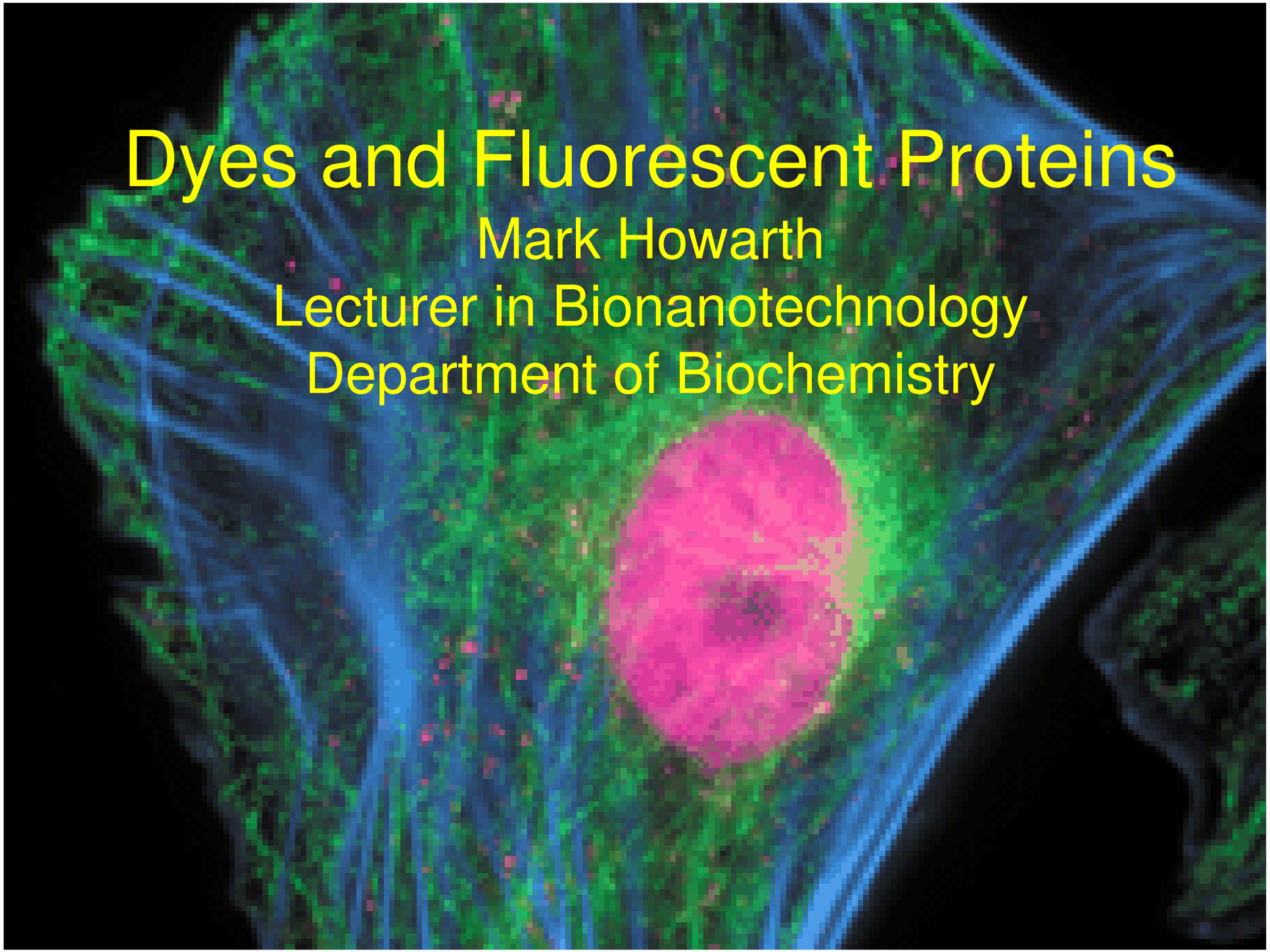
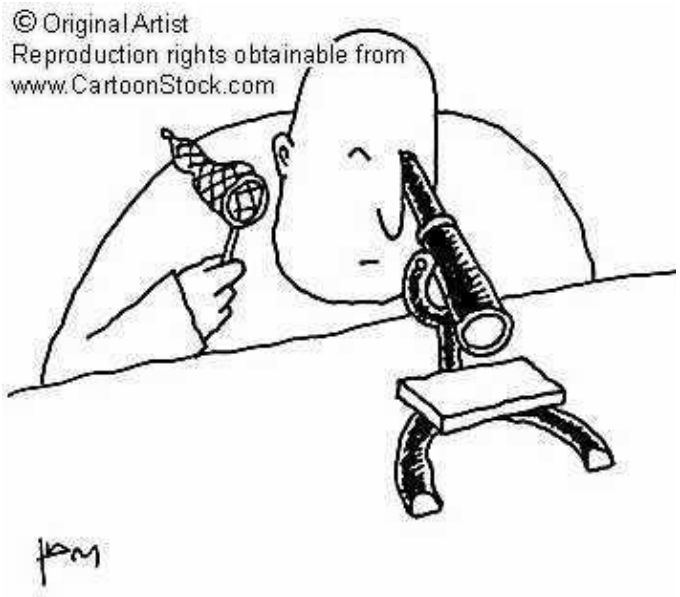


Dyes and Fluorescent Proteins

Mark Howarth
Lecturer in Bionanotechnology
Department of Biochemistry

Overview



1. What is fluorescence

2. What kind of structures are fluorescent

3. How to make and target fluorescent probes

4. Fluorescent probes for cellular structure and function

What is fluorescence?

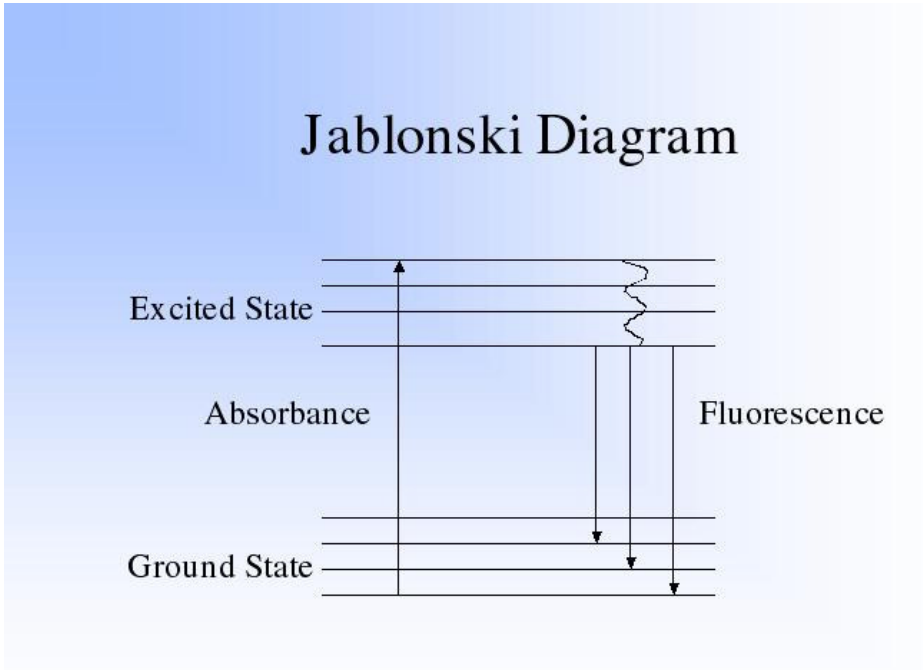
Absorption of photon leading to emission of a photon of a longer wavelength

Energy levels?

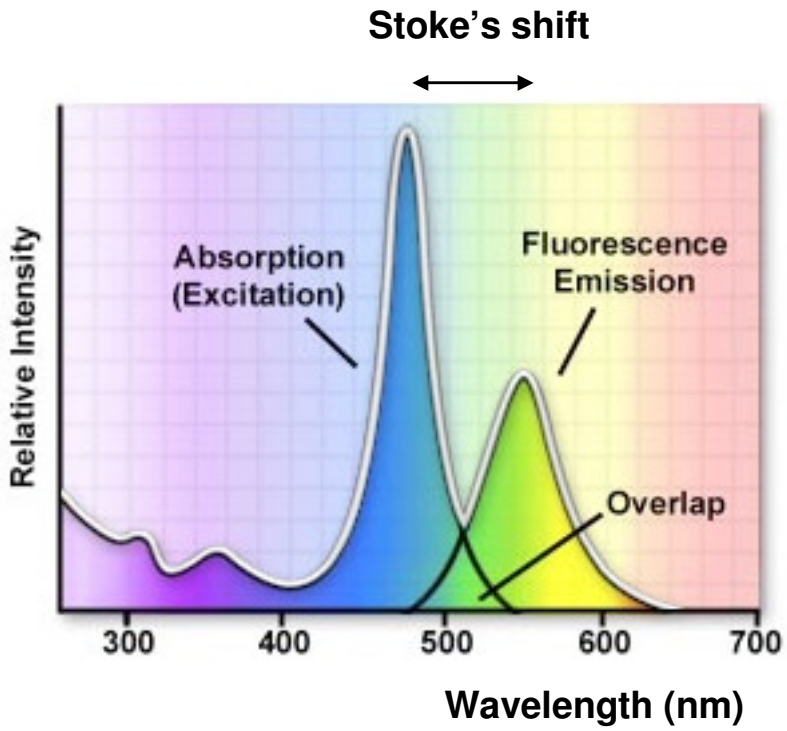
What is fluorescence?

Absorption of photon leading to emission of a photon of a longer wavelength

Energy levels?



Fluorescence spectrum



for fluorescein

We use fluorescence because of its sensitivity

Mountain by day

How can we see the stars??

We use fluorescence because of its sensitivity

Mountain by day

Small signal
High background

like absorbance

Same mountain by night

Small signal
Low background

like fluorescence

Overview

1. What is fluorescence

2. What kind of structures are fluorescent

3. How to make and target fluorescent probes

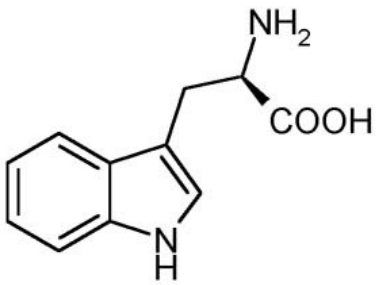
4. Fluorescent probes for cellular structure

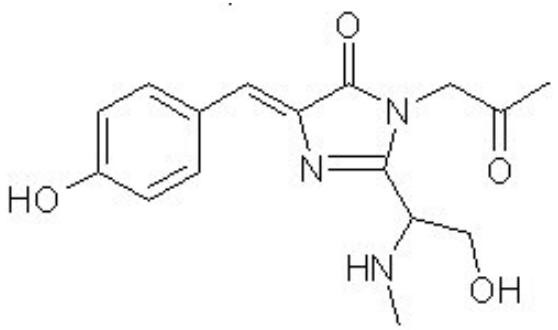
What sort of molecules are fluorescent?

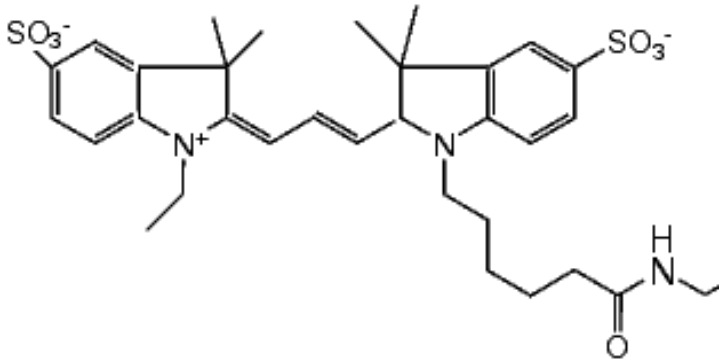
Organic fluorophores
especially
1. Intrinsic fluorophores (source of autofluorescence)
2. Dyes
3. Fluorescent proteins

Inorganic fluorophores
especially
1. Lanthanides
2. Quantum dots

What sort of molecules are fluorescent?
1. Organic fluorophores



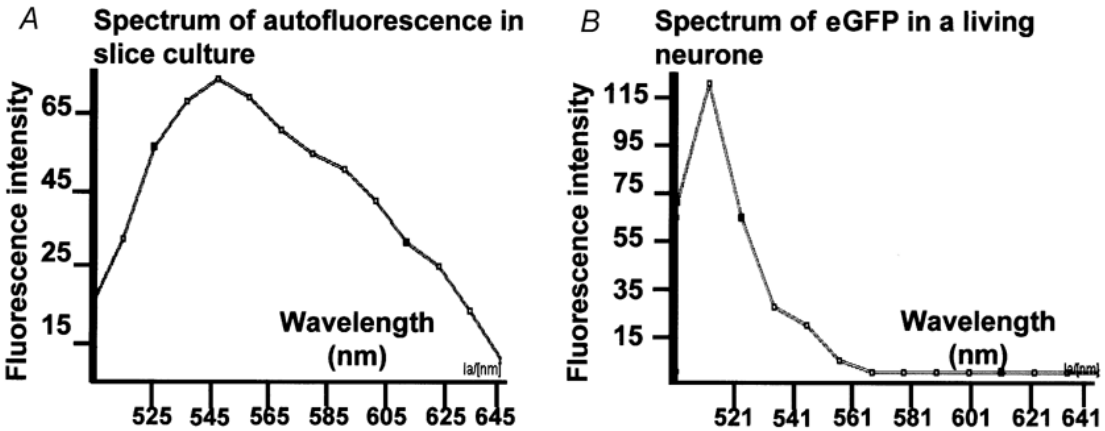


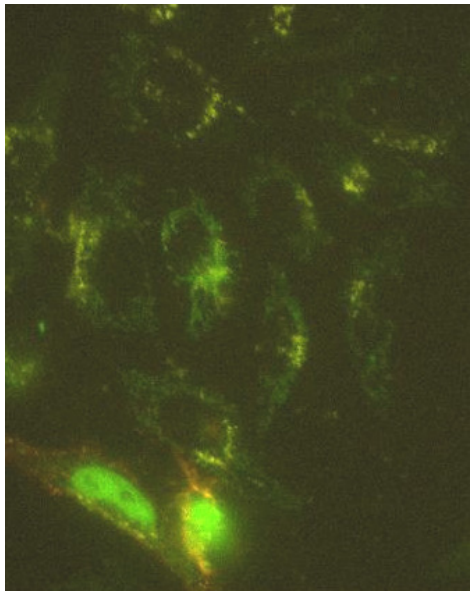


Chemical features:

1. Conjugation
2. Rigidity especially fused aromatic rings
3. Heteroatoms

What sort of molecules are fluorescent?
1. Endogenous organic fluorophores





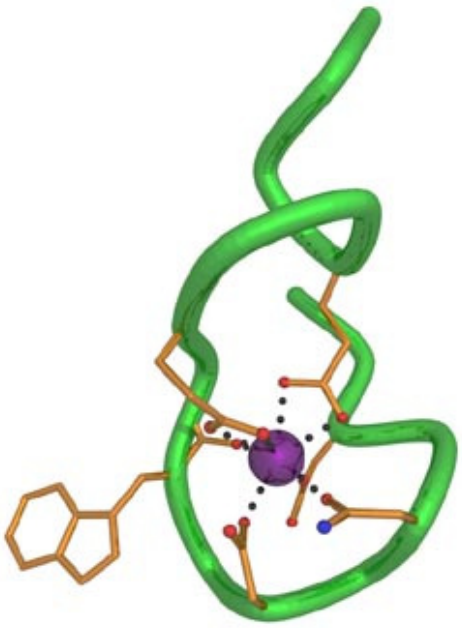
Most common autofluorescent molecules:
Flavins, NADH, NADPH, elastin, collagen, lipofuscin

Avoiding autofluorescence:
choose dye emitting in red with big Stokes shift
add quencher (Crystal violet)
add reducing agent to react with autofluorescent molecules
time-gate fluorescence

What sort of molecules are fluorescent?
2. Inorganic fluorophores

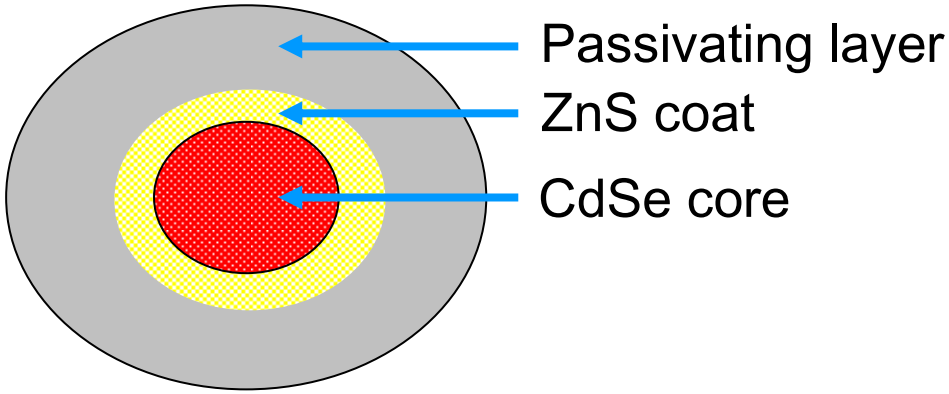
Lanthanides

Peptide sequence
binds Tb^{3+} and protects
from quenching by water



Curr Opin Chem Biol. 2010;14(2):247-54. Lanthanide-tagged proteins--an illuminating partnership. Allen KN, Imperiali B.

Quantum dots



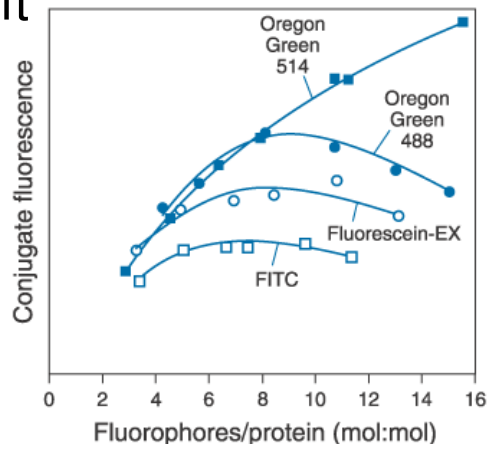
Michalet X, et al. Quantum dots for live cells, in vivo imaging, and diagnostics. Science. 2005 307(5709):538-44.

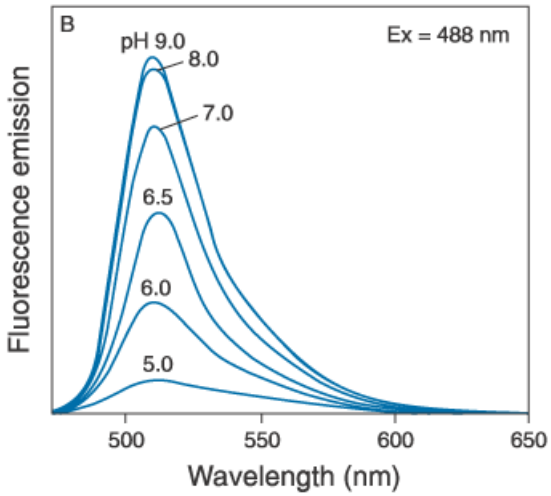
How good is a fluorophore?

1. Excitation and emission appropriate
   - background worse in UV + with small Stokes shift
   - good match to filters on your microscope
   - look at other fluorophores at same time
2. Bright
   - see small numbers of fluorophores,
   - low self-quenching, high QY and absorbance
3. Stable to photobleaching
   - exciting light damages fluorophore
4. Non-toxic
5. Environment-insensitive (especially to pH)
6. Little non-specific binding
7. Small
8. Little blinking

(9. Cost)

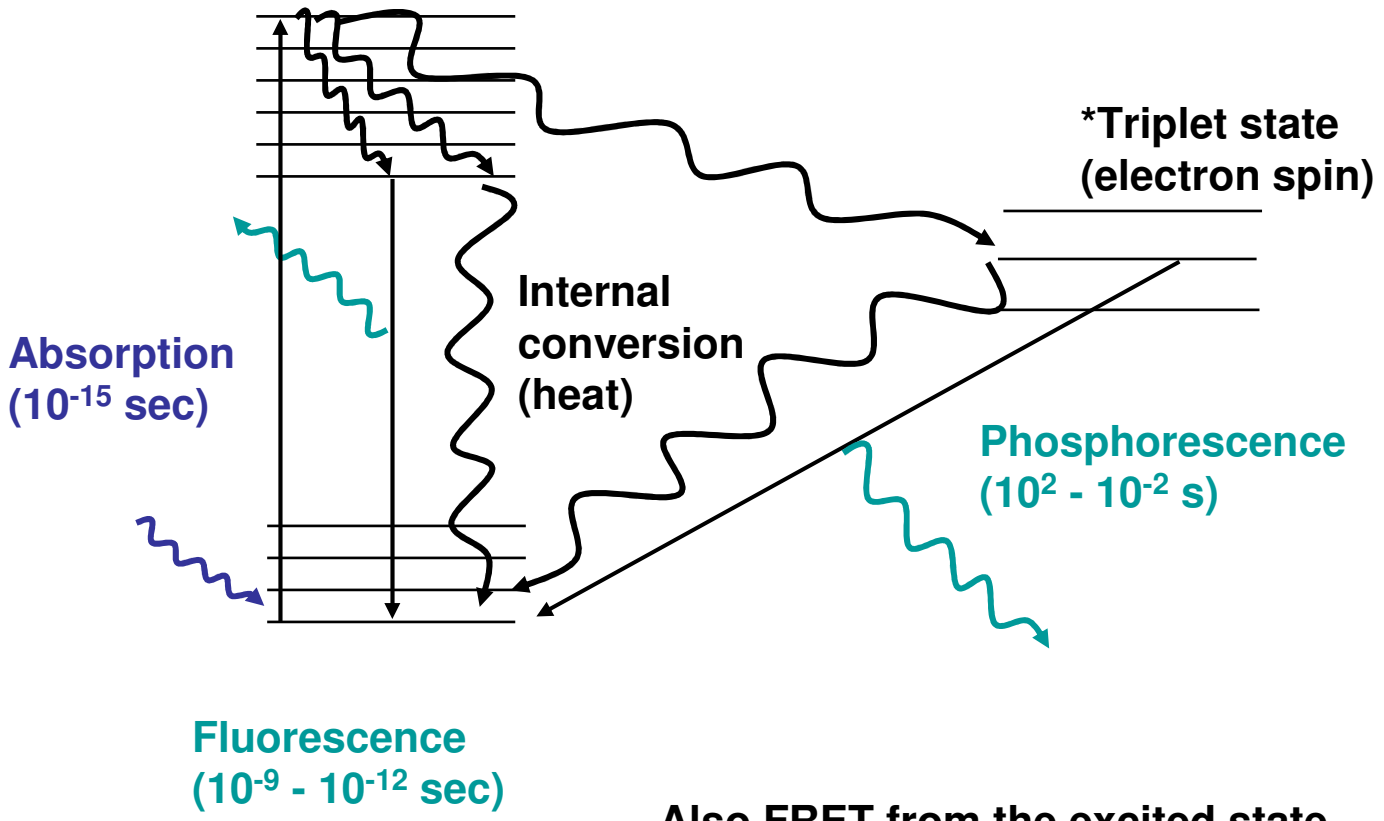
Green dye self-quenching





Fluorescein pH sensitivity

Not all energy emitted as fluorescence

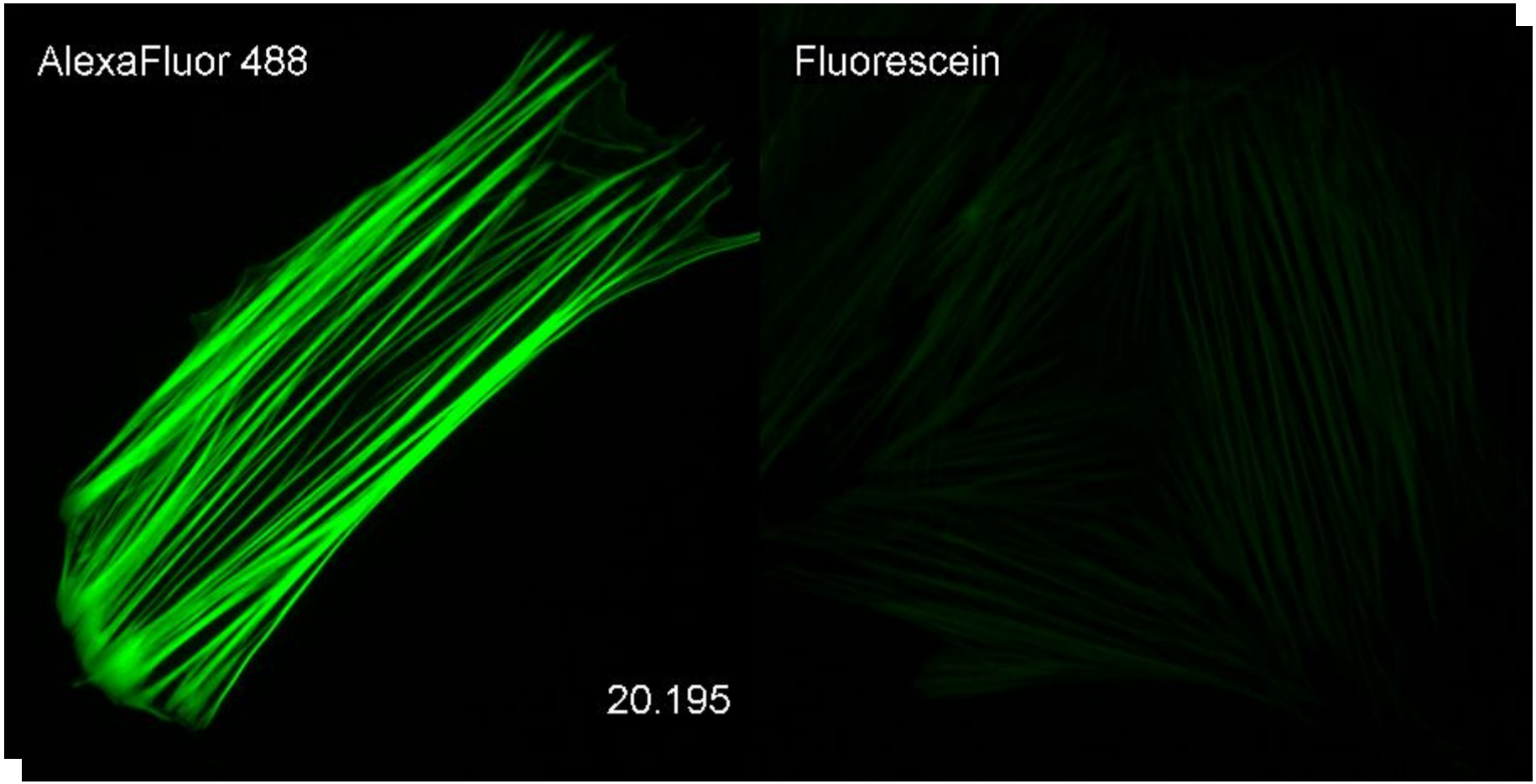


Also FRET from the excited state

***Triplet state-chemically reactive**
Photobleaching, reactive damaging free radicals

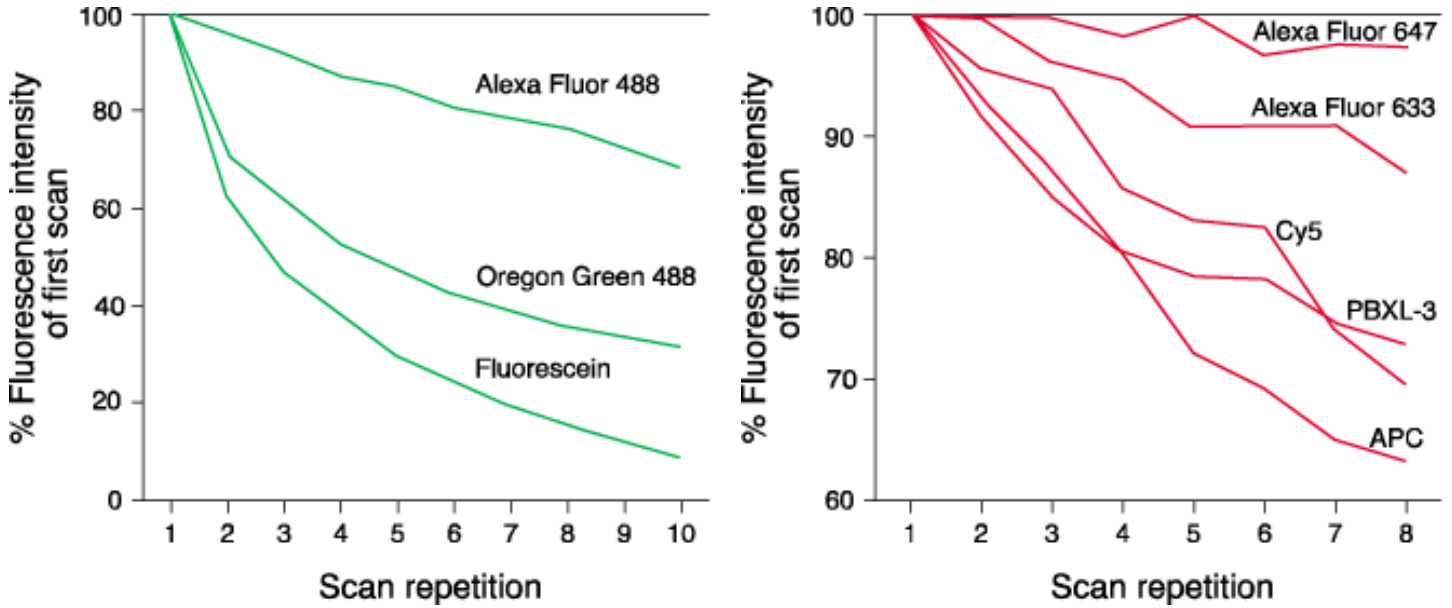
Alexa Fluor 488 vs Fluorescein Bleaching

2x Real Time



Alexa Fluor Dyes – Photostability

Laser-scanning cytometry
EL4 cells
biotin-anti-CD44 + streptavidin conjugates



Fluorescein is the commonest dye but has poor photostability.

Protecting the fluorescence signal - Antifade Reagents for fixed cells

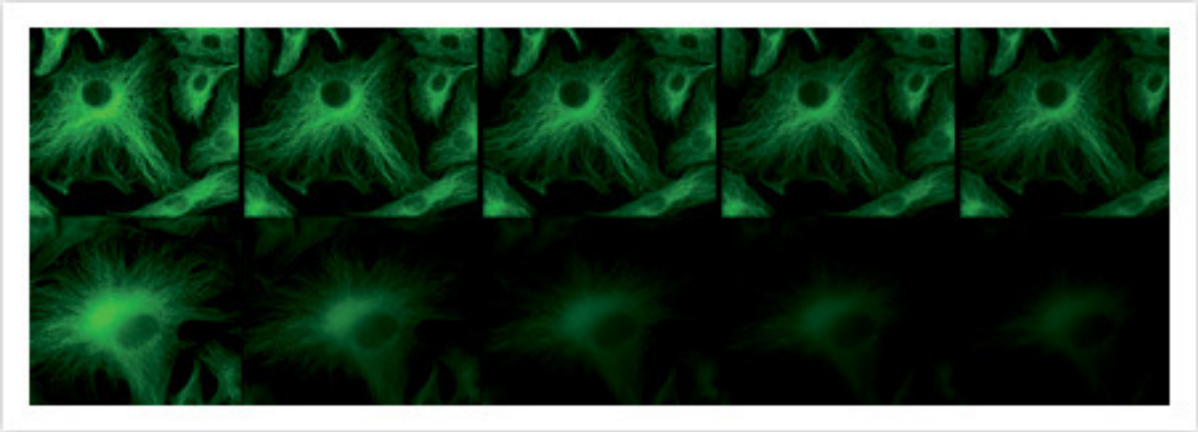
Scavenge and prevent reactive oxygen species from forming.

For fixed cells:

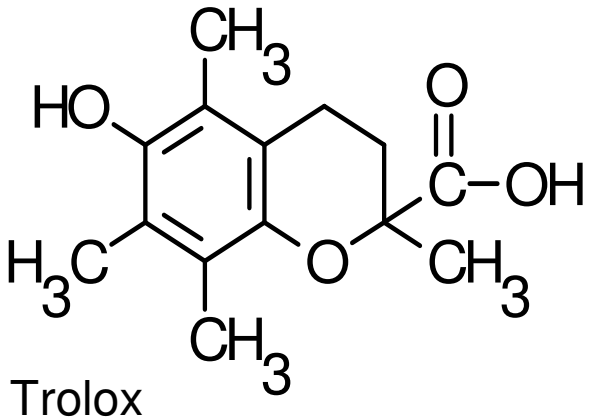
Home made: 0.3% p-phenylene-diamine (Sigma)
or Propyl Gallate
Vectashield: Proprietary, very effective all round, affects psf
Dabco
Prolong Gold®

+ Prolong Gold

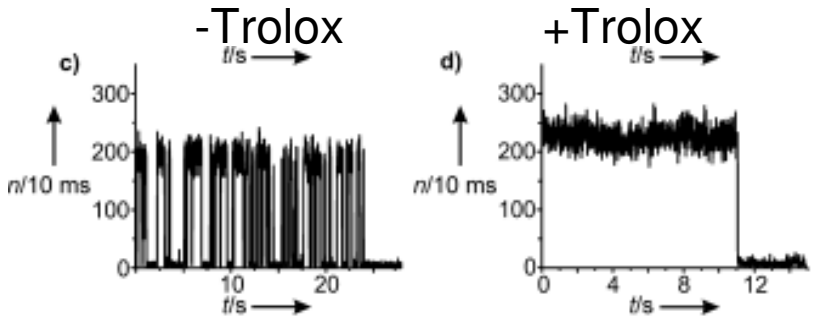
Untreated



Antifade Reagents for Live Cells



Trolox

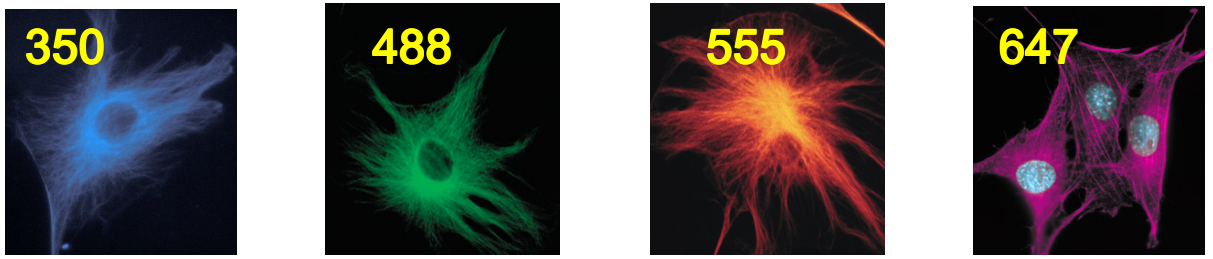


Blinking of single molecule of Atto647N on DNA, Vogelsang Tinnefeld Ang Chem 2008

- Trolox is an antioxidant that can reduce bleaching compatible with live specimens
  - water-soluble
  - working conc. ~100 µM
- Ascorbic acid is an alternative antioxidant
- Depleting oxygen (especially used for some single molecule experiments) with Glucose Oxidase and Catalase greatly reduces bleaching.
- Can stop not only bleaching but also blinking

Multiplexing- four main colours

Excitation wavelengths:



Emission wavelengths:

Blue green orange/red far red

350 400 450 500 550 600 650 700

DAPI/UV | FITC | TRITC | FAR RED

Alexa Fluor® 350
Coumarin, AMCA

Alexa Fluor® 488
Fluorescein (FITC)
Cy2

Alexa Fluor® 555
Rhodamine,
TAMRA, TRITC
Cy3

Alexa Fluor® 647
Cy5, APC

Alexa Fluor® 594
Texas Red, Cy3.5

Colour Selection ♦ Brightness ♦ Photostability

Overview

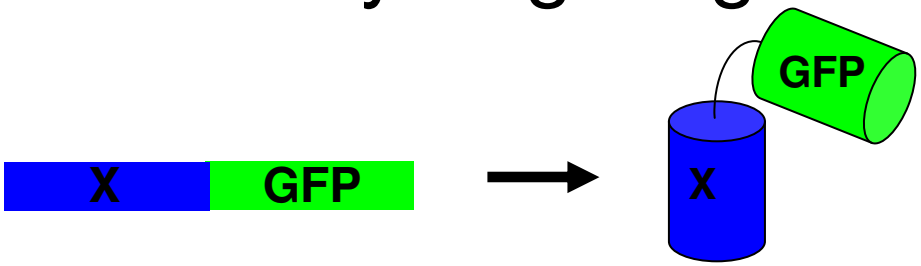
1. What is fluorescence

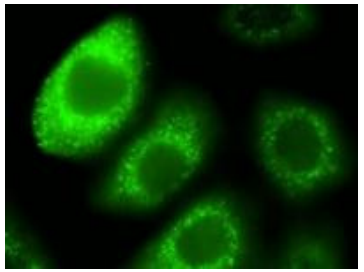
2. What kind of structures are fluorescent

3. How to make and target fluorescent probes

4. Fluorescent probes for cellular structure

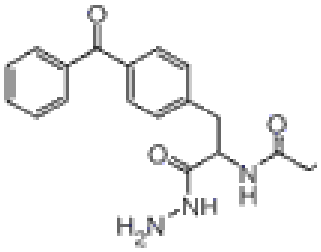
Major bottleneck to using new probes is difficulty targeting them



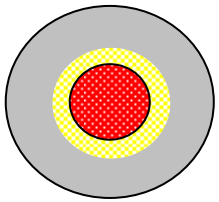


fluorescent proteins easy to target

photoaffinity probes

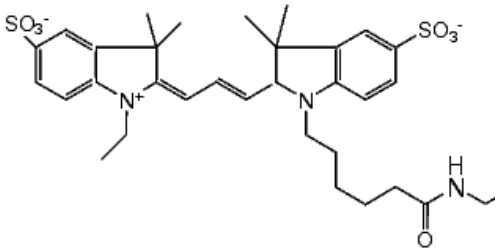


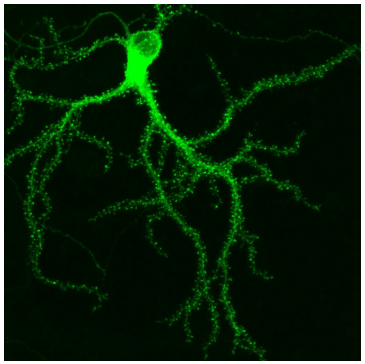
quantum dots



?

organic fluorophores

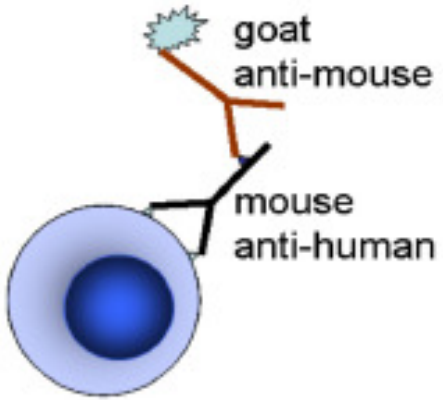




other probes hard to target

Antibodies for cellular imaging

Live cells



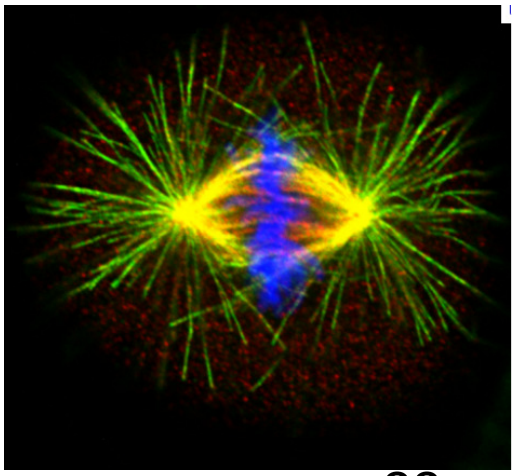
Label plasma membrane and secretory pathway
Penetrate plasma membrane
(microinjection, electroporation, pinosome lysis, streptolysin, cell permeable peptides, ester cage)

Get dynamics, avoid fixation artifacts

Fixed cells

Permeabilise

Still can give enormous amount of useful information

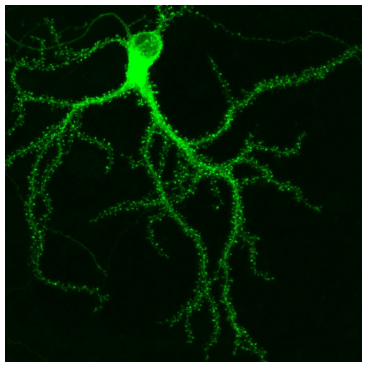


Not just antibodies for targeting

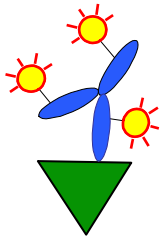
Other types of targeting agents:

Proteins
(especially antibodies, but also transferrin, insulin, EGF etc.)
Peptides (MHC class I pathway, proteasome function)
RNA (mRNA, molecular beacons, aptamers, siRNA)
DNA
lipids, lipoproteins
drugs

?

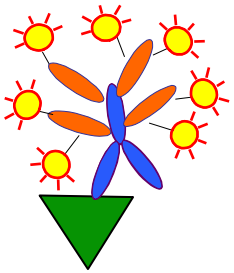


Getting fluorescence from antibody labelling



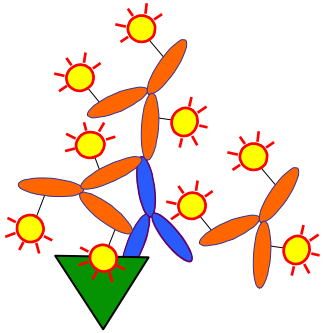
Directly-labelled Primary Antibody

- Lowest background
- Potentially low signal due to abundance of target or dye
- Dye could affect antigen recognition site



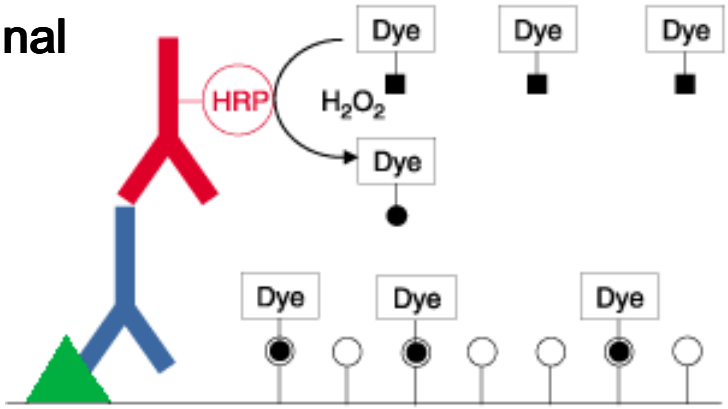
Zenon Technology

- **Brighter Signal**
- **Dye does NOT affect antigen recognition site**



Indirect-Labeled Secondary Antibody

- **Higher Background**
- **Brighter Signal**



Tyramide Signal Amplification (TSA™)

- **Higher background**
- **Brightest signal**

It is easy to attach dye to proteins

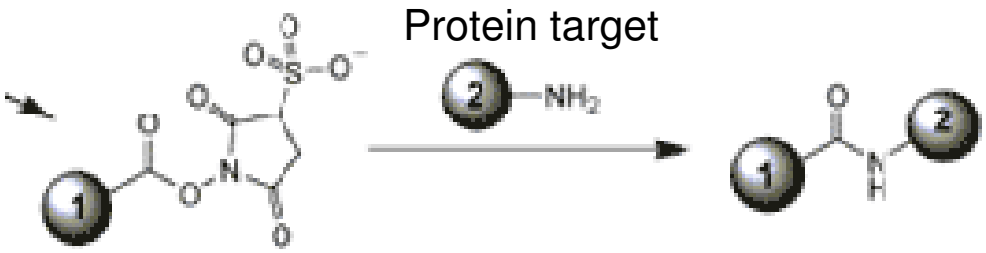
Multiple ways to modify proteins
(see Molecular Probes catalogue)

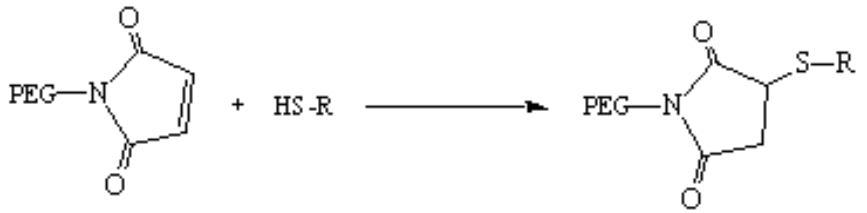
Most common ways are to modify:

1. Lysine

or

2. Cysteine



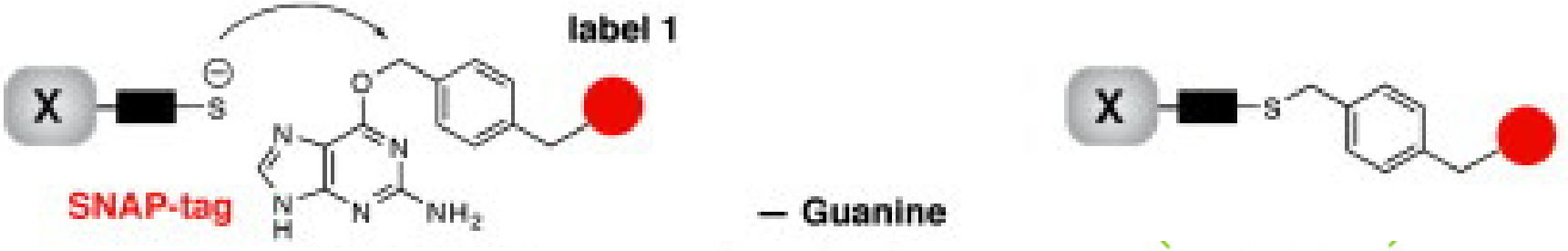


A Add dye to protein for 3 hr
B 1cm Sephadex column to remove most free dye (10 min)
C Dialyse away rest of free dye (24 hr)

Site-specific protein labelling methods

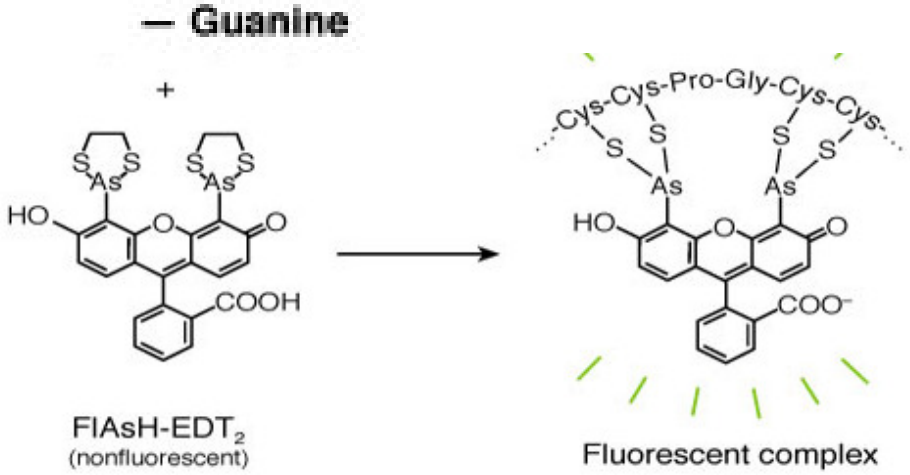
1. Binding domain

SNAP-tag (NEB), HaloTag (Promega)



2. Binding peptide

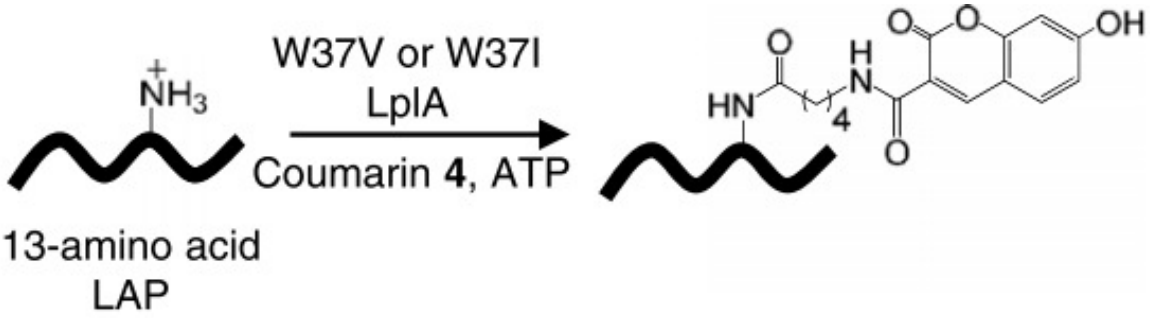
FlAsH (Invitrogen)



3. Enzymatic ligation to peptide

PRIME

AY Ting PNAS 2010



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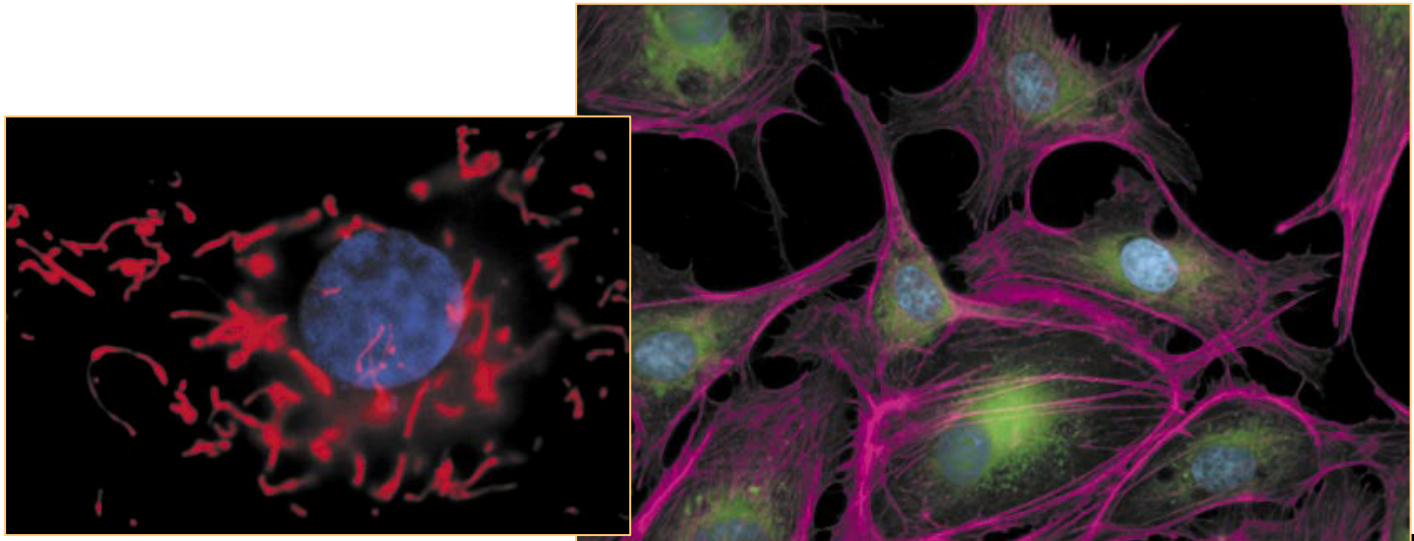
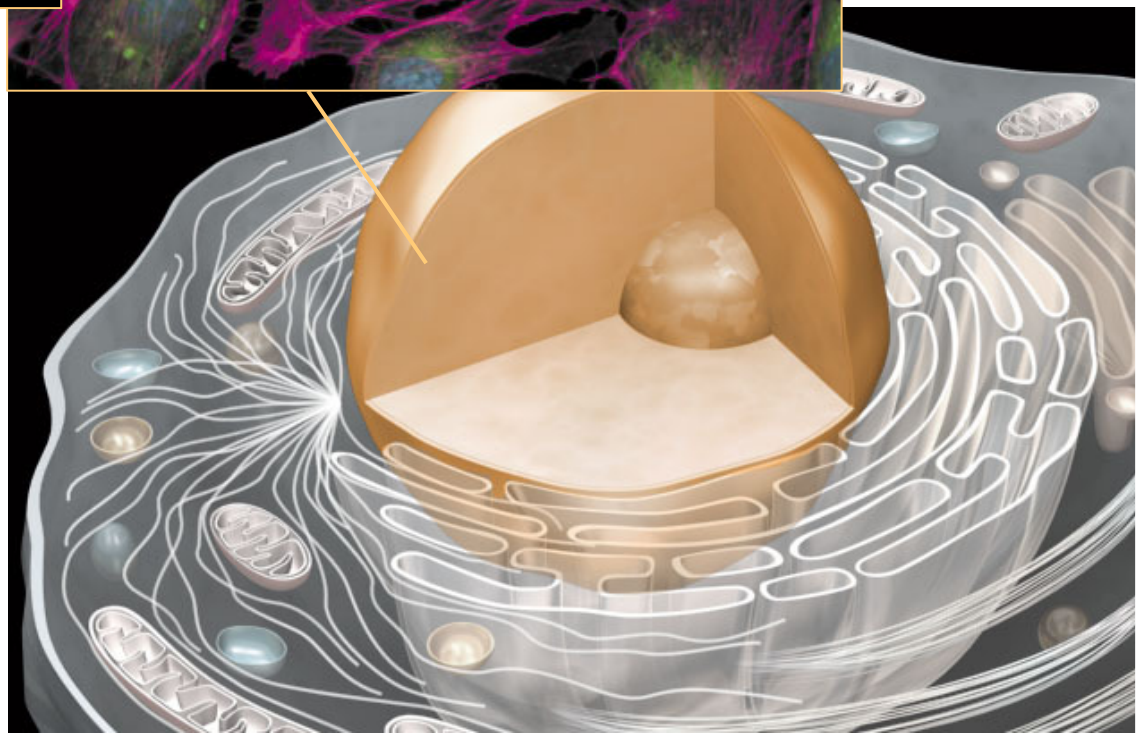




Nucleus

DAPI

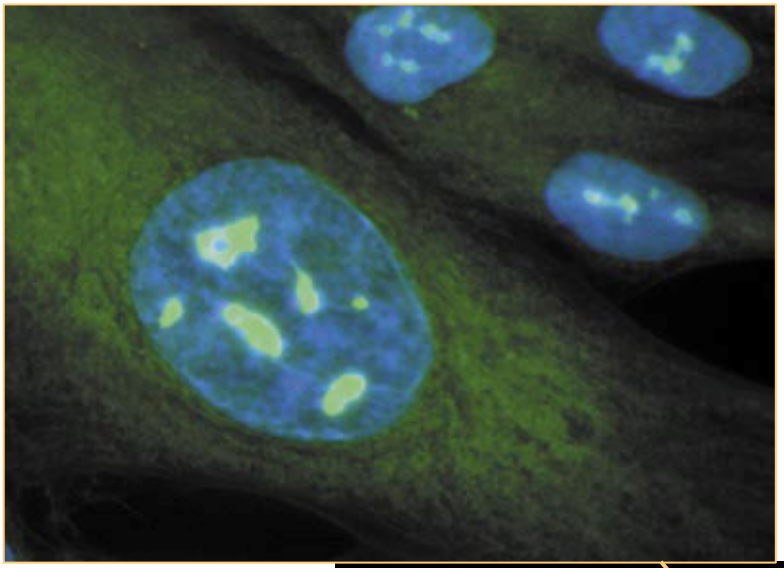
(well away from other channels)

Hoechst 33342

Live cells:

usually histone H2B-GFP

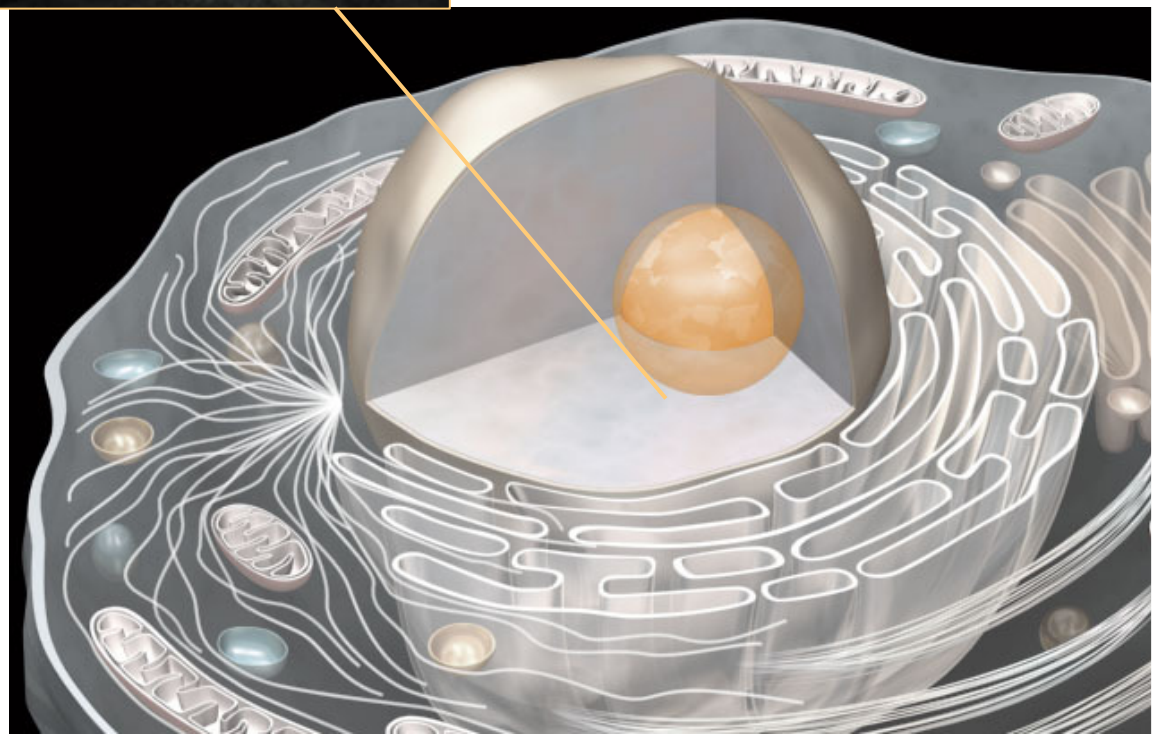
(or other monomeric FP)



Nucleoli

SYTO® RNASelect™

Live cells: GFP-Nopp140



Also note RNA-selective probe

RNA-selective, live cell imaging probes for studying nuclear structure and function. Chem. Biol. 2006, 13, 615-623.

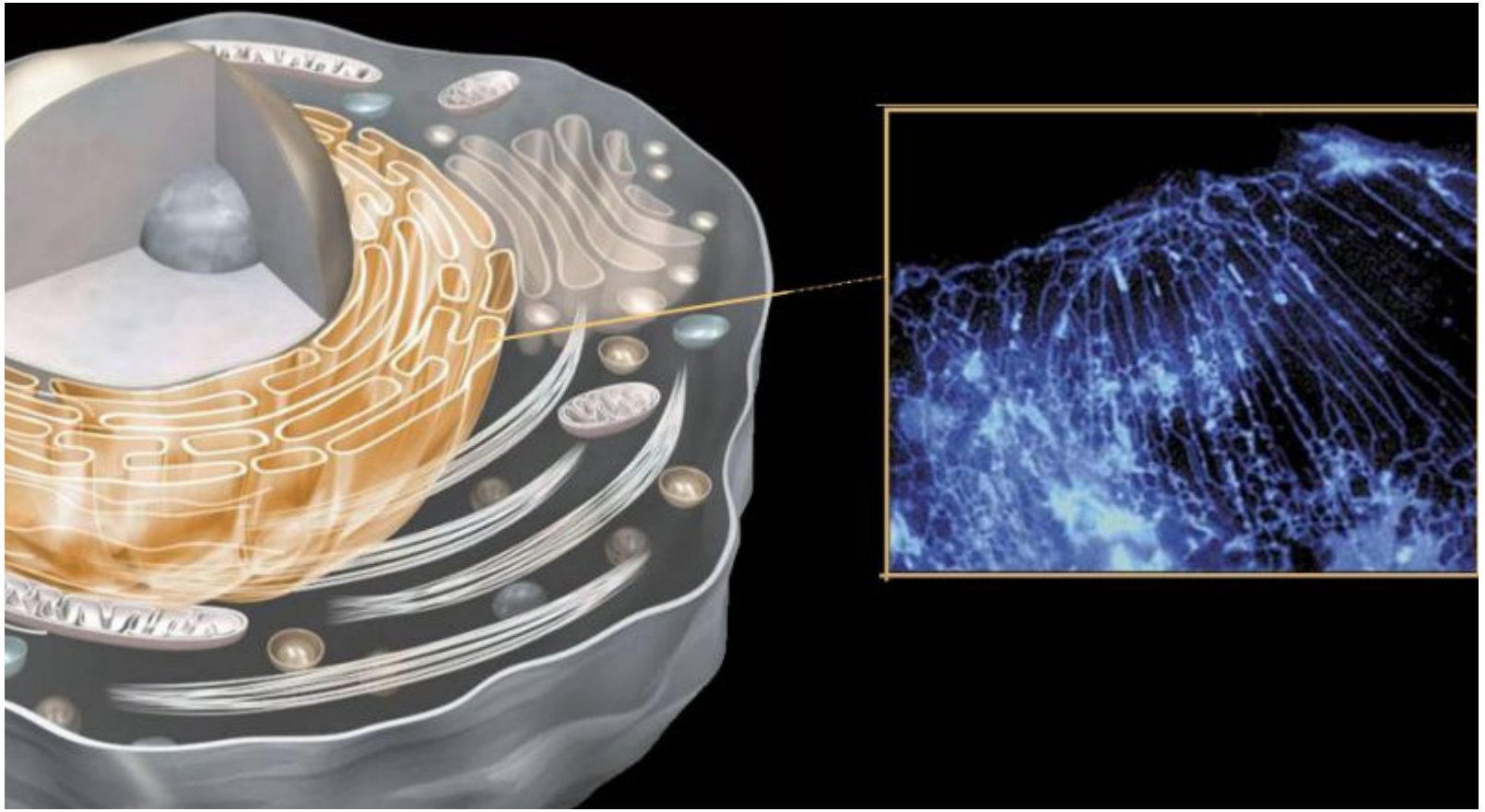
Endoplasmic Reticulum

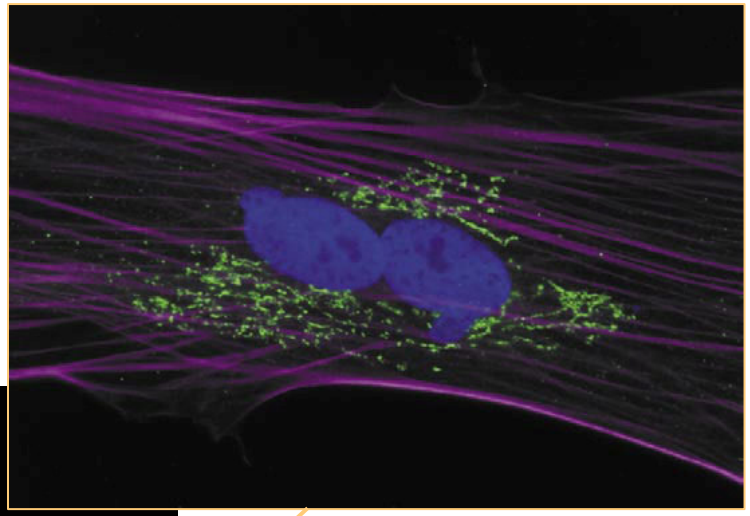
ER-Tracker™ Blue-White DPX

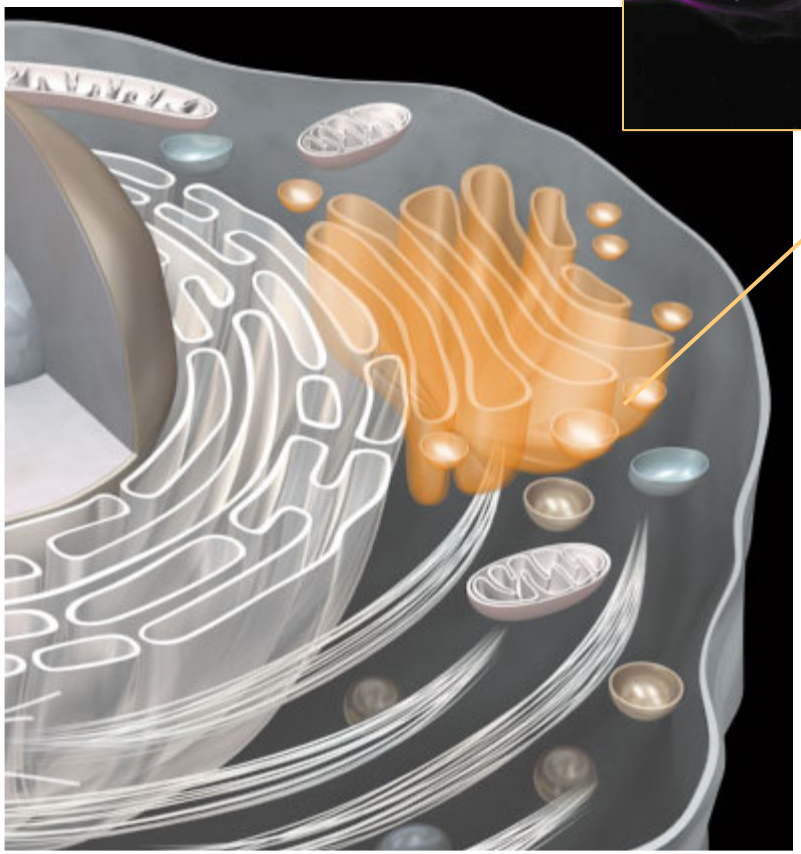
antibody to calnexin

Brefeldin A-BODIPY® 558 conjugate

Live cells: ss-GFP-KDEL





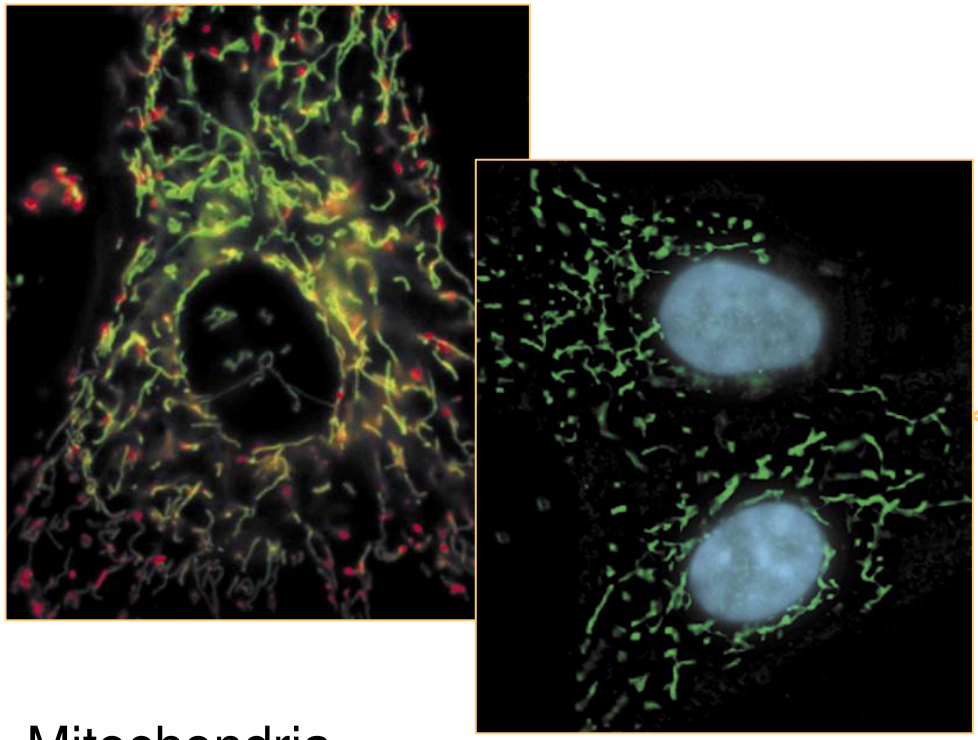


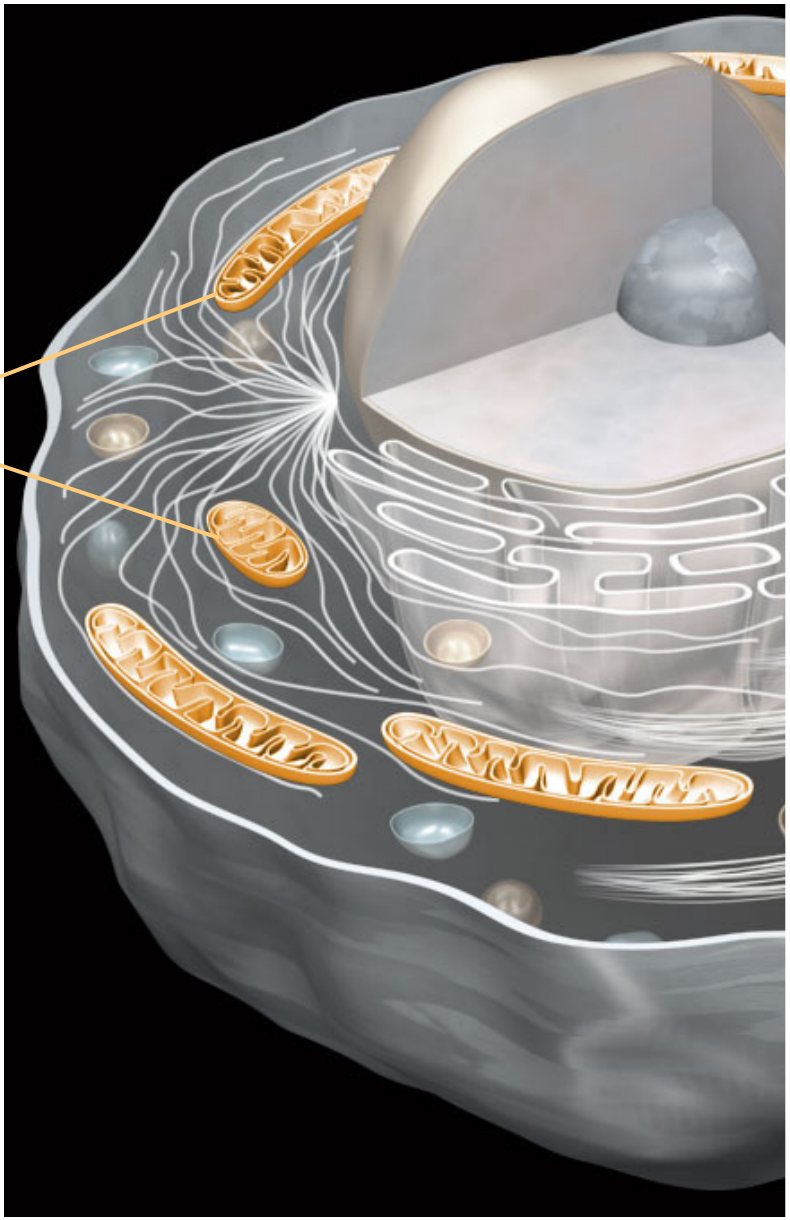
Golgi

anti–golgin-97 antibody

NBD C_6-ceramide complexed to BSA

Live cells: GalTase-GFP





Mitochondria

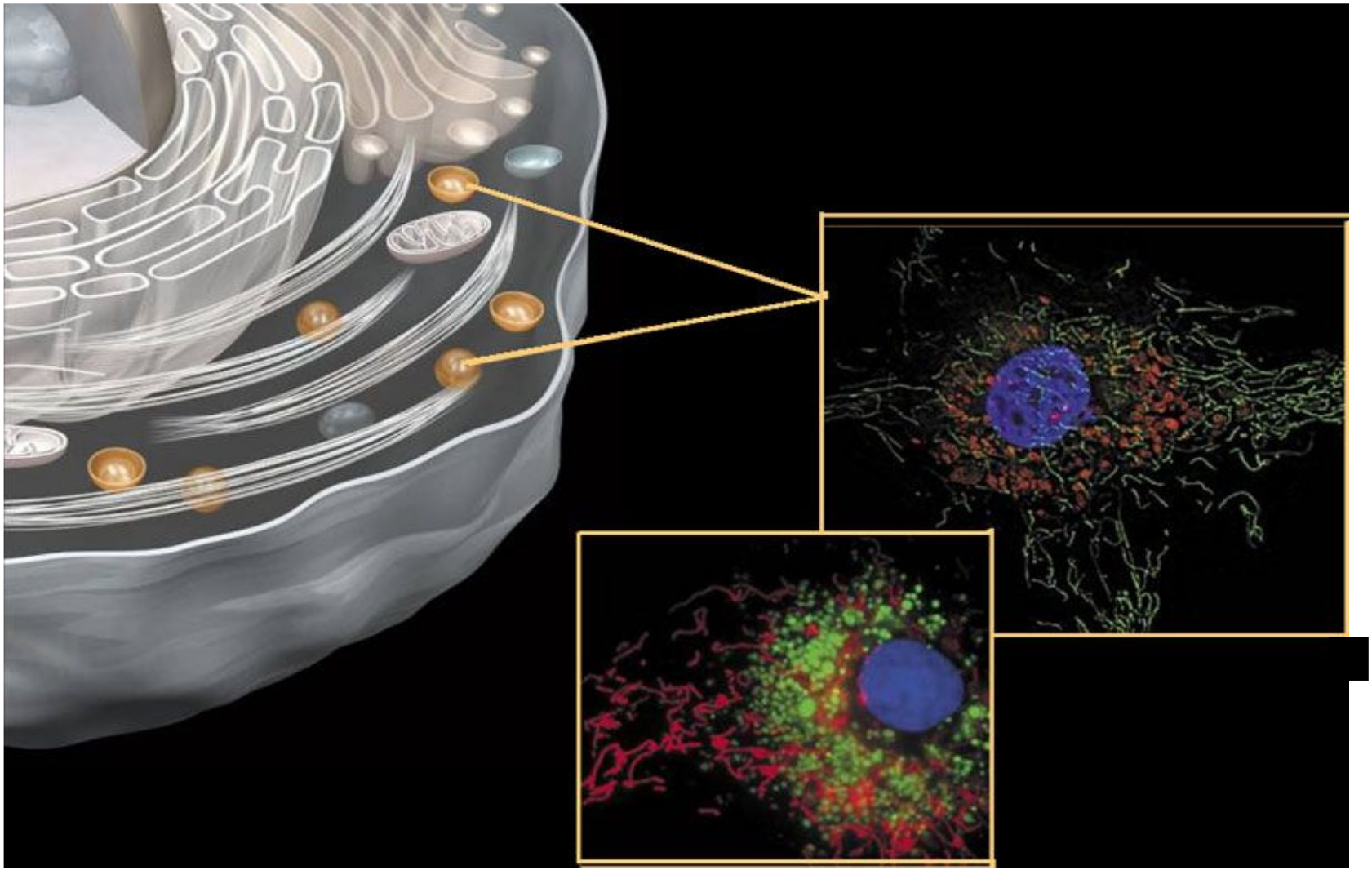
Fixed cells: anti–cytochrome oxidase subunit I Ab

Live cells: MitoTracker® Red/Green/Orange

CMTMRos

JC-1 (red J-aggregates at high conc., red to green depends on membrane potential)

Mitochondrial targeting sequence-GFP

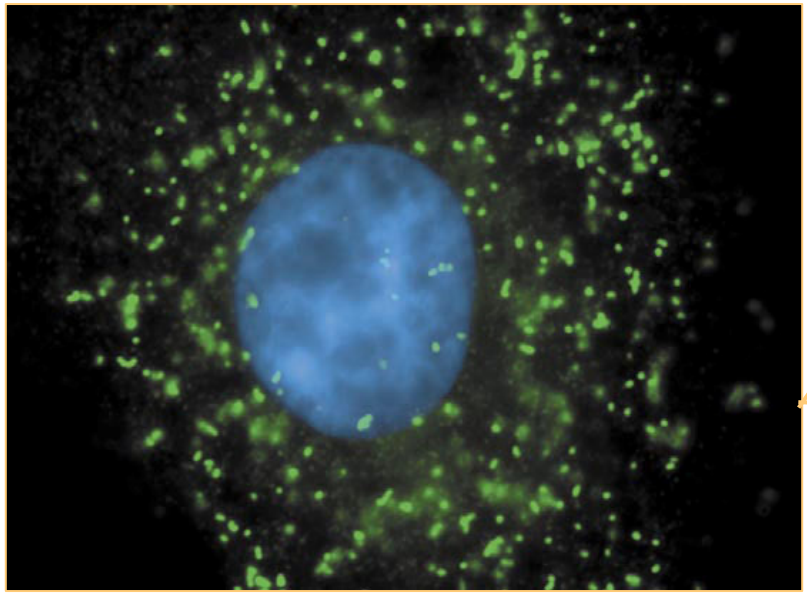


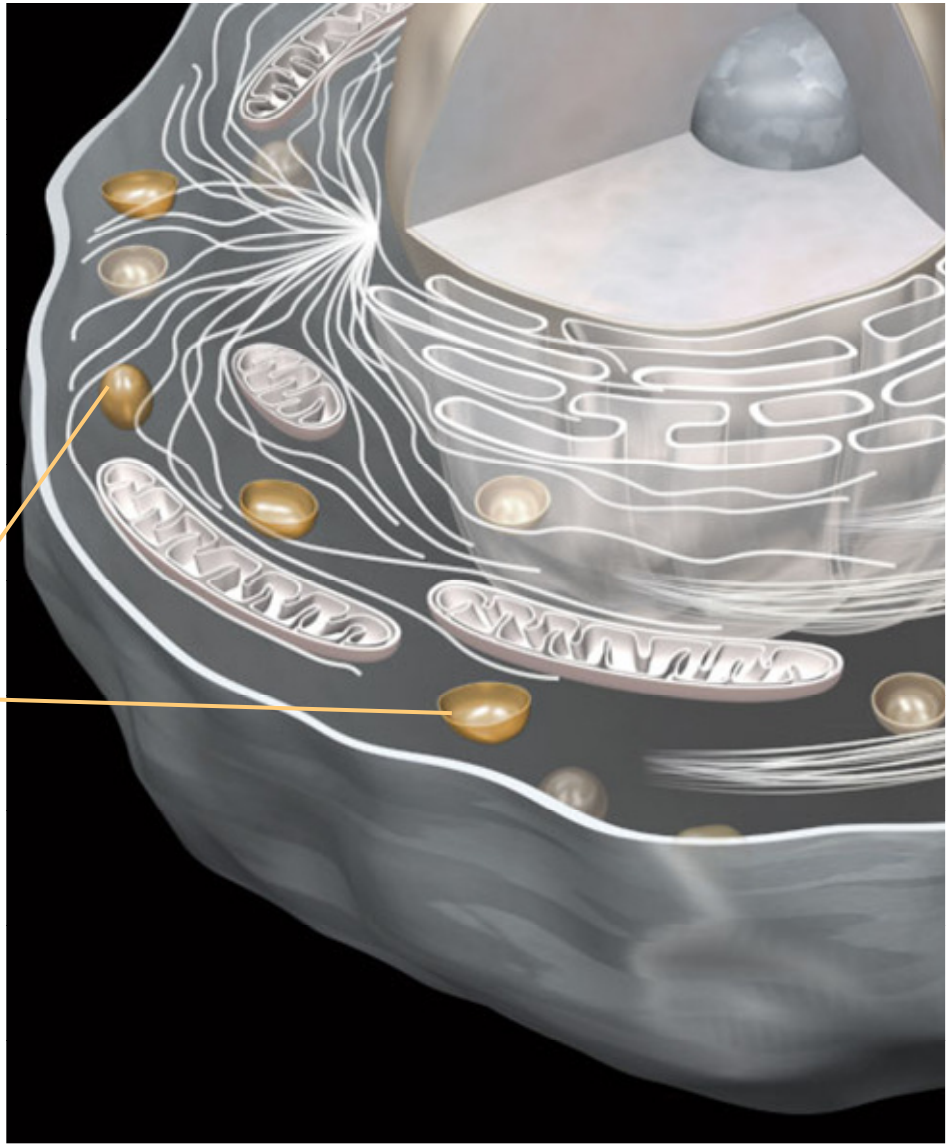
Lysosomes

Fixed cells: anti-LAMP1

Live cells: LysoTracker® Red /Green (weakly basic amines can accumulate in lysosomes)

LysoSensor™ Yellow/Blue DND-160, LAMP1-GFP



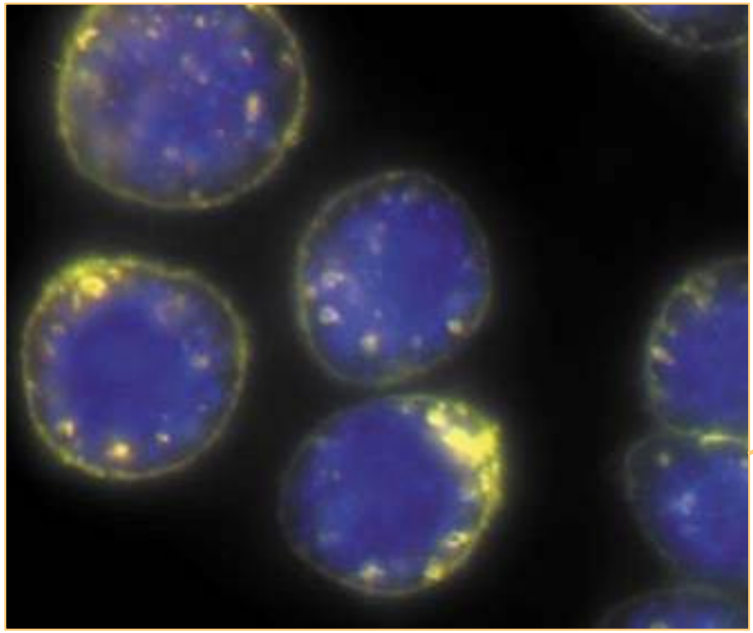


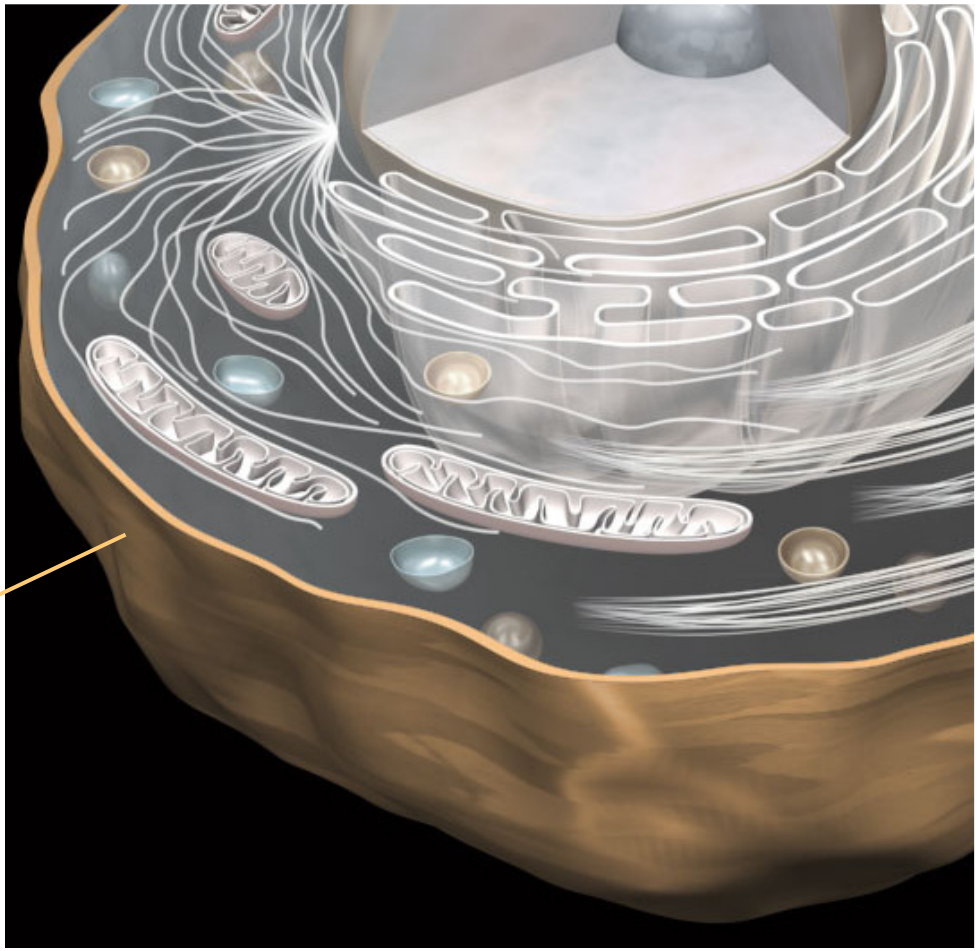
Peroxisomes

SelectFX™ Alexa Fluor® 488 Peroxisome Labeling Kit

(antibody to Peroxisomal membrane protein 70)

Live cells: GFP-SKL (tripeptide targeting sequence)

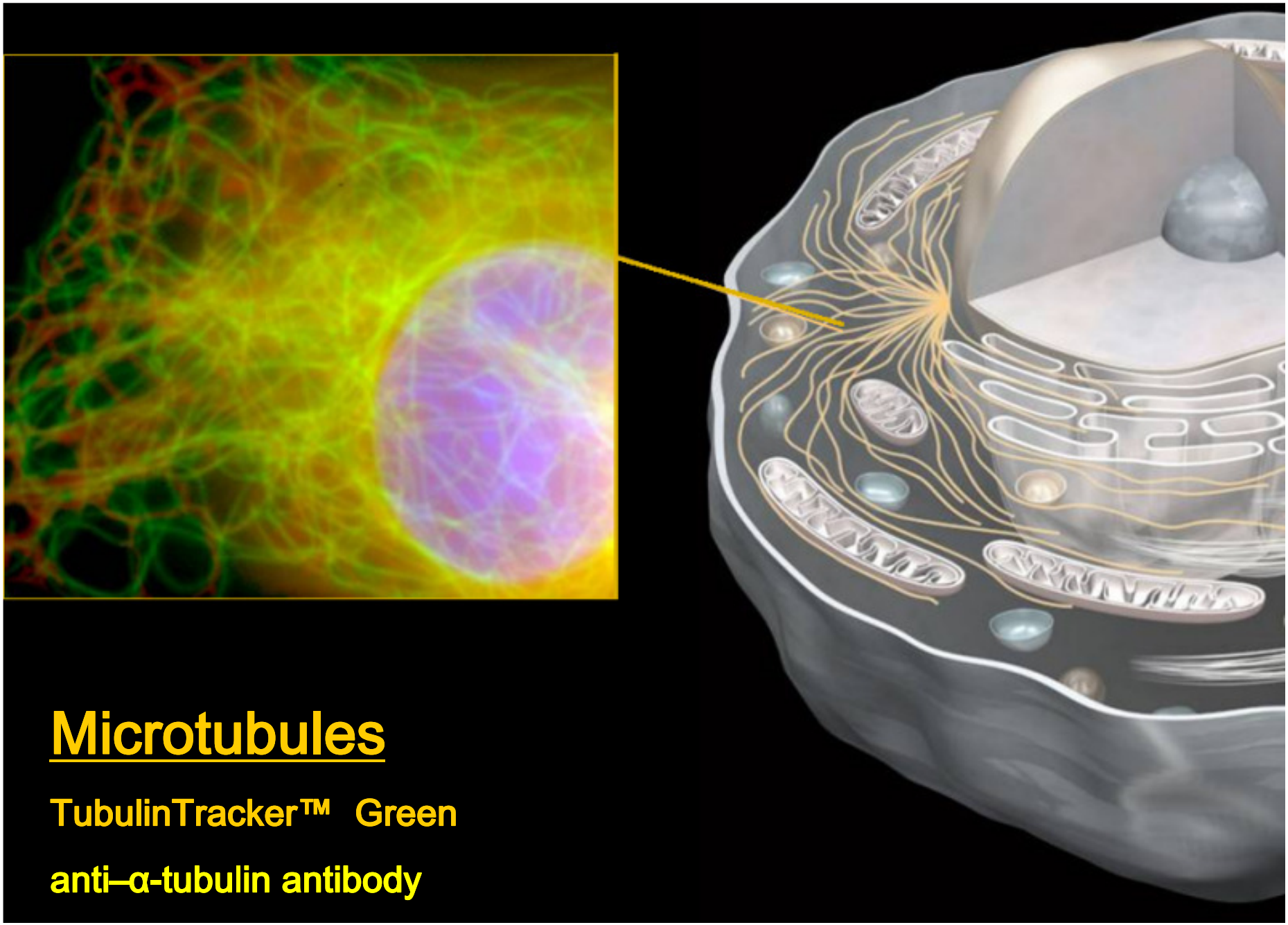


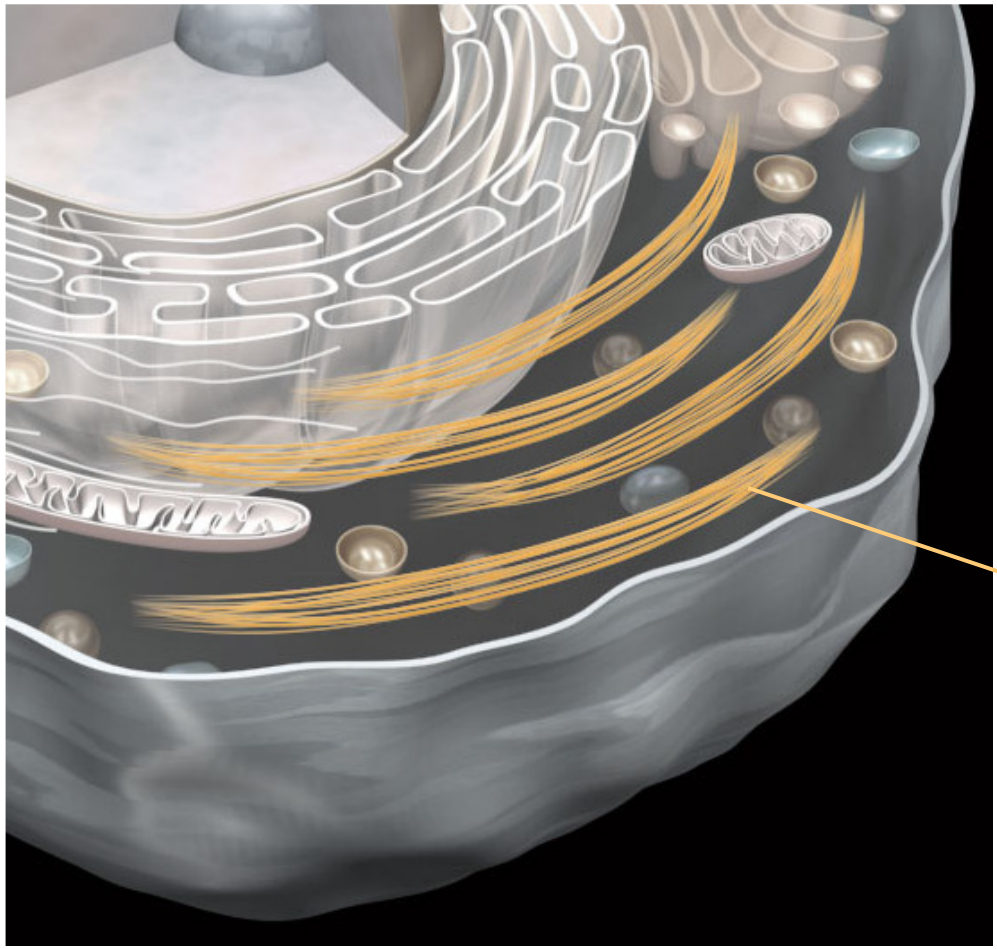


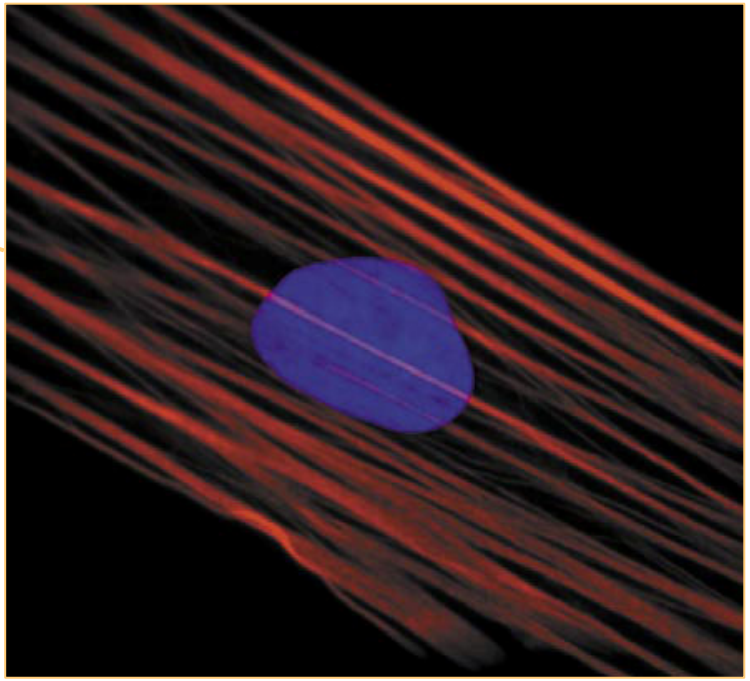
Lipid Rafts

BODIPY® FL C_5-ganglioside GM1

Fluorescent Cholera Toxin subunit B (CT-B)


Microtubules
TubulinTracker™ Green
anti–α-tubulin antibody

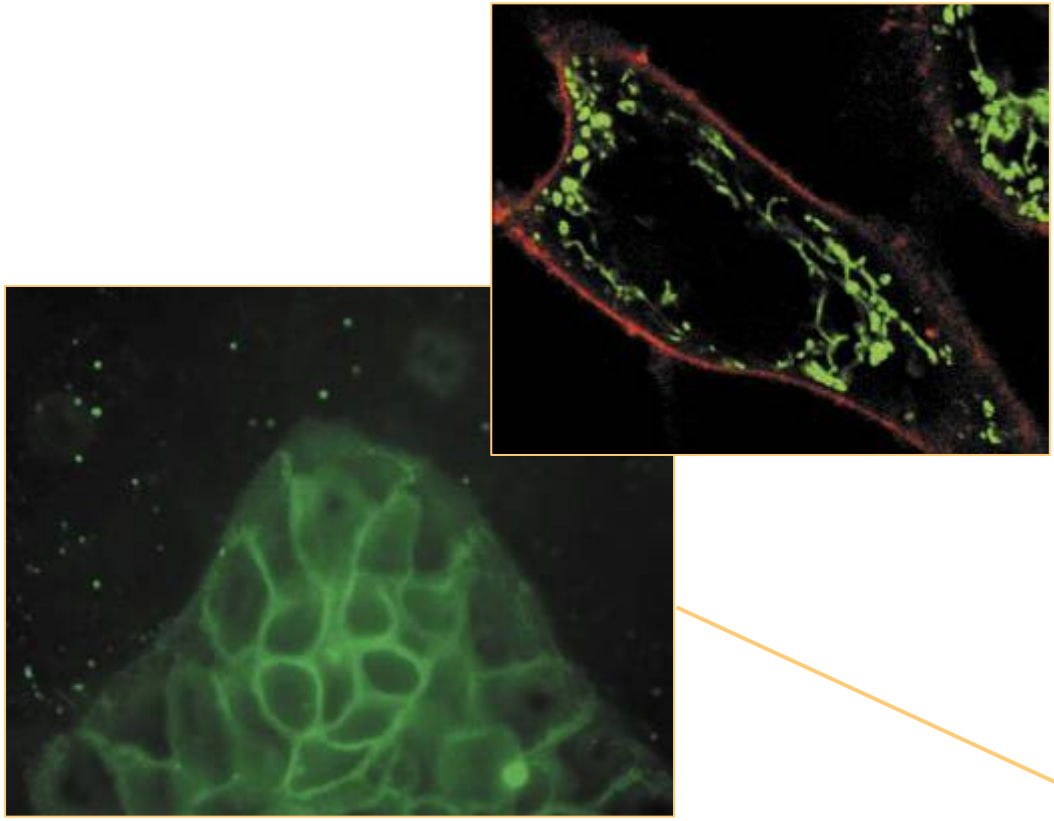


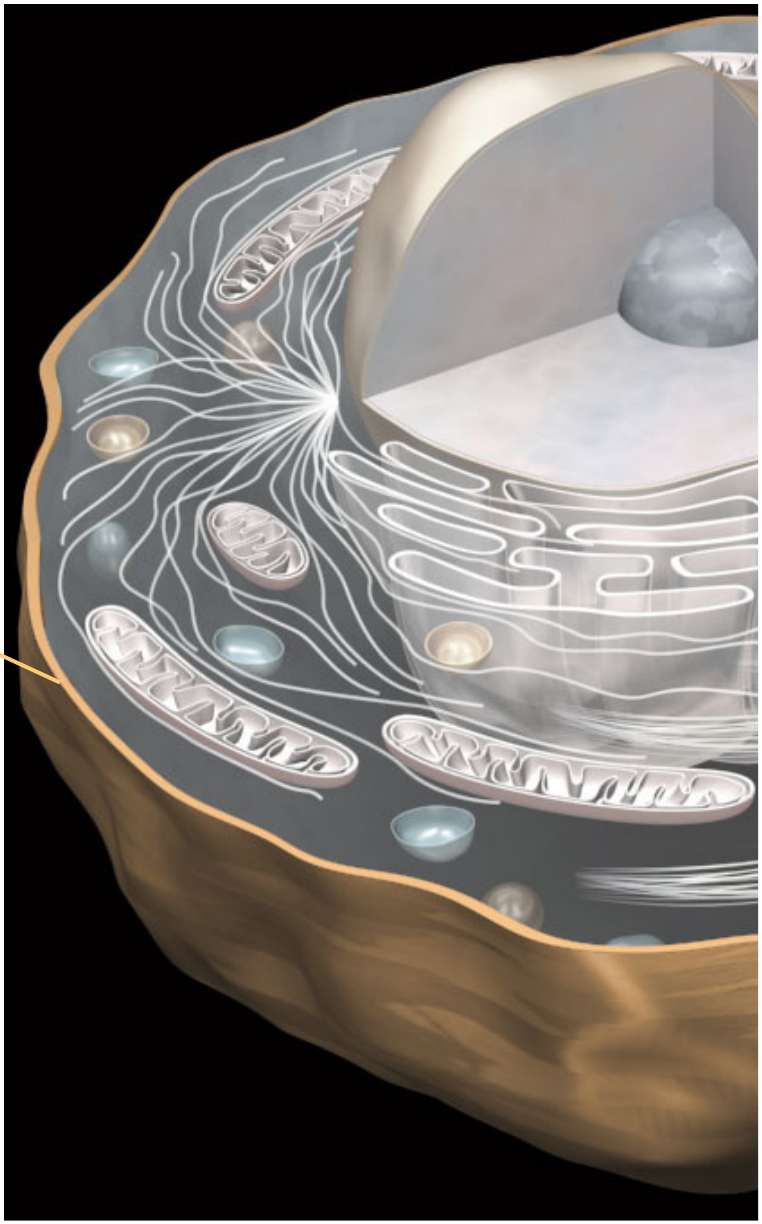


Actin cytoskeleton

Fixed cells: Alexa Fluor-phalloidin

Live cells: Lifeact-GFP (17 aa peptide binding actin)



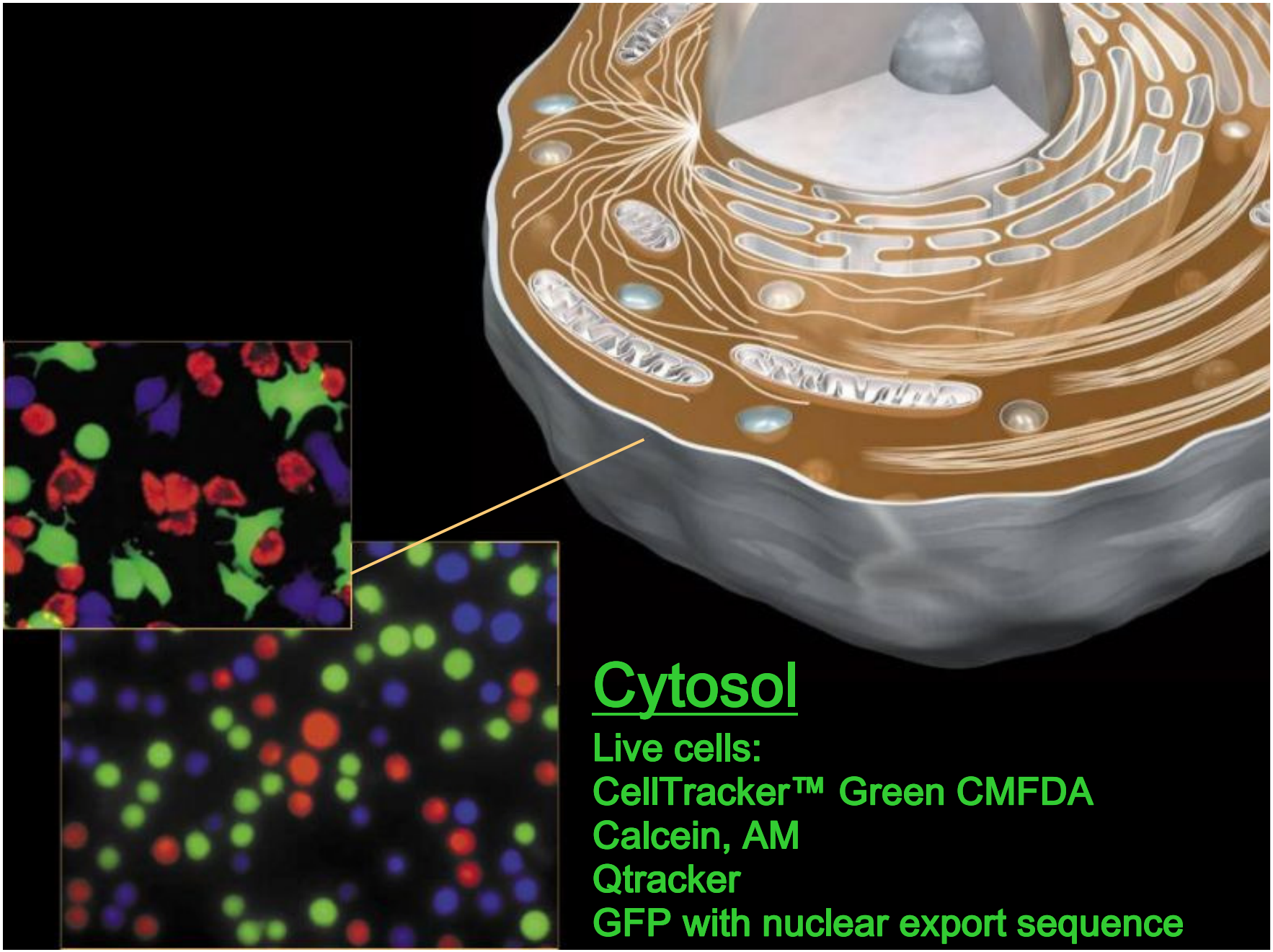


Plasma Membrane

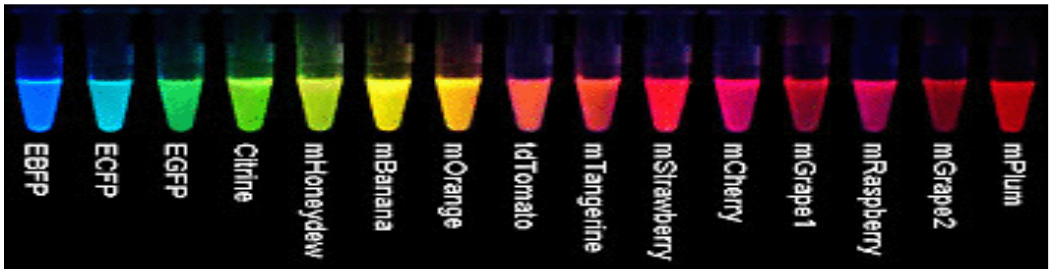
Wheat Germ Agglutinin

Live cells: FM dyes,

myristoyl+palmitoyl tagged-GFP


Cytosol
Live cells:
CellTracker™ Green CMFDA
Calcein, AM
Qtracker
GFP with nuclear export sequence

Fluorescent proteins are more than just labels

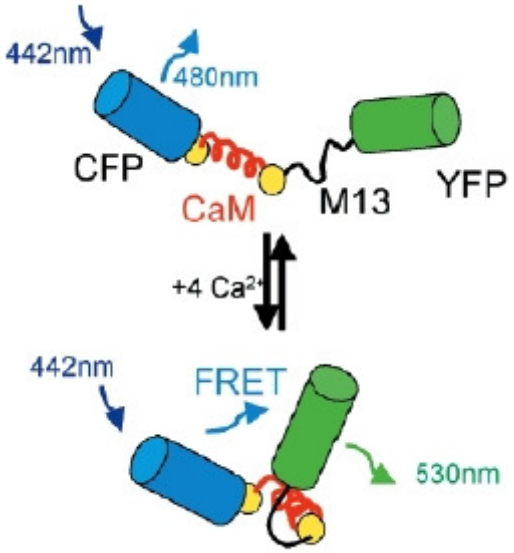


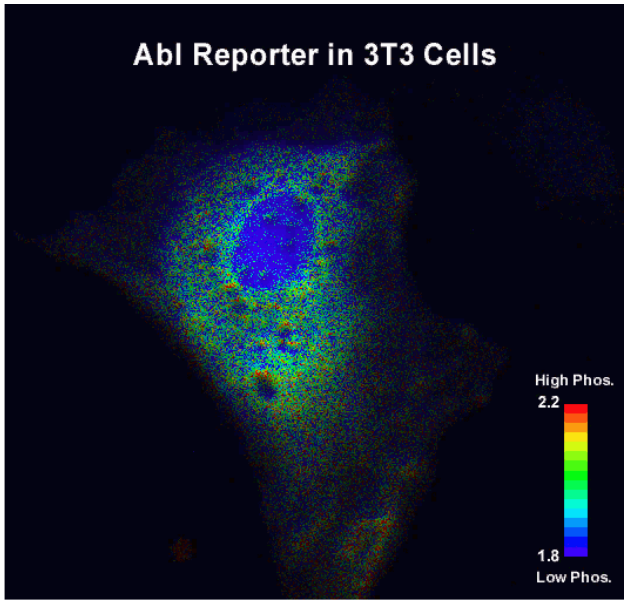
Photoactivation/Photoswitching
- PA-GFP, Dronpa, Eos

Reporting on environment
- Ca^{2+}, phosphorylation, cAMP, cGMP, pH, neurotransmitters, voltage, cell cycle, redox, ROS

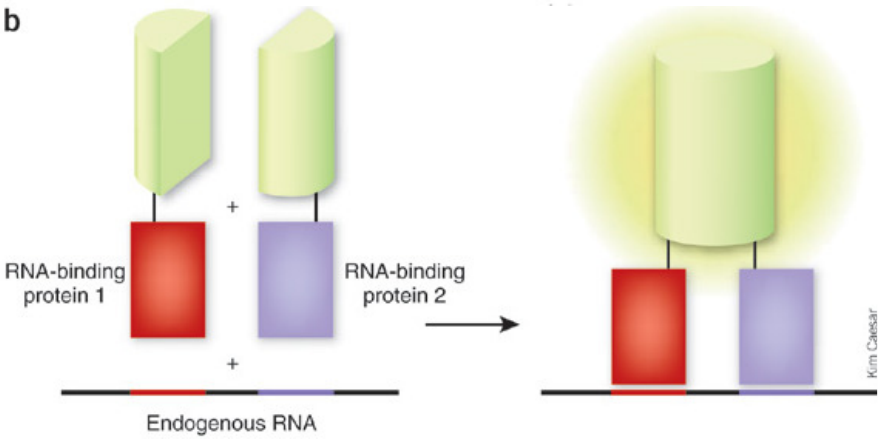
Reporting on protein-protein interaction
- CFP/YFP FRET, split fluorescent proteins

Modifying environment
- Singlet oxygen generation, Channelrhodopsin

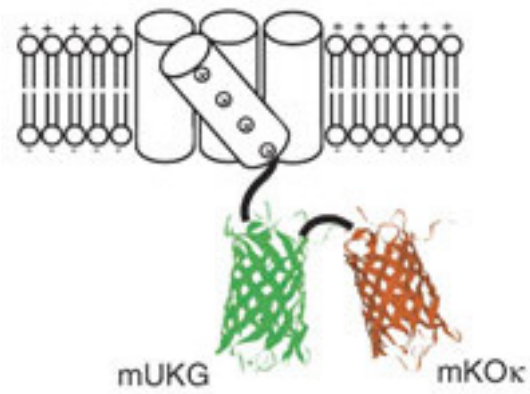




<u>Targeting advantage</u> to defined compartment, cell-type, developmental stage

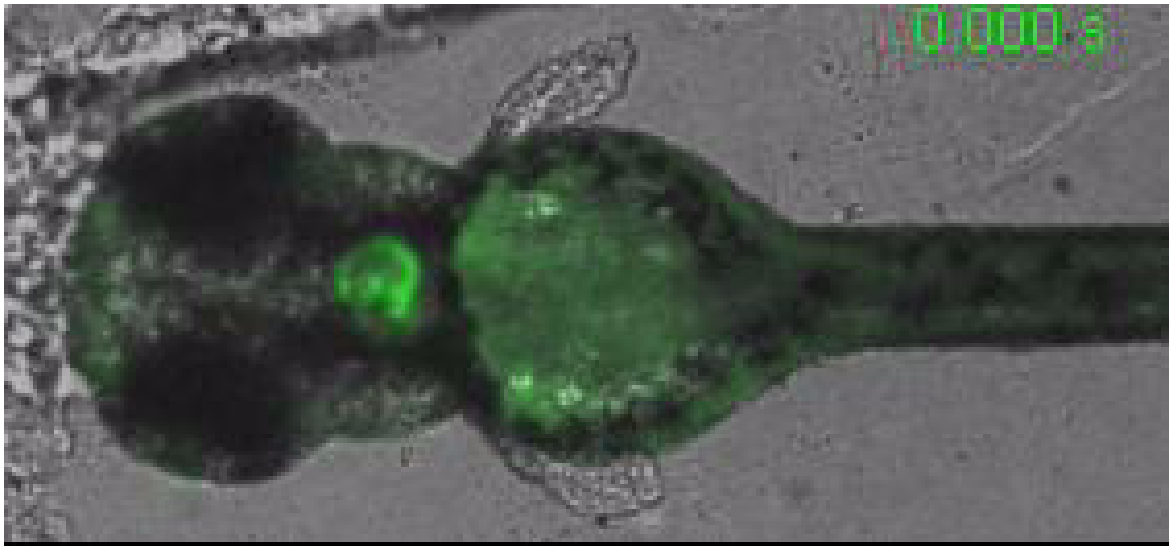


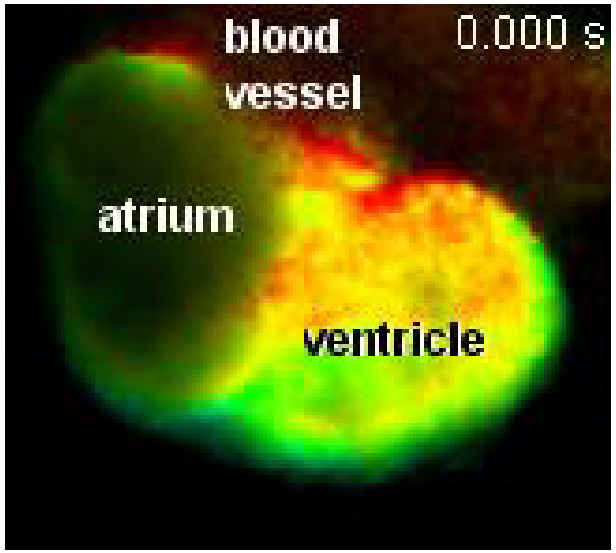
Sensing voltage with fluorescent protein



Mermaid FRET voltage-sensor
by FP fusion to voltage-sensing phosphatase

Expressed in zebrafish heart
Non-invasive testing of mutant phenotypes
and drug cardiotoxicity.
Tsutsui, Miyawaki J Physiol 2010

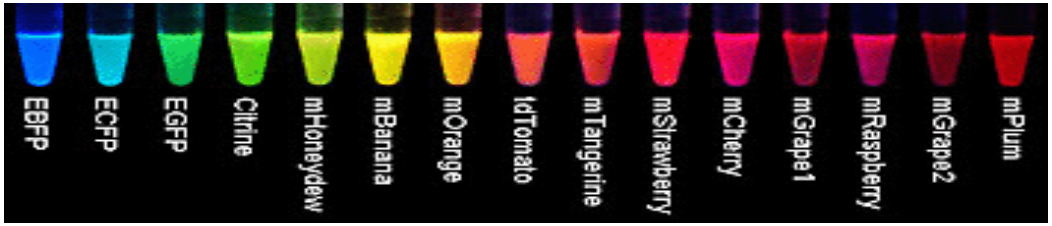




N.B. FRET sensor ratio crucial

best is YC2.60 cameleon: 600%,
if <20% then lost in cellular noise

How good is a fluorescent protein?



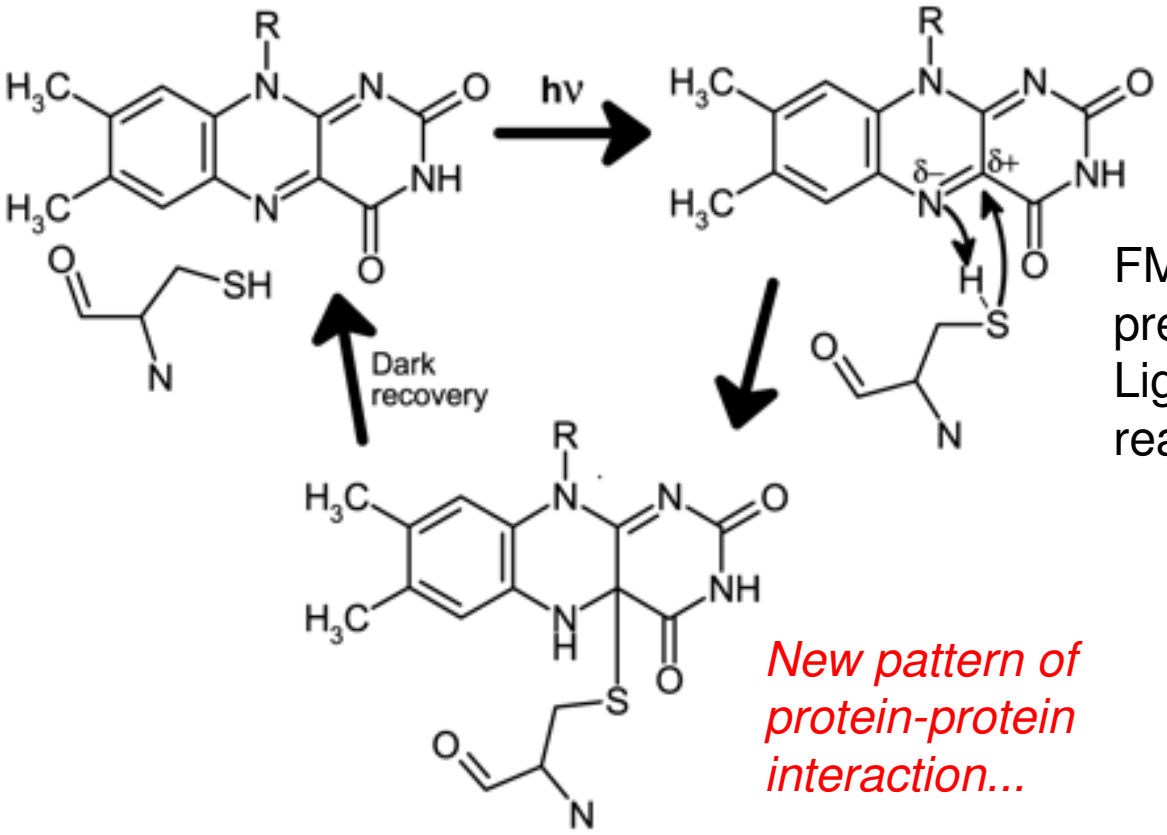
A. victoria GFP is terrible!

EGFP is OK, but there are now better...

1. Excitation and emission λ — good match to filters on your microscope
   look at other fluorophores at same time
2. Bright — ε x QY YPet 2.5 x EGFP
   TagRFP 2x mCherry
3. Stable to photobleaching — EBFP bad, mCherry and YPet good
4. Non-toxic — attach on right part of your protein
   all make H_2O_2, FPs can transfer electrons
5. Environment-insensitive — especially to pH, chloride
   CyPet does not fold at 37°C, all need O_2
   Photoactivatable FP did not work in ER
6. Little non-specific binding — fully monomeric, A206K non-dimerising
7. Maturation speed — Venus 2 min. Red FPs start off green!
   half-time 40 min mCherry, 100 min TagRFP

LOV domains react and switch conformation with light

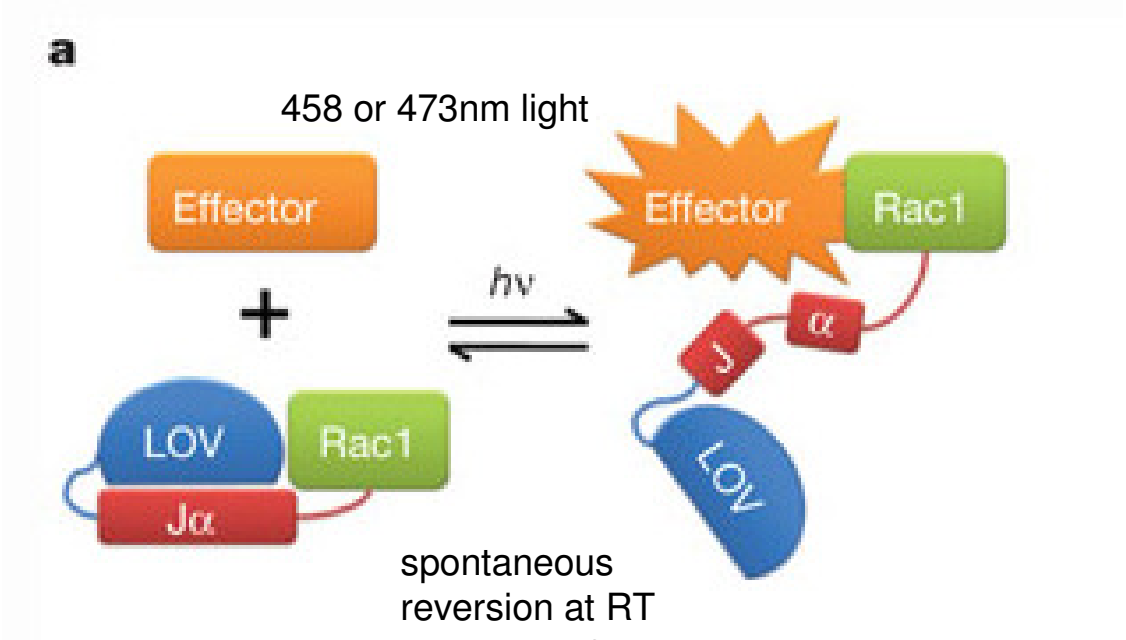
LOV domains:
light, oxygen, voltage responders
ones responding to blue light in bacteria, plants and fungi



FMN cofactor
present in all cells.
Light polarises to increase reactivity to Cys attack.

New pattern of protein-protein interaction...

Genetically-encoded photoactivation



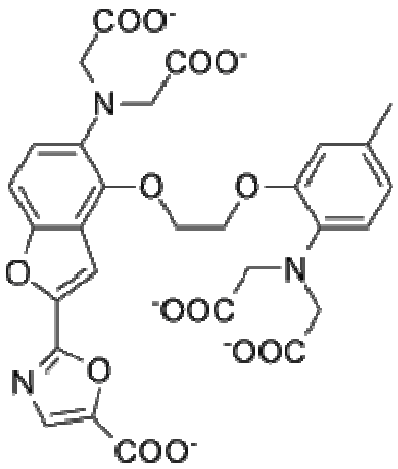
1. Constitutively active Rac mutant
2. Optimise LOV-Rac junction,
3. knockout GTP hydrolysis and GAP/GNDI/GEF interactions

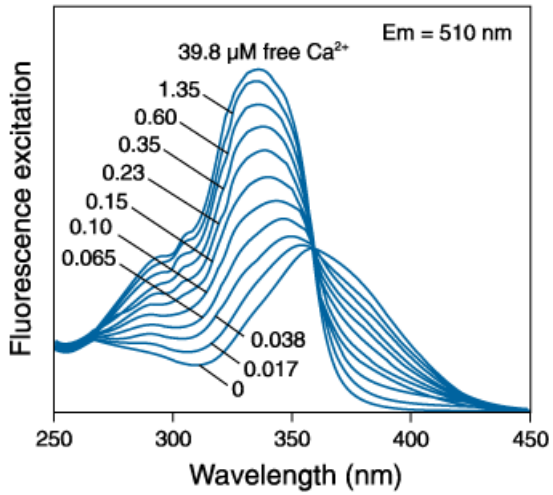
K_d for PAK 2 μM in dark, 200nM in light 10-fold ratio

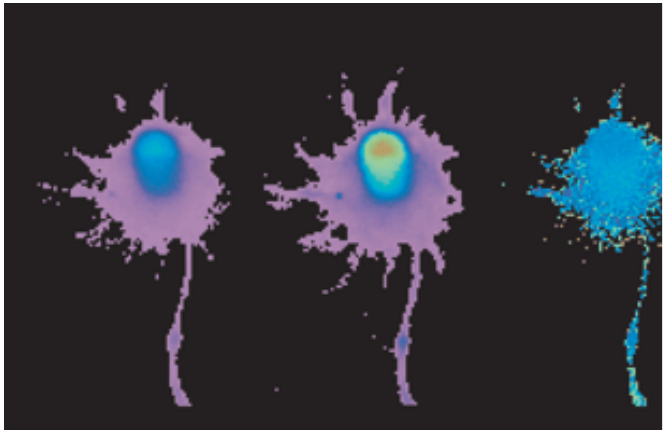
Interaction of Rac with PAK stimulates cell protrusion and migration.

K.Hahn et al. Nature Sept. 2009

Small molecule fluorescent sensors







Fura-2 sensing calcium

Metal ions: calcium, magnesium, zinc, sodium, potassium, chloride, mercury

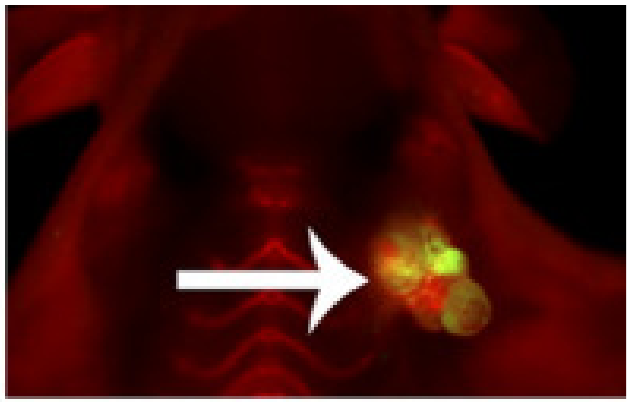
pH (also dyes to conjugate to proteins, CyPher from GE, SNARF from Invitrogen)

Reactive oxygen species, nitric oxide

Transmembrane potential

Why use small molecule rather than genetically-encoded probes?

1. No need to transfect
   - hard for some organisms and primary cells
   - easier to titrate
   - potential clinical application- e.g. image-guided surgery



MMP-activated Cy5 peptide labels tumour (RY Tsien 2010)

2. Probes often brighter, with bigger signal to noise
   - struggle to make GFP-based calcium reporter as good as fura-like dyes
3. Probes with entirely different fluorescent properties
   - QD photostability, probes with long fluorescence lifetimes, photouncaging
4. Smaller
   - e.g. calcium conc. right next to pore of ion channel

References

Fluorescence probes

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Principles of Fluorescence Spectroscopy 2nd edition, by Joseph R. Lakowicz.

Protein modification

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Chemical labeling strategies for cell biology, Marks KM, Nolan GP. Nat Methods. 2006 Aug;3(8):591-6.

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(i) as labels: A guide to choosing fluorescent proteins. Shaner NC, Steinbach PA, Tsien RY.
Nat Methods. 2005;2(12):905-9.
Poster: Fluorescent proteins illuminate cell biology Lin M, Miyawaki A, Tsien RY.
http://www.nature.com/nrm/posters/fluorescent/index.html
(ii) as sensors: Creating new fluorescent probes for cell biology. Zhang J, Campbell RE, Ting AY, Tsien RY. Nat Rev Mol Cell Biol. 2002 Dec;3(12):906-18.