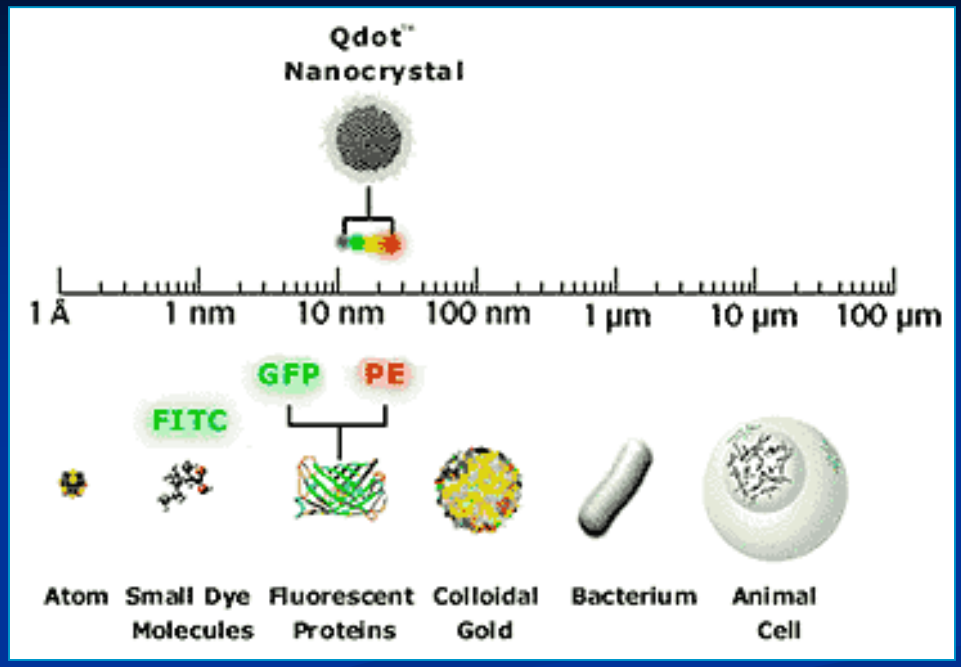
Use of **GFP** allows introduction of reporter into living cells by transfection

Courtesy of Stephen Adams, UCSD

Recent Advances: Quantum Dots



Nanocrystals consisting of semiconductor core of cadmium selenide coated with a shell of zinc sulfide



Quantum dot size

Additional polymer layer enhances water solubility and enables conjugation to other molecules

Optical properties of quantum dots:

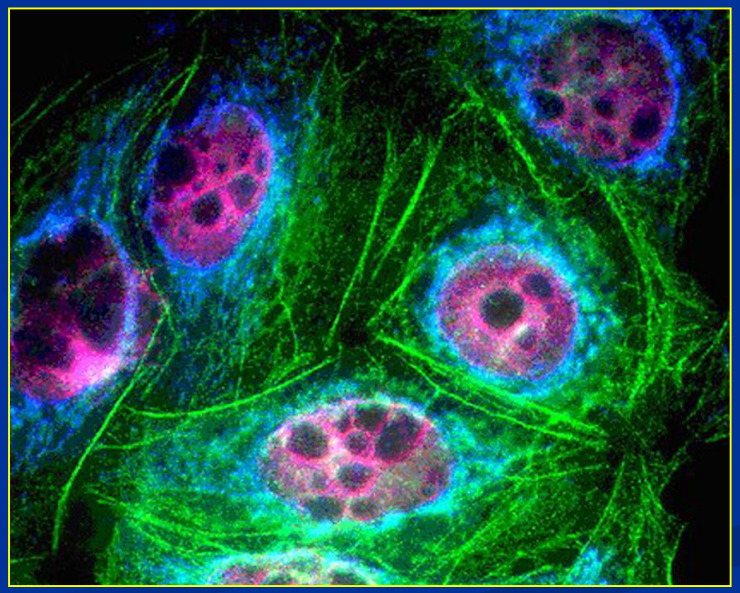
- high brightness (high signal to noise ratio)
- photostability (virtually no photobleaching)

Quantum Dots

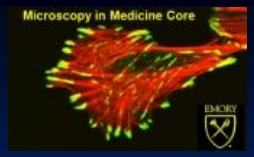
All colors excited by single excitation source, simplifying instrumentation

Slight changes in size of quantum dot semiconductor core change the emission spectra

Vials of quantum dots. Samples were excited with a single UV source. The colored spheres illustrate the relative sizes of the CdSe Qdots in the vials.



Multicolor labeling with Qdot anti-Mouse, Streptavidin, and Protein A Conjugates in HepG2 cells.



Future challenges in microscopic imaging

A Look "through the glass brightly" into the Future

The future of microscopic imaging is **bright** but there is a dilemma in "translational research" (from the lab to the clinic)

Microscopic methods: such as fluorescence, confocal and multiphoton light microscopy to electron microscopy provide highest resolution

Confocal and multiphoton resolution=100 **nm** **or Super resolution=30 nm**

TEM=few **nm**

Clinical methods: MRI, CT, PET, ultrasonography and optical imaging provide noninvasive, functionally relevant images but at a much lower resolution and specificity. These modalities reveal anatomical rather than molecular features.

MRI=100-500 **um**

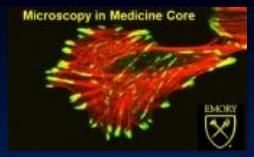
PET, ultrasonography, optical imaging=**mm**

Future challenges:

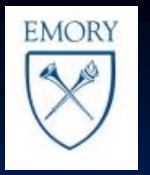
- **Develop new imaging methods that can bridge resolution gap**
- **Determine which microscopic techniques are best benchmarks for interpreting clinical images and can relate disease to molecular entities such as proteins or expressed genes**

For discussion, see the following article:

McDonald and Choyke. Imaging of angiogenesis: from microscope to clinic. 2003. Nature Medicine 9:713-725.



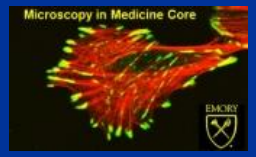
Microscopy Websites



http://micro.magnet.fsu.edu/primer/index.html

http://microscopyu.com/

http://olympusmicro.com/



http://medicine.emory.edu/MIMCore