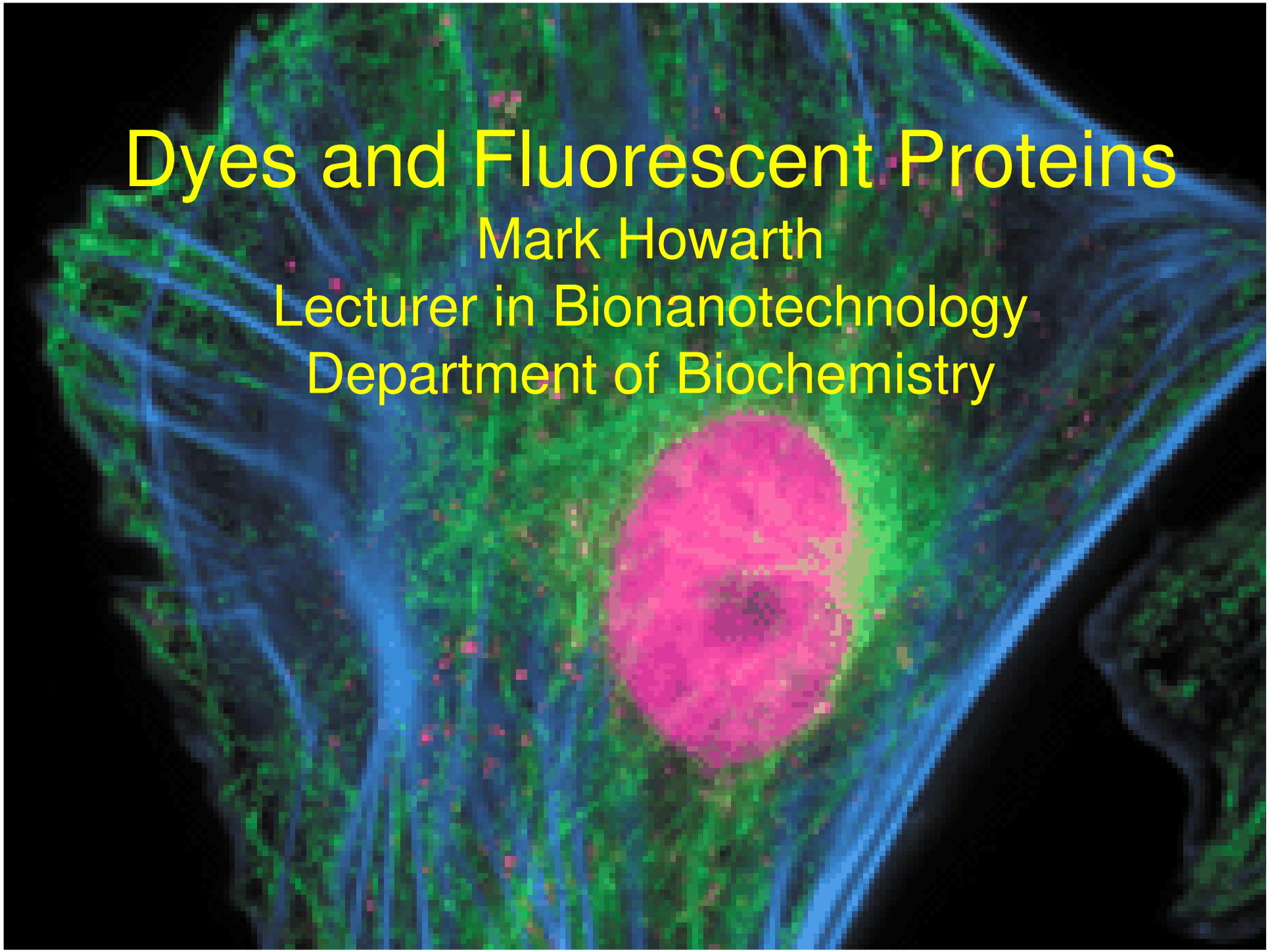
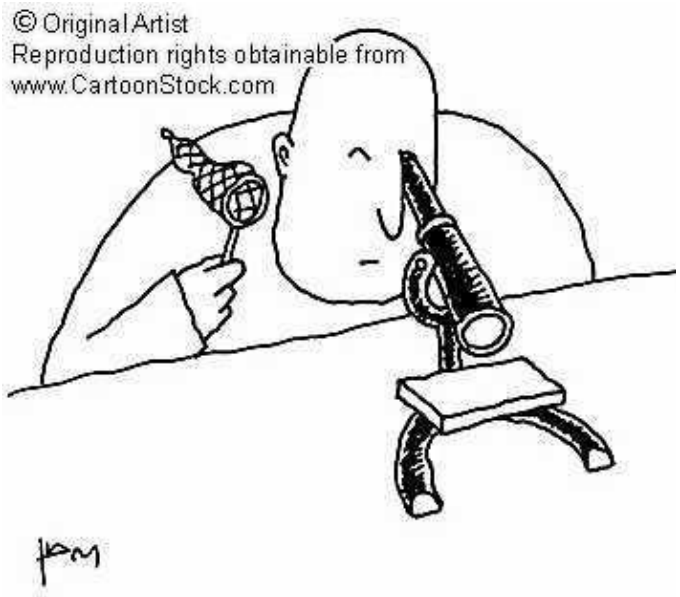


Dyes and Fluorescent Proteins

Mark Howarth
Lecturer in Bionanotechnology
Department of Biochemistry

Overview



1. What is fluorescence

2. What kind of structures are fluorescent

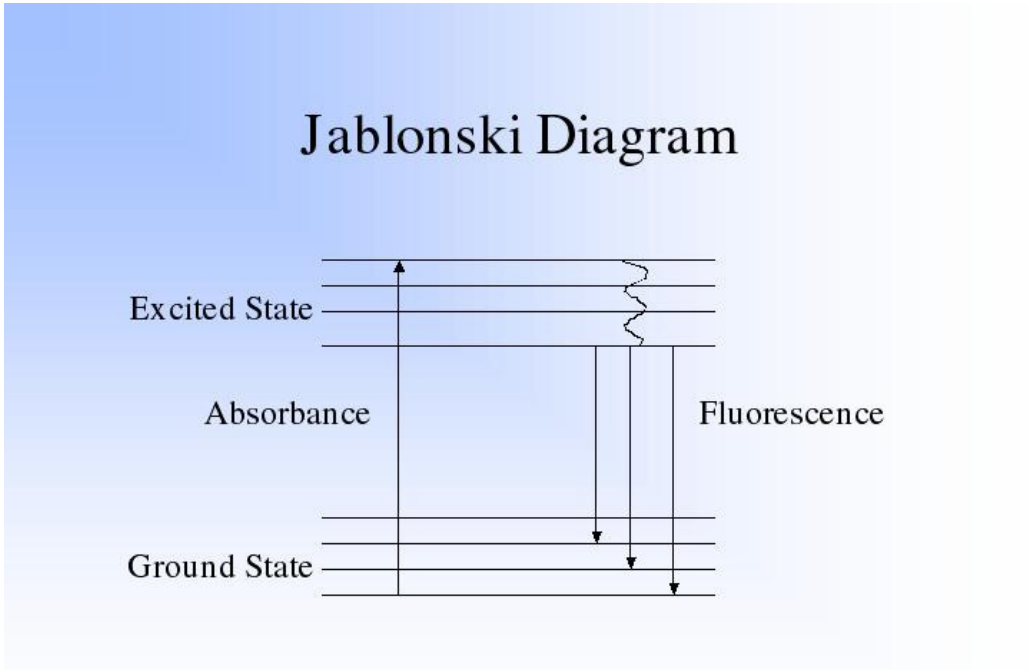
3. How to make and target fluorescent probes

4. Fluorescent probes for cellular structure and function

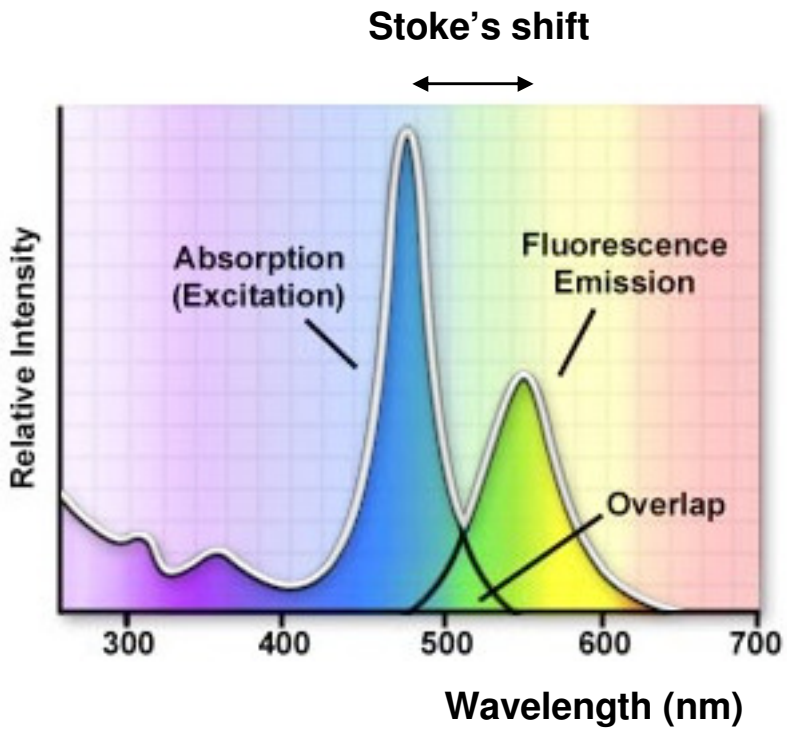
What is fluorescence?

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

Energy levels?

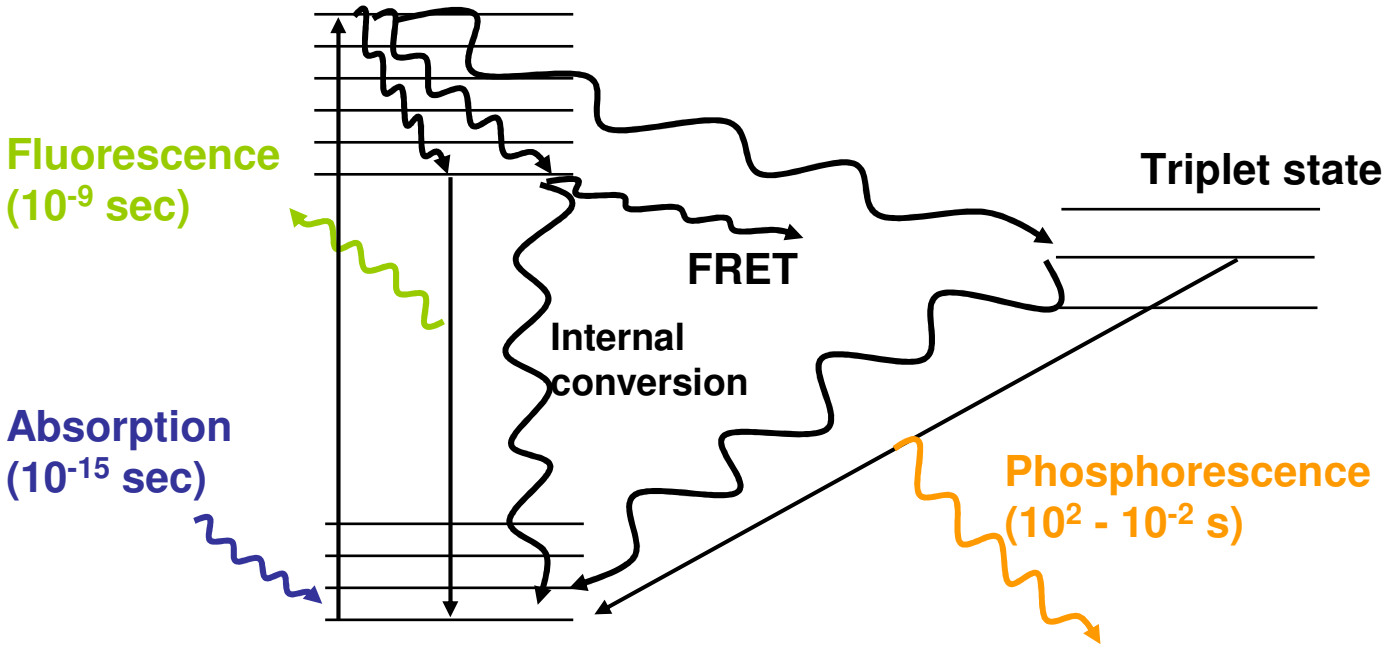


Fluorescence spectrum



for fluorescein

Not all energy emitted as fluorescence



$$\text{Quantum yield} = \frac{\text{no. of fluorescent photons emitted}}{\text{no. of photons absorbed}}$$

e.g. EGFP QY=0.6 For every 10 photons absorbed, 6 are emitted.
(at optimal temp, pH etc.)

Overview

1. What is fluorescence

2. What kind of structures are fluorescent

3. How to make and target fluorescent probes

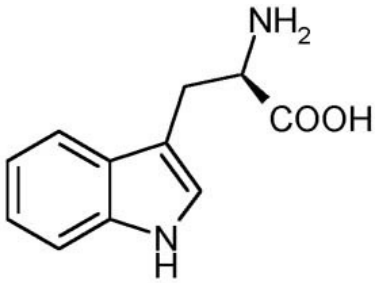
4. Fluorescent probes for cellular structure

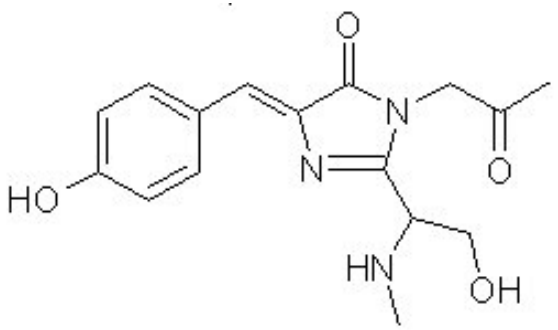
What sort of molecules are fluorescent?

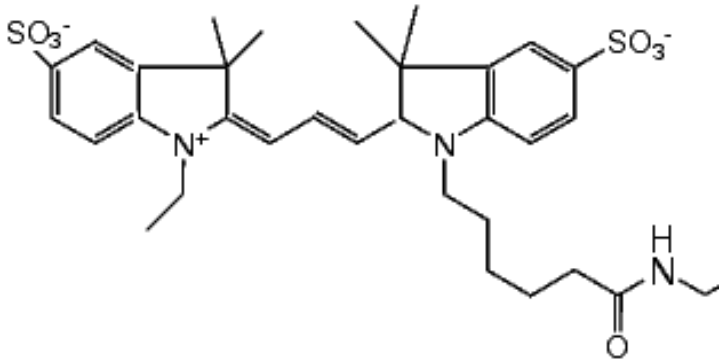
Organic fluorophores
especially
1. Intrinsic fluorophores (source of autofluorescence)
2. Dyes
3. Fluorescent proteins

Inorganic fluorophores
especially
1. Lanthanides
2. Quantum dots

What sort of molecules are fluorescent?
1. Organic fluorophores



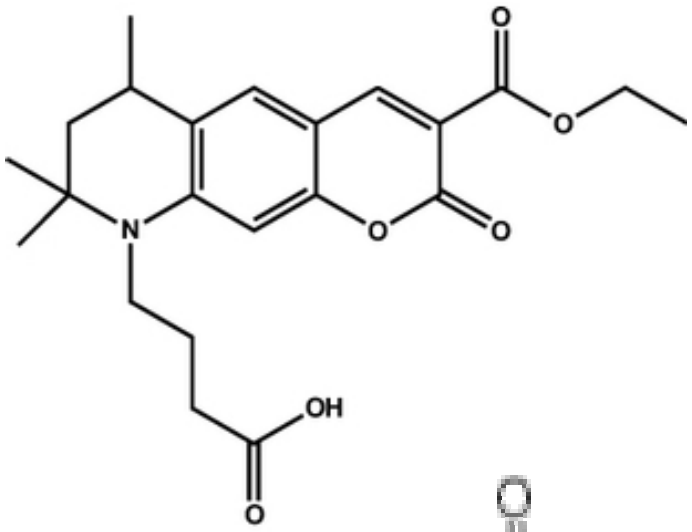


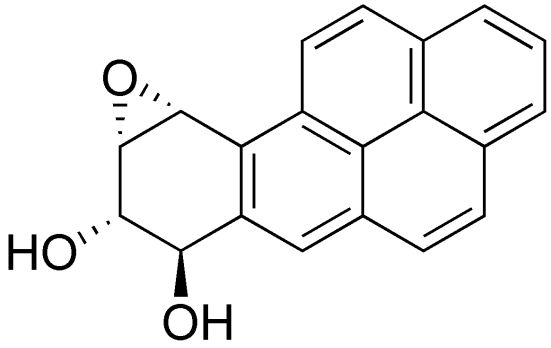


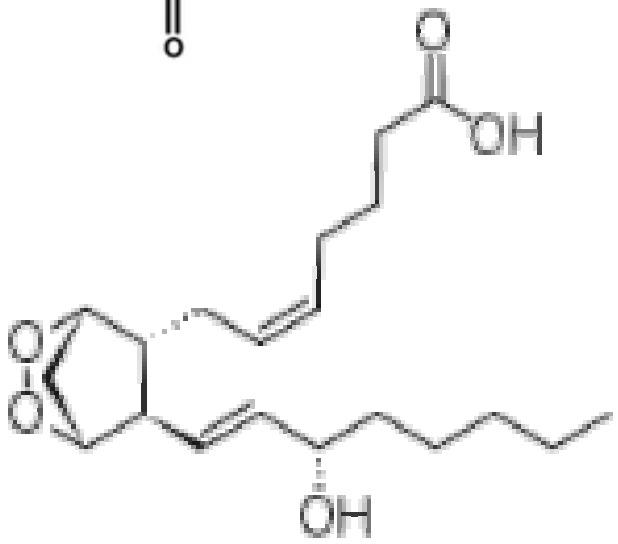
Chemical features:

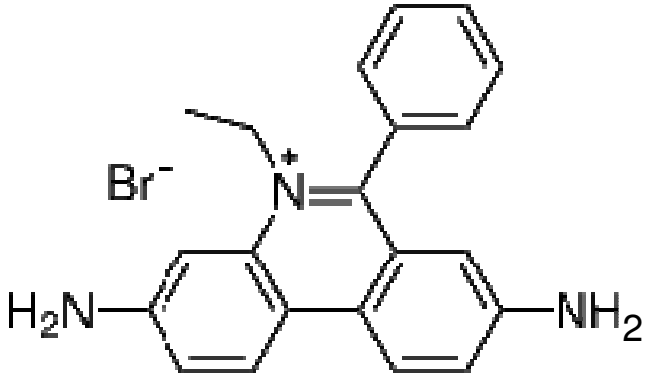
1. Conjugation
2. Rigidity especially fused aromatic rings
3. Heteroatoms

Please rank these in order of fluorescence

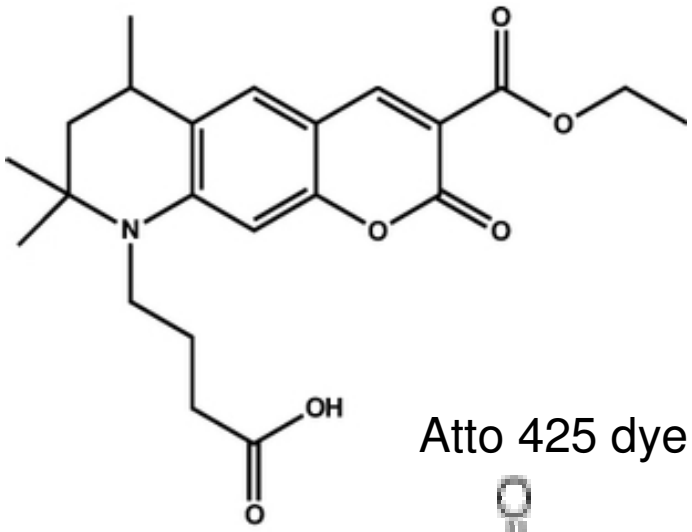




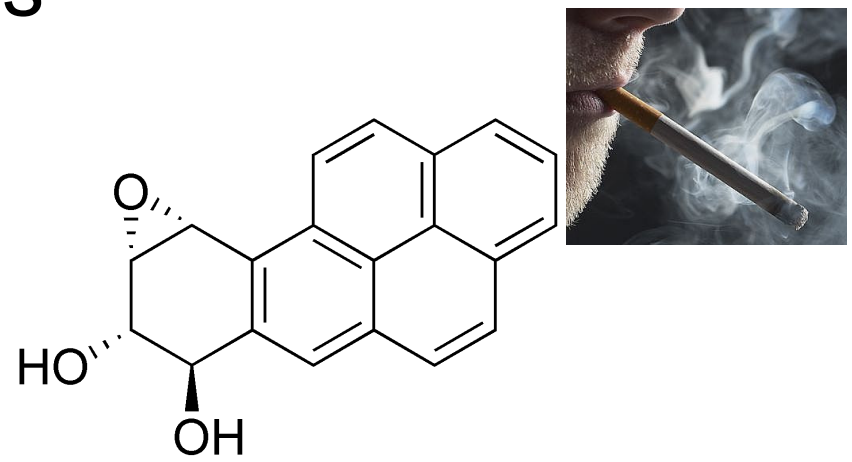




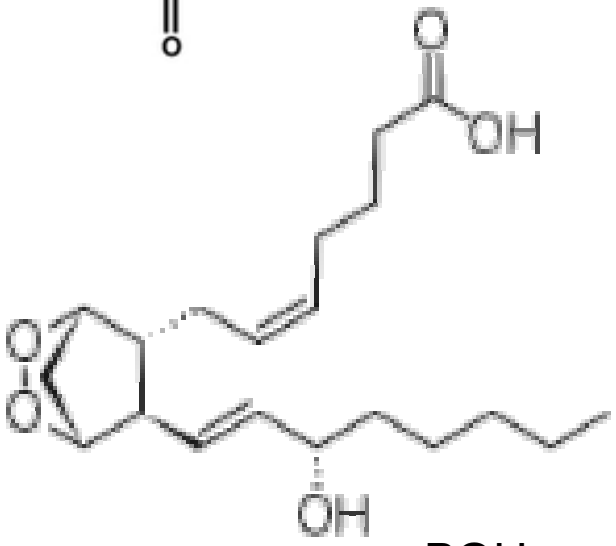
Rank these in order of fluorescence- answers



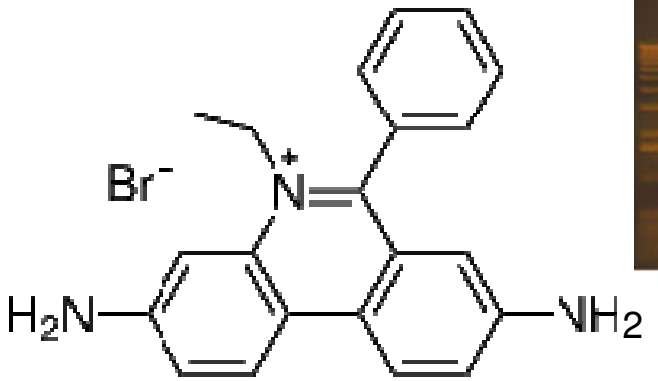
Atto 425 dye +++

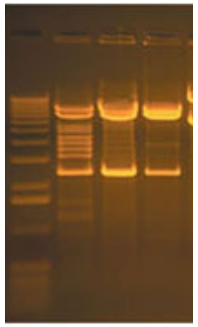


benzopyrene diol epoxide +?



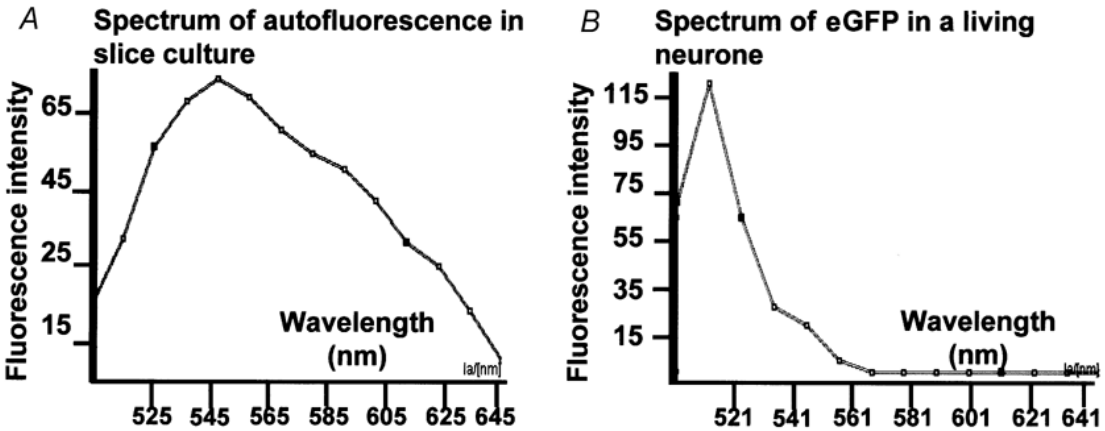
PGH_2 -

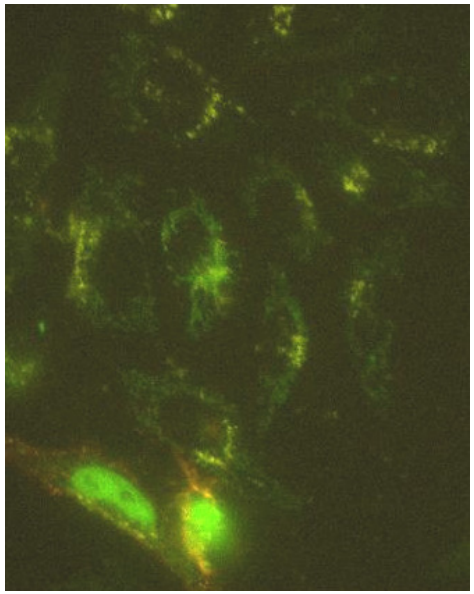




ethidium bromide ++

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





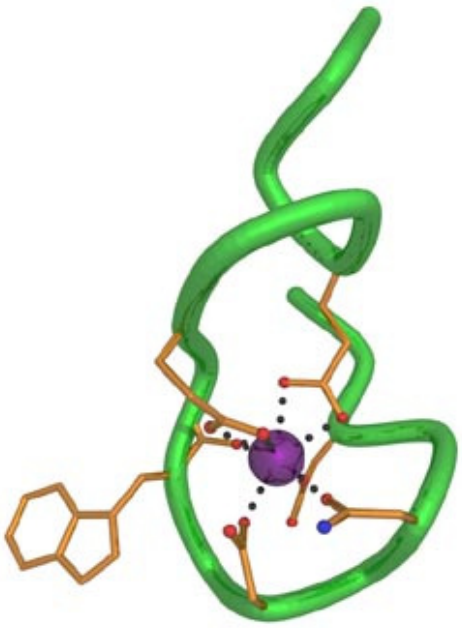
Most common autofluorescent molecules:
Flavins, NADH, NADPH, elastin, collagen, lipofuscin

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

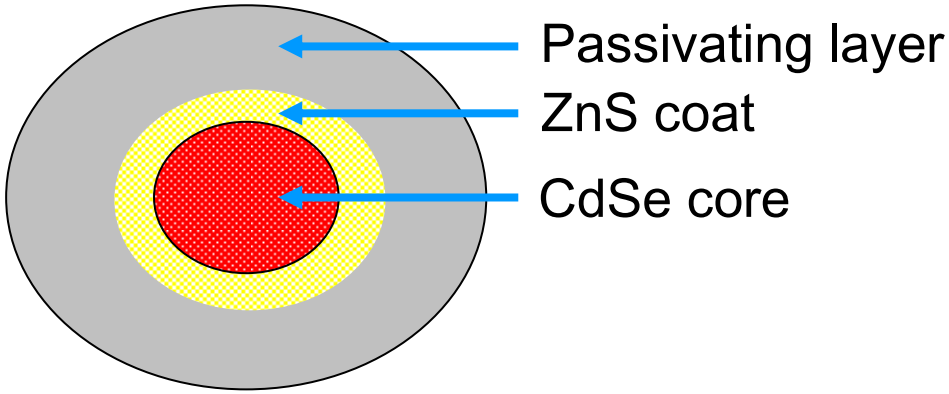
Lanthanides

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



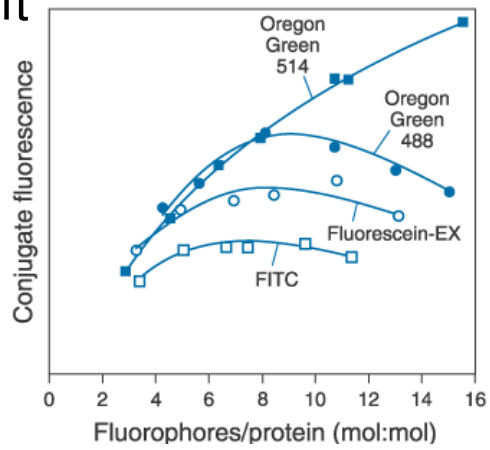
Michalet X, et al. Quantum dots for live cells, in vivo imaging, and diagnostics. Science. 2005 307(5709):538-44.

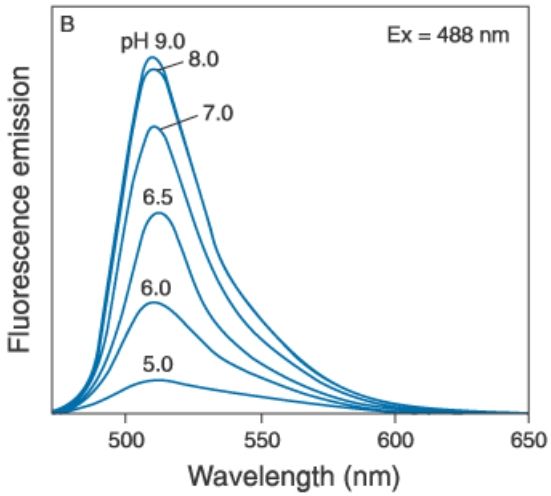
How good is a fluorophore?

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

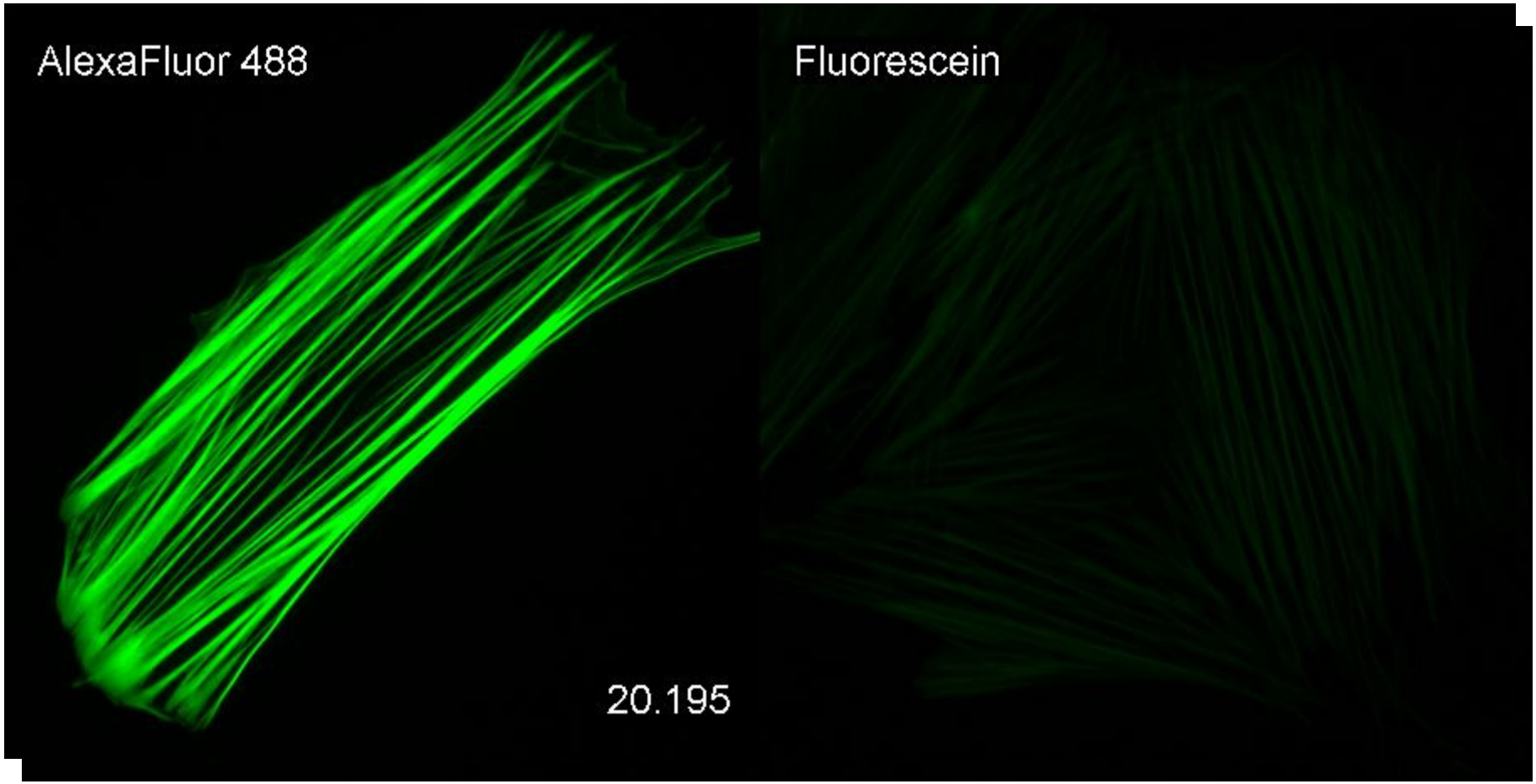




Fluorescein pH sensitivity

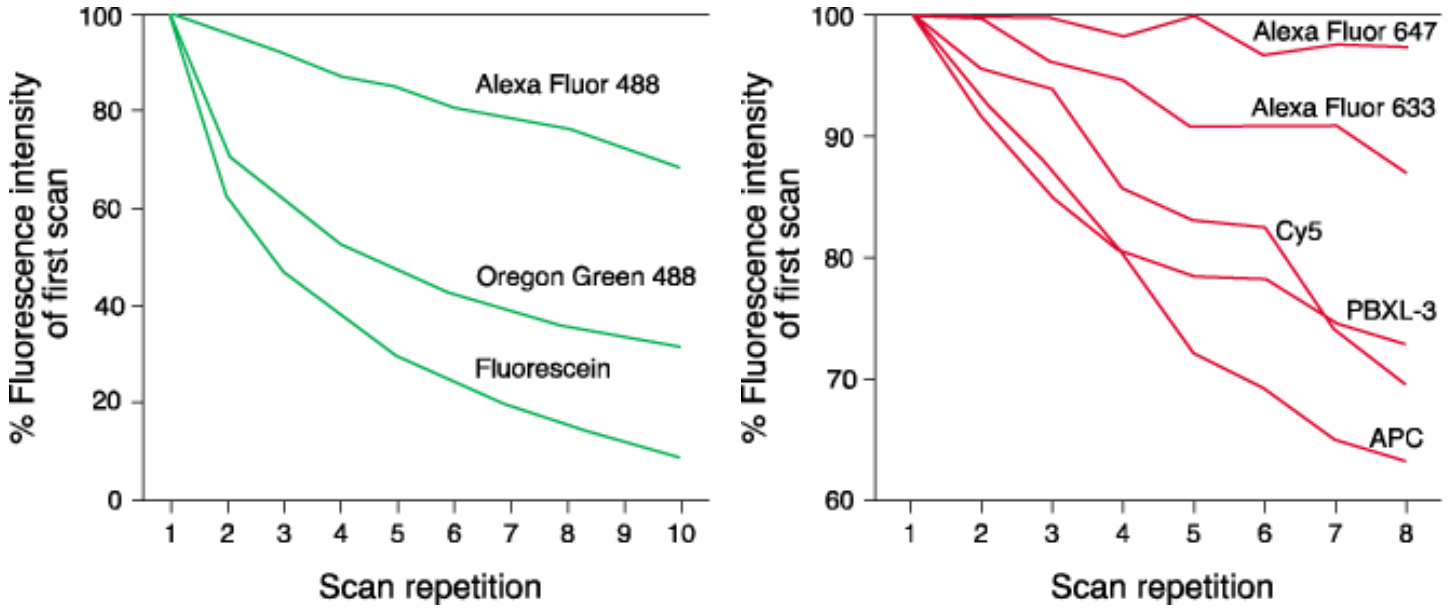
Alexa Fluor 488 vs Fluorescein Bleaching

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

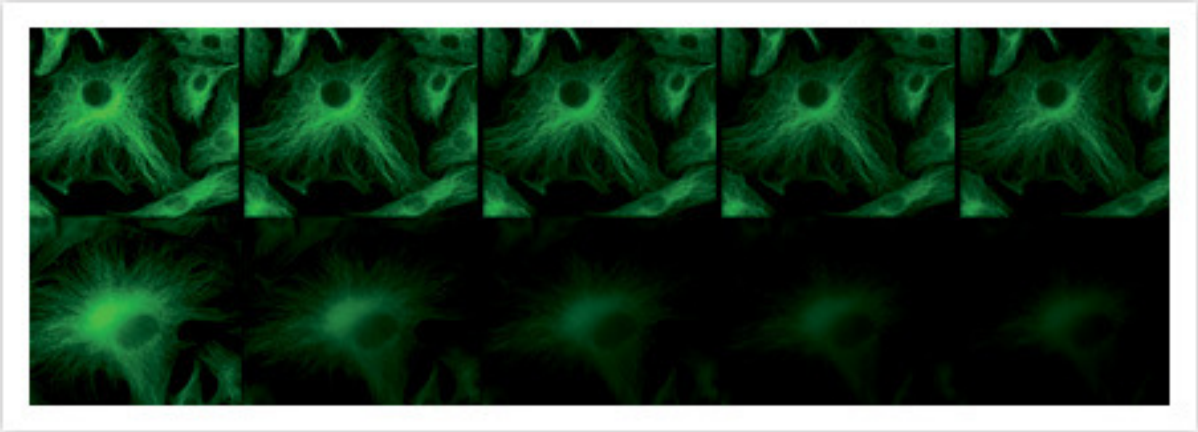
Scavenge and prevent reactive oxygen species from forming.

For fixed cells:

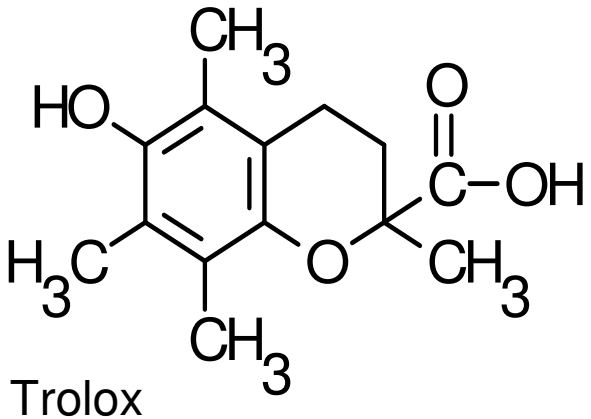
Home made: 0.3% p-phenylene-diamine (Sigma)
or Propyl Gallate
Vectashield: Proprietary, very effective all round, affects psf
Dabco
Prolong Gold®

+ Prolong Gold

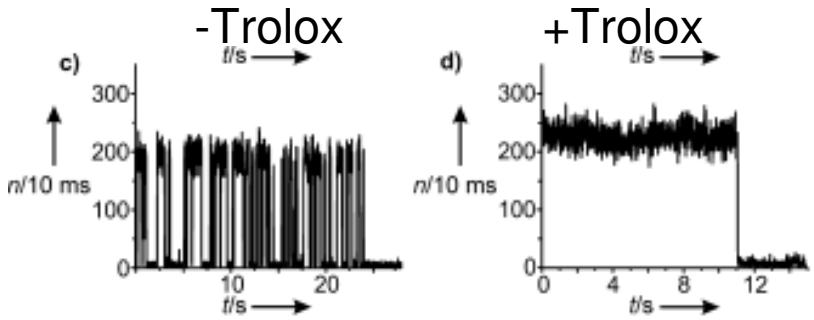
Untreated



Antifade Reagents for Live Cells



Trolox

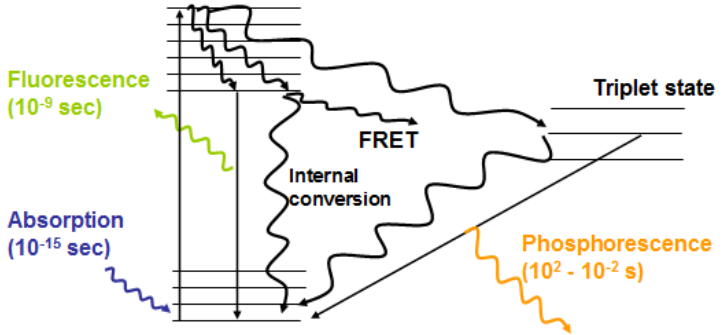


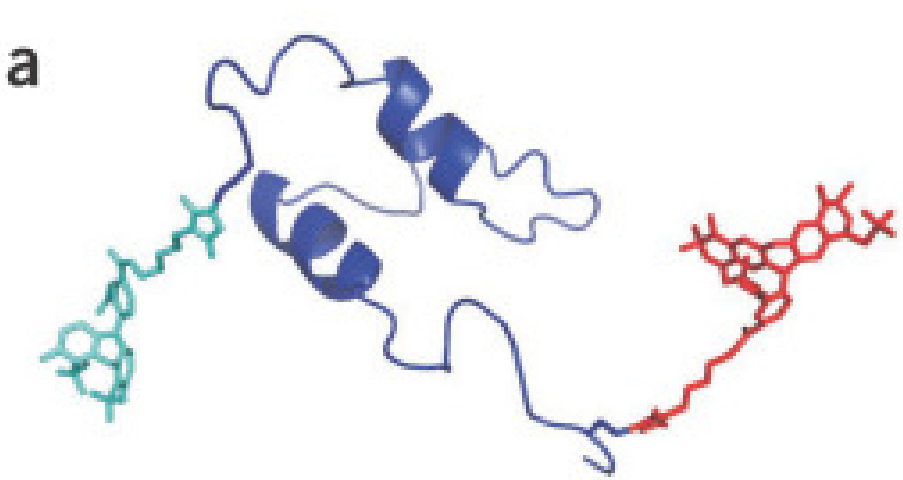
Blinking of single molecule of Atto647N on DNA, Vogelsang Tinnefeld Ang Chem 2008

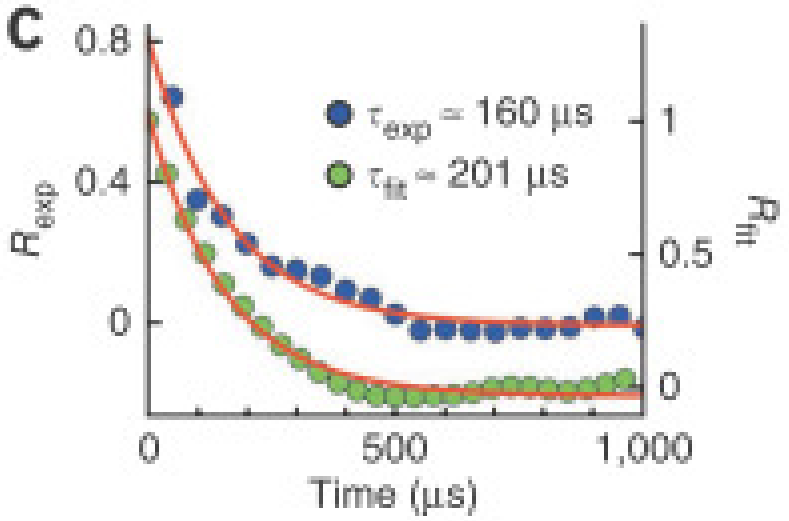
- Trolox is an antioxidant that can reduce bleaching
  compatible with live specimens
  water-soluble
  working conc. ~100 µM
- Ascorbic acid is an alternative antioxidant
- Depleting oxygen (especially used for some single molecule experiments) with Glucose Oxidase and Catalase greatly reduces bleaching.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

Microsecond! fluorescent measurements with Trolox + cysteamine

Oxygen helps stop triplet-state build-up
BUT oxygen promotes photobleaching
For rapid photon cycling-
1. leave oxygen in
2. add Trolox to further quench triplet state
3. include cysteamine (a thiol) to protect from singlet oxygen and hydroxyl radicals





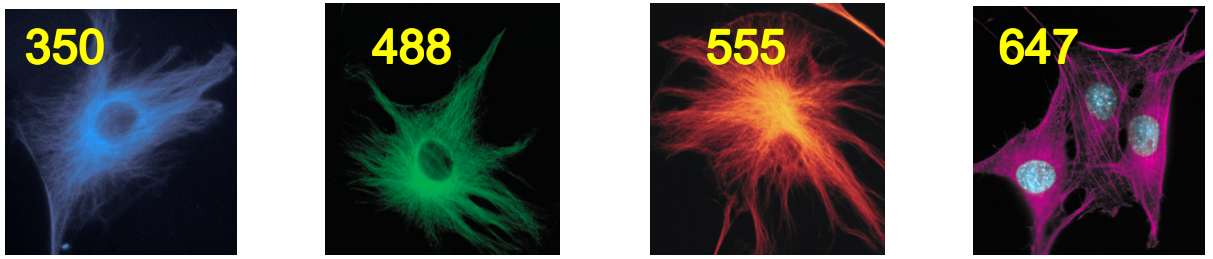


Green/Red Alexa dye FRET
on rapid folding protein

V. Munoz Nat Meth 2011

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

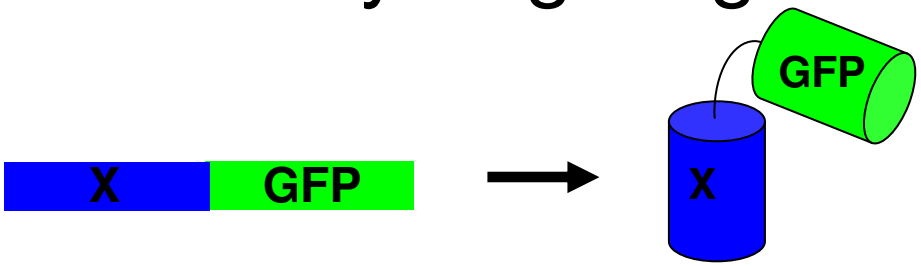
1. What is fluorescence

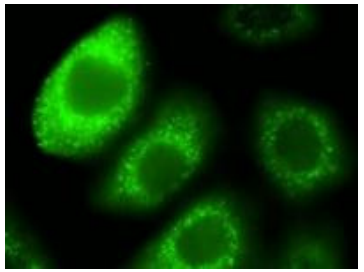
2. What kind of structures are fluorescent

3. How to make and target fluorescent probes

4. Fluorescent probes for cellular structure

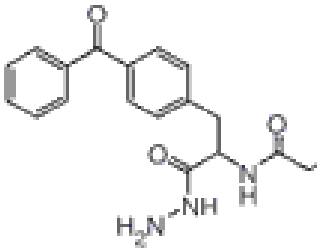
Major bottleneck to using new probes is difficulty targeting them



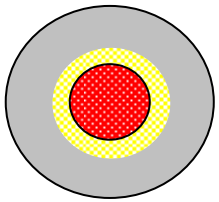


fluorescent proteins easy to target

photoaffinity probes

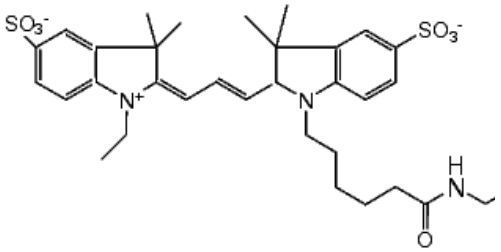


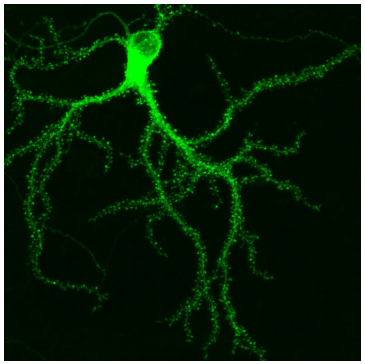
quantum dots



?

organic fluorophores

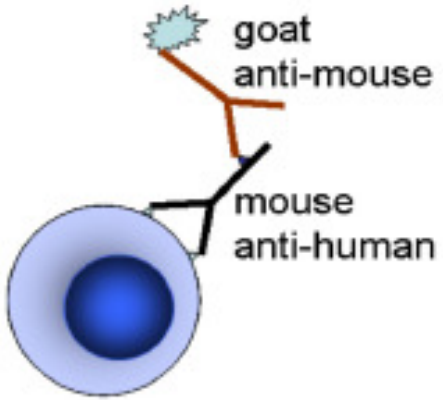




other probes hard to target

Antibodies for cellular imaging

Live cells



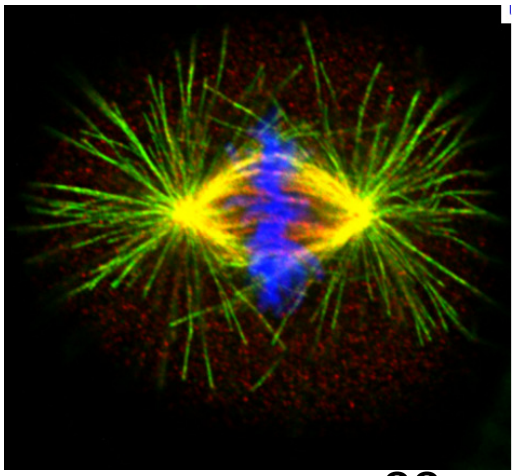
Label plasma membrane and secretory pathway
Penetrate plasma membrane
(microinjection, electroporation, pinosome lysis, streptolysin, cell permeable peptides, ester cage)

Get dynamics, avoid fixation artifacts

Fixed cells

Permeabilise

Still can give enormous amount of useful information

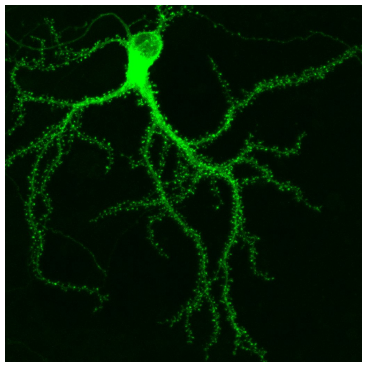


Not just antibodies for targeting

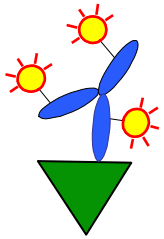
Other types of targeting agents:

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

?

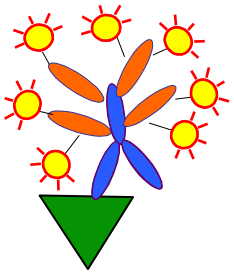


Getting fluorescence from antibody labelling



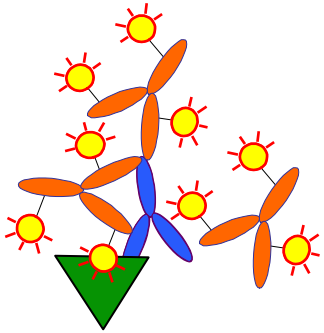
Directly-labelled Primary Antibody

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



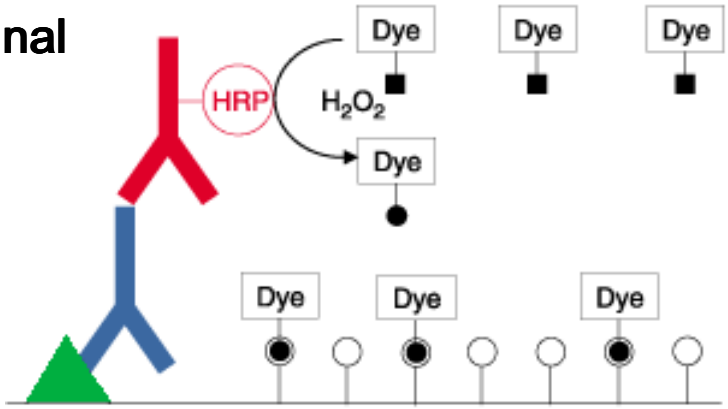
Zenon Technology

- **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**

How to dye: it is easy

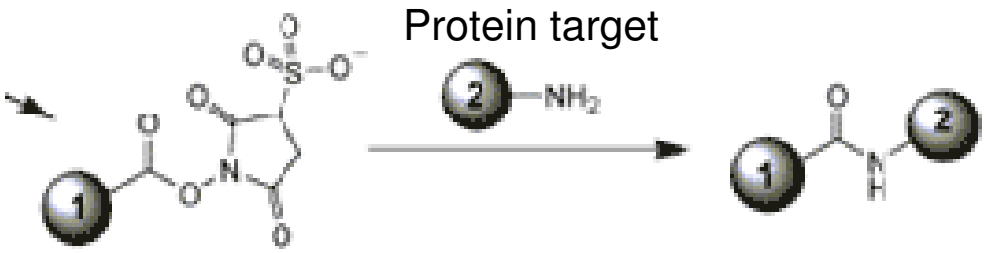
Multiple ways to modify proteins
(see Molecular Probes catalogue)

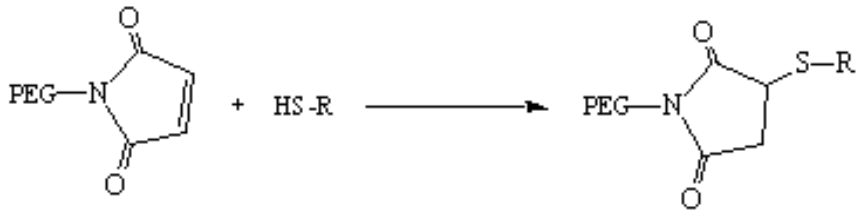
Most common ways are to modify:

1. Lysine

or

2. Cysteine



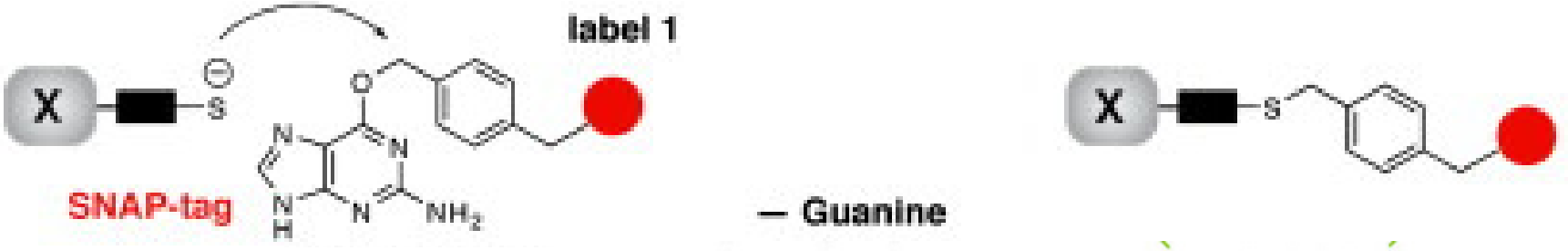


A	Add dye to protein for 3 hr
B	1cm Sephadex column to remove most free dye (10 min)
C	Dialyse away rest of free dye (24 hr)

Site-specific protein labelling methods

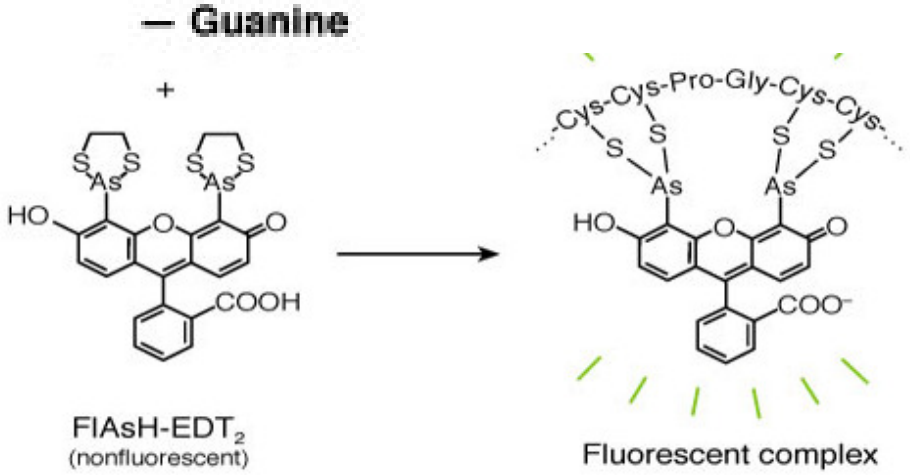
1. Binding domain

SNAP-tag (NEB), HaloTag (Promega)



2. Binding peptide

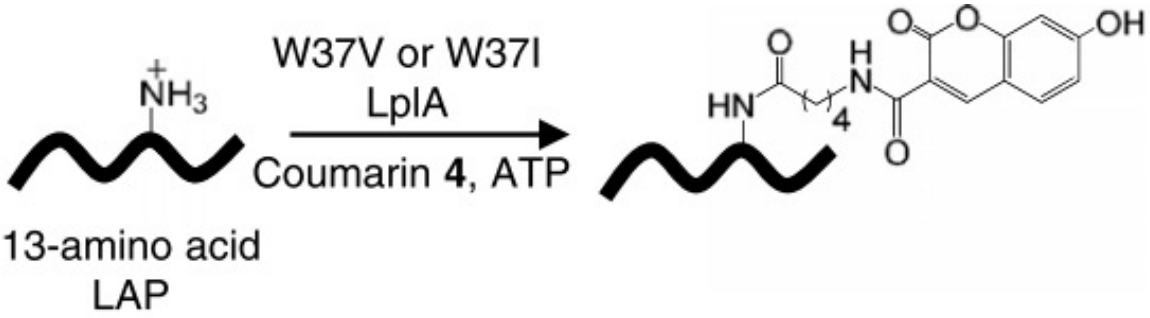
FlAsH (Invitrogen)



3. Enzymatic ligation to peptide

PRIME

AY Ting PNAS 2010



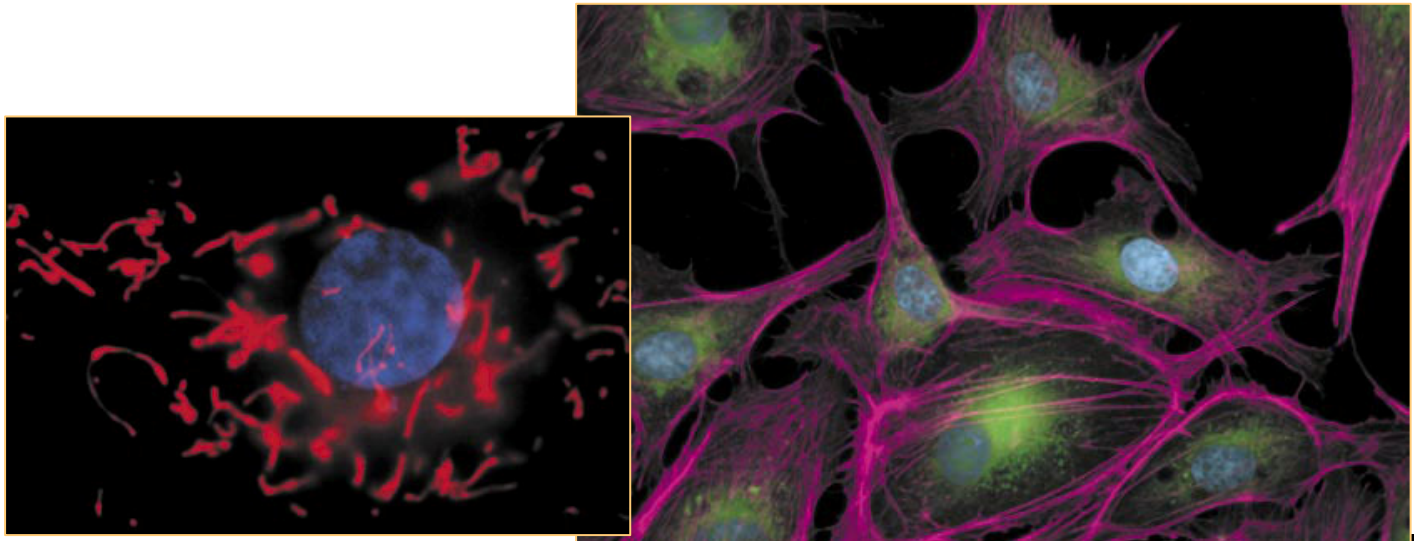
Overview

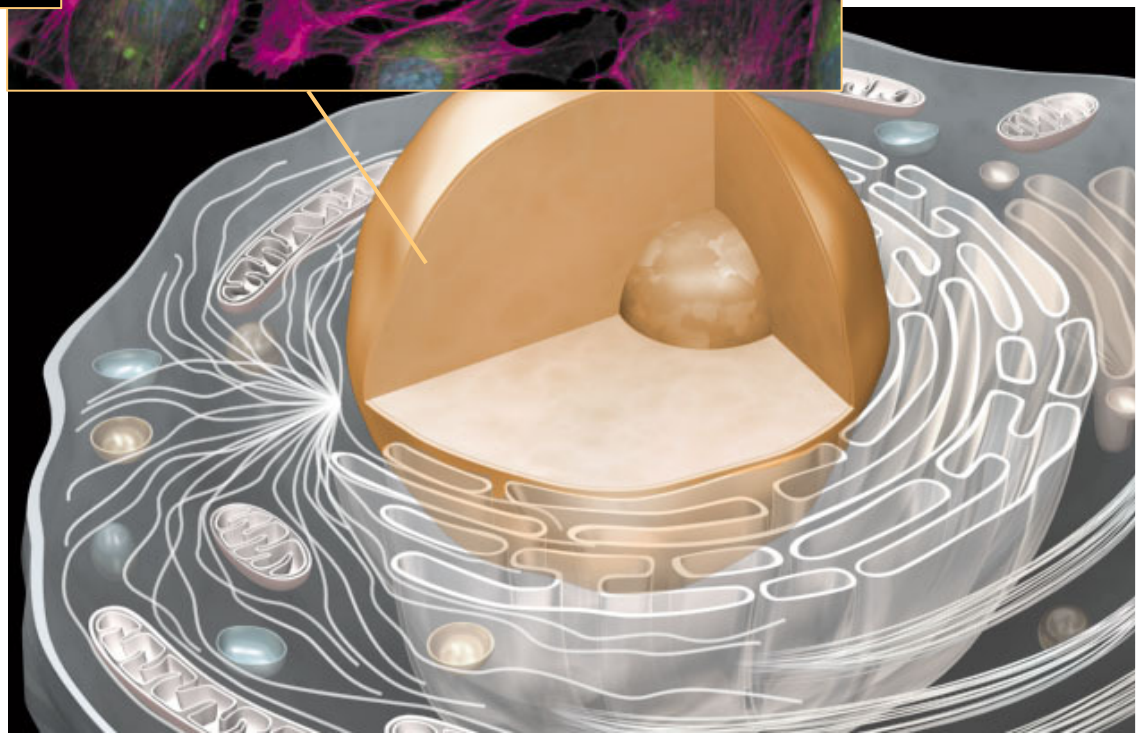
1. What is fluorescence

2. What kind of structures are fluorescent

3. How to make and target fluorescent probes

4. Fluorescent probes for cellular structure





Nucleus

DAPI

(well away from other channels)

Hoechst 33342

Live cells:

usually histone H2B-GFP

(or other monomeric FP)

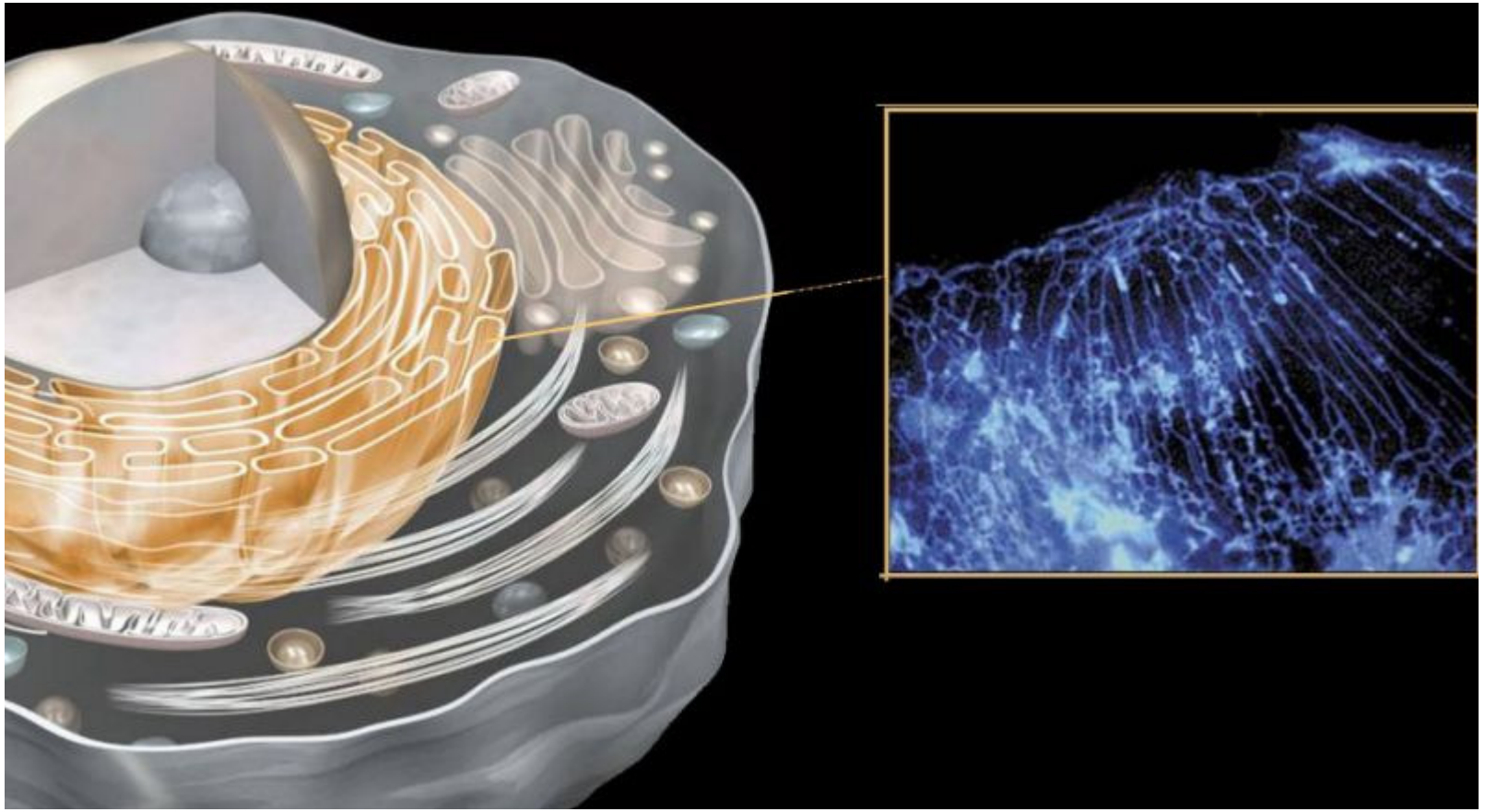
Endoplasmic Reticulum

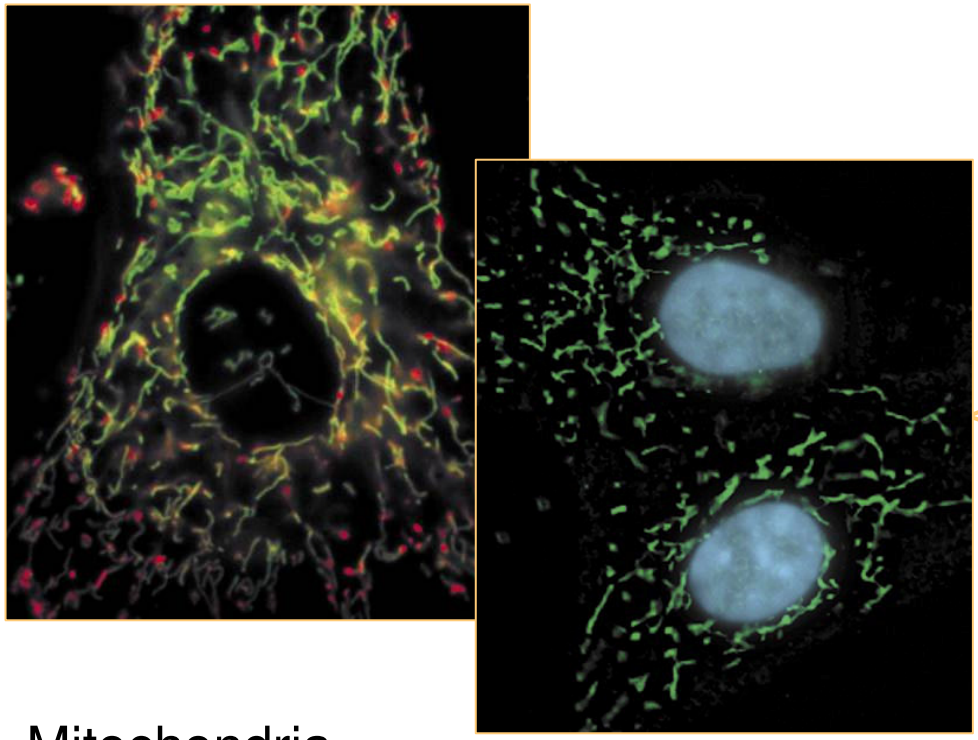
ER-Tracker™ Blue-White DPX

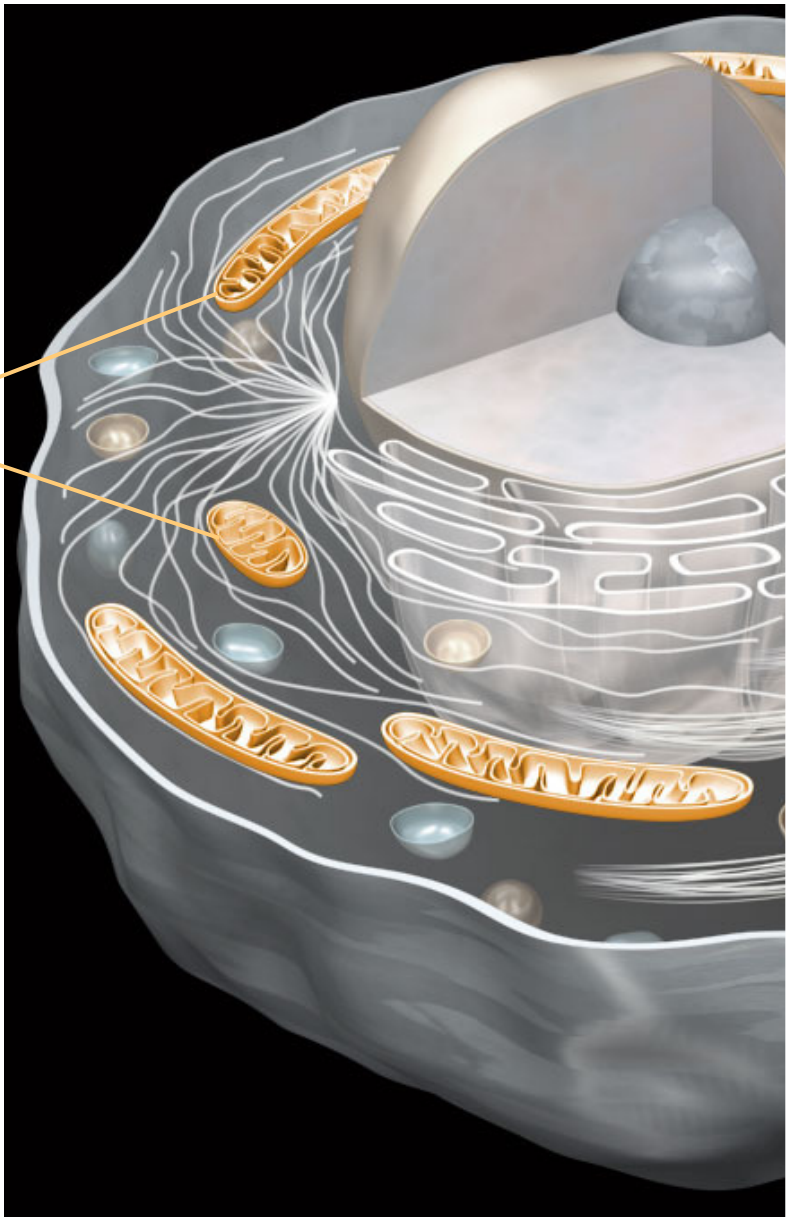
antibody to calnexin

Brefeldin A-BODIPY® 558 conjugate

Live cells: ss-GFP-KDEL







Mitochondria

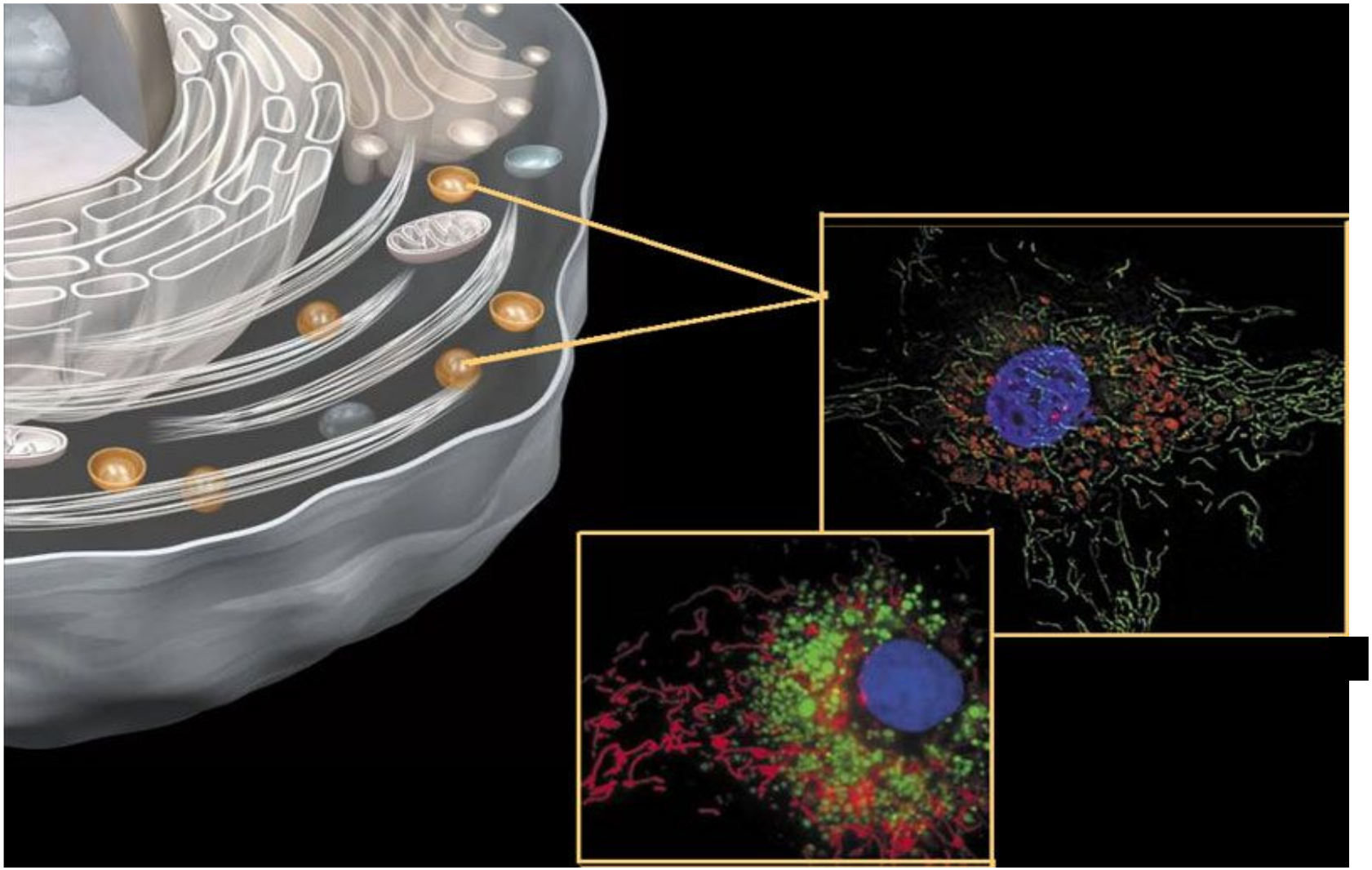
Fixed cells: anti–cytochrome oxidase subunit I Ab

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

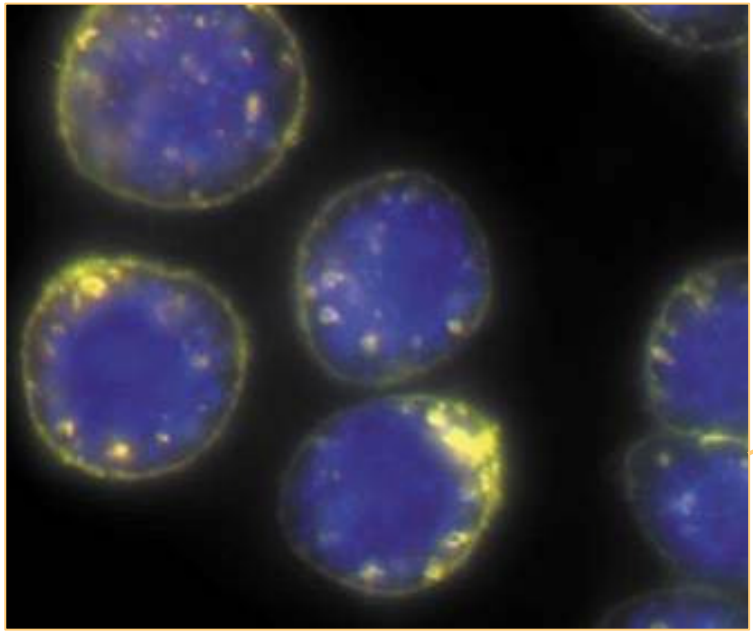


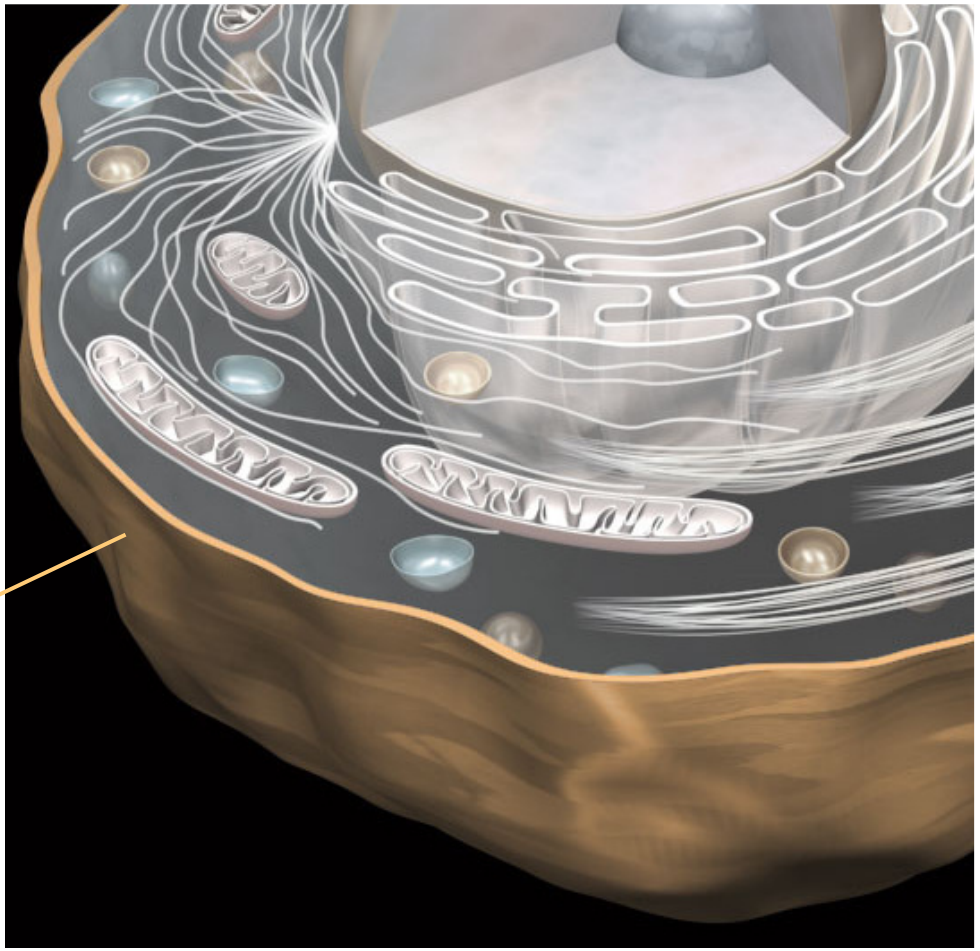
Lysosomes

Fixed cells: anti-LAMP1

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

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

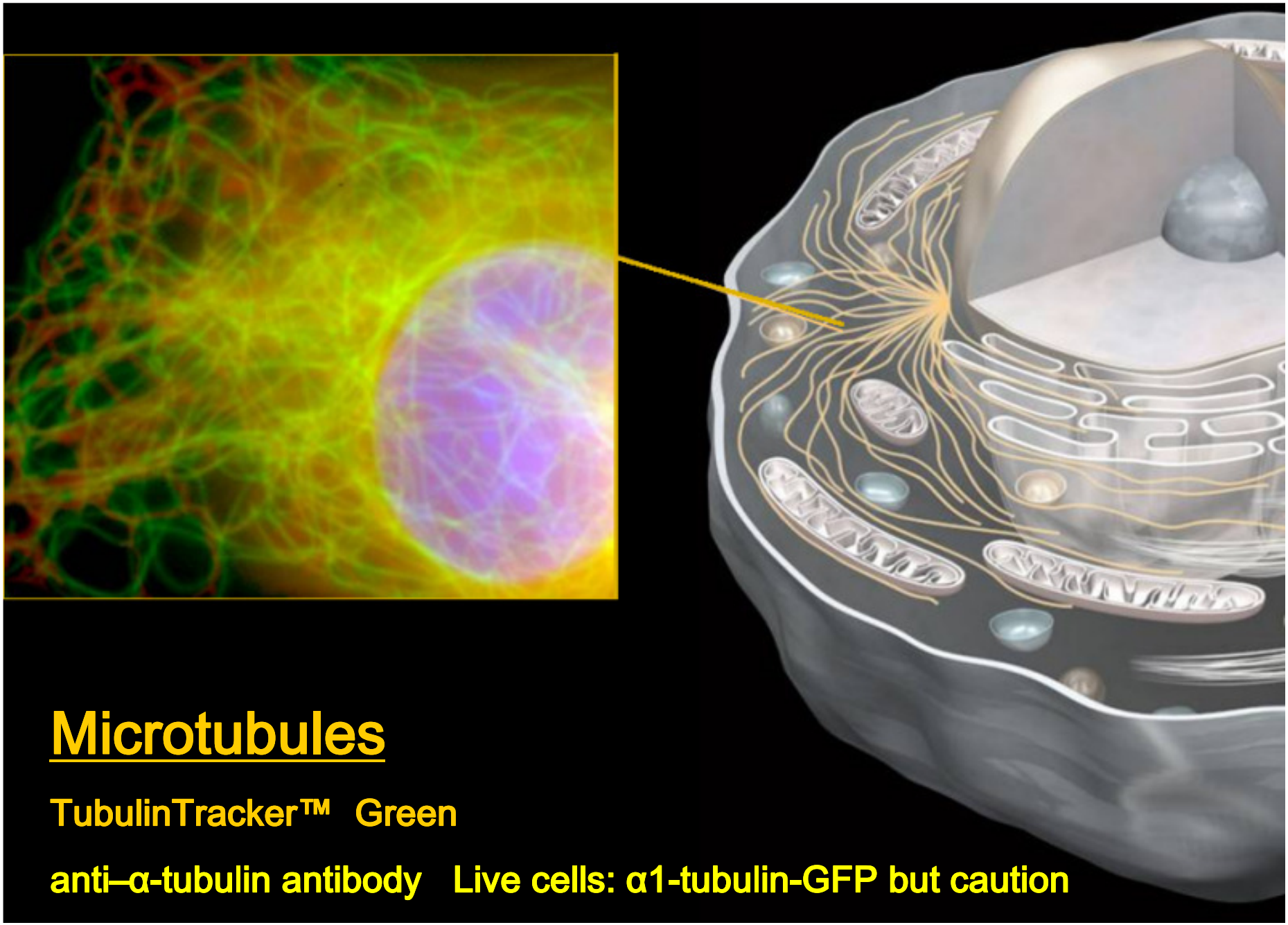




Lipid Rafts

BODIPY® FL C_5-ganglioside GM1

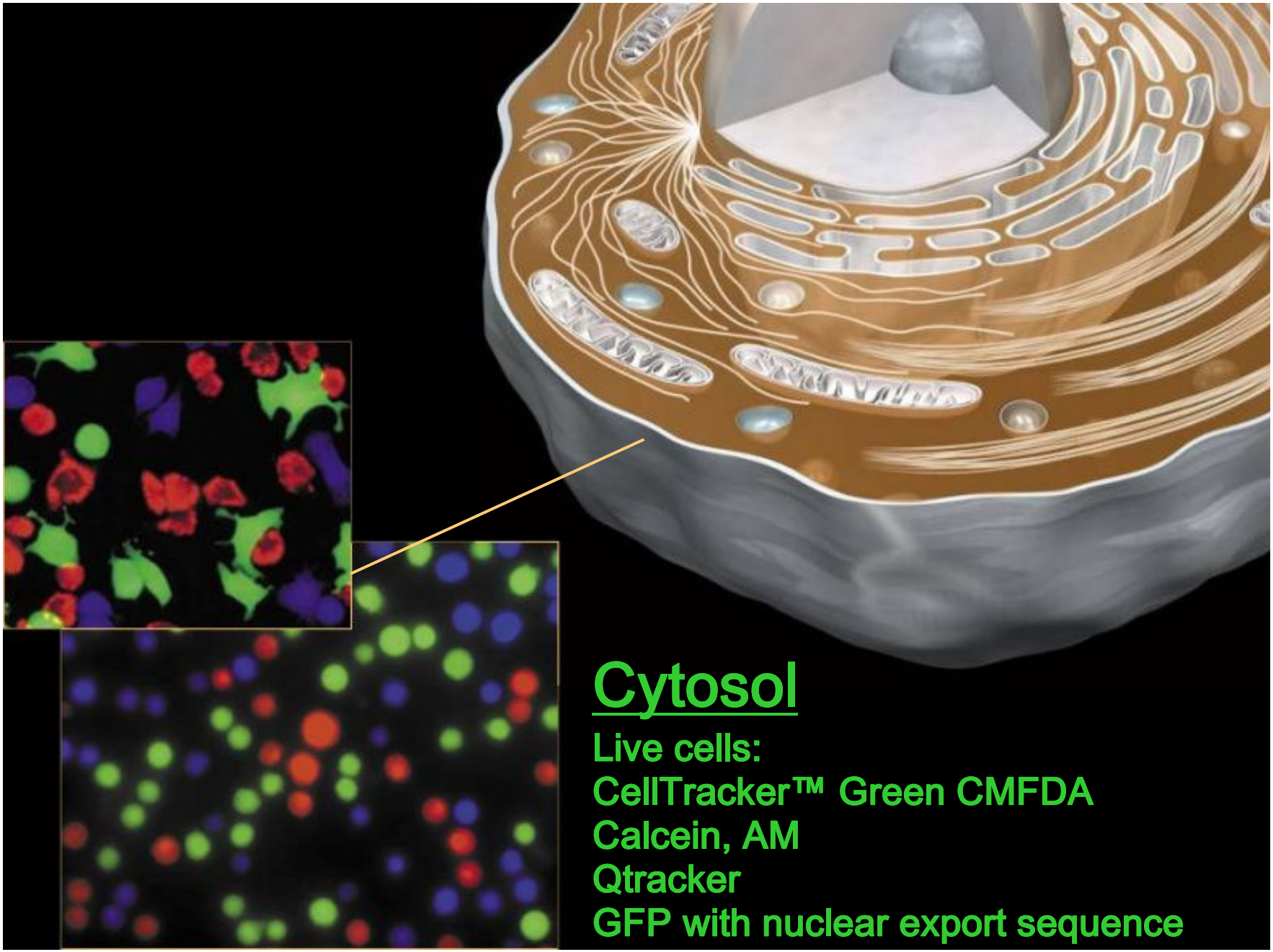
Fluorescent Cholera Toxin subunit B (CT-B)



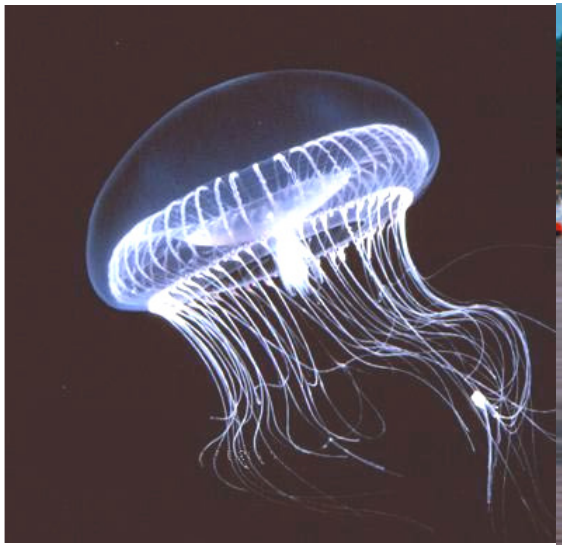
Microtubules

TubulinTracker™ Green

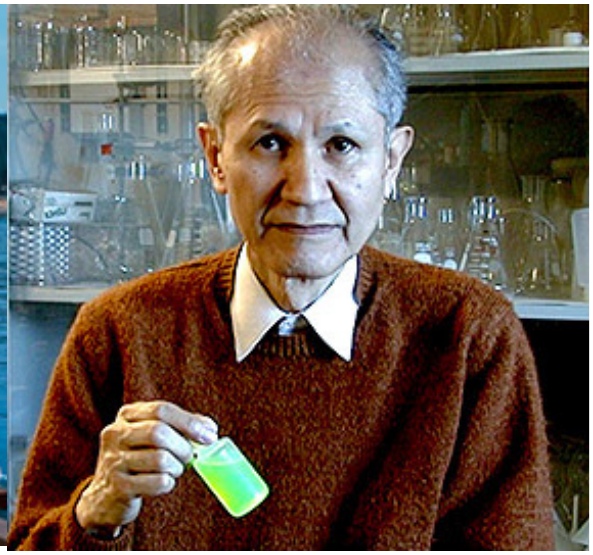
anti–α-tubulin antibody Live cells: α1-tubulin-GFP but caution


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

The breakthrough of fluorescent proteins from jellyfish

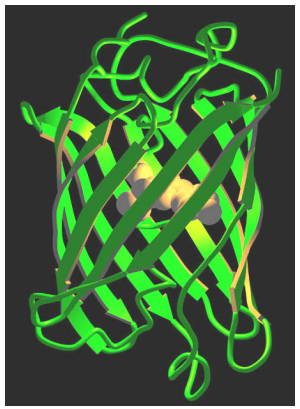


Aequorea victoria

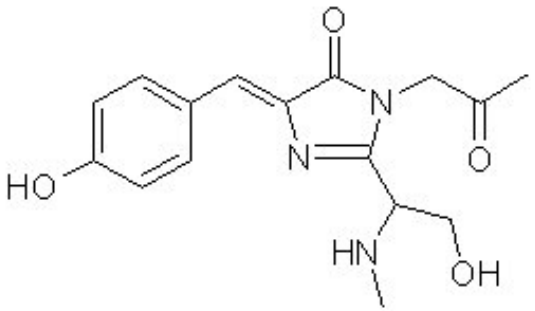


Osamu Shimomura

The breakthrough of fluorescent proteins for live cell imaging

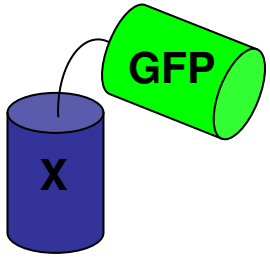


GFP fold
β-can

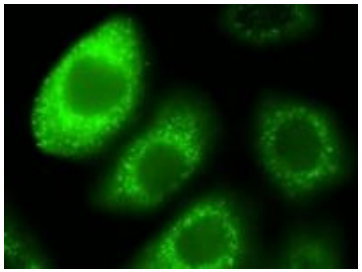


GFP chromophore
from Ser-Tyr-Gly

Link GFP sequence to gene of your favourite protein

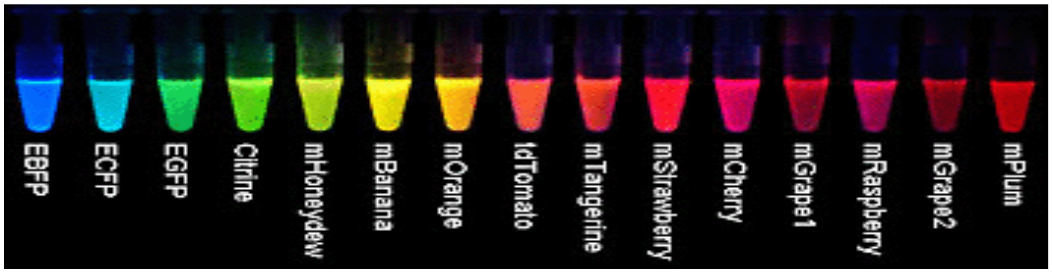


GFP folds
and becomes
fluorescent

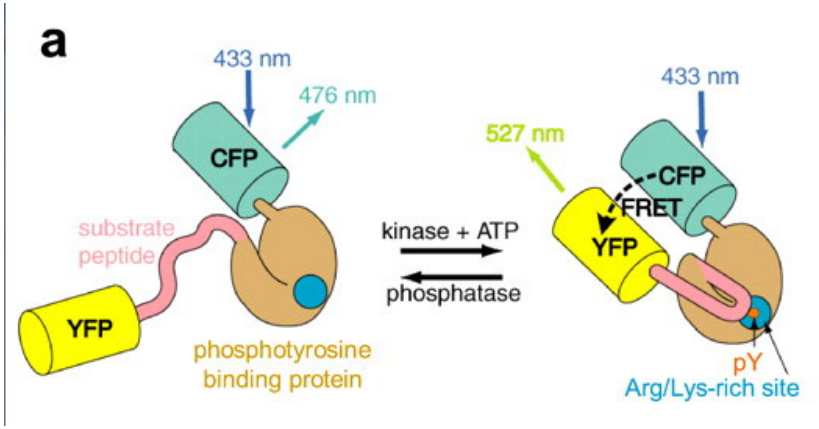


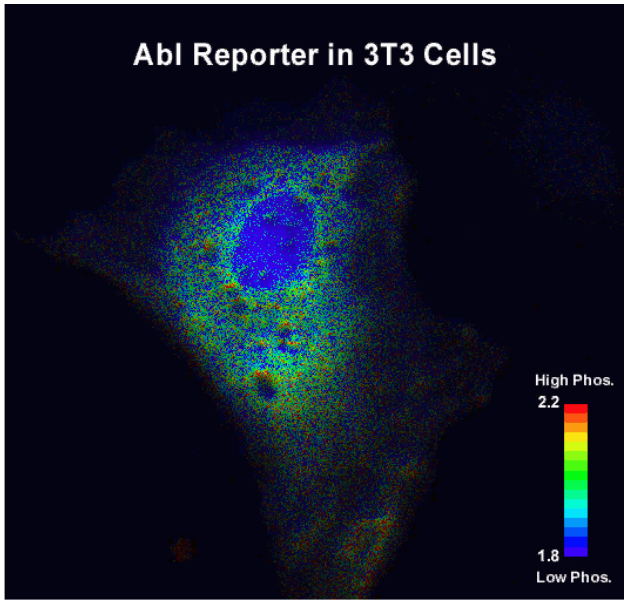
GFP lights up your
favourite protein in cell

Fluorescent proteins are more than just labels

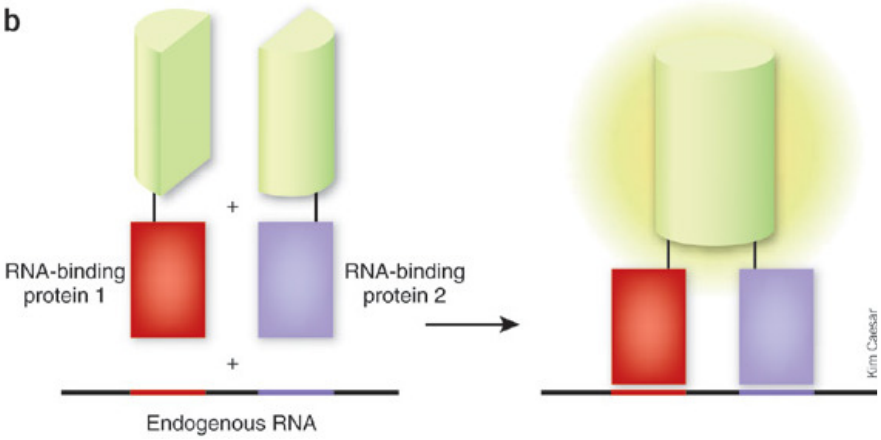


Photoactivation/Photoswitching
PA-GFP, Dronpa, Eos
Reporting on environment
Ca^{2+}, phosphorylation, cAMP, cGMP, pH, neurotransmitters, voltage, cell cycle, redox
Reporting on protein-protein interaction
CFP/YFP FRET, split fluorescent proteins
Modifying environment
Singlet oxygen generation, Channelrhodopsin

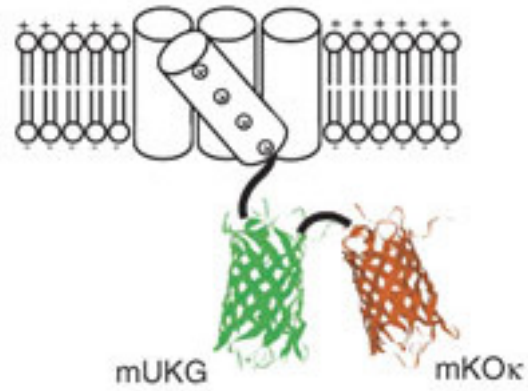




<u>Targeting advantage</u>
to defined compartment, cell-type, developmental stage

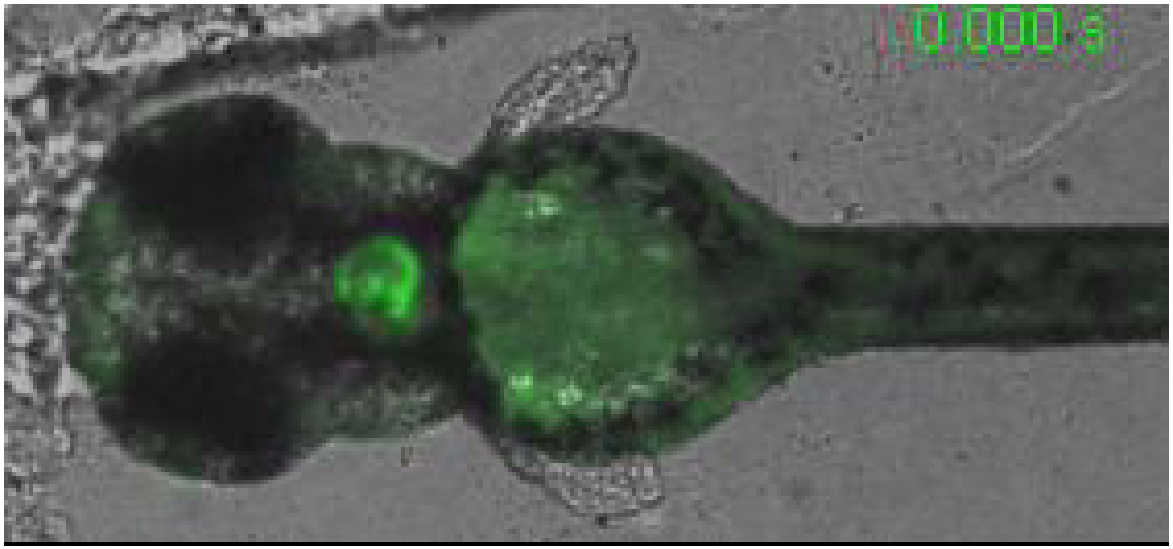


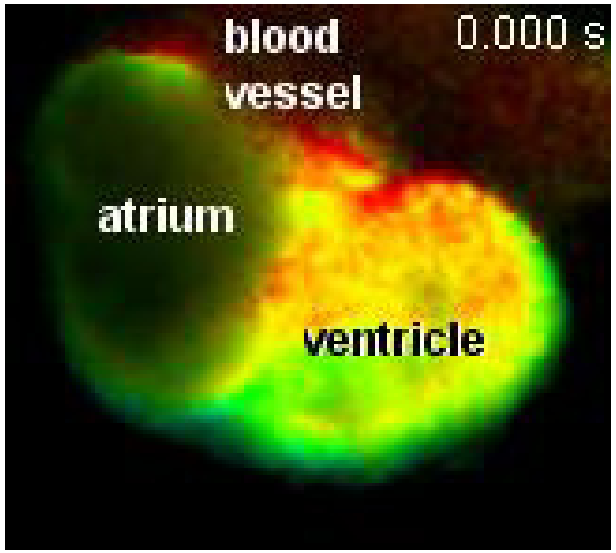
Sensing voltage with fluorescent protein



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

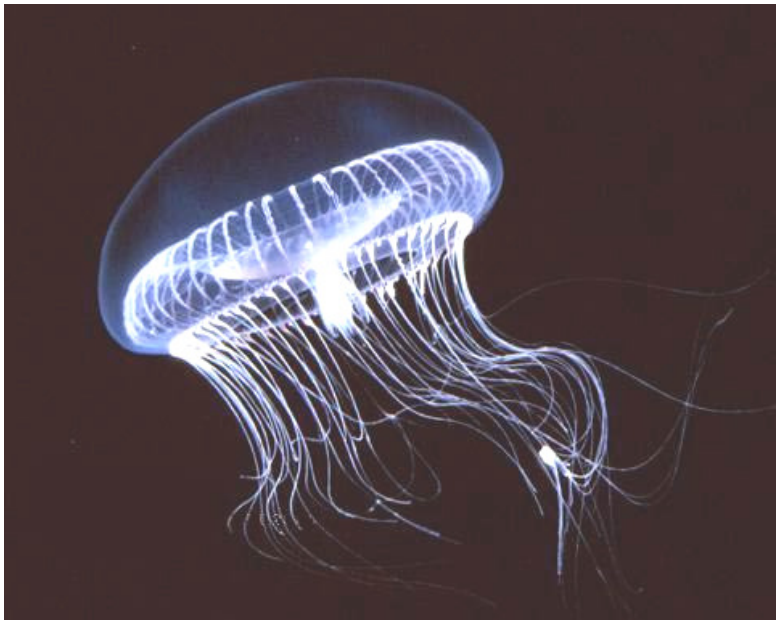




N.B. FRET sensor ratio crucial

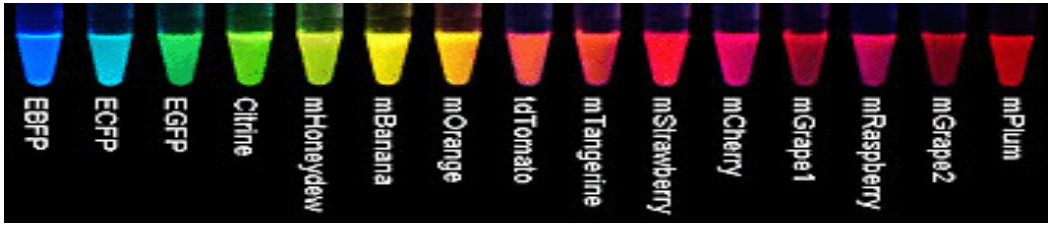
best is YC2.60 cameleon: 600%,
if <20% then lost in cellular noise

How good is a fluorescent protein?



A. victoria GFP is good for jellyfish,
but not great for cell biologists!

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. Fast Maturating — Venus 2 min. Red FPs can start off green!
   half-time 40 min mCherry, 100 min TagRFP

Problems with GFP in cells

- **GFP with light can donate electrons to different acceptors**
  (FMN, FAD, NAD^+, cyt. c)
  GFP reddens after transfer: photobleaching and phototoxicity
  use DMEM lacking e^- acceptors

(riboflavin or all vitamins) for less bleaching
(HEK 293T happy for 1 week)
effect for EGFP and PA-GFP, not RFPs
Lukyanov Nat Meth 2009

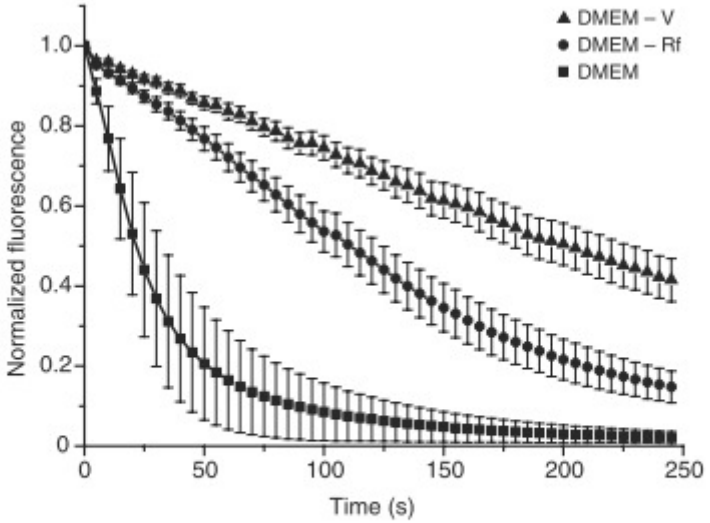
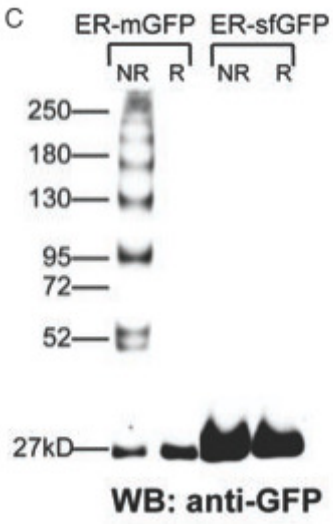


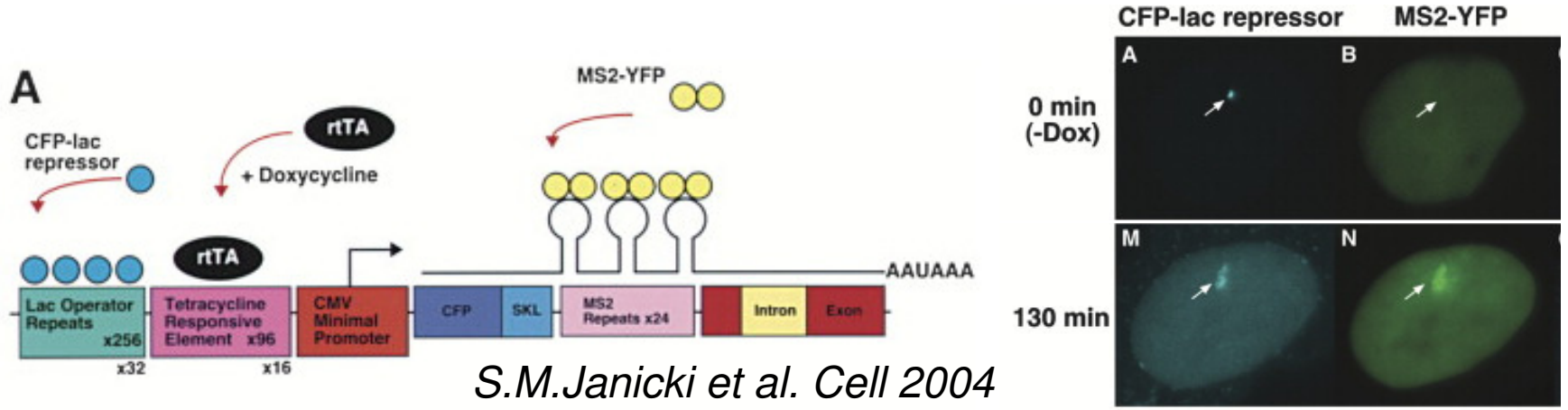
Figure 1 | Influence of cell medium on fluorescent protein photostability. Normalized bleaching curves for EGFP in live HEK293T cells maintained in DMEM, DMEM – Rf or DMEM – V. Error bars, s.d. (n = 20 cells).

- **EGFP not good in secretory pathway**
  mixed disulfide oligomers in ER and non-fluorescent in *E. coli* periplasm
  (superfolder GFP behaves fine)
  Erik Snapp, Traffic 2011



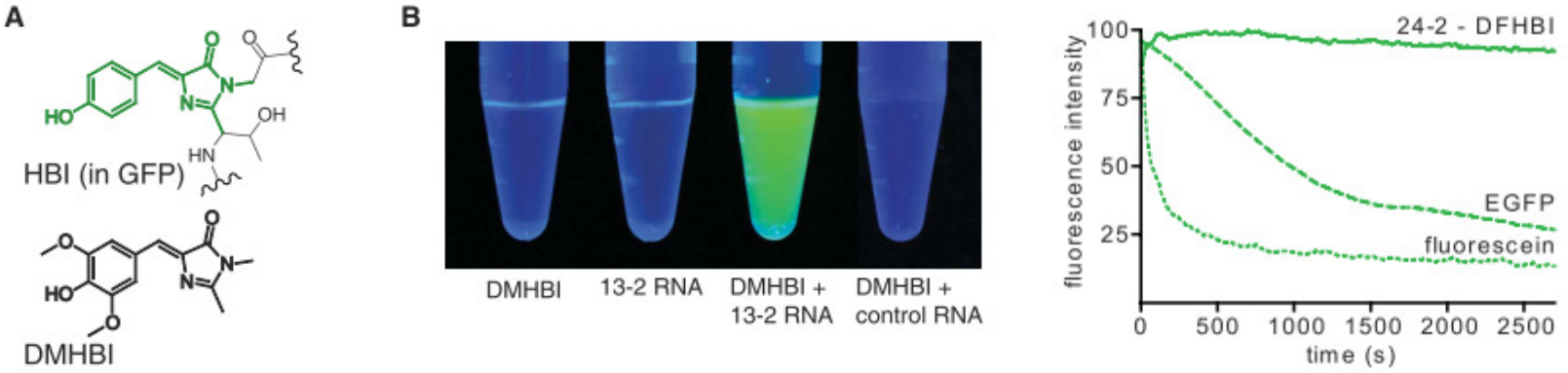
Fluorescent RNA imaging

See single mRNA: mRNA stem-loops bound by MS2-YFP
See product of translation: mRNA encodes CFP-SKL which goes to peroxisomes



S.M.Janicki et al. Cell 2004

Spinach RNA 60 nt aptamer binds cell-permeable fluorogenic dye
Photostable. Used to label 5S RNA in HEK cells. *Samie Jaffrey Science 2011*



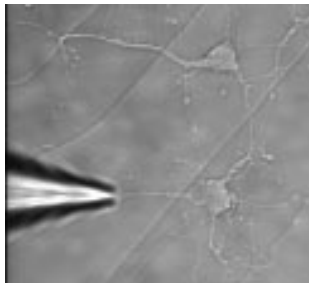
Why use light to control biology?

Light control allows extreme temporal and spatial control.

Temporal control

genes< chemicals < light

min-hr s-min µs-s



Spatial control

chemicals / genes < light

one or many cells 1 µm part of cell

(note micropipettes for precise small molecule delivery)

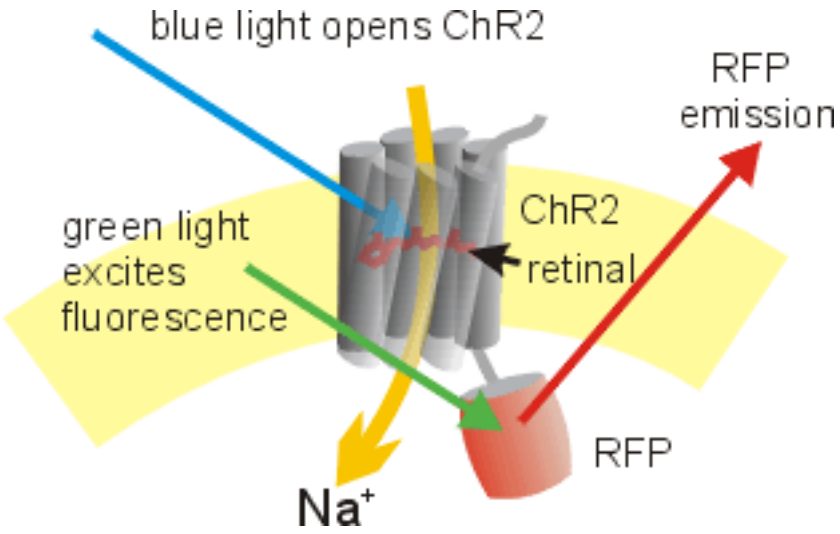
(often combine chemical/light control or gene/light control)
optogenetics/chemogenetics

Limitations of light? $$$$$

and usually data on one cell at a time

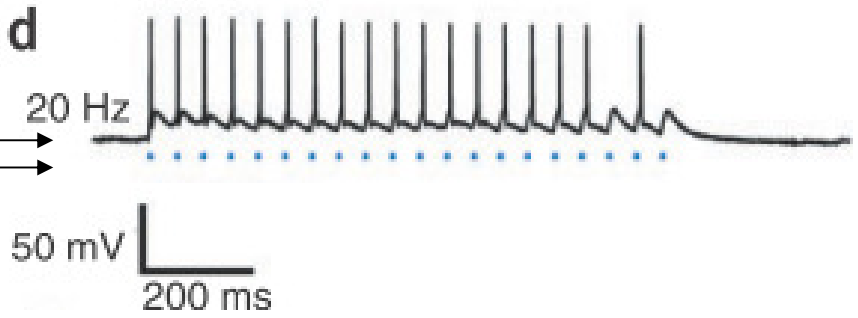
Controlling biology with light: light-gated ion channels

Channelrhodopsin from an alga, like rhodopsin, undergoes retinal isomerisation in response to light, and changes conformation, but opens a Na^+ channel. This allows light to control membrane voltage and trigger neuron firing.



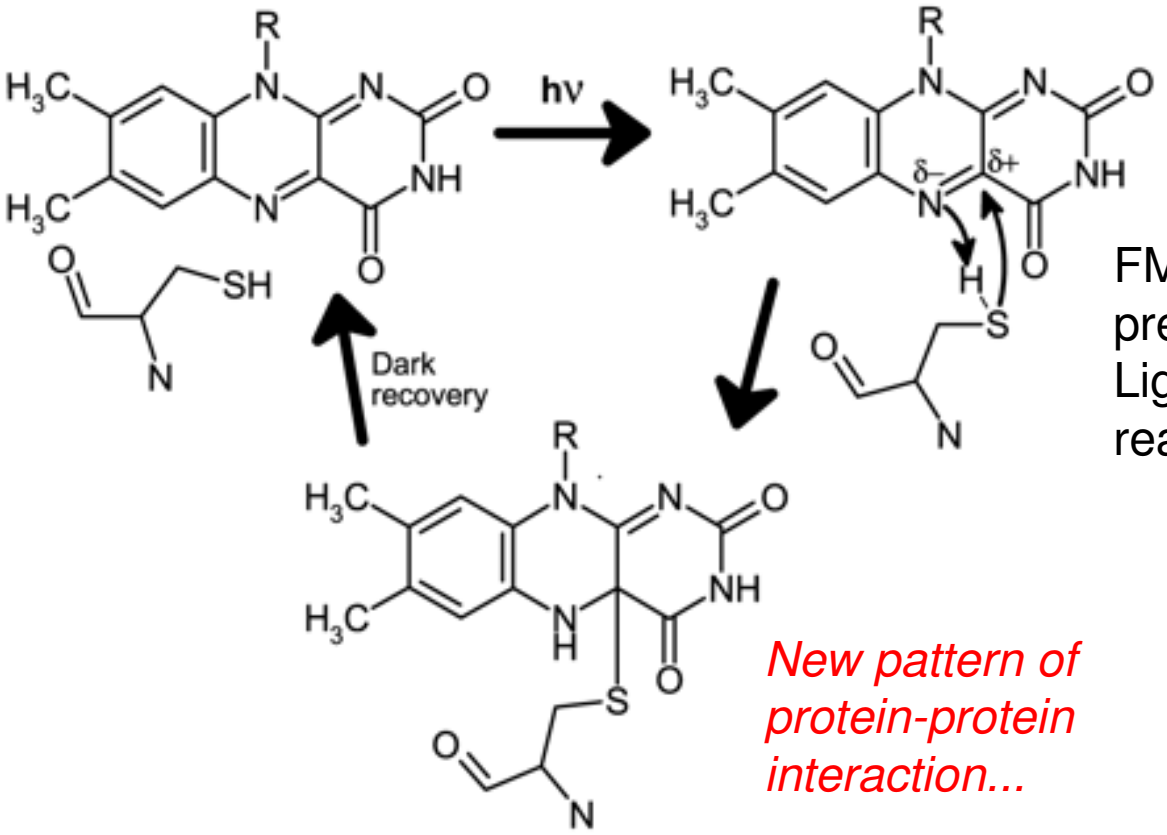
to understand neuronal firing patterns
to control secretion in diabetes
potentially in fixing neural diseases?
e.g. damping down overactivity in epilepsy

trains of electrical firing
flashes of light



LOV domains react and switch conformation with light

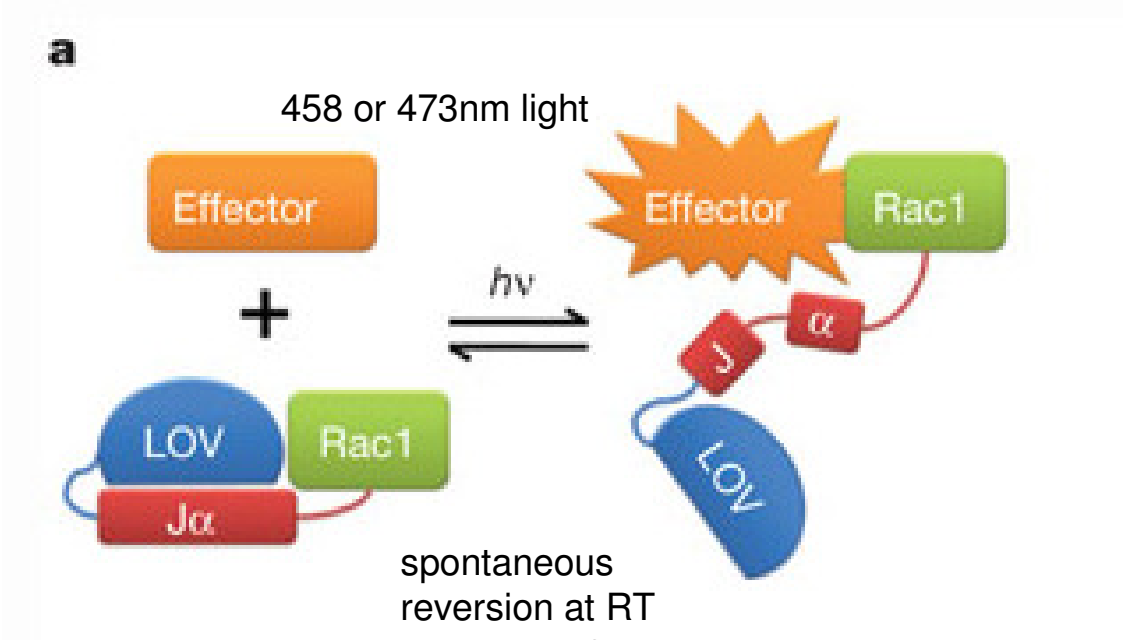
LOV domains:
light, oxygen, voltage responders
ones responding to blue light in bacteria, plants and fungi



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

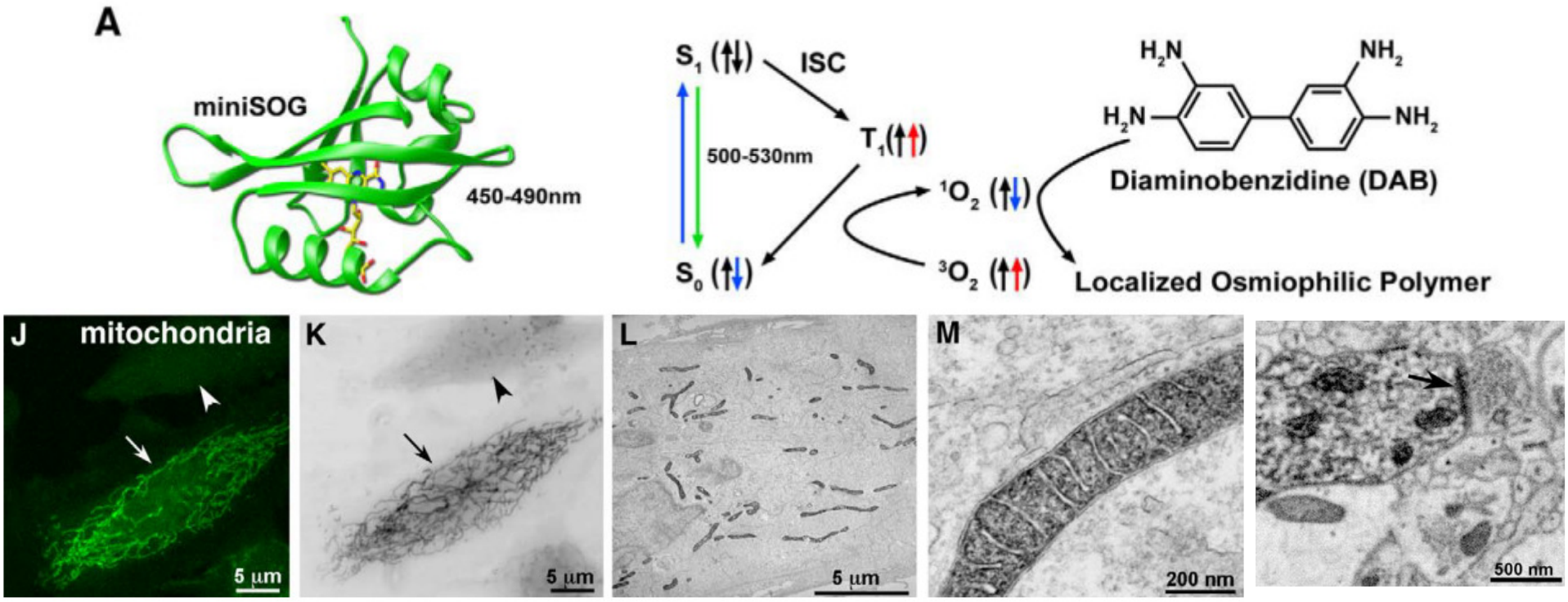
K_d for PAK 2 μM in dark, 200nM in light 10-fold ratio

Interaction of Rac with PAK stimulates cell protrusion and migration.

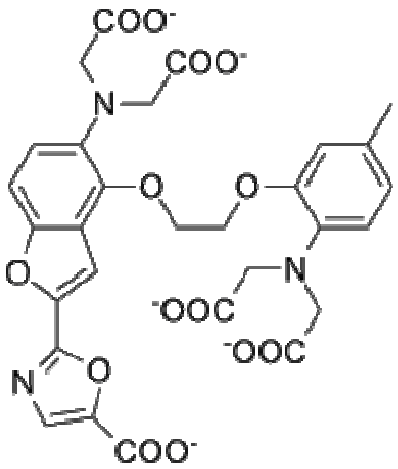
K.Hahn et al. Nature Sept. 2009

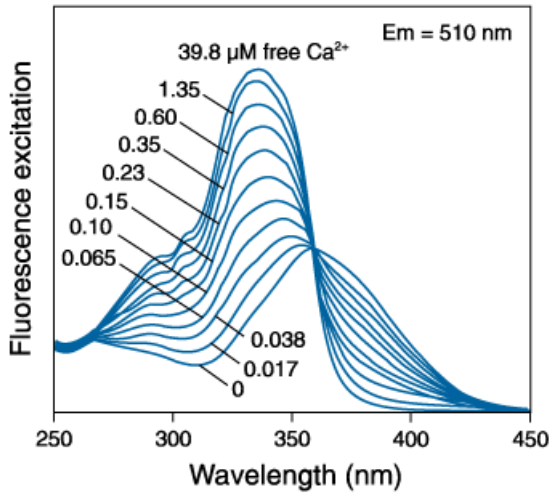
Correlated Light Microscopy/ Electron Microscopy

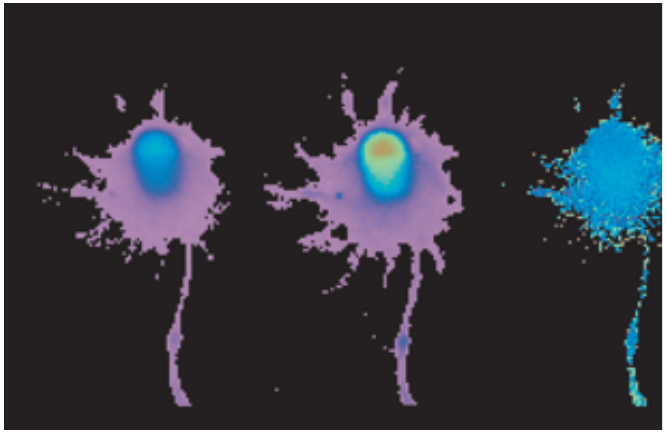
MiniSOG (Shu, Tsien PLoS Biol 2011)
Light causes generation of singlet oxygen-> DAB polymerized-> binds OsO_4
106 aa monomer, engineered from Arabidopsis LOV domain
tested in cell-lines, worms and transgenic mice
Overcomes trade-off between thorough fixation and penetration of labeling reagent



Small molecule fluorescent sensors







Fura-2 sensing calcium

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

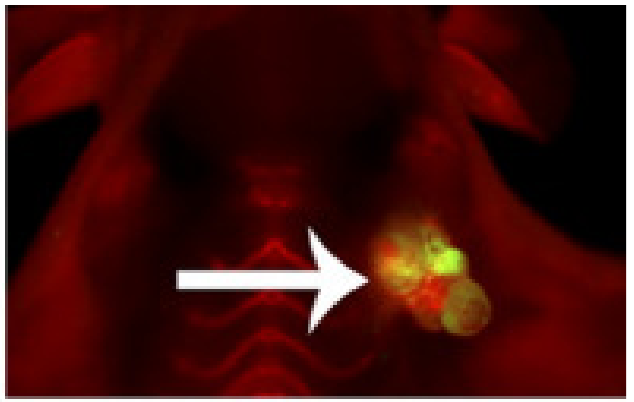
pH (also dyes to conjugate to proteins, CyPher from GE, SNARF from Invitrogen)

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

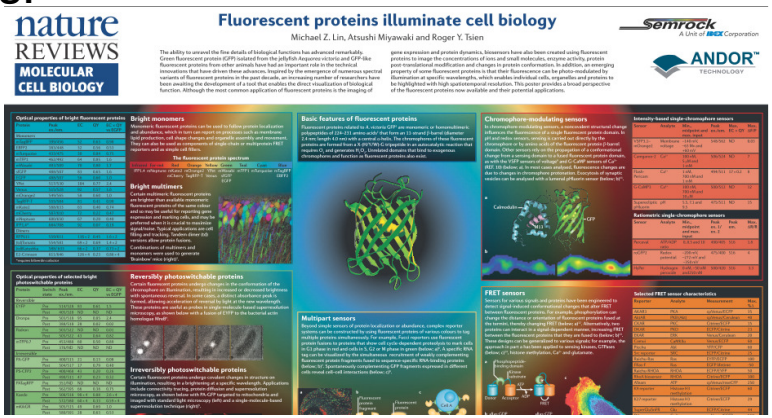
Molecular Probes Handbook, free from Invitrogen.
Principles of Fluorescence Spectroscopy 2nd edition, by Joseph R. Lakowicz.

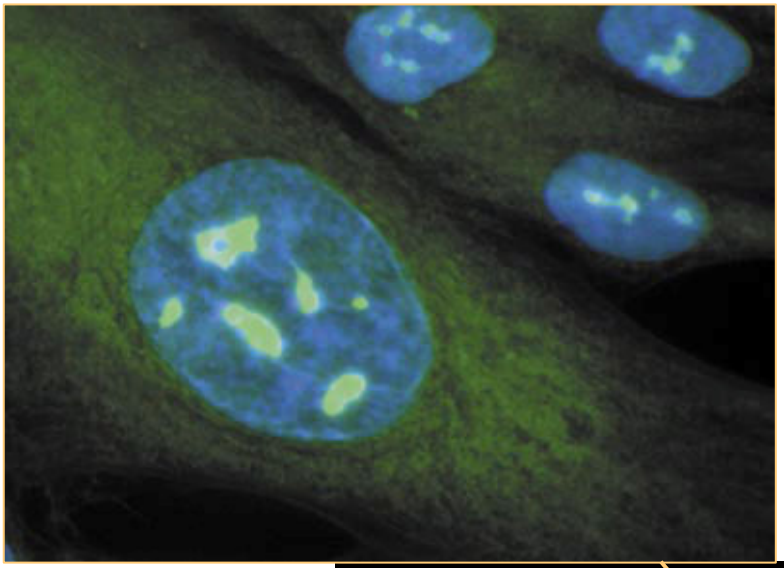
Protein modification

Bioconjugate Techniques, 2nd Edition
by Greg T. Hermanson.
Chemical labeling strategies for cell biology, Marks KM, Nolan GP. Nat Methods. 2006 Aug;3(8):591-6.

Fluorescent proteins

(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.

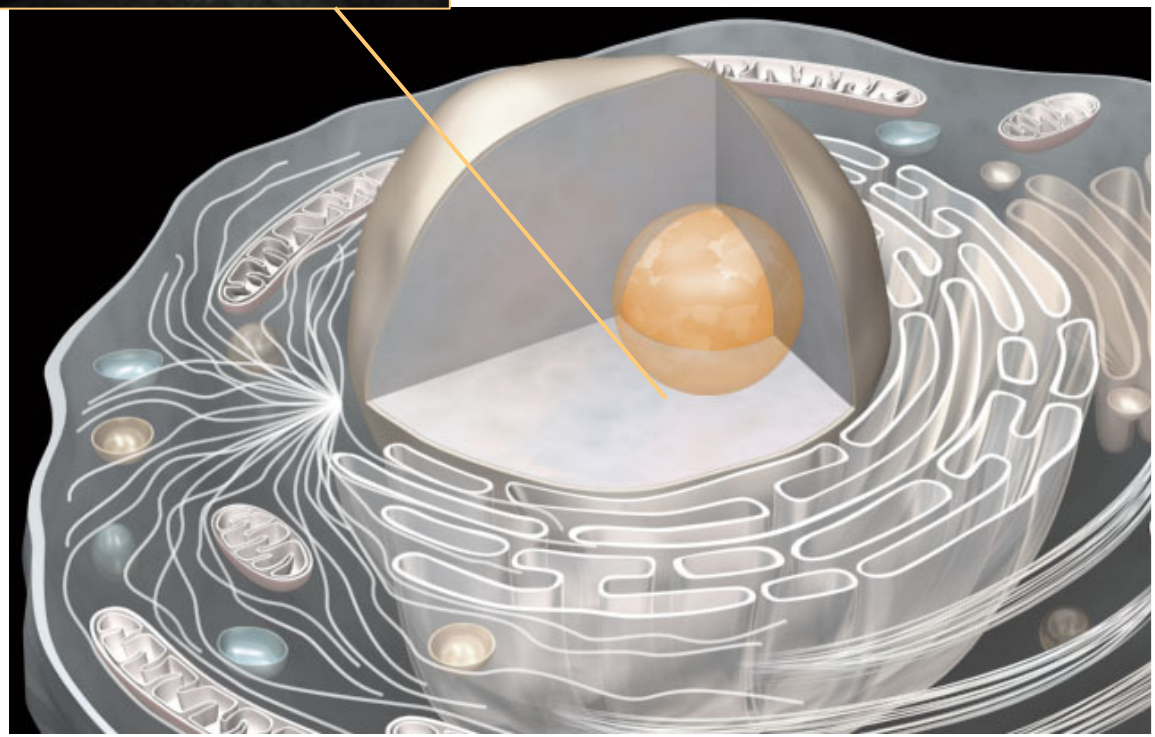




Nucleoli

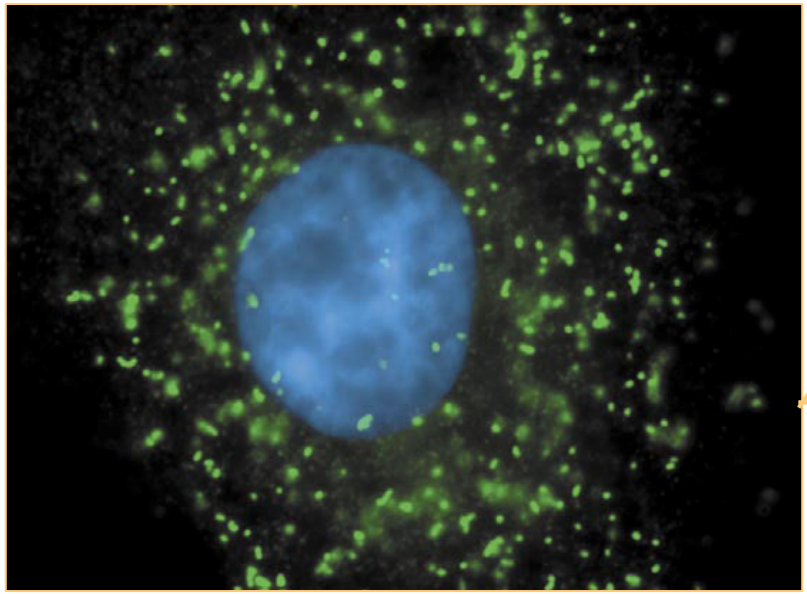
SYTO® RNASelect™

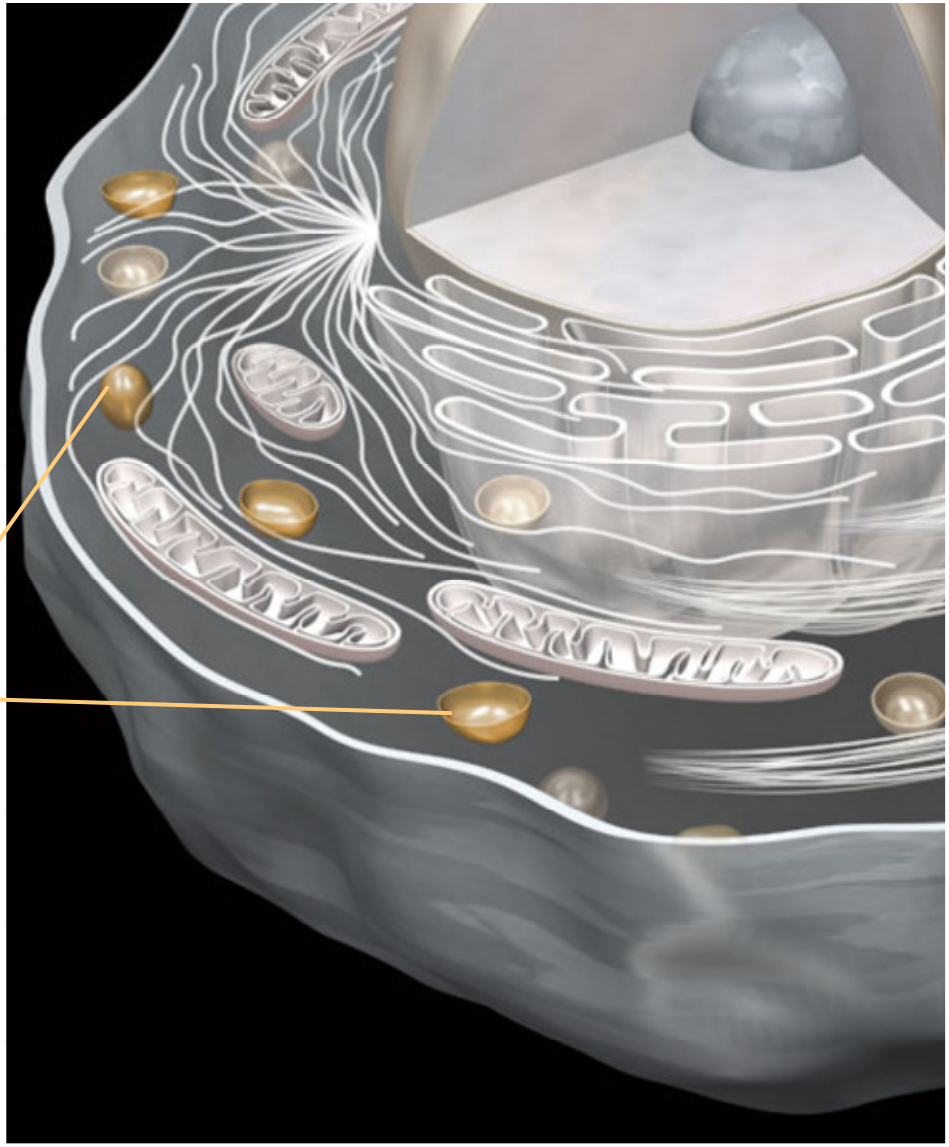
Live cells: GFP-Nopp140



Also note RNA-selective probe

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



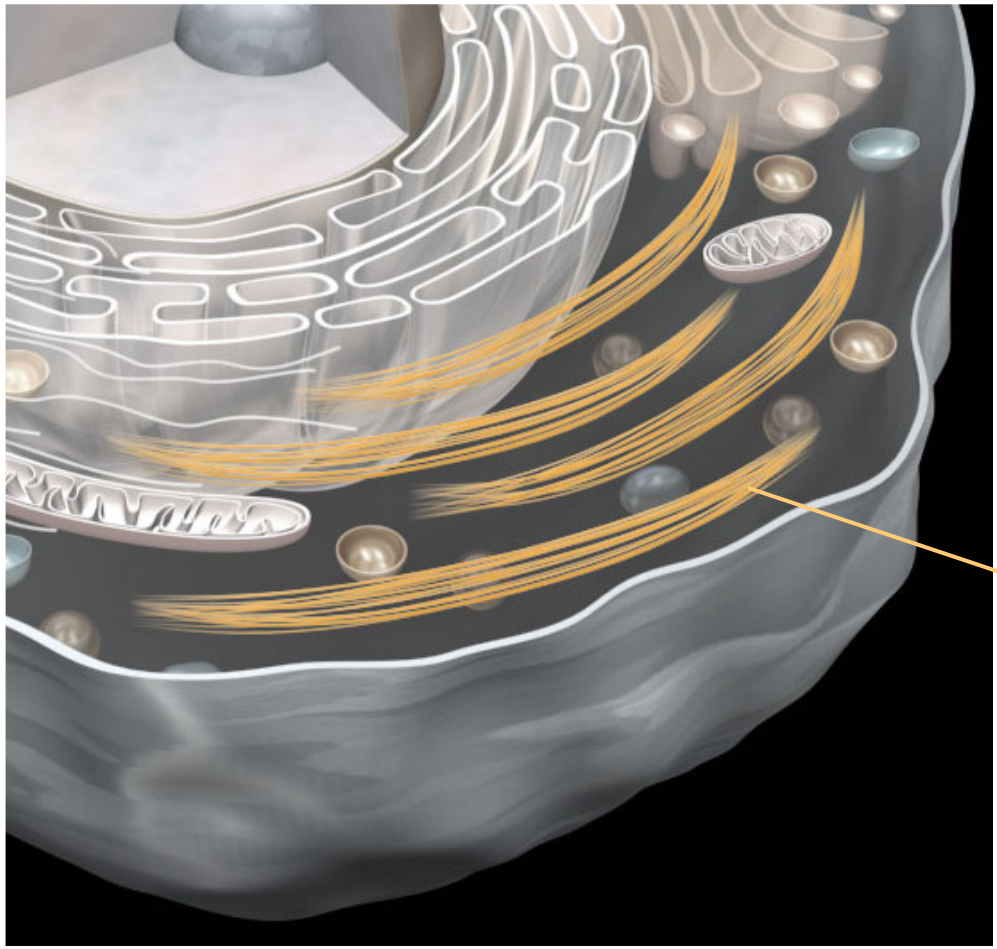


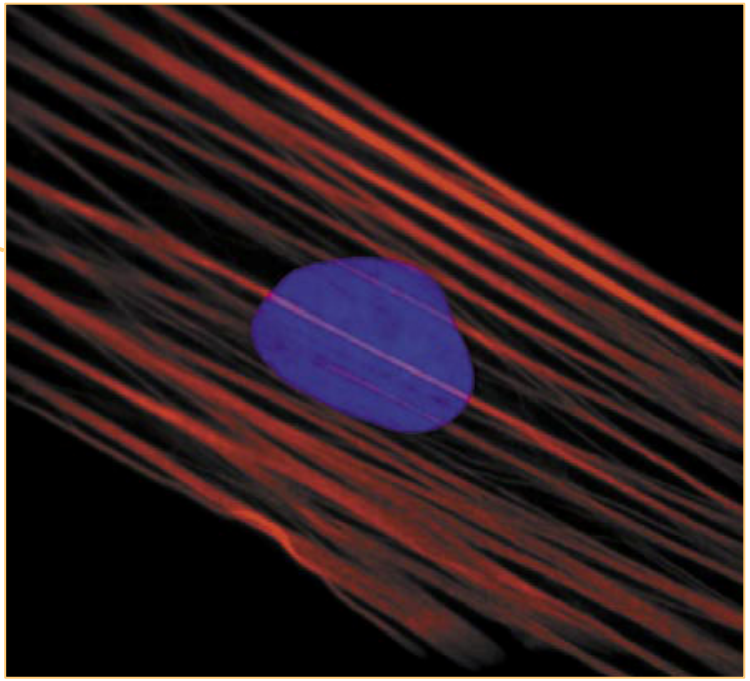
Peroxisomes

SelectFX™ Alexa Fluor® 488 Peroxisome Labeling Kit

(antibody to Peroxisomal membrane protein 70)

Live cells: GFP-SKL (tripeptide targeting sequence)

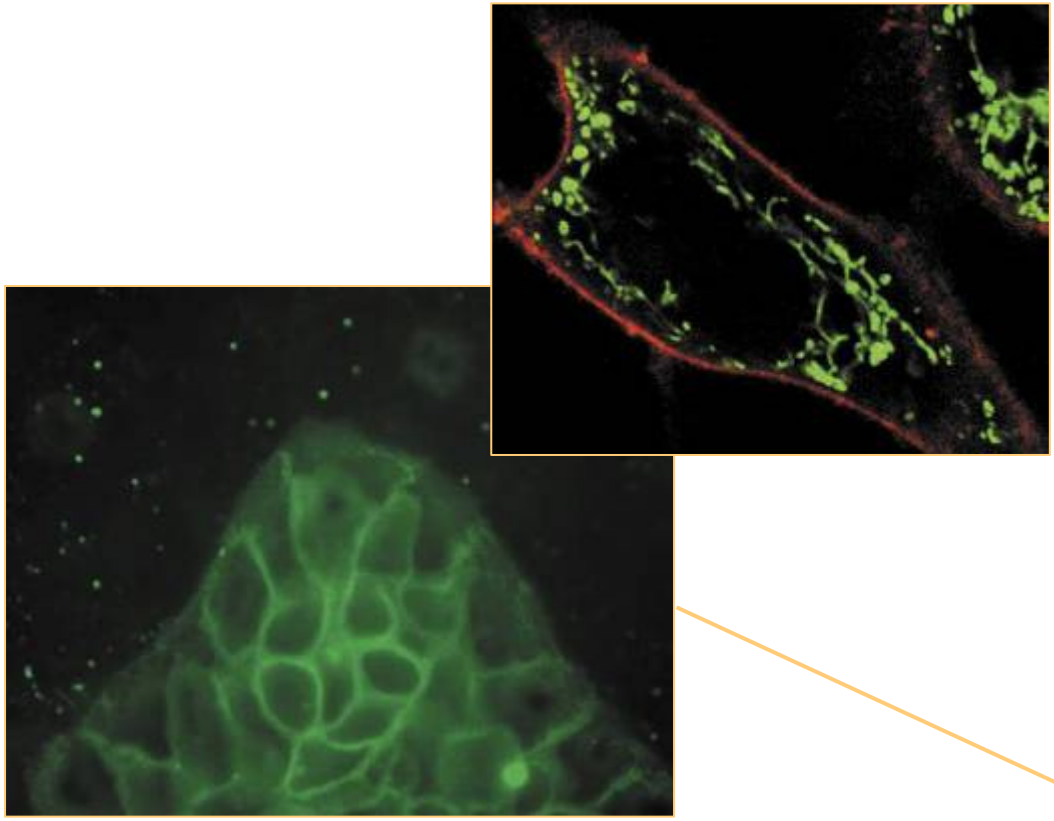


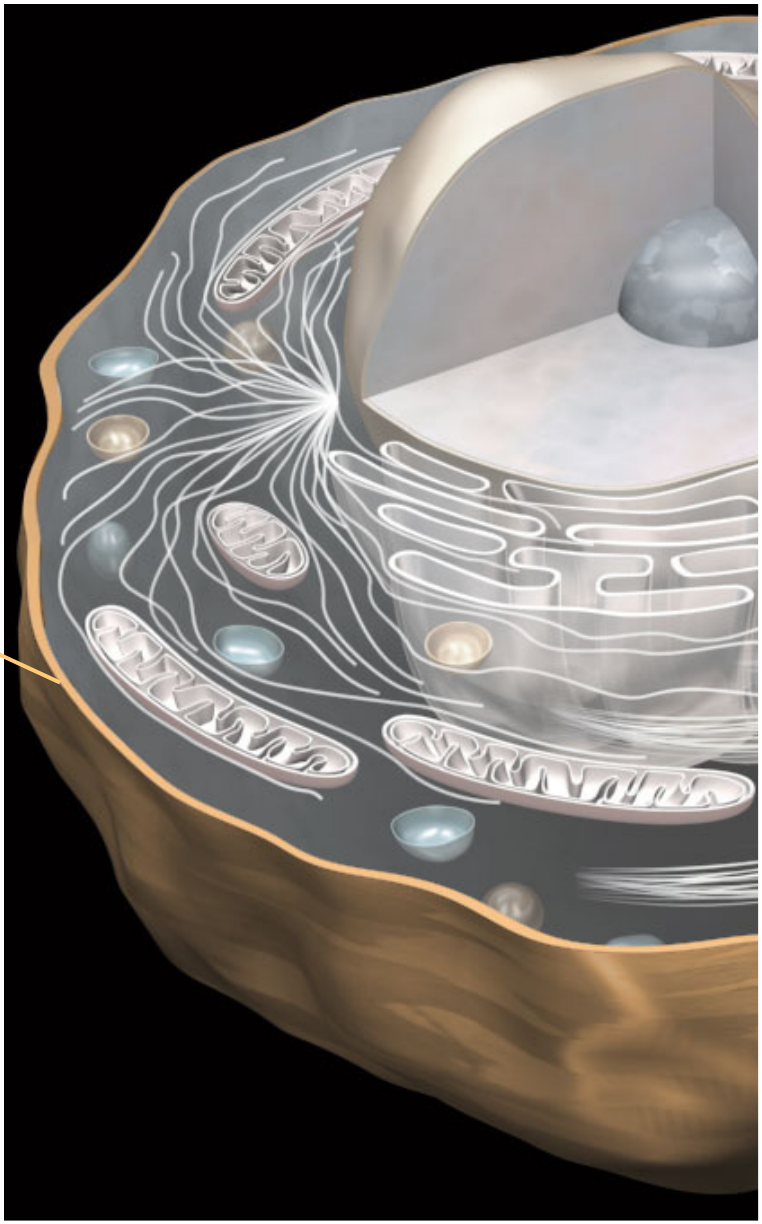


Actin cytoskeleton

Fixed cells: Alexa Fluor-phalloidin

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



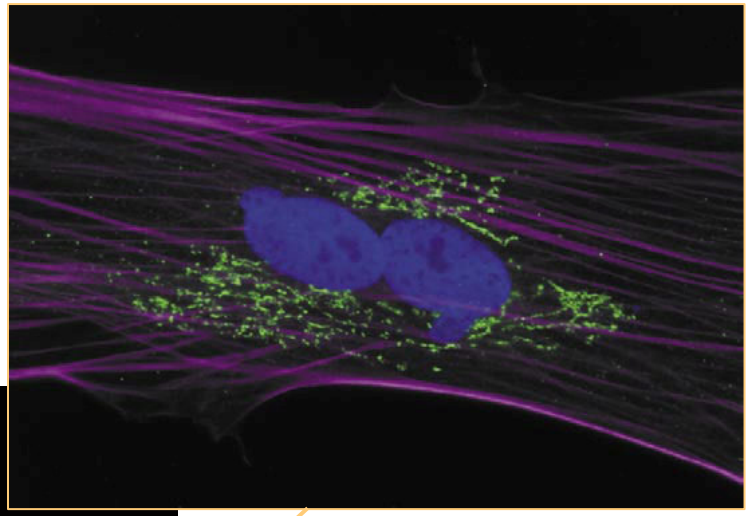


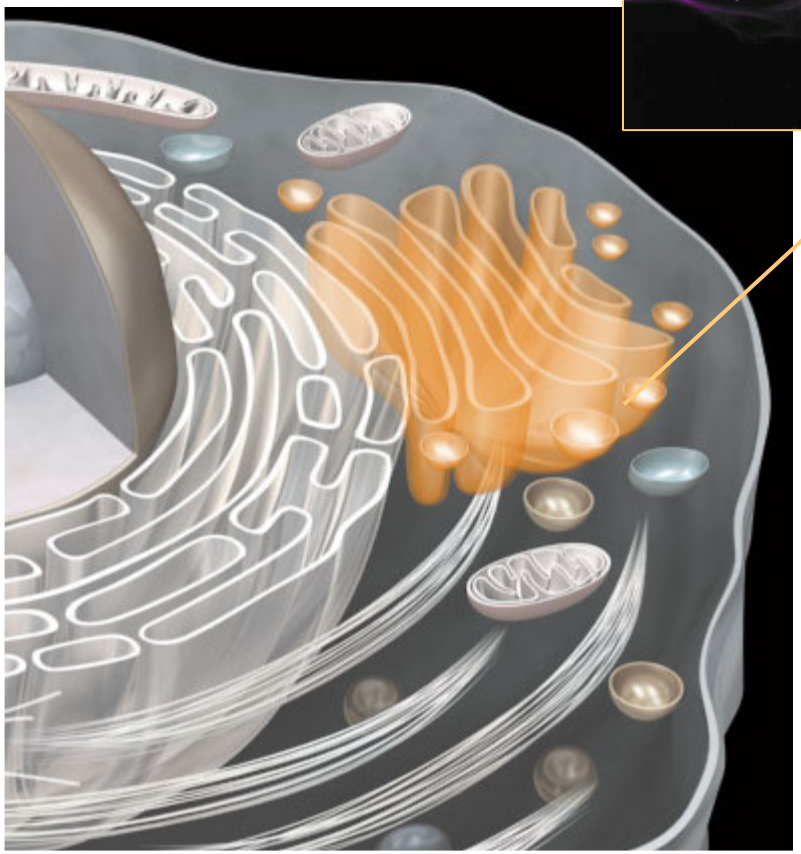
Plasma Membrane

Wheat Germ Agglutinin

Live cells: FM dyes,

myristoyl+palmitoyl tagged-GFP





Golgi

anti–golgin-97 antibody

NBD C_6-ceramide complexed to BSA

Live cells: GalTase-GFP