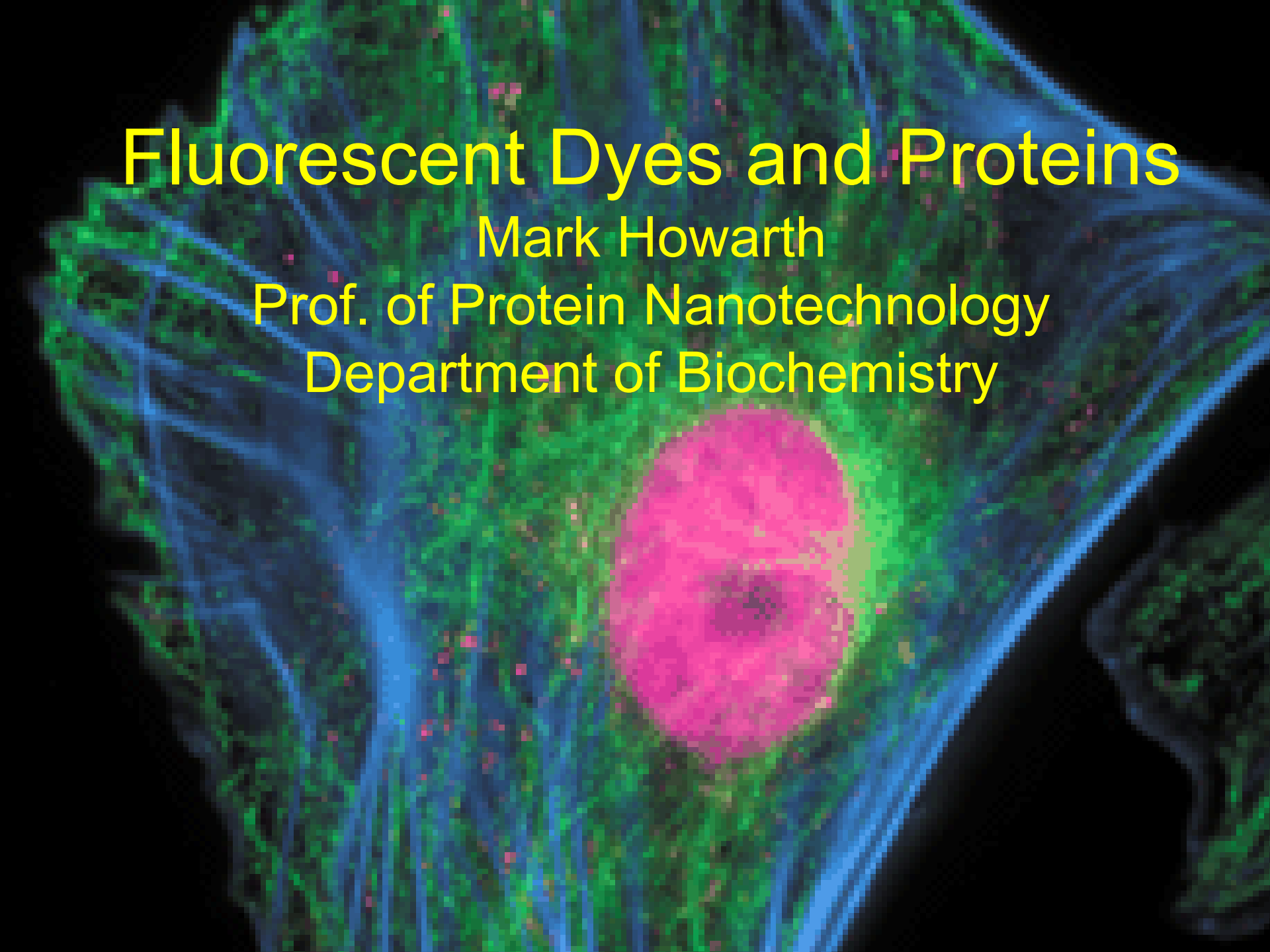
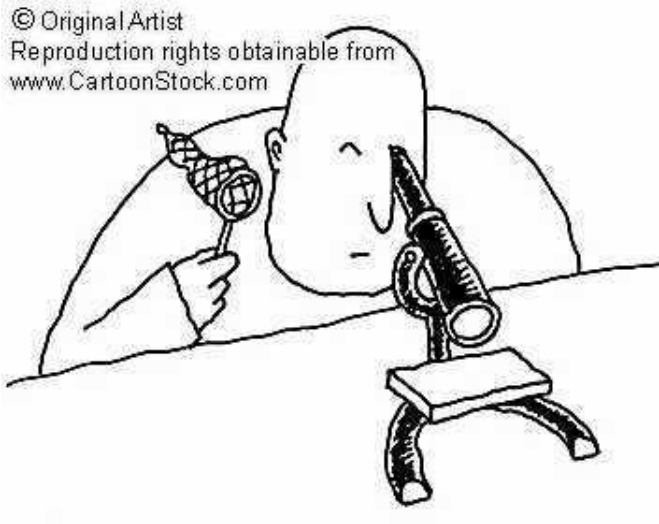


Fluorescent Dyes and Proteins

Mark Howarth
Prof. of Protein Nanotechnology
Department of Biochemistry

Overview

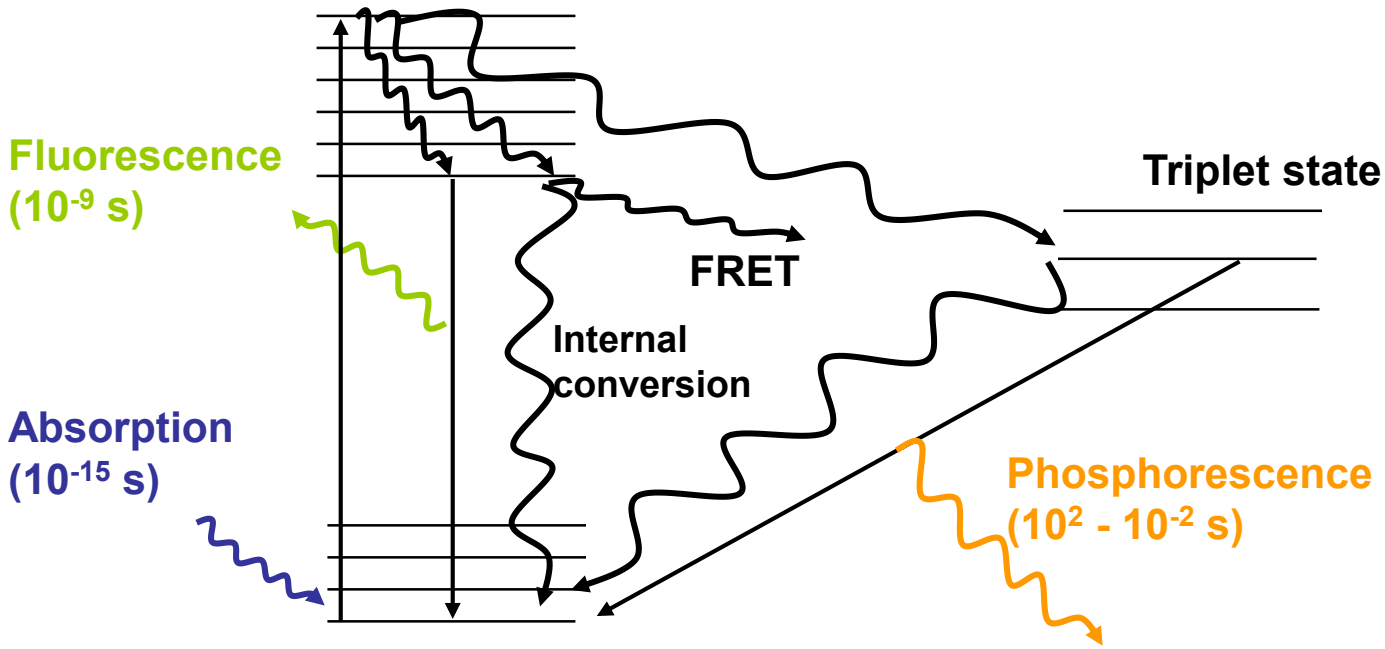


1. What kind of structures are fluorescent

2. How to make and target fluorescent probes

3. Fluorescent probes for cellular structure and function

Not all energy emitted as fluorescence



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

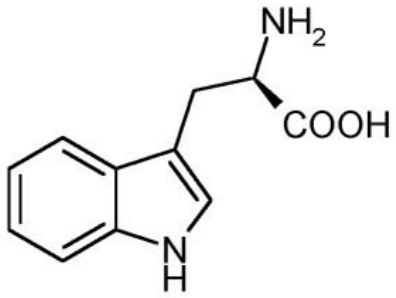
e.g. EGFP QY=0.6 For every 10 photons absorbed, 6 are emitted. (at optimal temp, pH etc.)

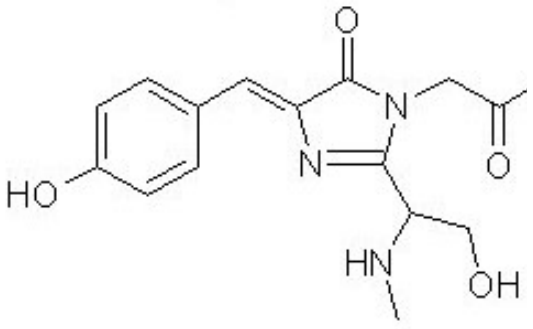
What sort of molecules are fluorescent?

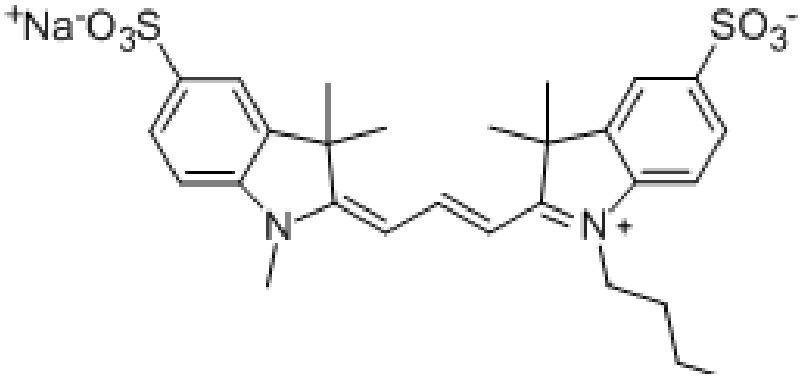
Organic fluorophores
especially
1. Intrinsic fluorophores (source of autofluorescence)
2. Dyes
3. Fluorescent proteins

Inorganic fluorophores
especially
1. Lanthanides
2. Quantum dots

What sort of molecules are fluorescent?
1. Organic fluorophores





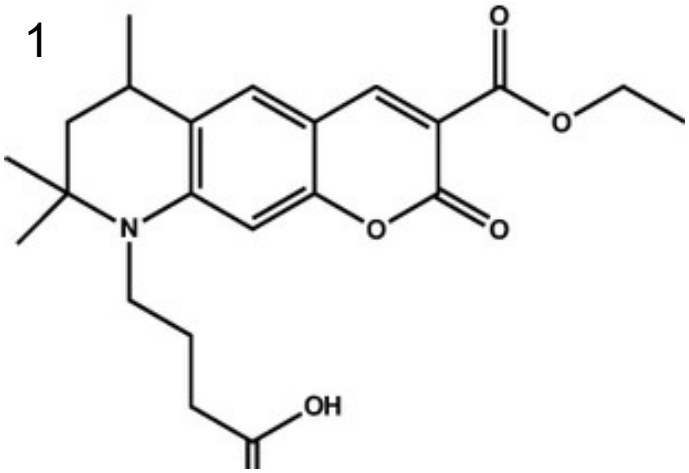


Chemical features:

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

Relating structure to fluorescence properties

1



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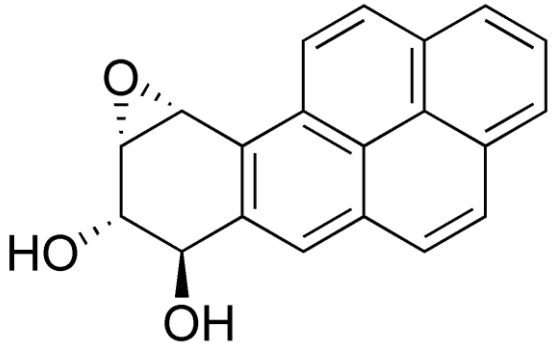
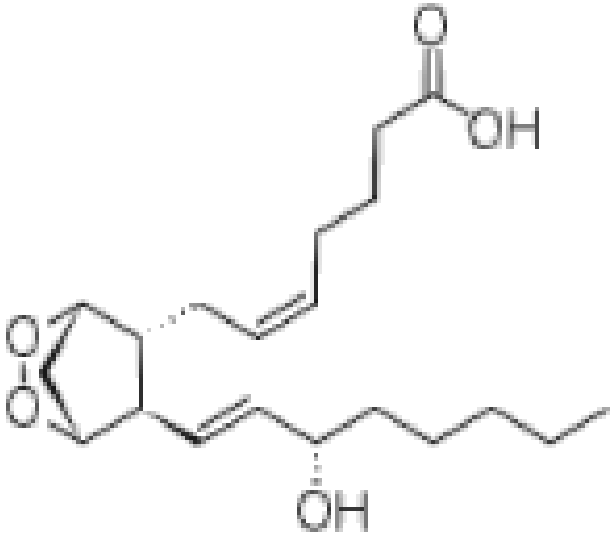
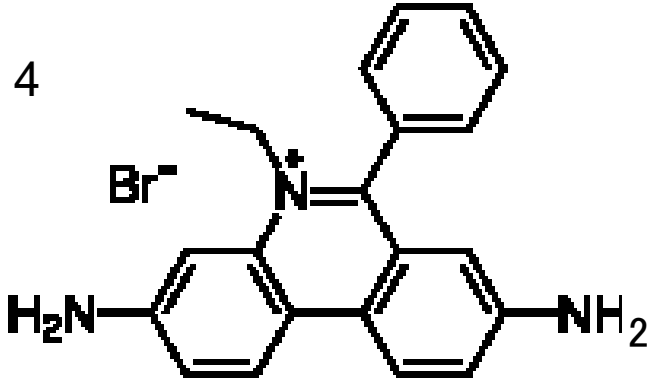


3

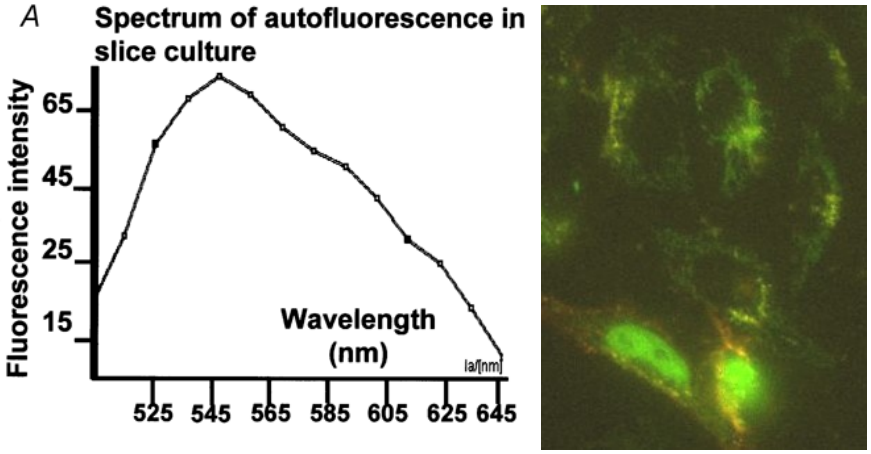


4

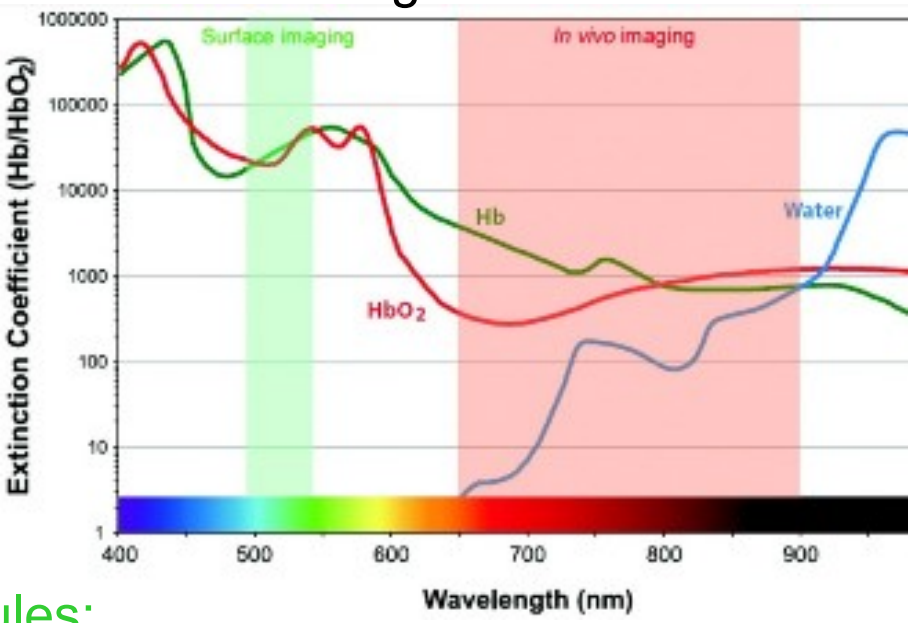


What sort of molecules are fluorescent?
1. Endogenous organic fluorophores

Isolated cells



Living animals



Most common autofluorescent molecules:

Flavins, NADH, NADPH, elastin, collagen, lipofuscin

Avoiding autofluorescence:

choose dye emitting in red with big Stokes shift

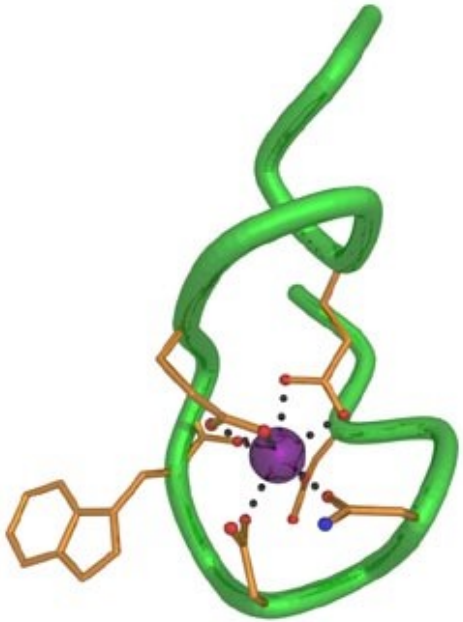
add quencher (Crystal violet)

time-gate fluorescence

What sort of molecules are fluorescent?
2. Inorganic fluorophores

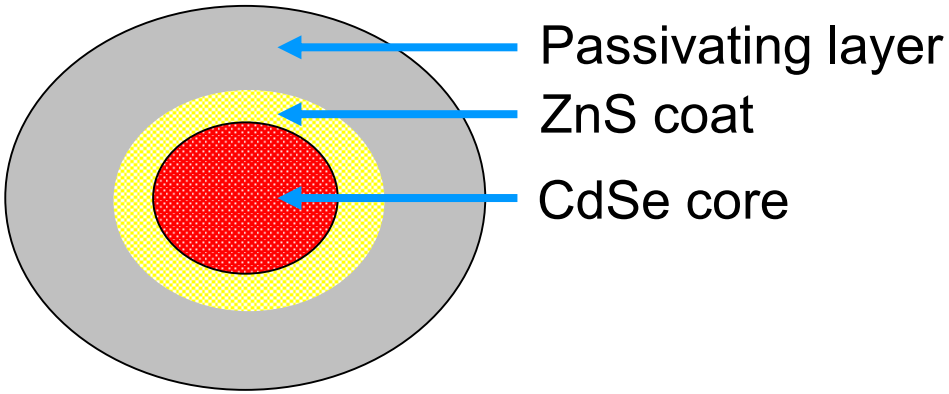
Lanthanides

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



+ bright, photostable, narrow emission
- large (~20 nm), expensive,
hard to target specifically

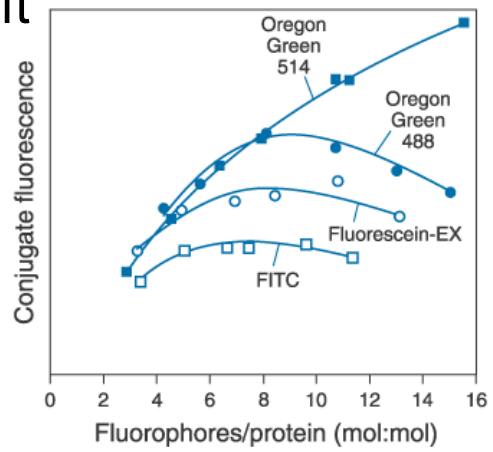
Michalet X, et al. Quantum dots for live cells, in vivo imaging, and diagnostics. Science. 2005 307(5709):538-44.

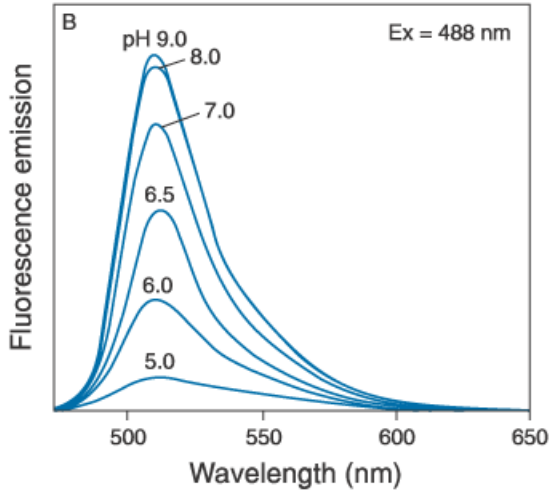
How good is a fluorophore?

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

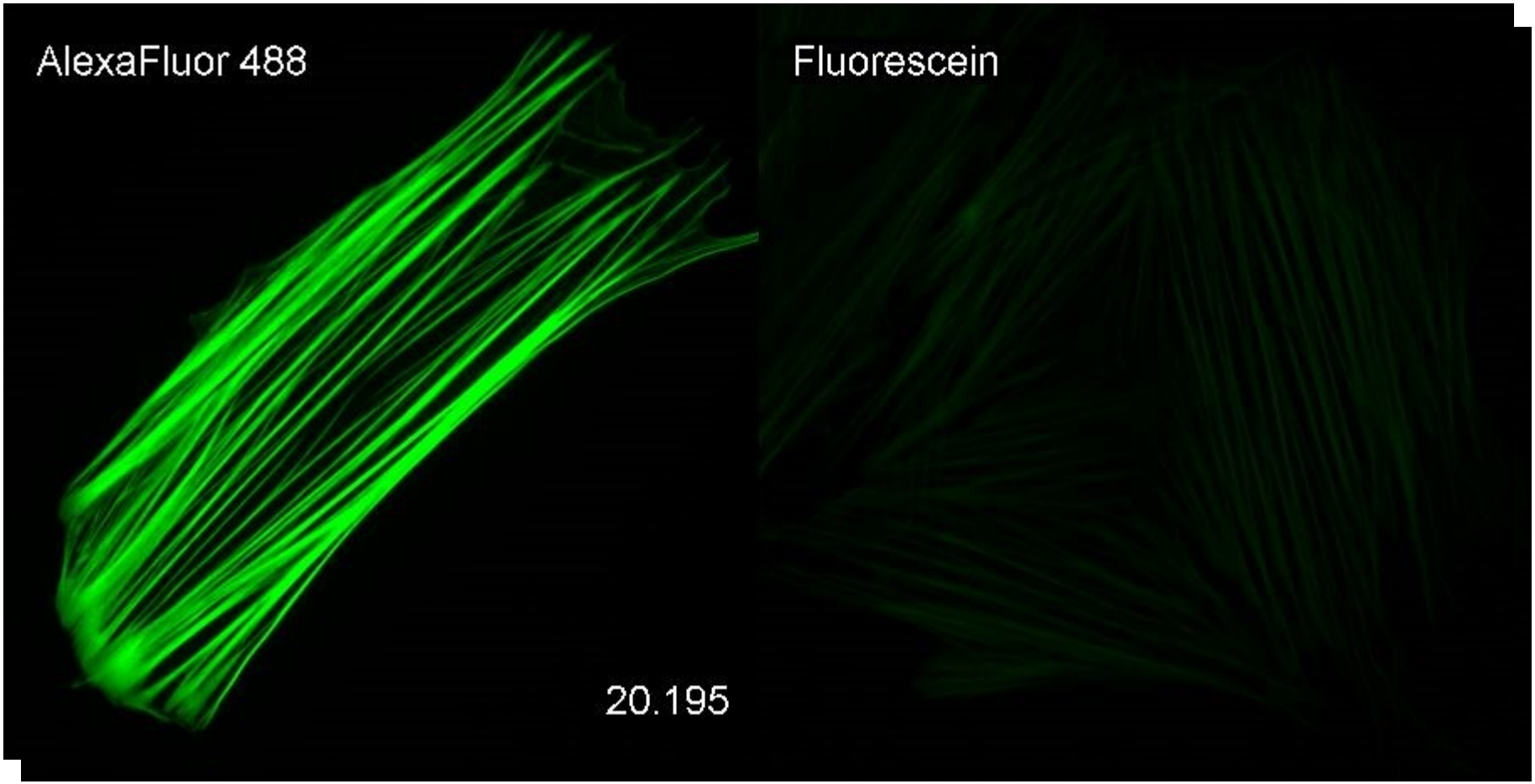




Fluorescein pH sensitivity

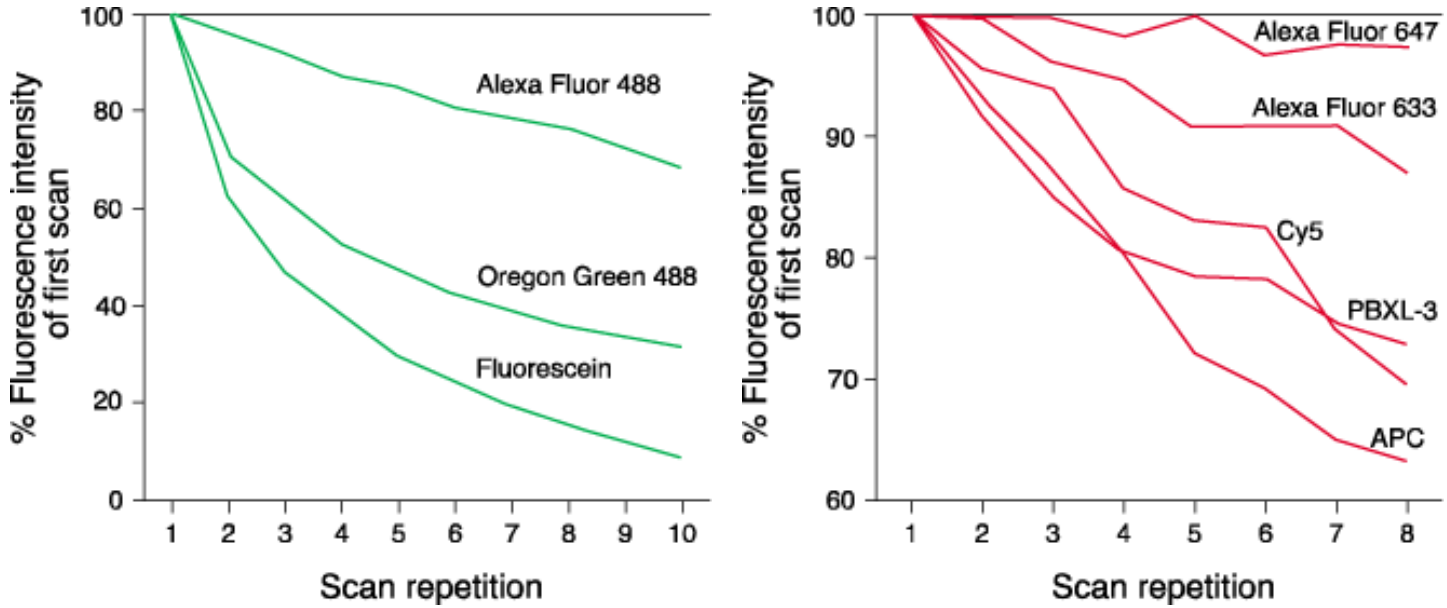
Alexa Fluor 488 vs Fluorescein Bleaching

2x Real Time



Alexa Fluor Dyes – Photostability

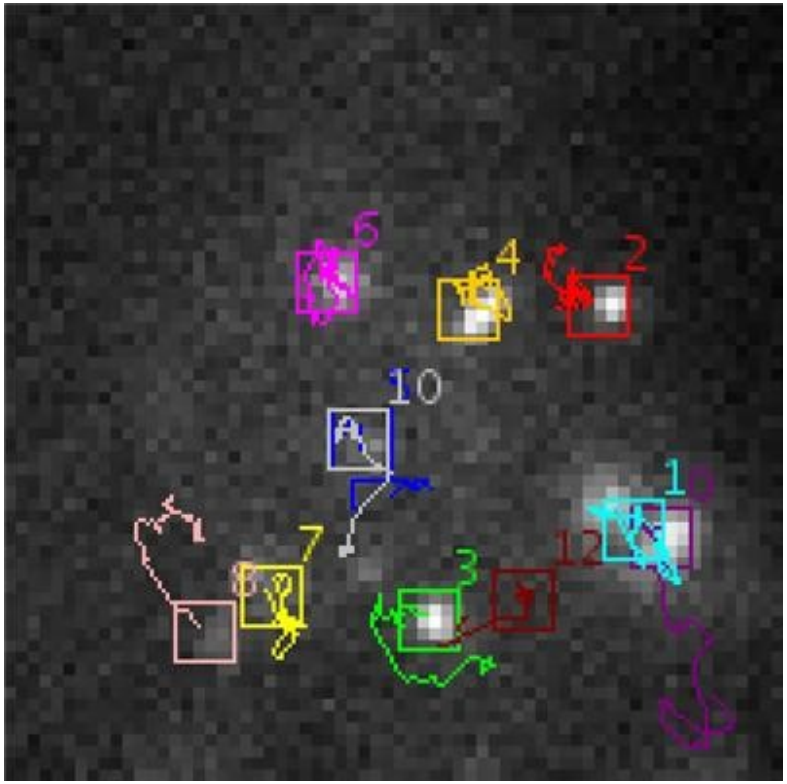
Laser-scanning cytometry
EL4 cells
biotin-anti-CD44 + streptavidin conjugates

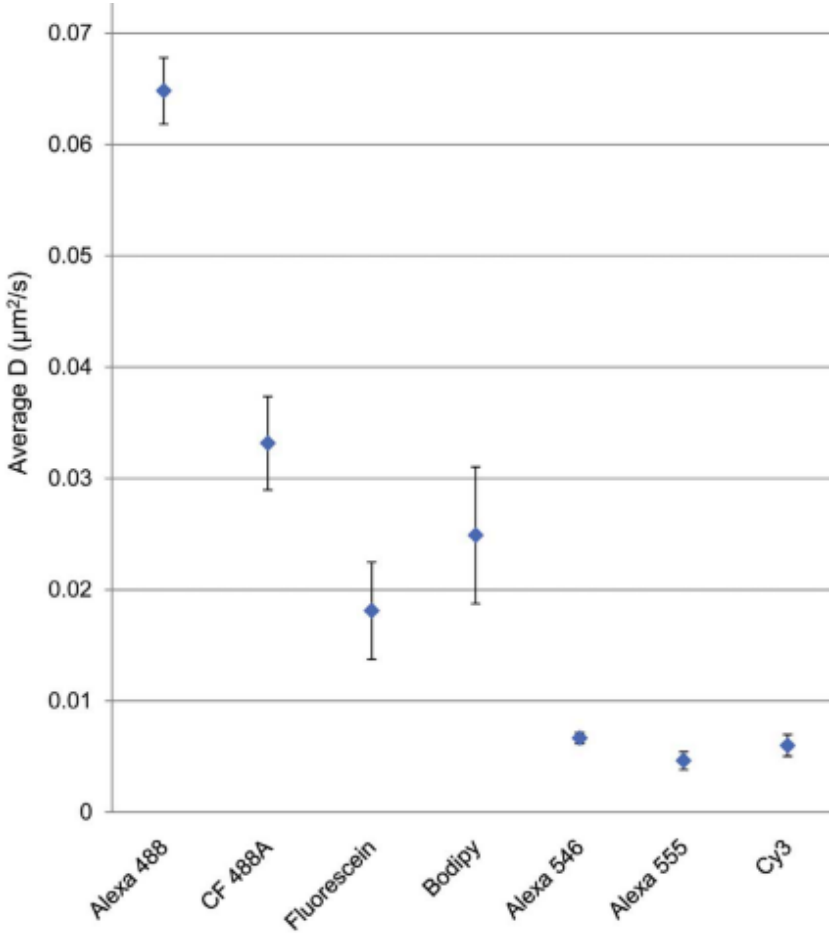


Fluorescein is the commonest dye
but has poor photostability.
Also consider Atto dyes (Sigma) and Dyomics dyes

Dye affects non-specific binding and receptor mobility

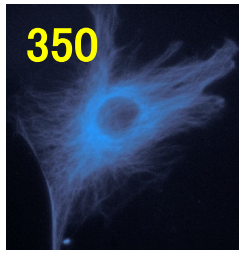
Dye makes a big difference to non-specific binding
Diffusion coefficient for dye-labelled Affibody against EGFR varies 10-fold with hydrophilic versus more hydrophobic dye!
L. Zanetti-Domingues et al.
PLoS one 2013

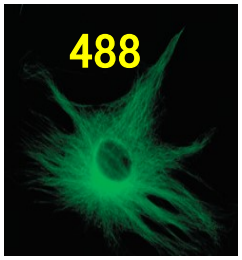


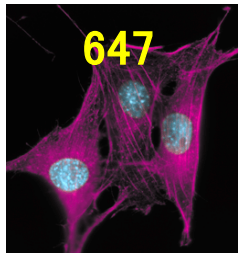


Multiplexing– four main colours

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

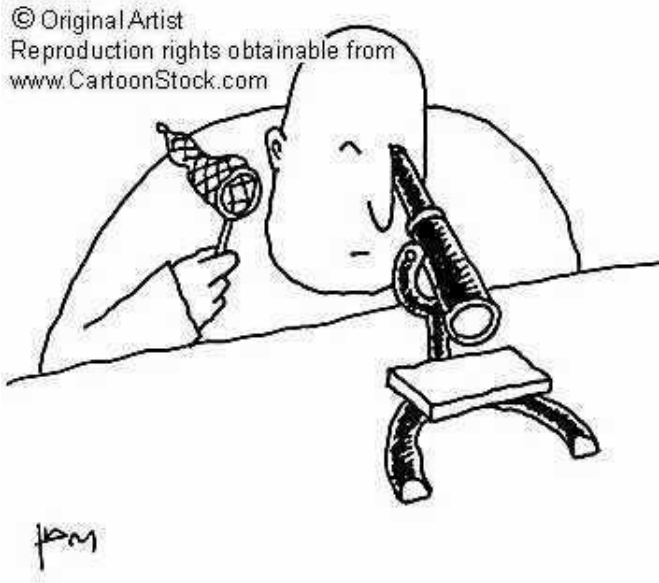
Alexa Fluor® 555
Rhodamine,
TAMRA, TRITC
Cy3

Alexa Fluor® 647
Cy5, APC

Alexa Fluor® 594
Texas Red, Cy3.5

Colour Selection ♦ Brightness ♦ Photostability

Overview



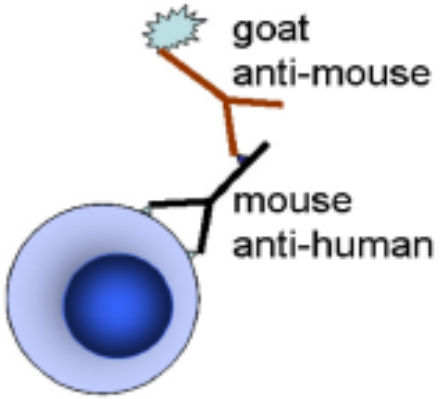
1. What kind of structures are fluorescent

2. How to make and target fluorescent probes

3. Fluorescent probes for cellular structure and function

Antibodies for cellular imaging

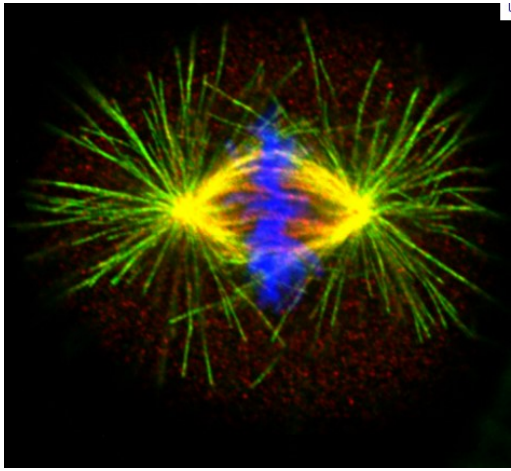
Live cells



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

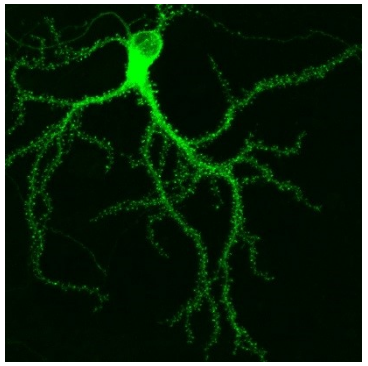
Still can give enormous amount of useful information

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

?



How to dye: it is easy

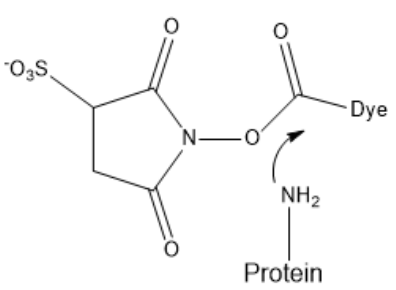
Multiple ways to modify proteins
(see Molecular Probes catalogue)

Most common ways are to modify:

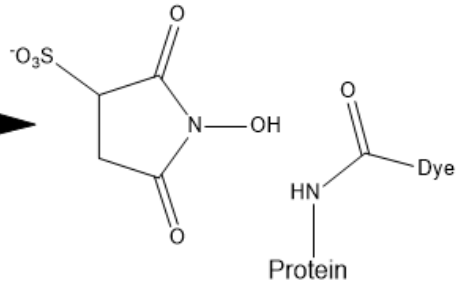
1. Lysine

or

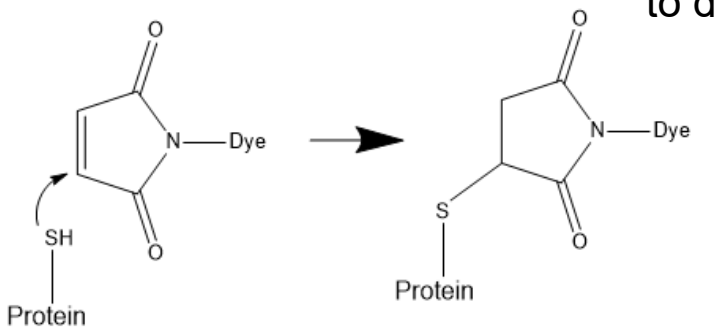
2. Cysteine



sulfoNHS-dye



Amide bond
to dye



maleimide-dye

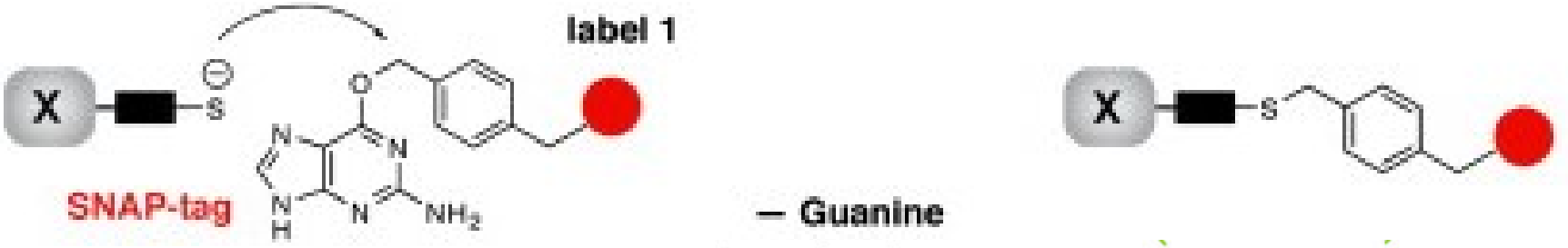
Thioether bond
to dye

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

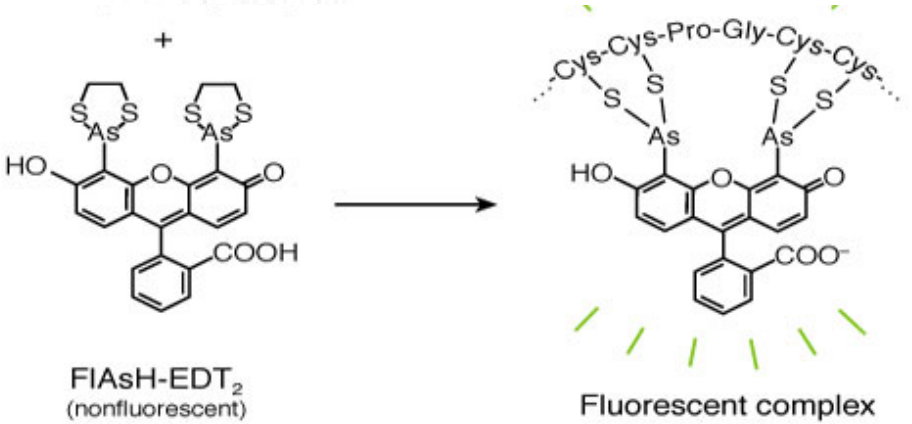
1. Binding domain

SNAP-tag (19 kDa NEB), HaloTag (34 kDa, Promega)



2. Binding peptide

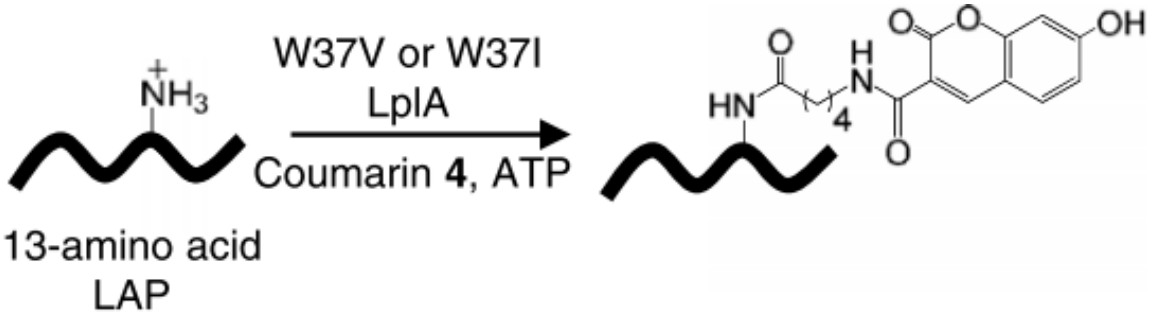
FlAsH (Invitrogen)



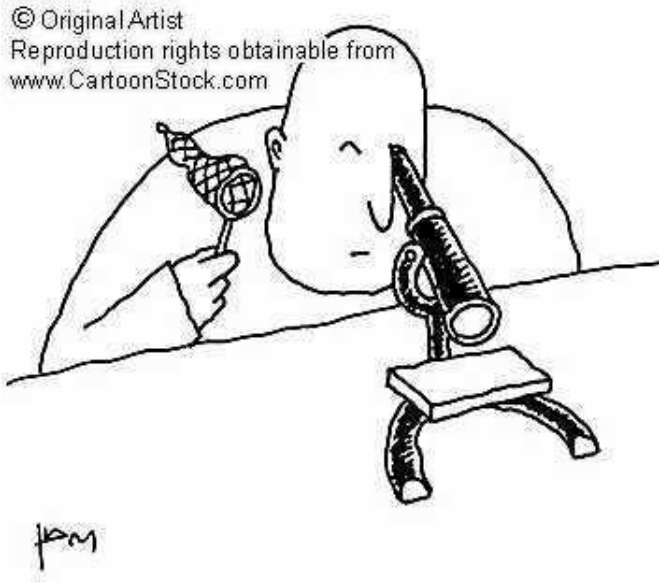
3. Enzymatic ligation to peptide

PRIME

AY Ting PNAS 2010



Overview



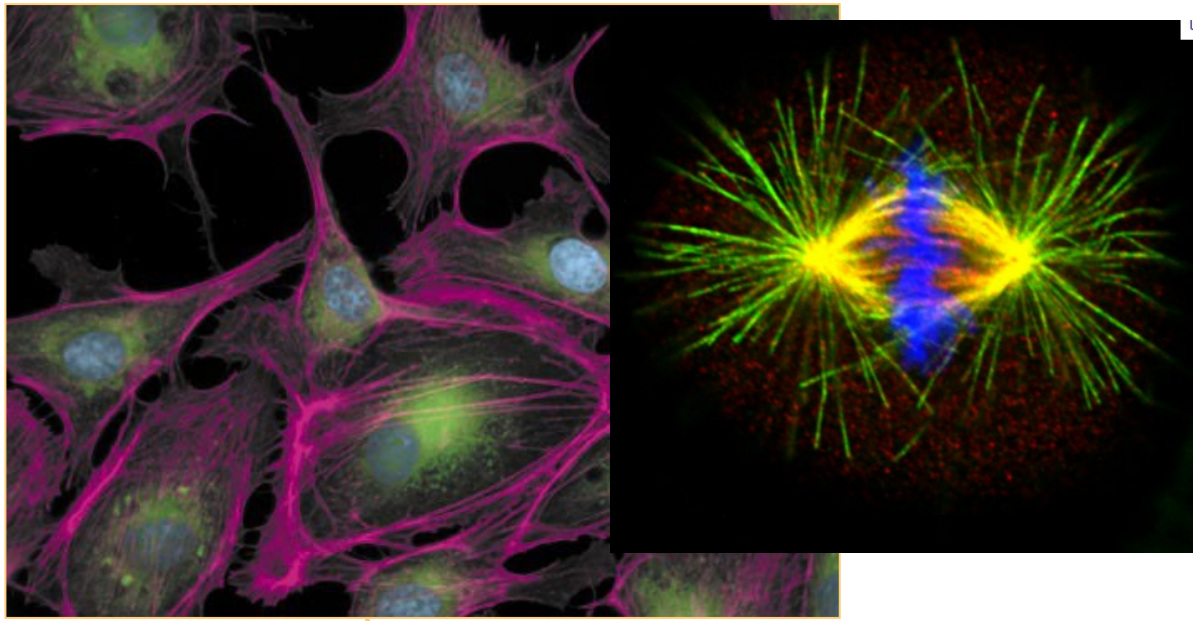
1. What kind of structures are fluorescent

2. How to make and target fluorescent probes

3. Fluorescent probes for cellular structure and function

Putting the signal in context: nuclear labelling

(follow DNA even when nucleus breaks down)



Fixed cells:

Intercalate into DNA

DAPI

(well away from other channels)

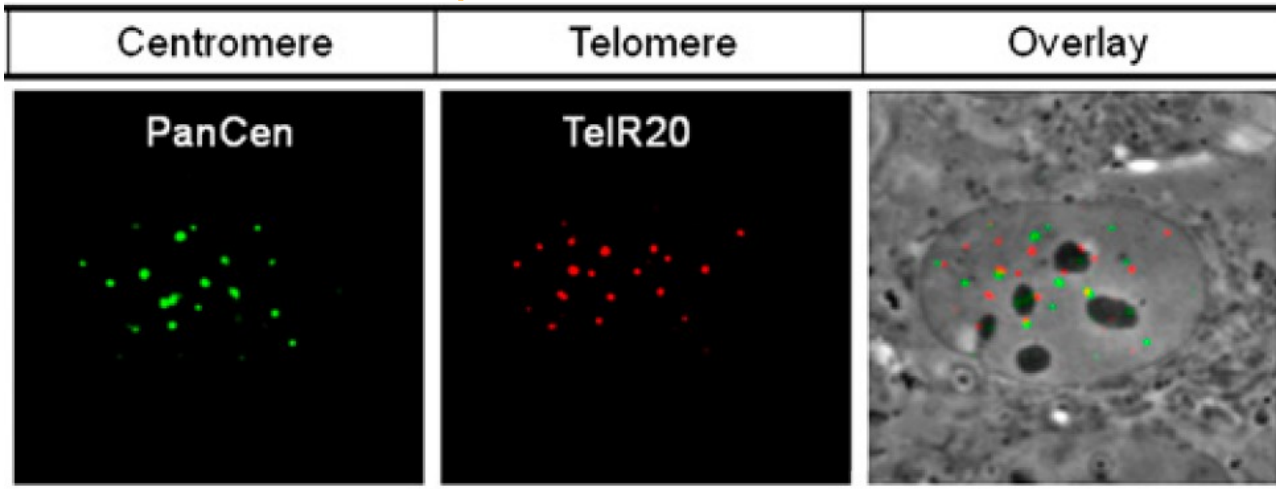
Hoechst 33342

Live cells:

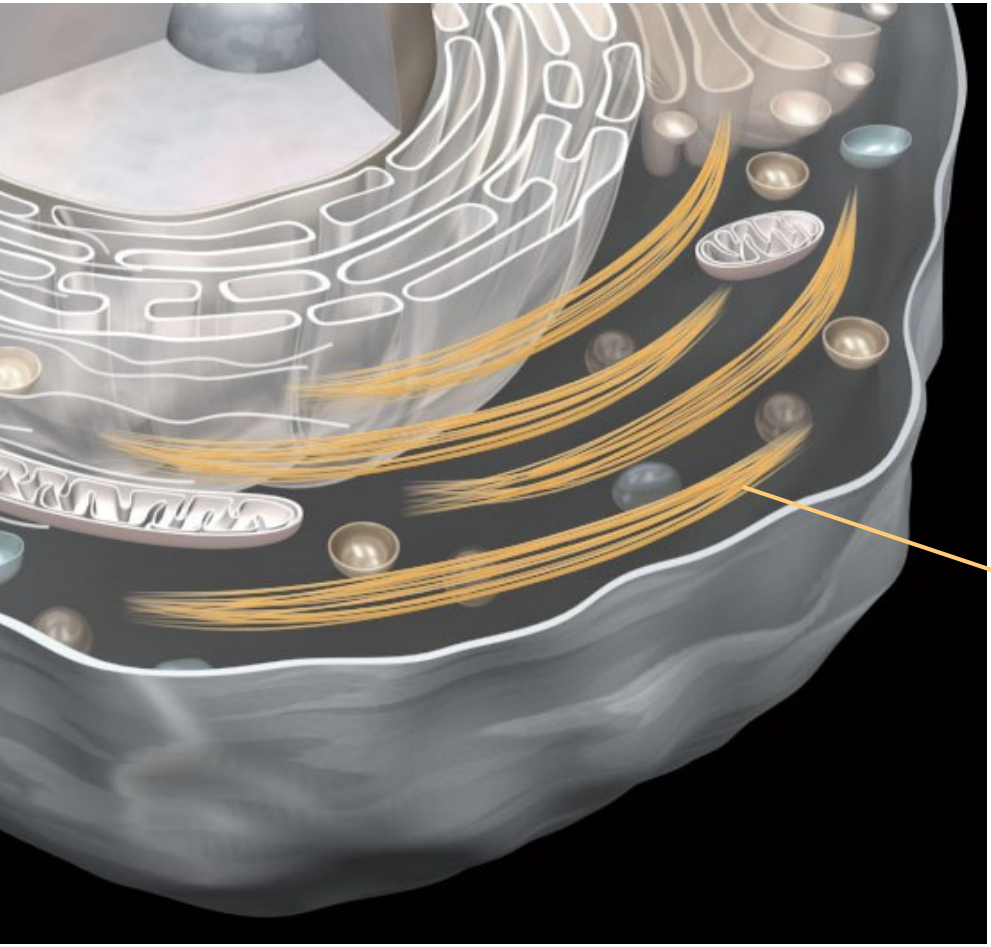
histone H2B-GFP

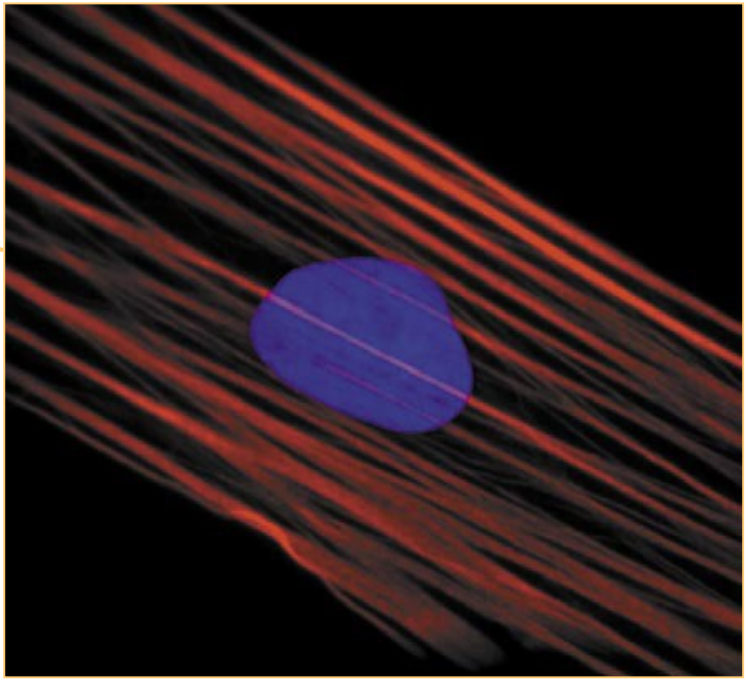
TALEN-XFP

CRISPR/Cas9-GFP (B. Chen et al. Cell 2013 155:1479)



Putting the signal in context: actin labelling





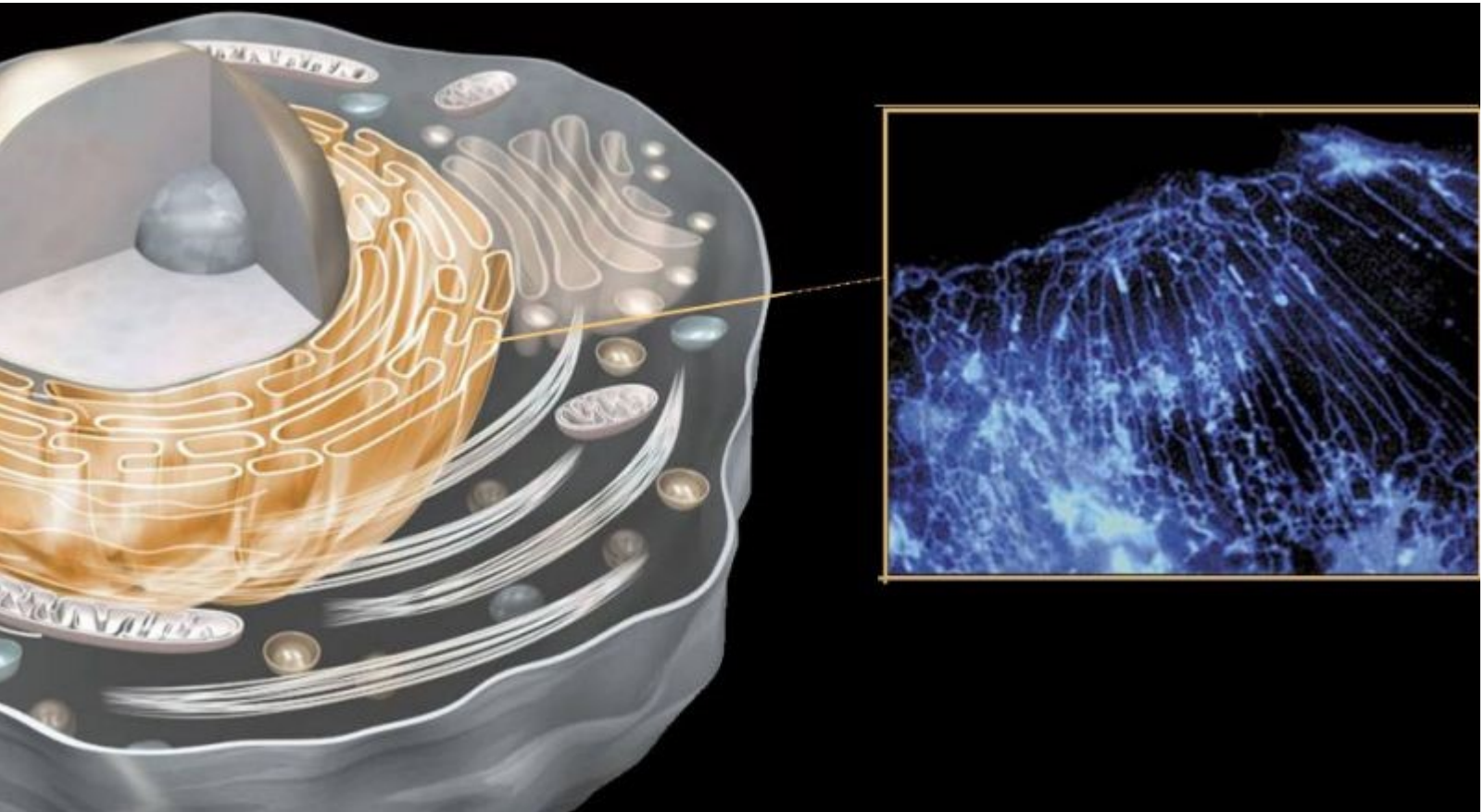
Fixed cells: phalloidin-dye

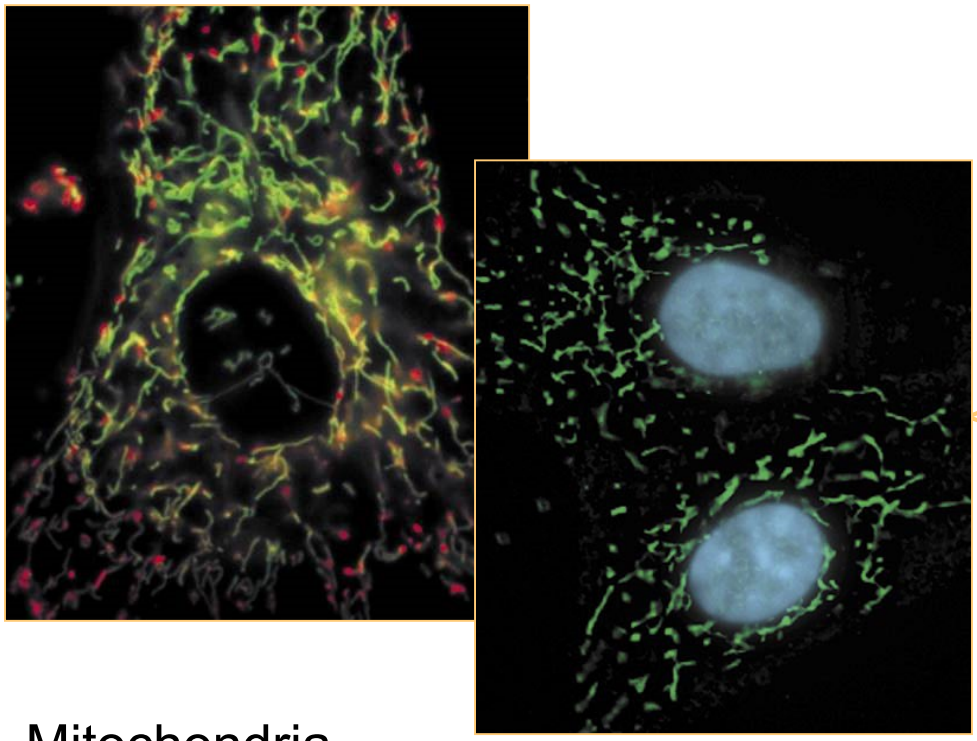
Endoplasmic Reticulum

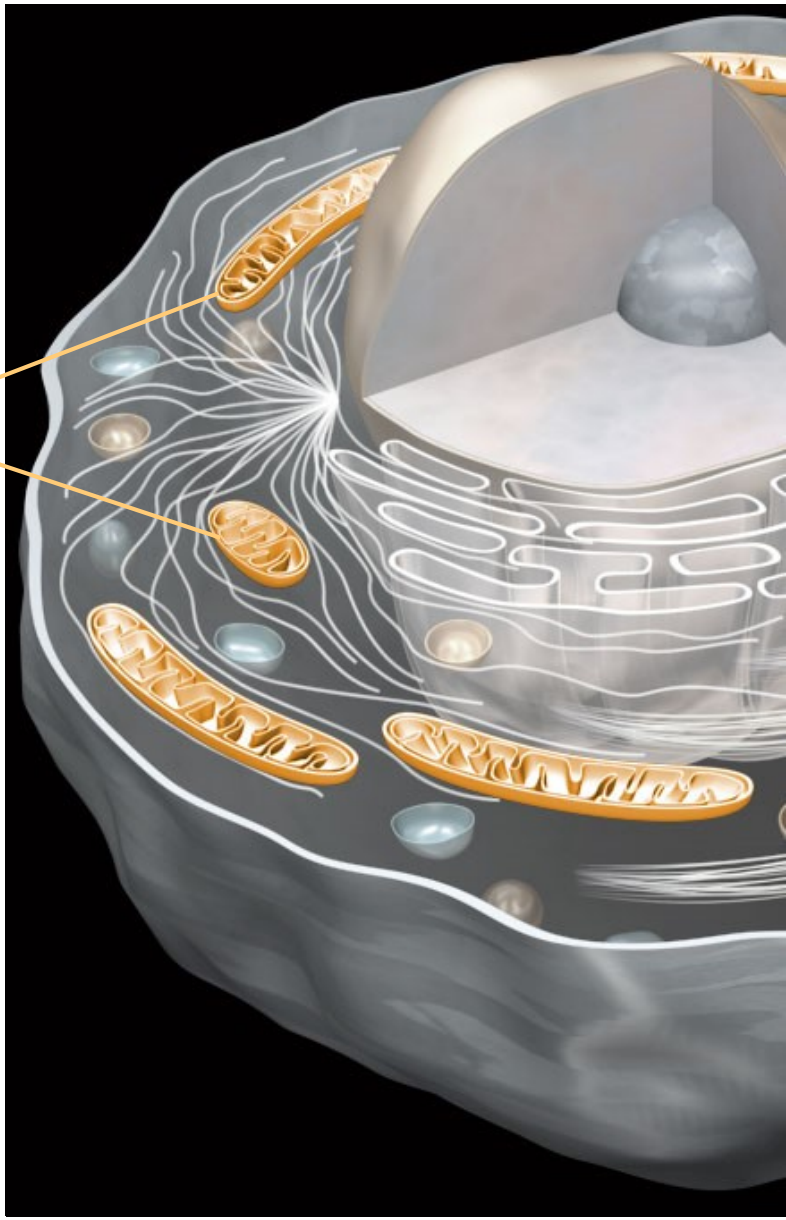
ER-Tracker™ Blue-White DPX

antibody to calnexin

Live cells: ss-GFP-KDEL







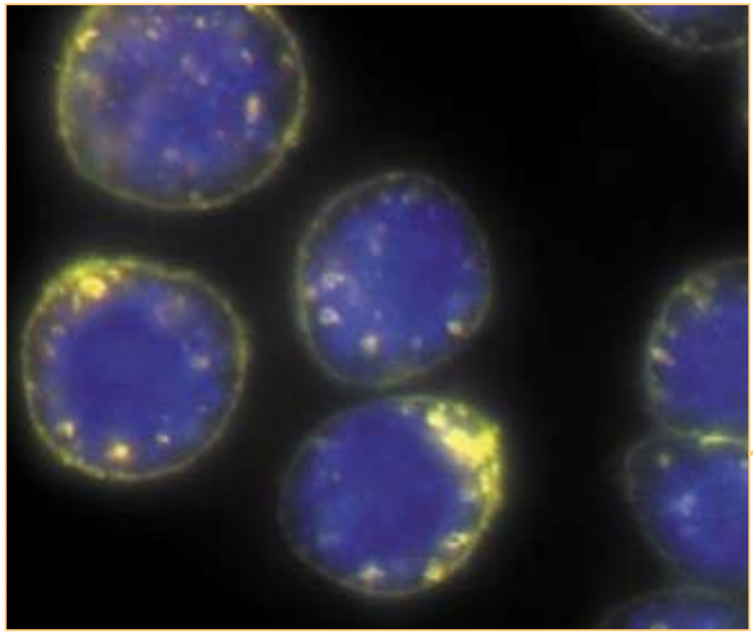
Mitochondria

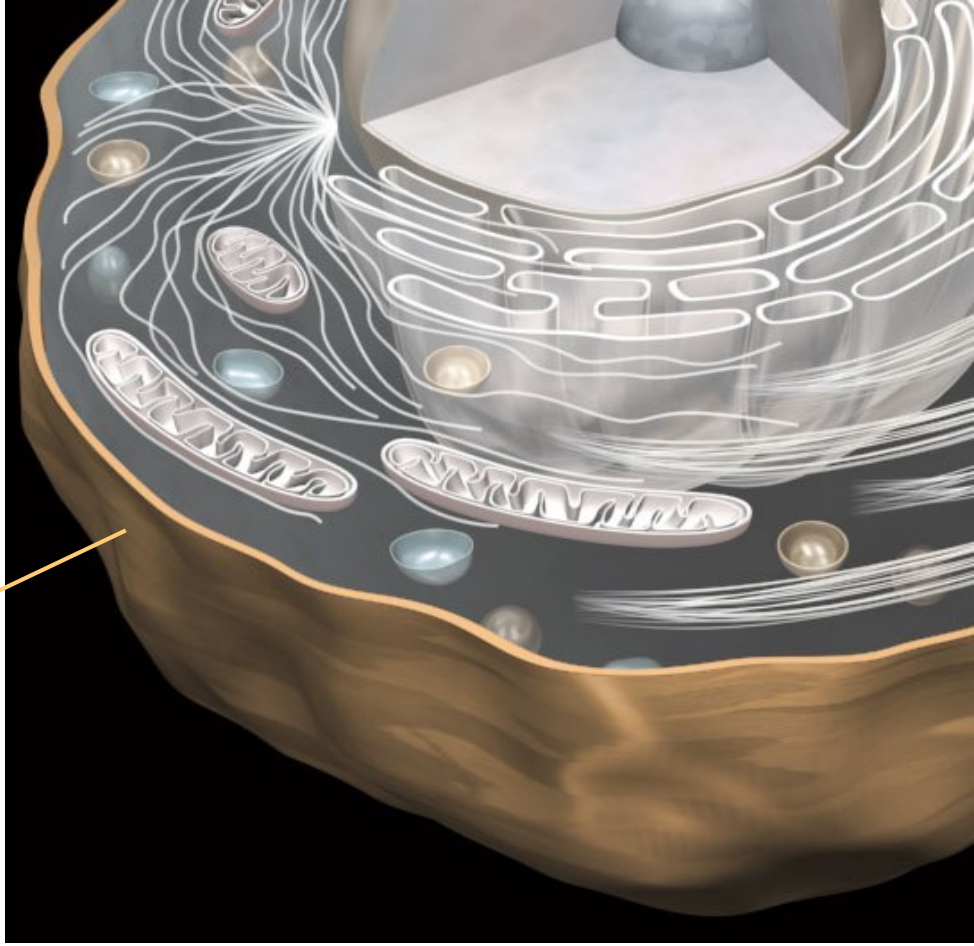
Fixed cells: anti–cytochrome oxidase subunit I Ab

Live cells: MitoTracker® Red/Green/Orange

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

Mitochondrial targeting sequence-GFP

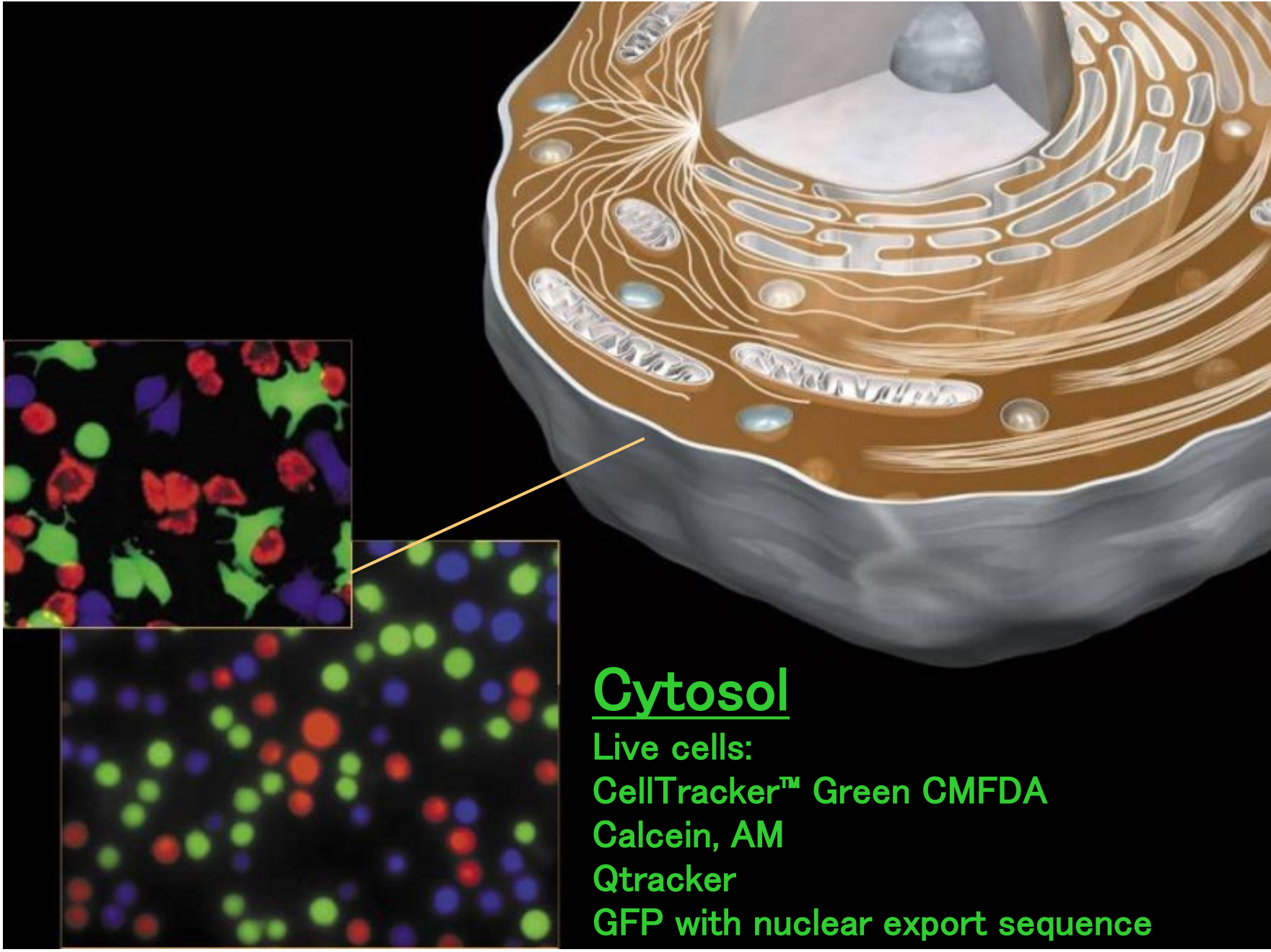




Lipid Rafts

BODIPY® FL C_5-ganglioside GM1

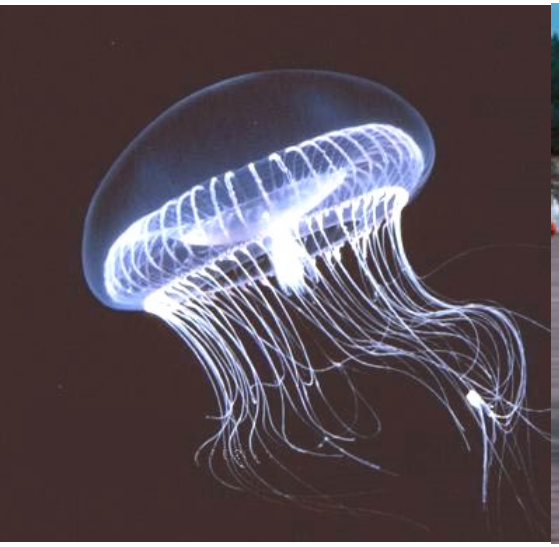
Fluorescent Cholera Toxin subunit B (CT-B)



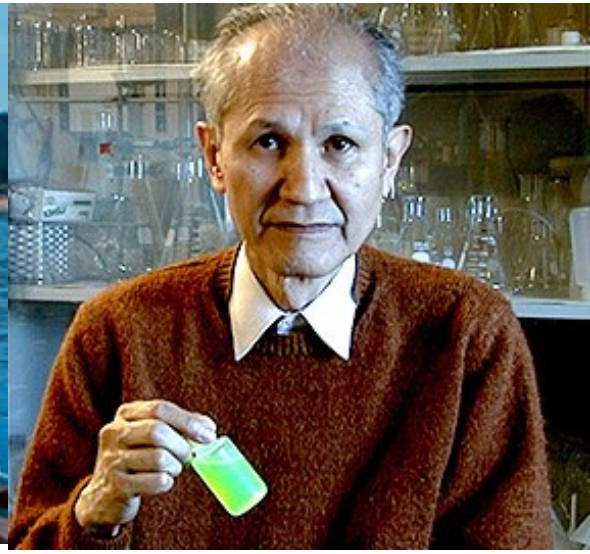
Cytosol

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

The breakthrough of fluorescent proteins from jellyfish

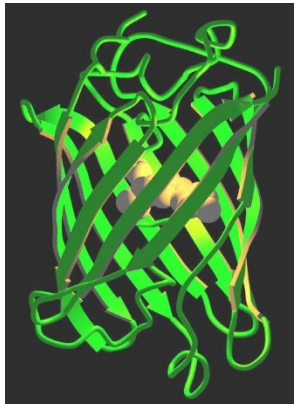


Aequorea victoria

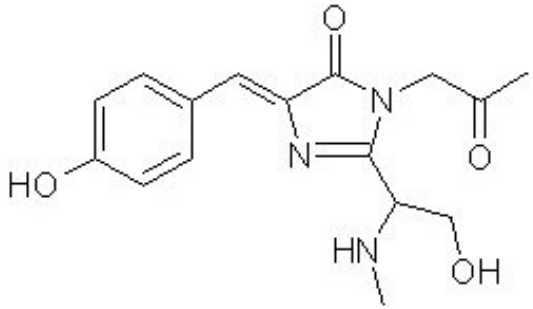


Osamu Shimomura

The breakthrough of fluorescent proteins for live cell imaging

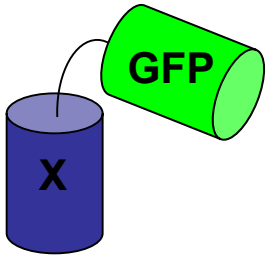


GFP fold
β-can

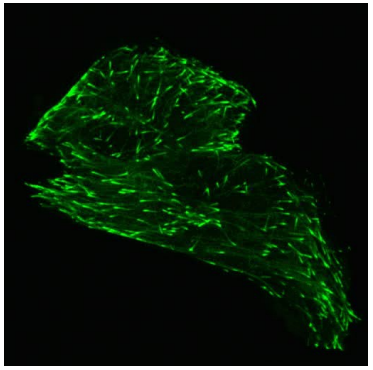


GFP chromophore
from Ser-Tyr-Gly

Link GFP sequence to gene of your favourite protein

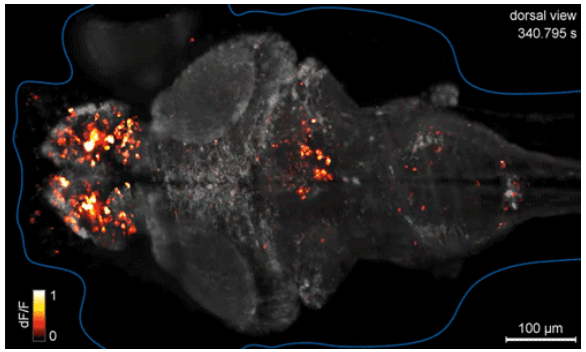


GFP folds
and becomes
fluorescent



GFP lights up your
favourite protein in cell

Fluorescent proteins are more than just labels

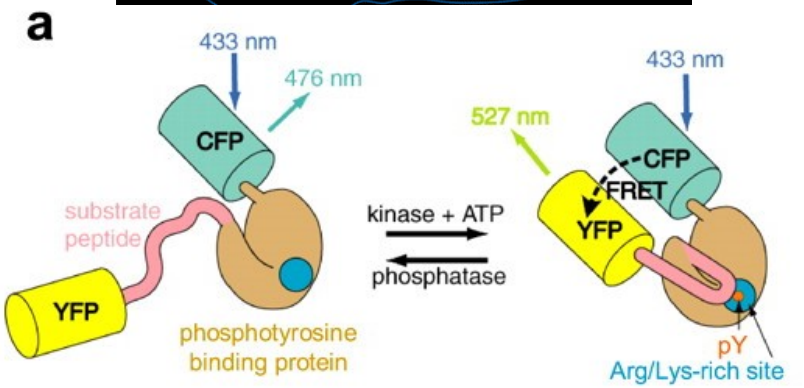


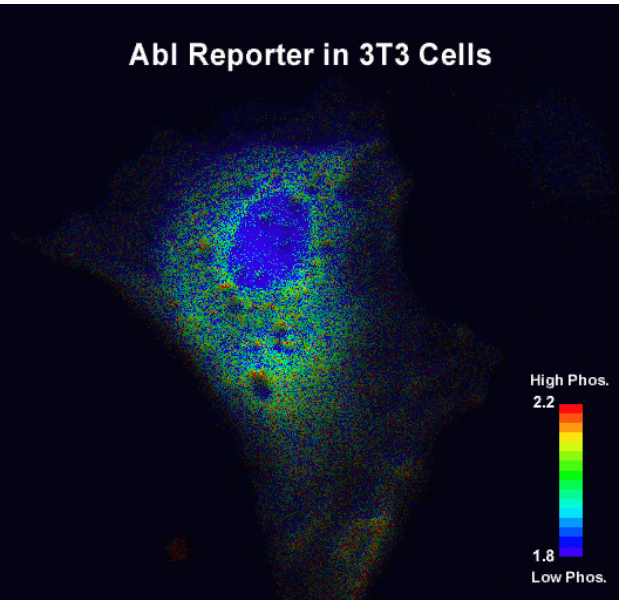
Photoactivation/Photoswitching
- PA-GFP, Dronpa, Eos

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

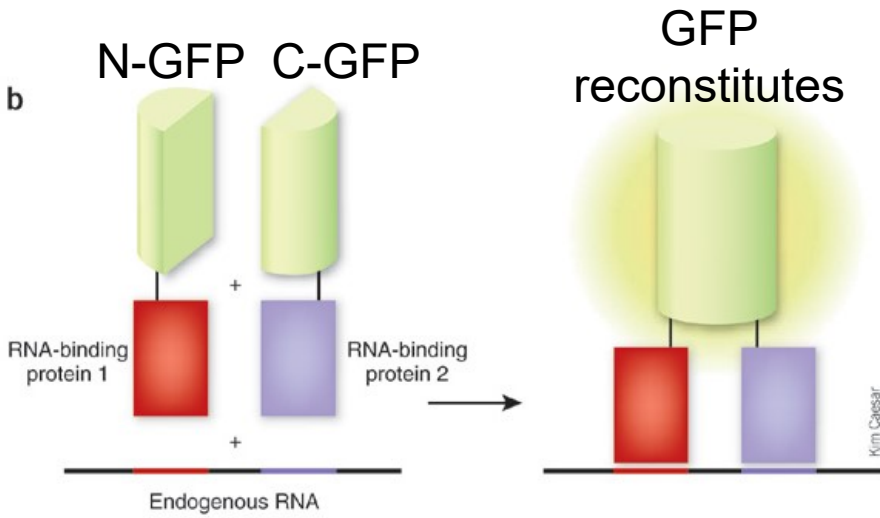
Reporting on protein-protein interaction
- CFP/YFP FRET, split fluorescent proteins

Modifying environment
- Singlet oxygen generation, Channelrhodopsin





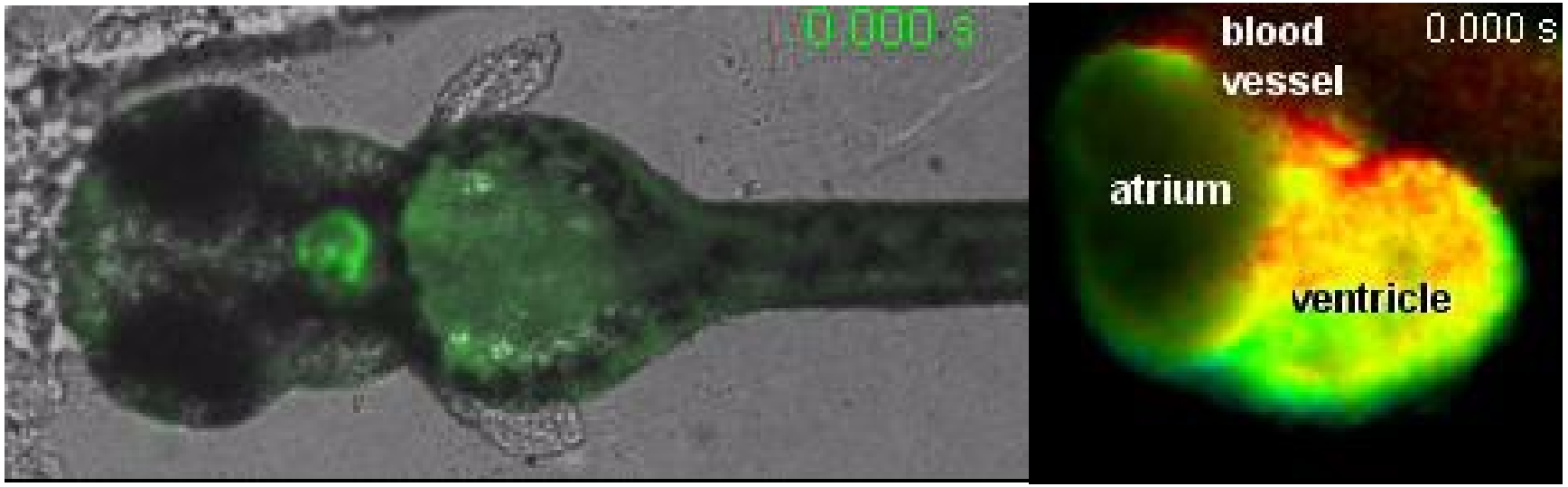
<u>Targeting advantage</u> to defined compartment, cell-type, developmental stage



Sensing voltage with fluorescent protein

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

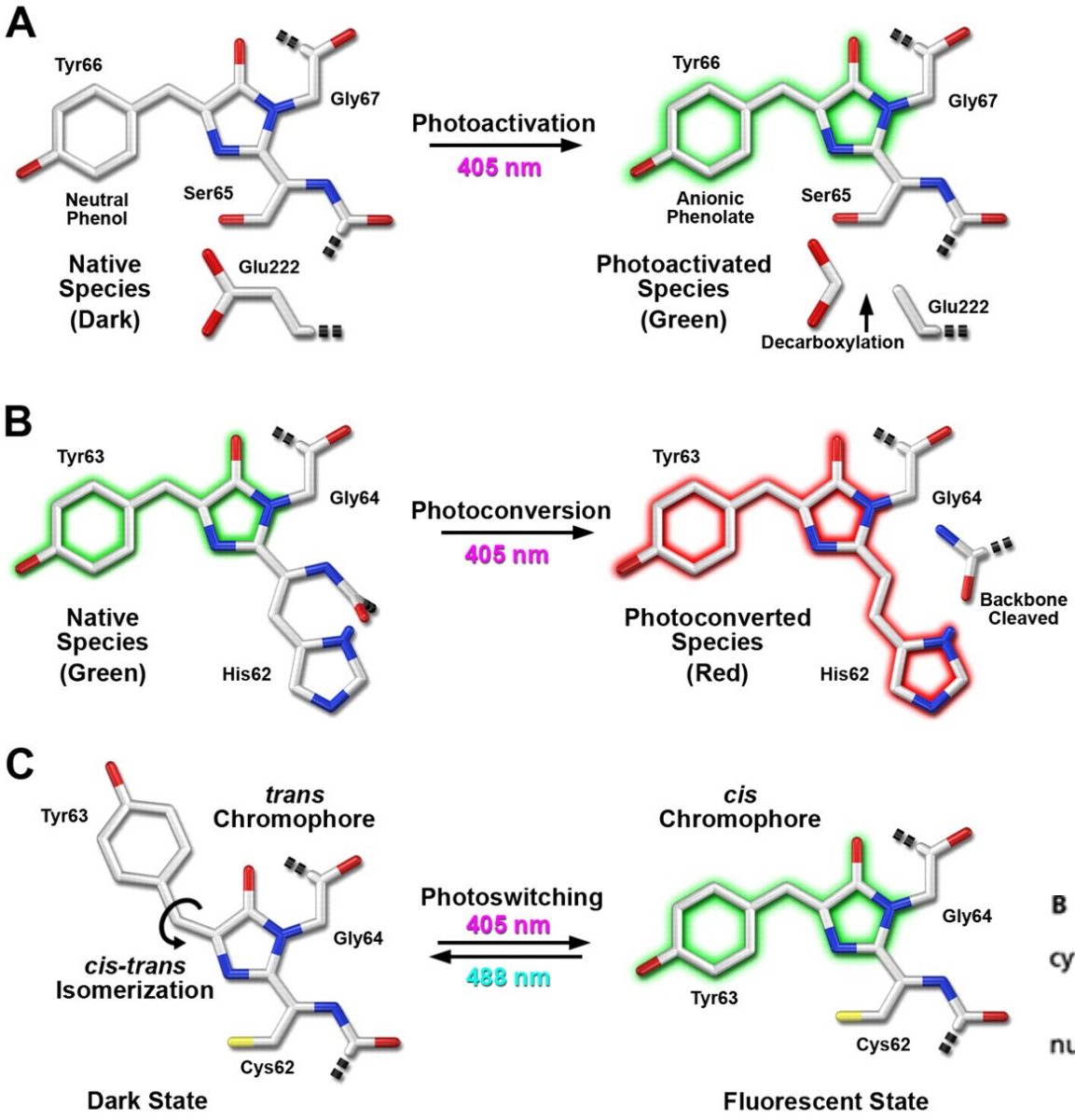
Expressed in zebrafish heart
Non-invasive testing of mutant phenotypes
and drug cardiotoxicity.
Tsutsui, Miyawaki J Physiol 2010



FRET sensor ratio crucial

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

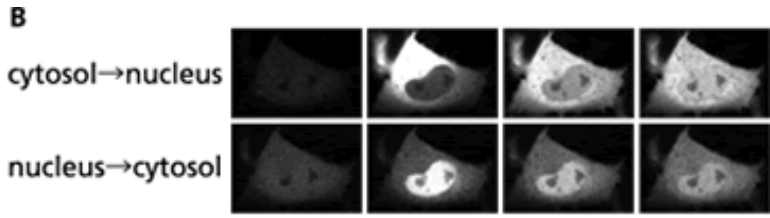
Chromophores in switching



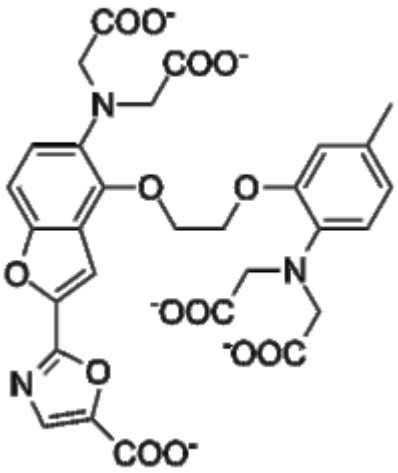
PA-GFP

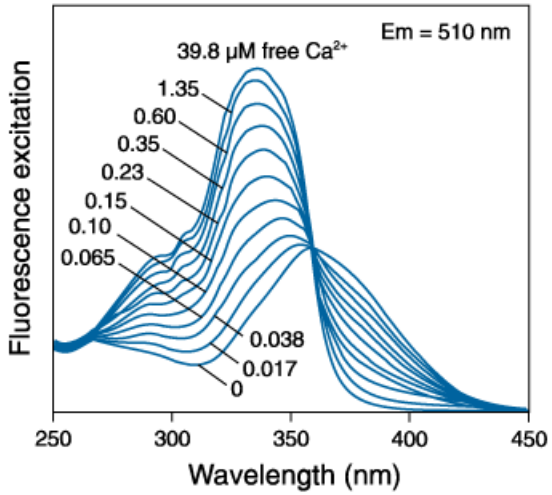
Dendra, Eos

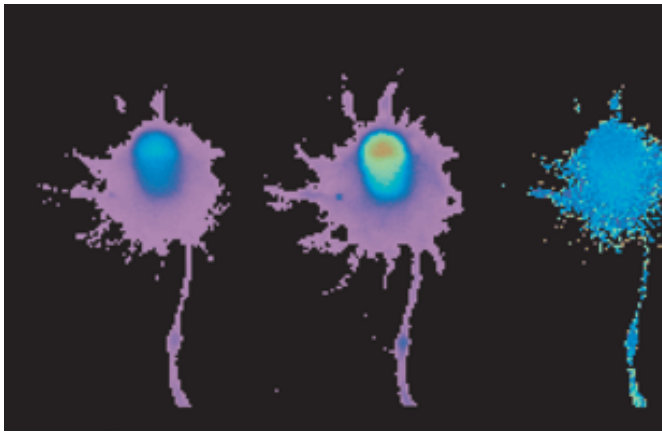
Dronpa



Small molecule fluorescent sensors







Fura-2 sensing calcium

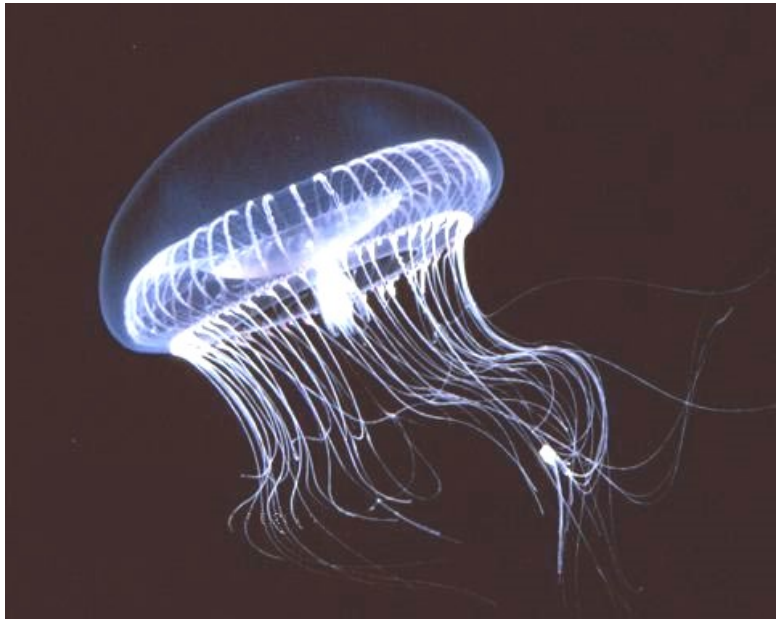
Metal ions: calcium, magnesium, zinc, sodium, potassium, chloride, mercury

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

Reactive oxygen species, nitric oxide

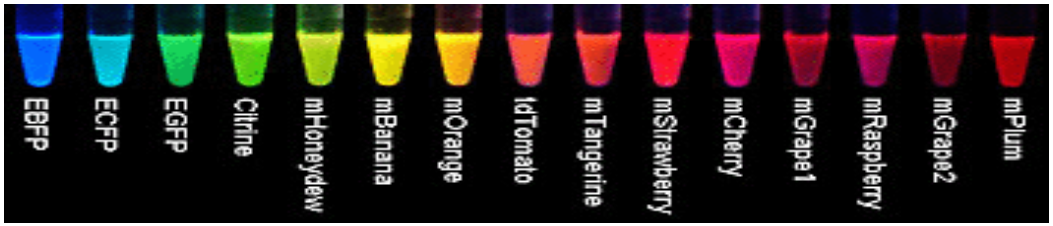
Transmembrane potential

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?



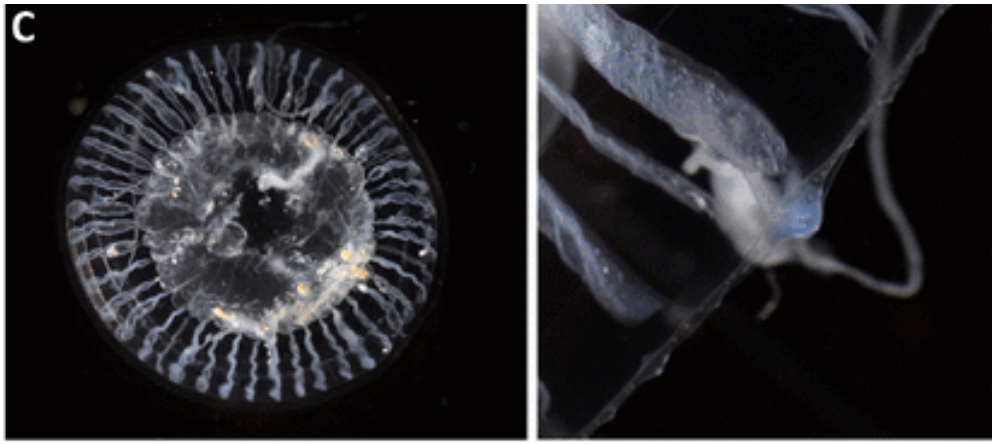
A. victoria GFP is terrible!

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 Clover, YPet 2.5 x EGFP
   mRuby2 3x 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 Maturation — Venus 2 min. Red FPs can start off green
   half-time ~15 min mCherry, 100 min TagRFP

Revenge of the jellyfish

mRNA-seq on *A. victoria* gave 9 undiscovered FPs
Also on related *Aequorea australis*
AausFP1 brightest ever,
5x brighter than EGFP (QY 0.97)
Narrow excitation, narrow emission, folds at 37 ºC.
They monomerized it. Much better starting point than GFP!
“Aequorea victoria’s secrets” Nathan Shaner, bioRxiv 2019



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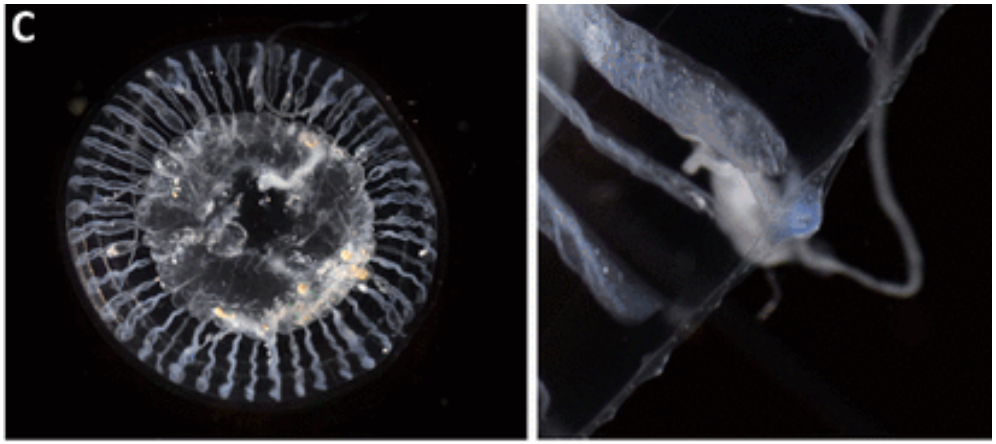
"Aequorea victoria's secrets" Nathan Shaner, bioRxiv 2019

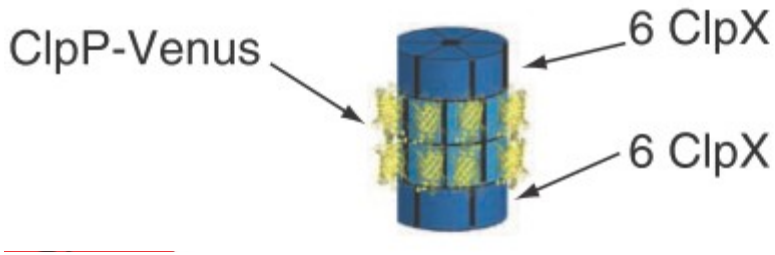
"Aequorea's secrets revealed: New fluorescent proteins with unique properties for bioimaging and biosensing."
Lambert GG, Shaner NC. PLoS Biol. 2020 Nov



You MUST worry about FP multimerization!

Tag multimerizing protein with FP and sometimes see foci-

are these real or caused by the tag?



With hexameric barrel involved in *E. coli* protein degradation,

many commonly used FPs induce artifactual foci

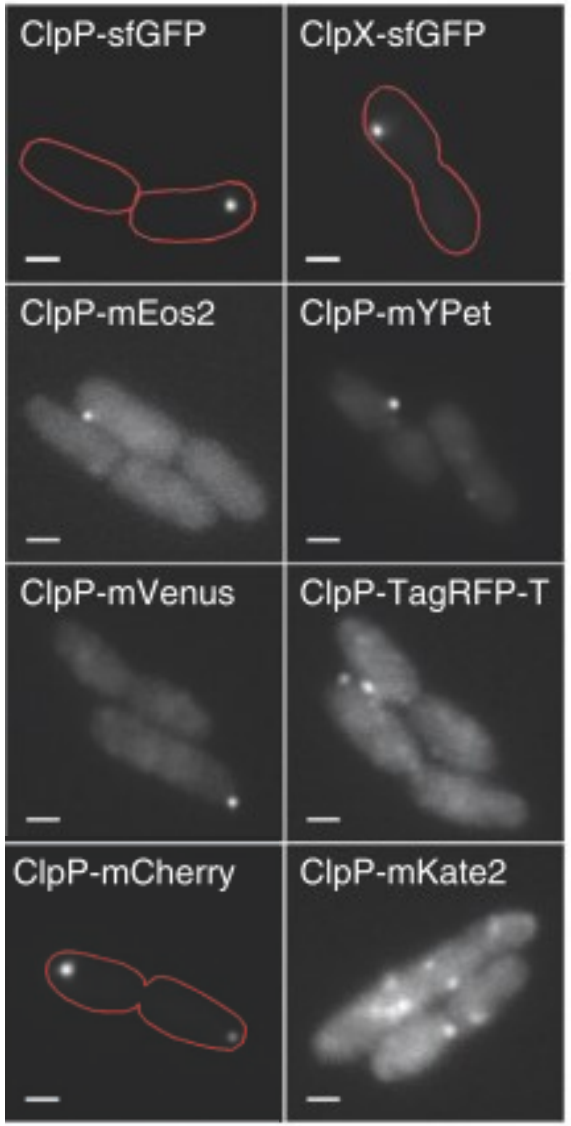
(no cluster with Ab or SNAP-Tag)

as well as affecting daughter cell inheritance of proteolysis ability

mCherry, sfGFP, mYPet poor!

mGFPmut3, Dronpa OK

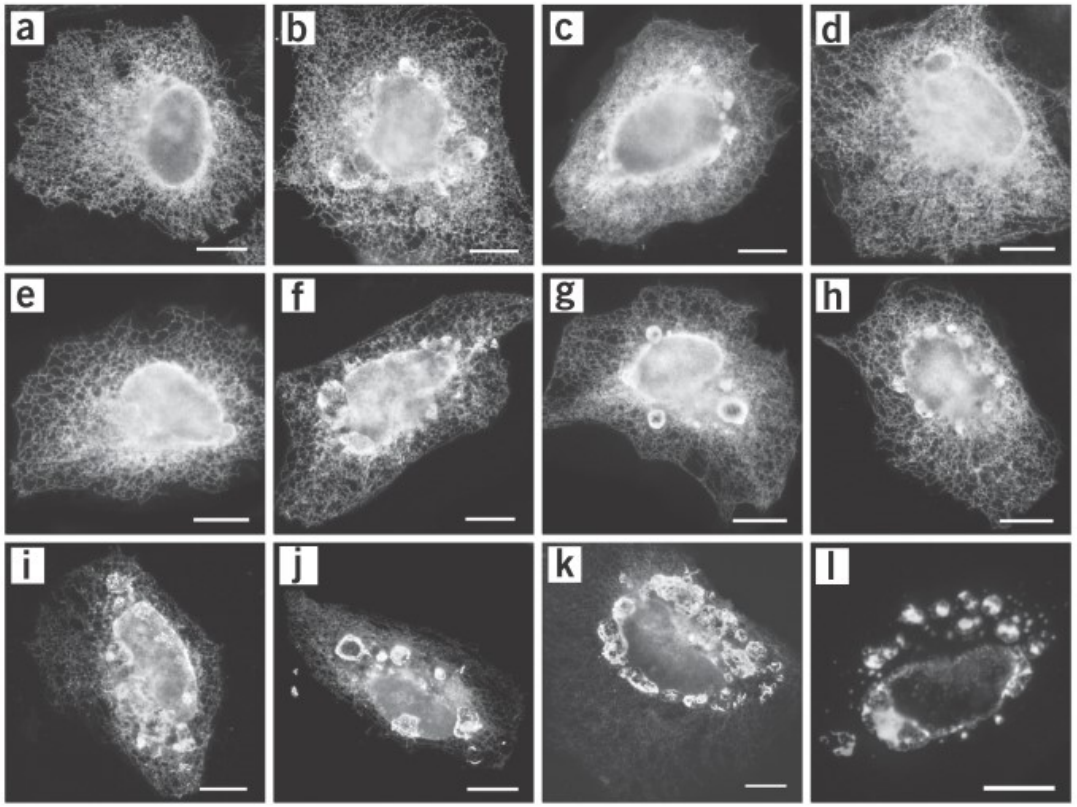
D. Landgraf et al. Nature Meth 2012



You MUST worry about FP multimerization!

In mammalian cells,
linked to ER membrane protein,
some FPs cause organized smooth ER (OSER) whorls
PJ Cranfill et al. Nature Meth 2016

mEGFP



dsRed2

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
  Lukyanov Nat Meth 2009
  HAM F-12 medium 6x better EGFP stability in cells than DMEM, RPMI!
  Lukyanov Biotechniques 2015
- **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

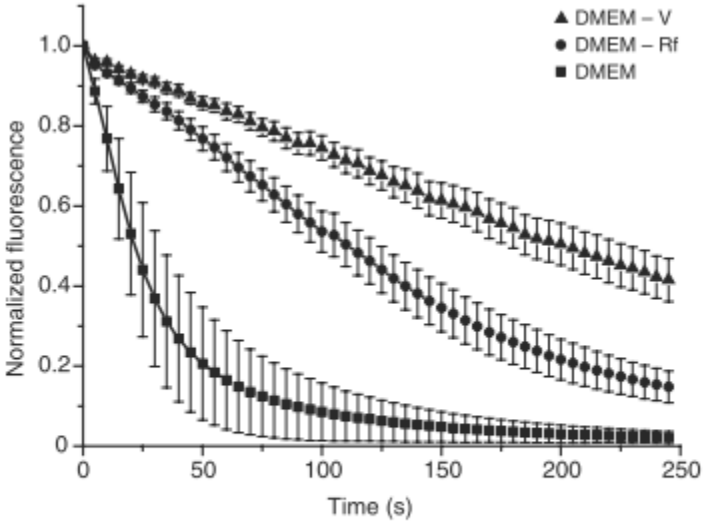
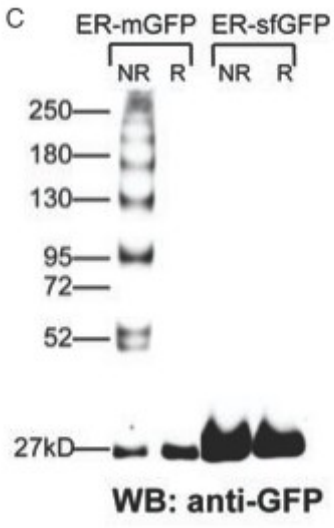


Figure 1 | Influence of cell medium on fluorescent protein photostability. Normalized bleaching curves for EGFP in live HEK293T cells maintained in DMEM, DMEM – Rf or DMEM – V. Error bars, s.d. (n = 20 cells).



Compare at fpbase.org

Fluorescent Protein Table

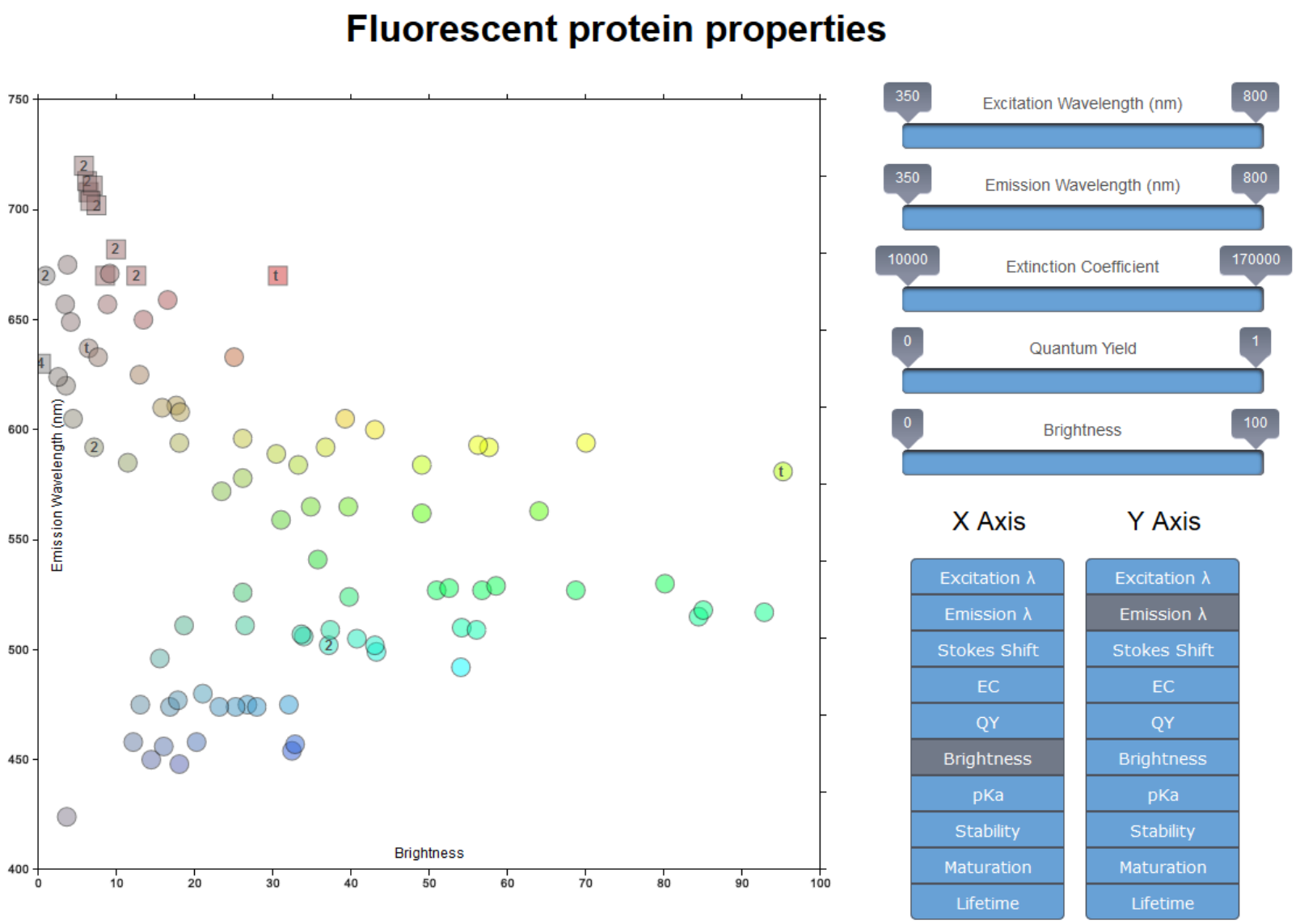
This is a comprehensive table of all proteins in the database. You may sort by clicking the column headers (multi-sort by shift-clicking th
field. ***Tip:*** *click the* (+) *button to add a protein to the comparison slider on right.*

Switch Type: All Oligomerization Type: All

Show 20 entries

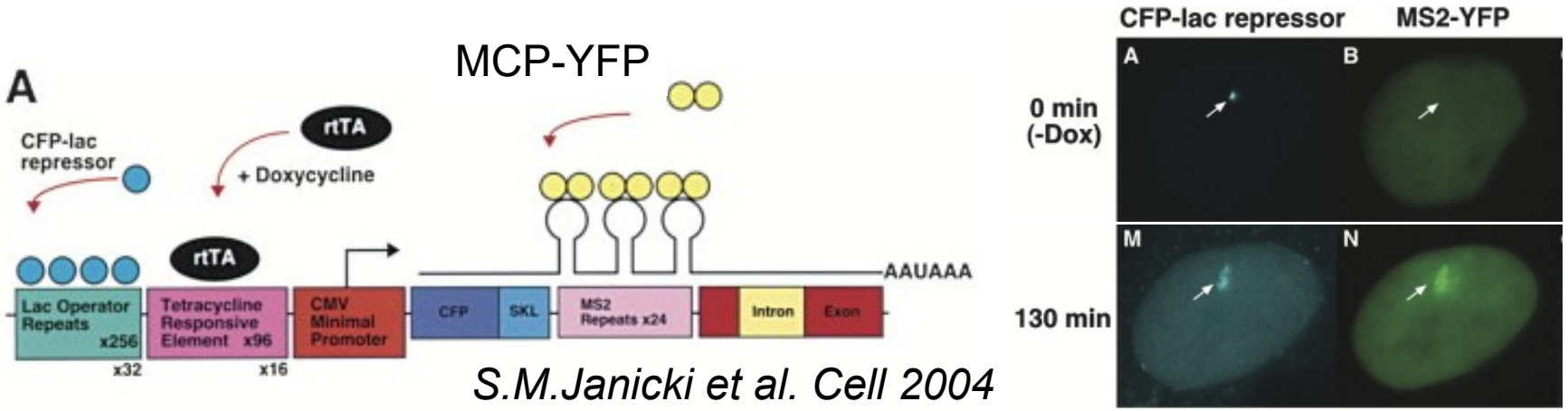
Name	λ_{ex}	λ_{em}	Stokes	EC	QY	Brightness	pKa	Aggregation
AausFP1	504	510	6	170,000	0.97	164.9	4.4	d
vsfGFP-0	485	510	25	209,916	0.76	159.54	4.84	d
LanYFP	513	524	11	150,000	0.95	142.5	3.5	t
YGFPdp (Default)	502	513	11	173,000	0.7	121.1		d
bfloGFPa1	500	512	12	120,900	1.0	120.9	3.0	d
RRvT	556	583	27	134,000	0.88	117.92	3.9	td
dLanYFP	513	524	11	125,000	0.9	112.5		d
dVFP	491	503	12	107,000	1.0	107.0		d

Compare at fpbase.org/chart



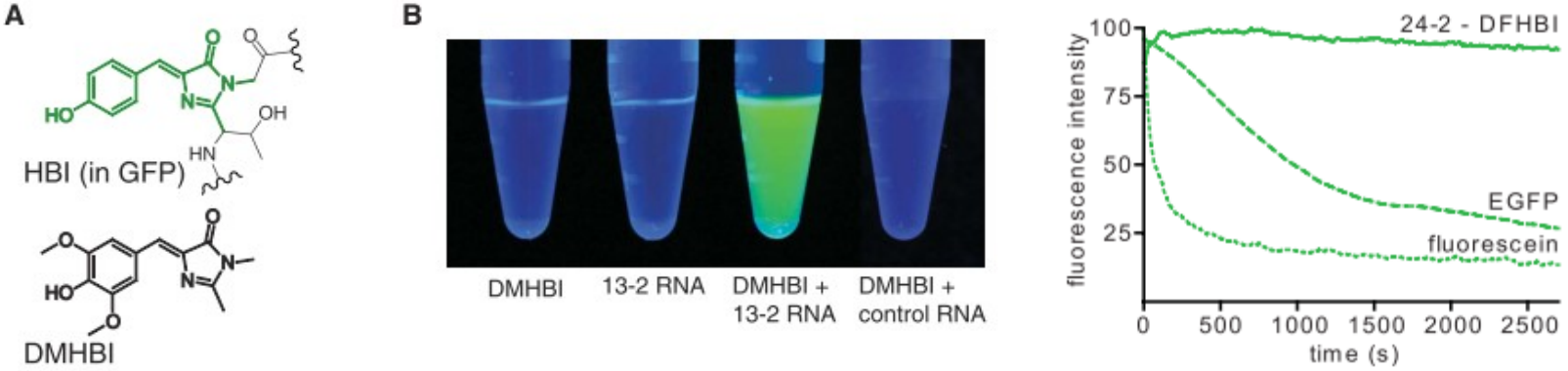
Fluorescent RNA imaging

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



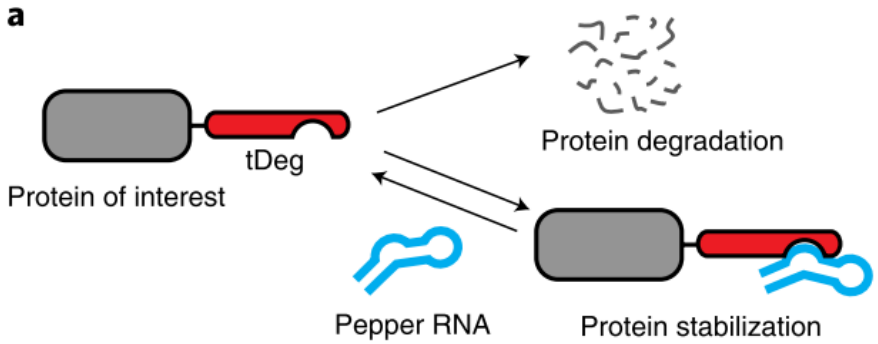
S.M.Janicki et al. Cell 2004

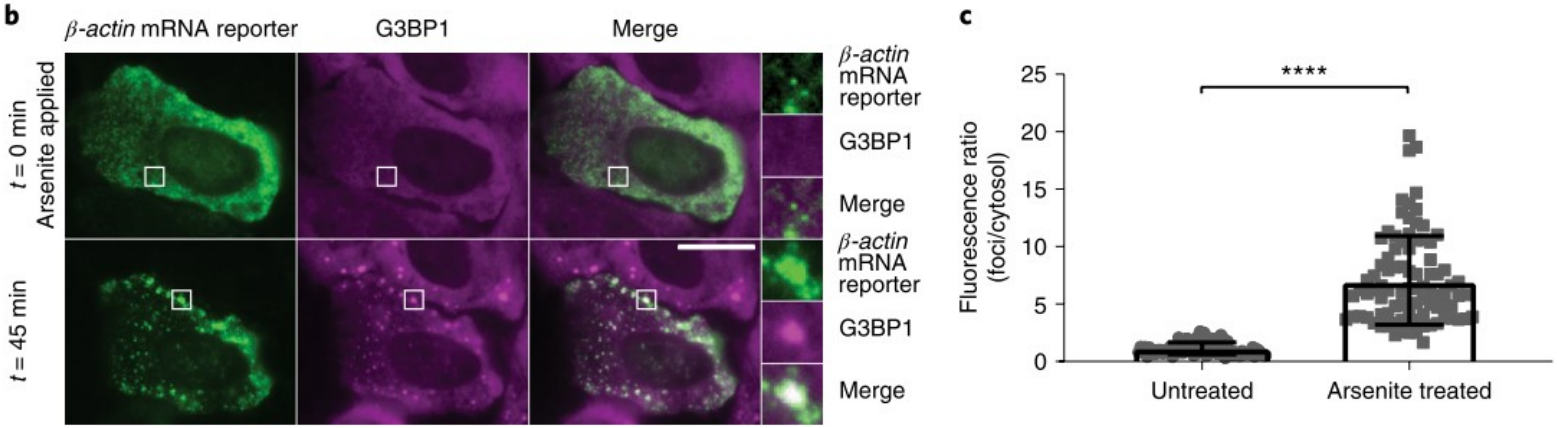
Spinach RNA 60 nt aptamer binds cell-permeable fluorogenic dye
Photostable. Used to label 5S RNA in HEK cells. *Samie Jaffrey Science 2011*
Also new bright panel of dyes binding RNA aptamers: Y. Yang Nat Biotech 2019



Degradation to improve signal:noise

J. Wu, SR Jaffrey, Nature Methods 2019





Conclusions

Choosing the right dye or fluorescent protein can make a big difference for:

sensitivity
signal stability
modification to molecule/cell function
by size or multimerization

Fluorescent probes allow more than just following location:

reporting cellular events
uncaging biomolecule function
controlling interactions and ion flux

References

Fluorescence probes

Molecular Probes Handbook, from Thermo Fisher.

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

Protein modification

Bioconjugate Techniques, 3rd 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) See table at
www.fpbase.org/chart

(ii) Quantitative assessment of fluorescent proteins
PJ Cranfill, DW Piston et al. Nature Methods 2016

(iii) as sensors: Designs and applications of fluorescent protein-based biosensors.
Ibraheem A, Campbell RE. Curr Opin Chem Biol 2010;14:30-6

Designing a rigorous microscopy experiment: Validating methods and avoiding bias.
Anna Payne-Tobin Jost and Jennifer C. Waters. J Cell Biol 2019, 218:1452–1466