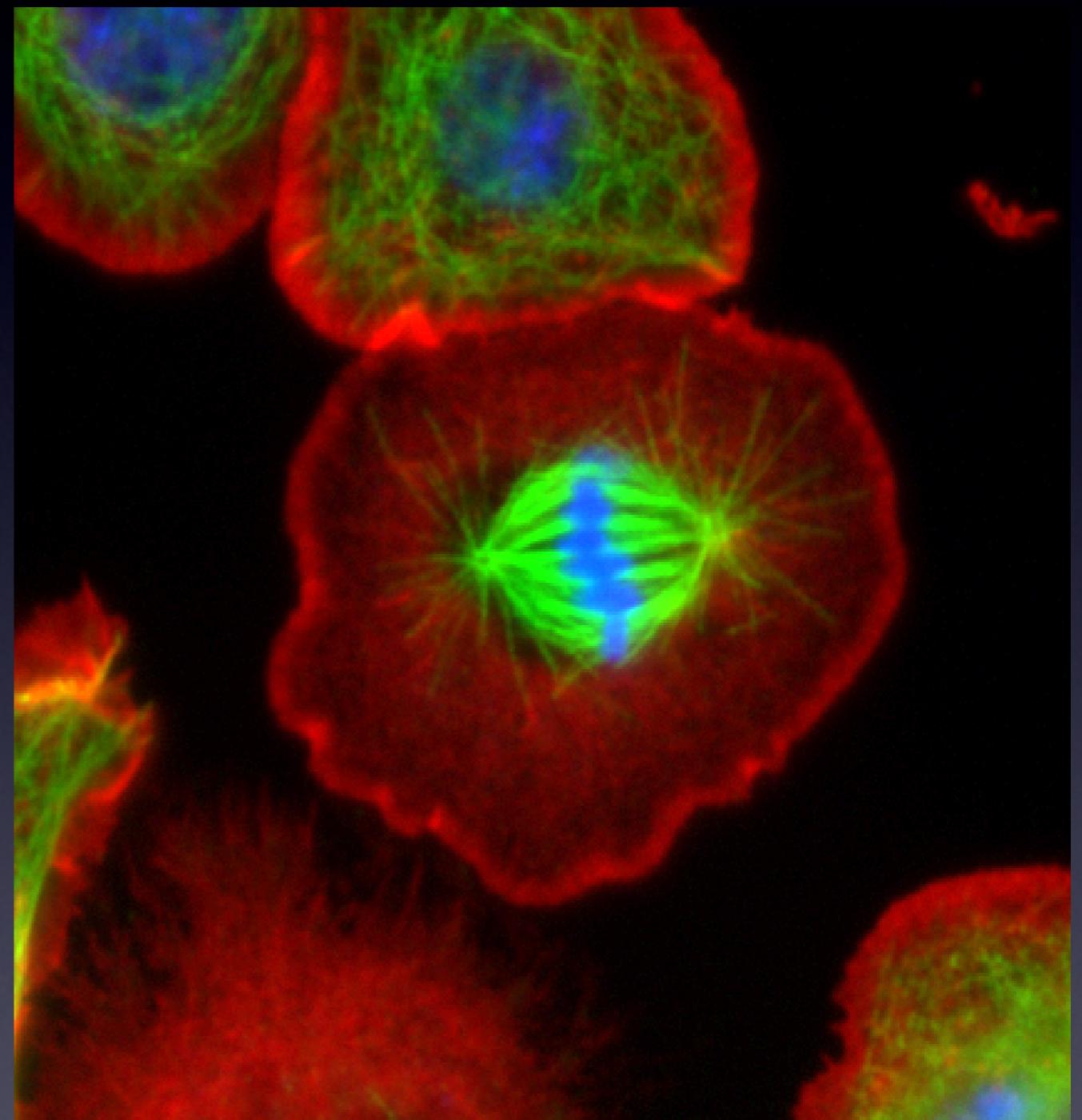


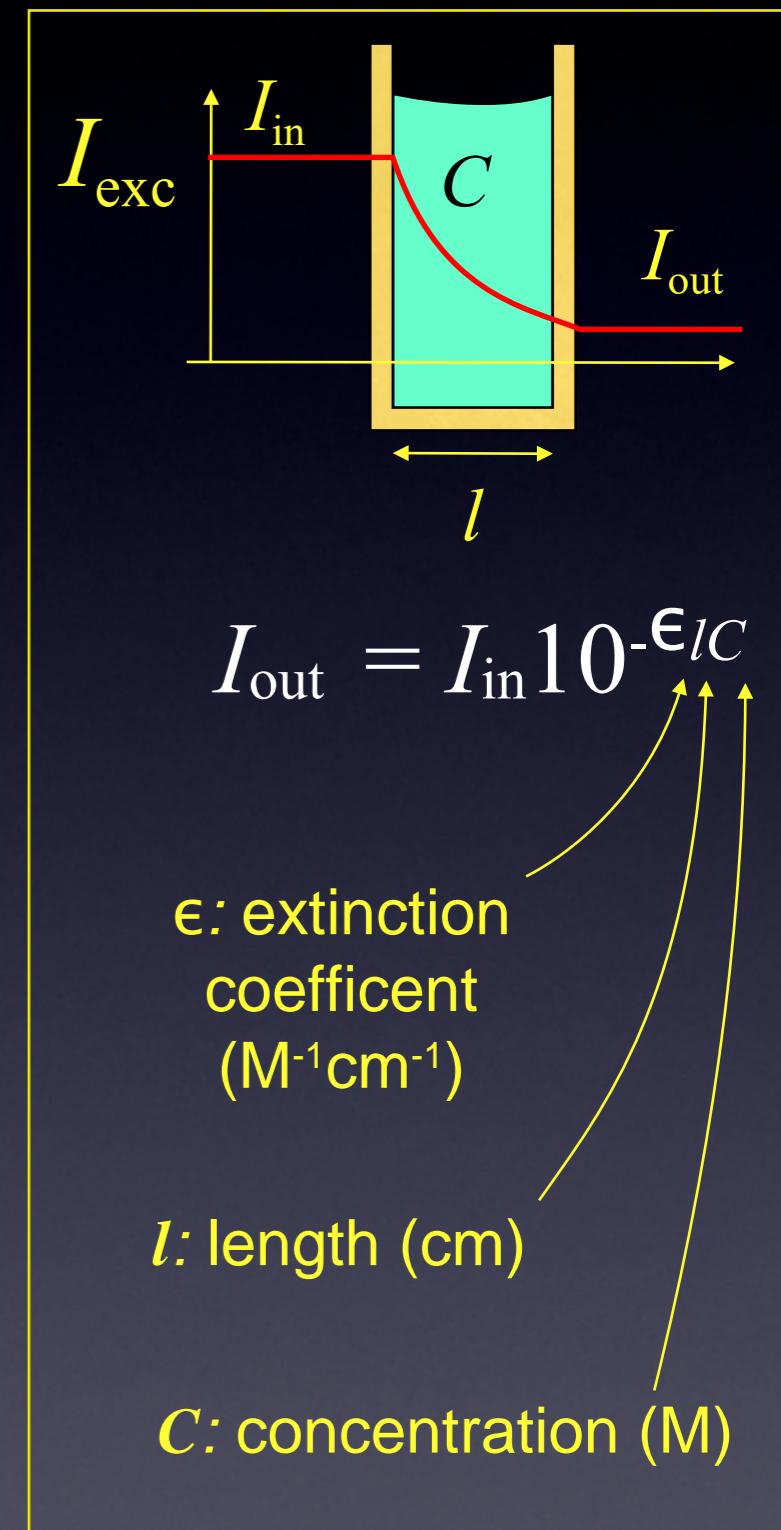
Fluorescent dyes and their use as biological probes

Nico Stuurman
Microscopy Course UCSF
April 19, 2010



Parameters of fluorescent dyes

- Excitation & emission maxima
- Extinction coefficient ϵ
 - \propto absorption cross section
 - $\epsilon \approx 50,000\text{--}100,000 \text{ M}^{-1}\text{cm}^{-1}$
- Fluorescence quantum yield Q_f
 - $= \# \text{ Photons emitted} / \# \text{ photons absorbed}$
 - $Q_f \approx 25\text{--}90\%$
 - Brightness $\propto \epsilon Q_f$
- Photo-bleaching quantum yield Q_b
 - $=$ average # of photons emitted per molecule before bleaching.
 - Depends on environment.
 - $\propto Q_f / Q_b$



Fluorescent dyes/probes

Other important factors

- **Lifetime**

Typically nanoseconds, dye specific

Strongly influenced by environment

- **Sensitivity to environment**

pH, Ca-ions,

- **Coupling**

*Amino- or cystein coupling, number of dyes per molecule,
expression as fusion protein*

- **Delivery Method**

Cell permeability, micro-injection, genetic expression, etc.

Fluorescent Dye Types

- Organic dyes
- Phycobiliproteins
- Lanthanide Chelates
- Fluorescent Nanocrystals
- Fluorescent Proteins

Organic Dyes

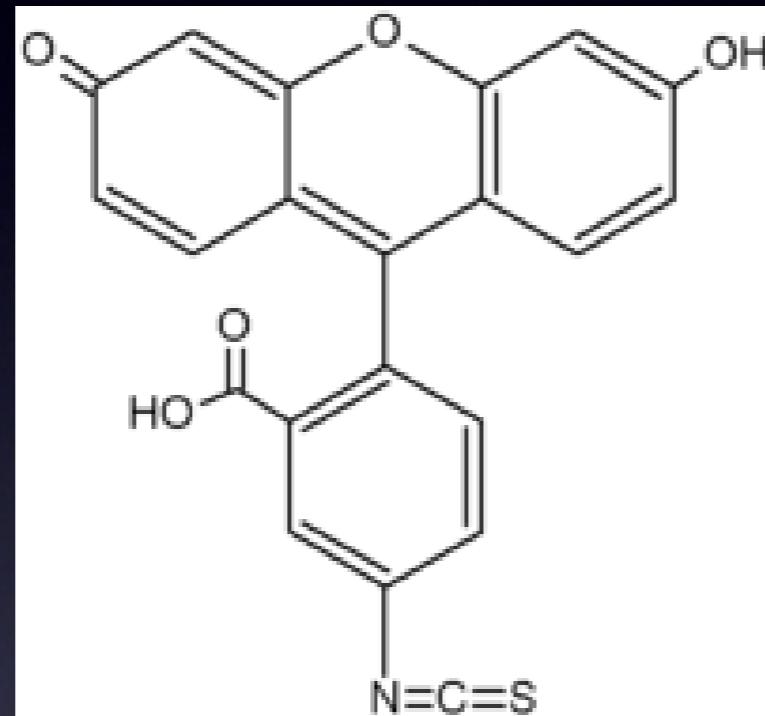
The Classics

Coumarin

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

332/456
QY 0.77

Fluorescein



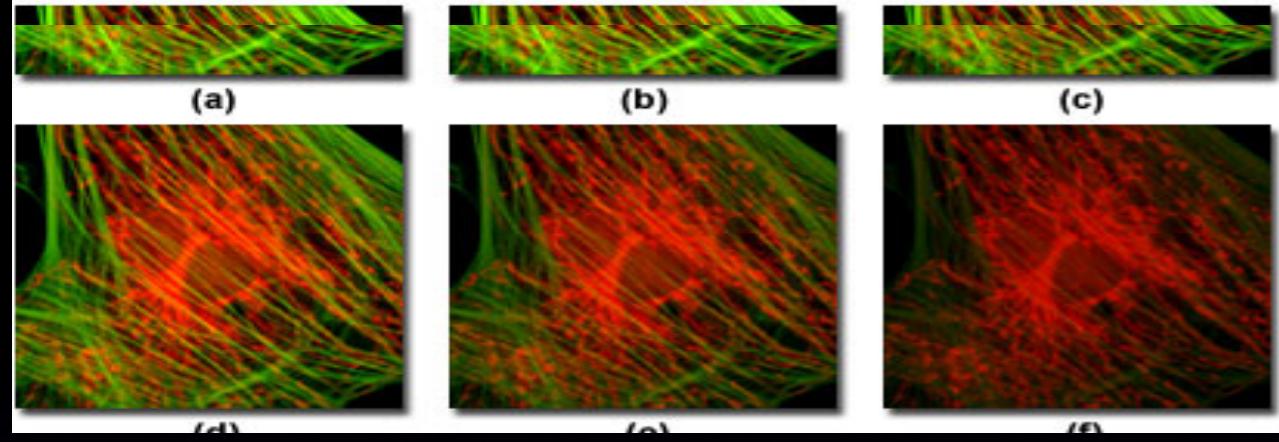
490/520
QY 0.925

Rhodamine

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

554/573
QY 0.28

- Systems of conjugated bonds that share electrons
- Larger system → longer wavelength

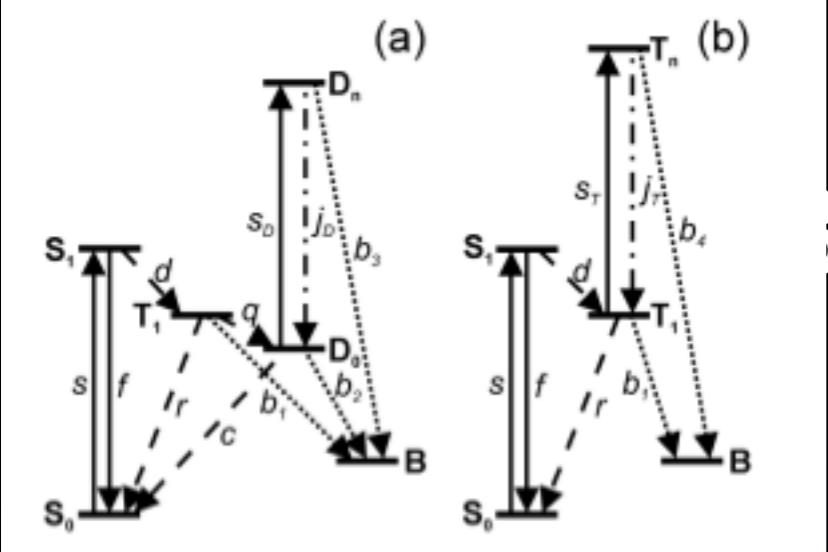


Enemy: bleaching

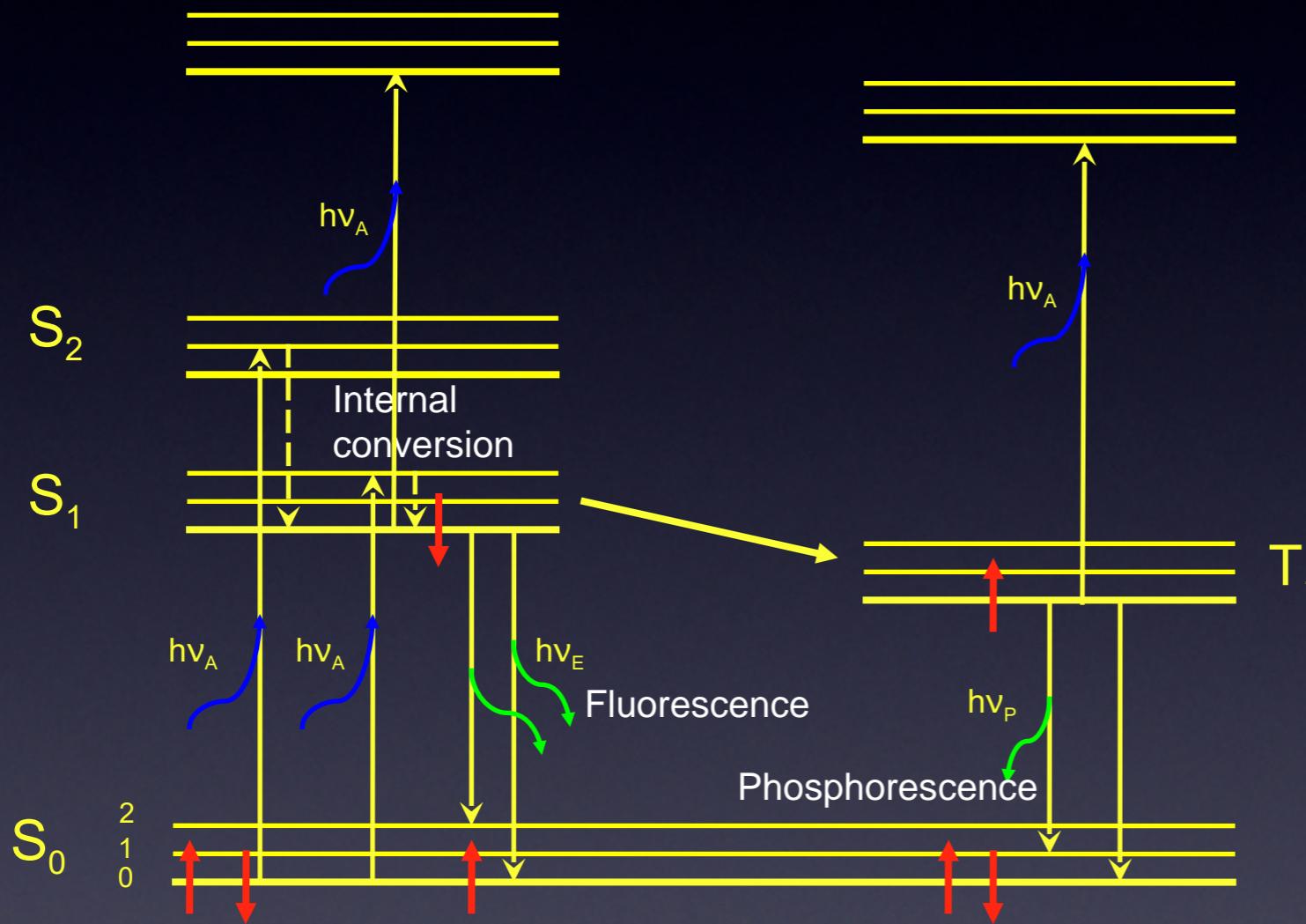
Decrease in emission intensity after exposure

Exciting a molecule once has a probability Q_b of killing it

Each molecule will emit only a finite number of photons



Bleaching - Mechanisms



Zondervan et al., J. Phys. Chem. A , 2004, 108:1657–1665.

O₂

•O₂ Singlet Oxygen

Except when used in CALI
(Chromophore-assisted light inactivation)

What to do about photo-bleaching?

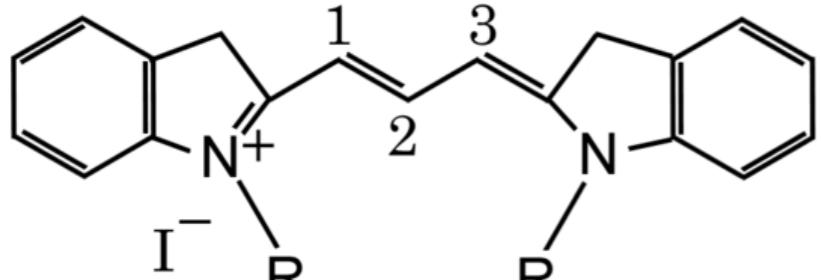
- Select fade-resistant dyes
- Label densely
- Decrease bleaching by anti-fade mounting media
 - Glycerol
 - Oxygen scavengers
 - Free-radical scavengers
 - Triplet state quenchers

Note: some anti-fade agents quench some dyes.

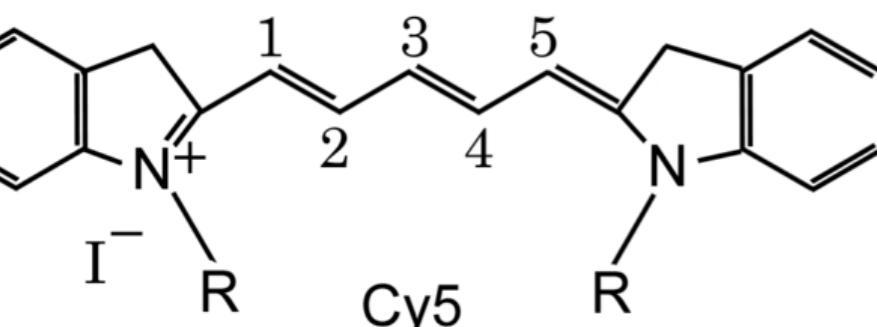
- Budget the photons you have
- Only expose when observing
- Minimize exposure time & excitation power
- Use efficient filter combinations
- Use highly QE, low noise camera
- Use simple light path

Organic Dyes

Cyanine dyes



Cy3

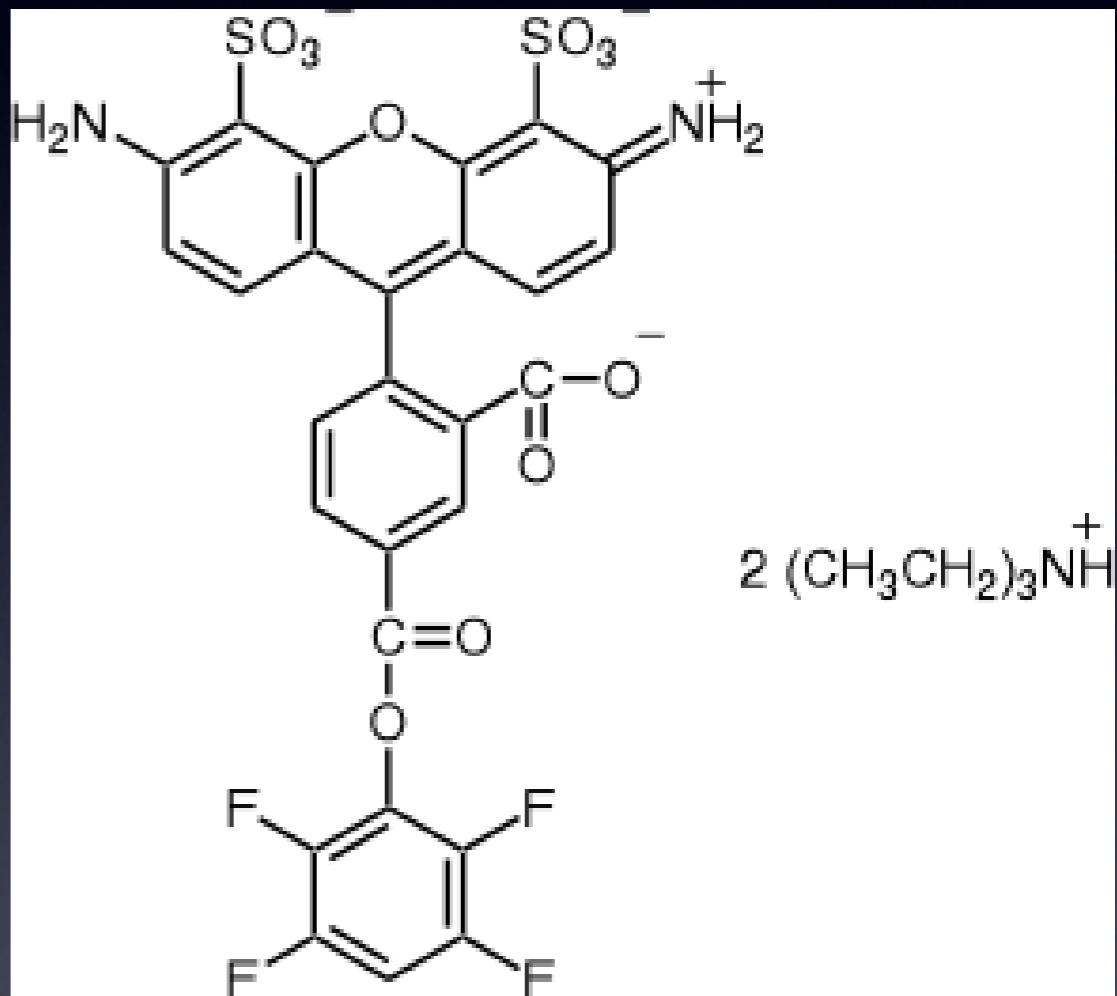


Also, Cy2, Cy5.5

554/568
QY 0.14

652/672
QY 0.18

Alexa dye series



$$2 (\text{CH}_3\text{CH}_2)_3\text{NH}^+$$

499/517
QY 0.60

Organic Dyes

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

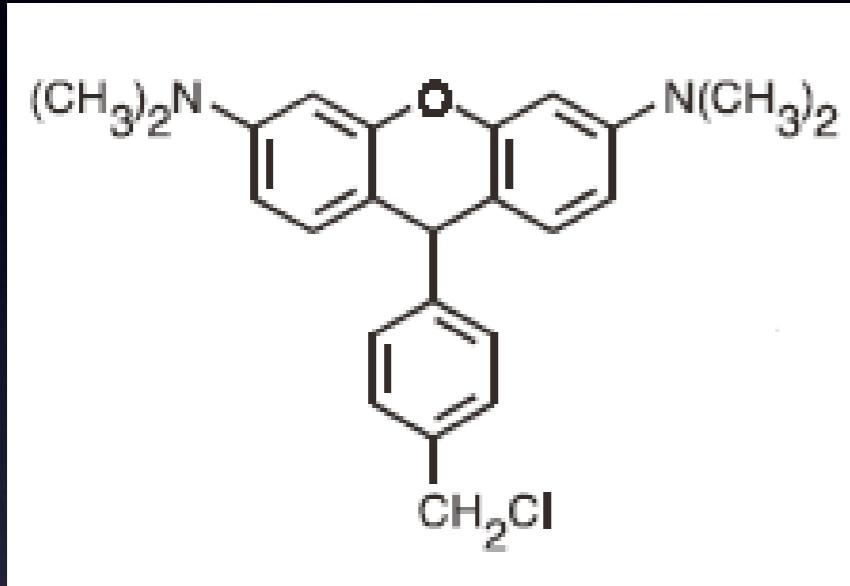
Parameters of some fluorophores

Dye	I_{ex}	I_{em}	ε	QY	brightness
DAPI	350	470	27000	0.58	15.7
Fluorescein	490	520	67000	0.71	47.6
Alexa 488	494	517	73000	0.6	43.8
Rhodamine	554	573	85000	0.28	23.8
Cy3	554	568	130000	0.14	18.2
Cy5	652	672	200000	0.18	36
GFP	488	507	56000	0.6	33.6
mCherry	587	610	72000	0.22	15.8
CFP	433	475	32500	0.4	13
YFP	516	529	77000	0.76	58.5

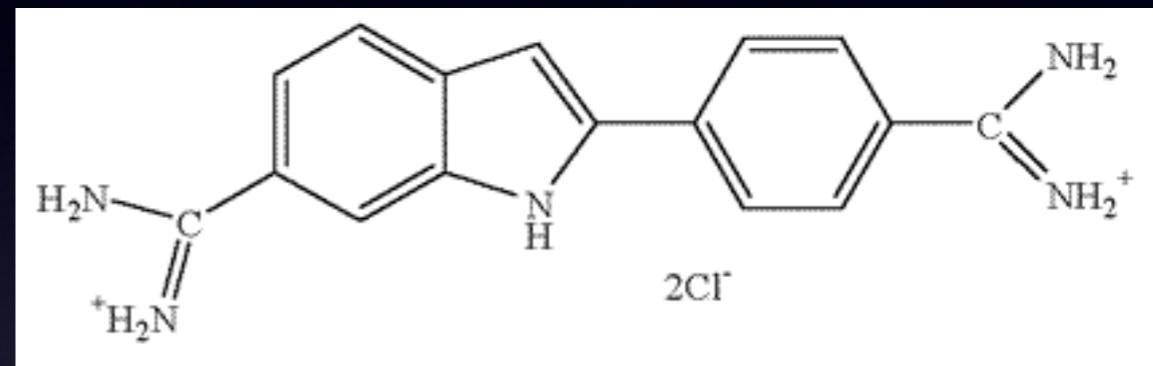
From Dye to Probe:

Small dyes that are Probes

Small dyes that are Probes



Mitotracker
Oxidized in mitochondria in
fluorescent compound



DAPI
Hoechst33258
Hoechst 33342
~20 fold enhancement
TOTO, YOYO

Conjugation of organic dyes

Chemistry/Method

Amino groups (lysine): succinimidyl ester or isothiocyanate

Example:

Dynein driven gliding of microtubules labelled with TMR on lysine residues.

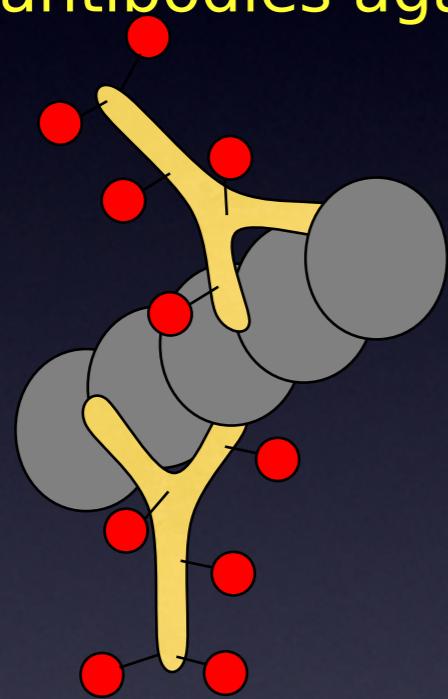
QuickTime™ and a decompressor are needed to see this picture.

Targets

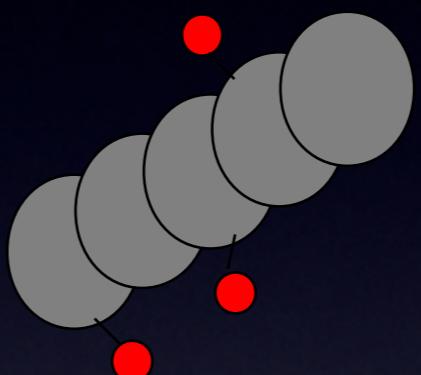
- Small molecules, i.e. phalloidin, taxol
- Proteins: labeling site unspecific
- Antibodies: direct/indirect labeling (Label density)

Fluorescent labeling

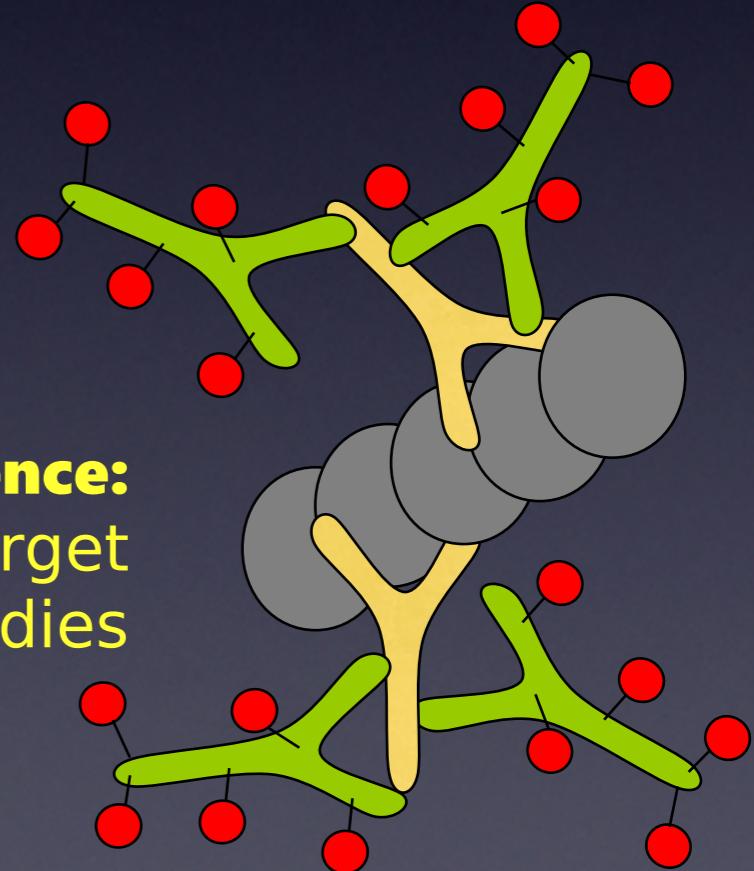
Direct immunofluorescence:
labeled antibodies against target

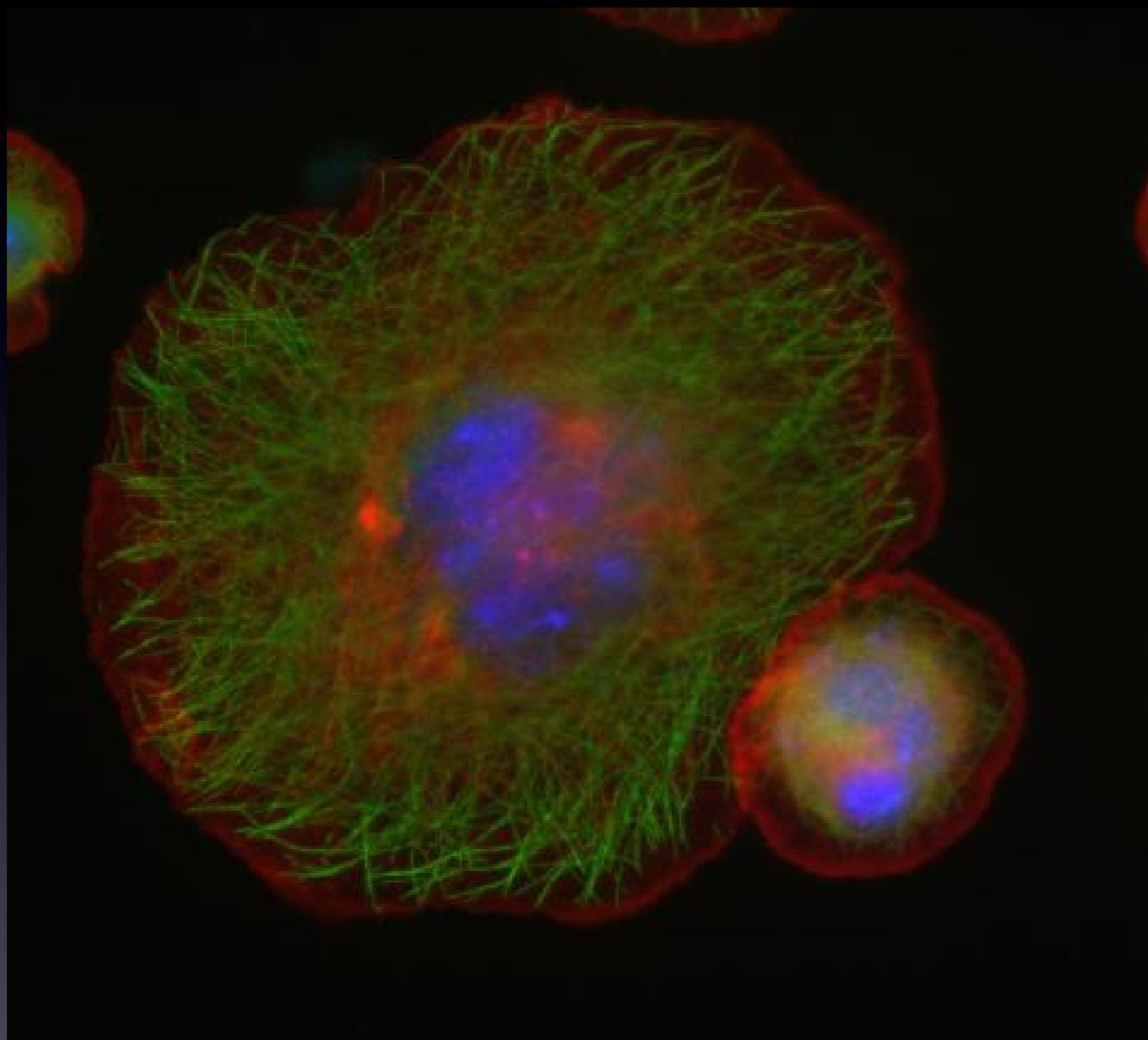


Direct labeling (& microinjection)
of target molecules



Indirect immunofluorescence:
Unlabeled antibodies against target
Labeled antibodies against those antibodies



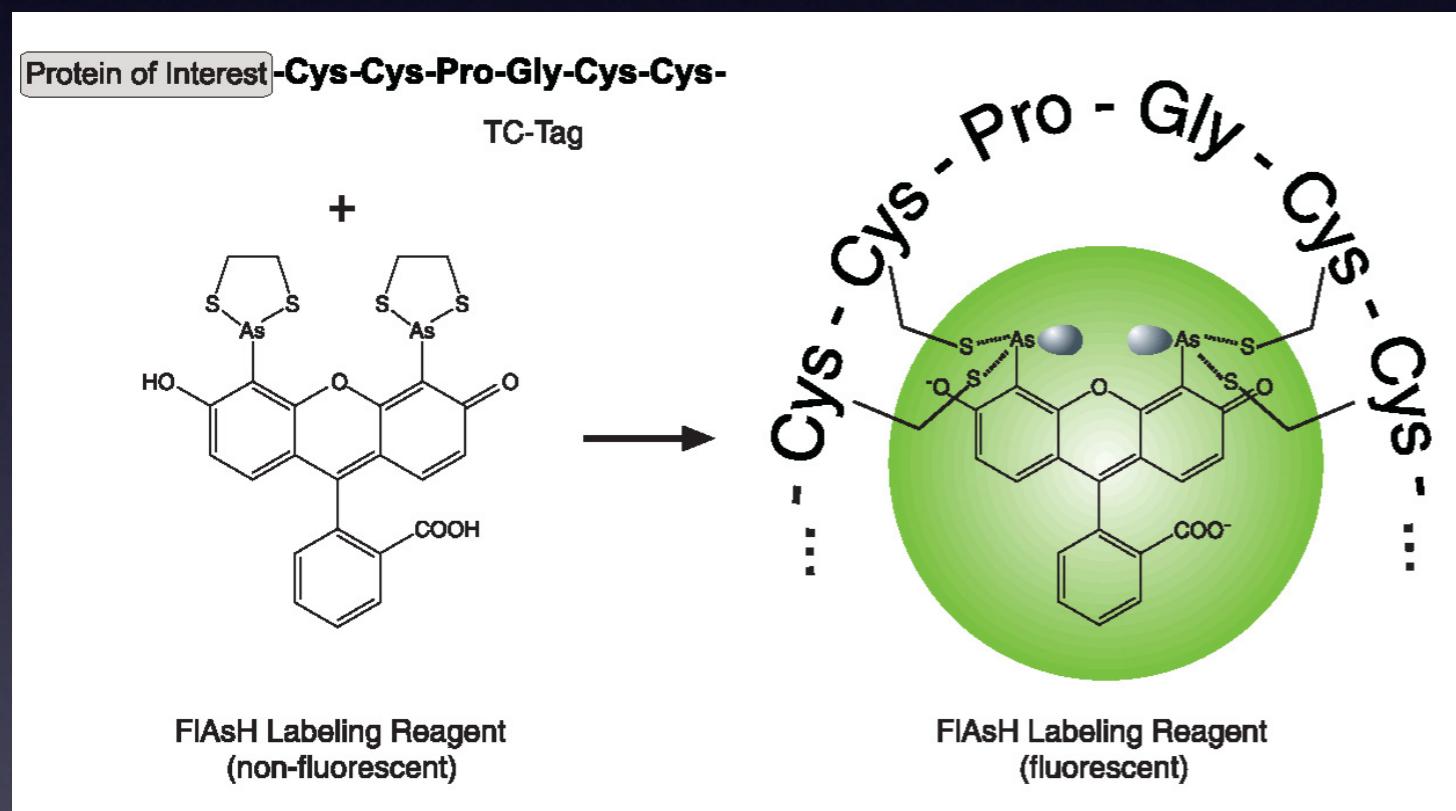


Site-Specific Labeling

Chemistry/Method

Sulfhydryl groups (cystein): maleimide

Engineer Cys-light version of target protein

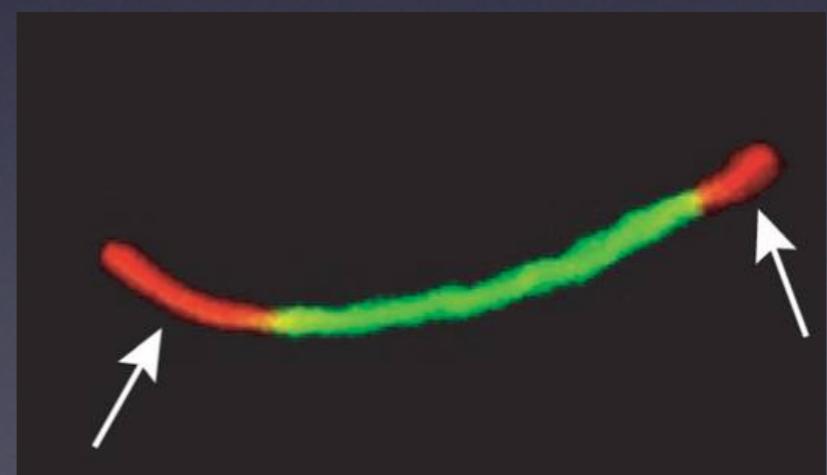


Example:

Newly synthesised connexins (ReAsH:Red) are added to the outer edges of existing gap junctions (FlAsH:Green). Gaietta et al 2002

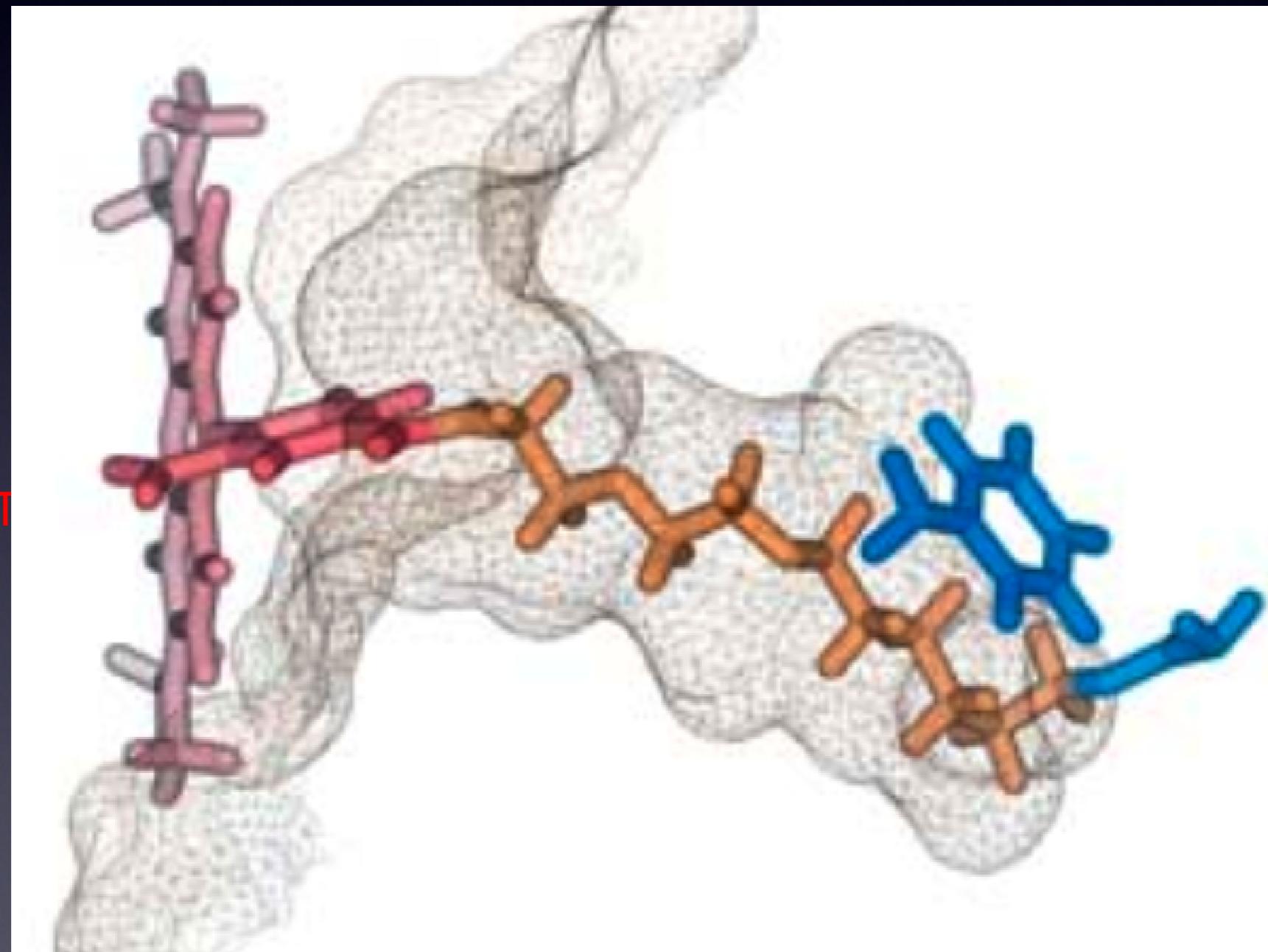
FlAsH/ReAsH

Labeling protein with tetra-cysteine motifs (Tsien lab/Invitrogen):



Covalent attachment of dyes to genetic tags

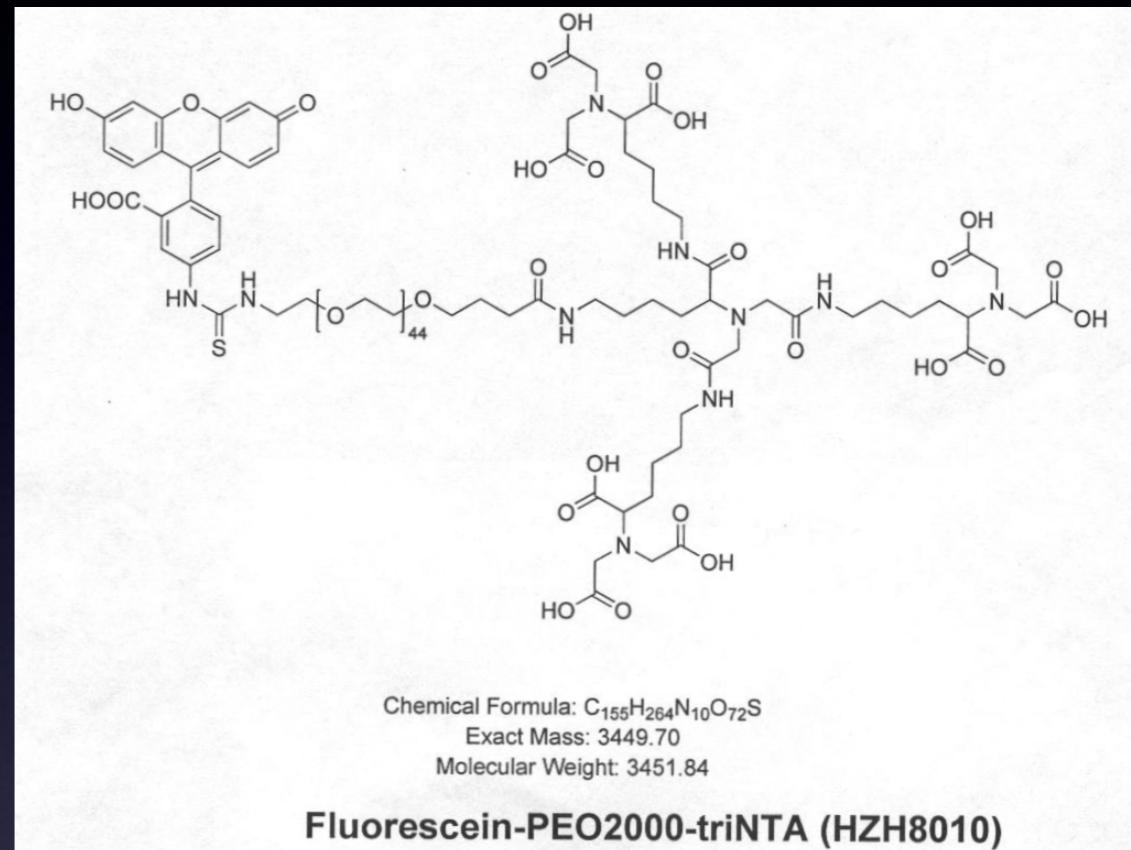
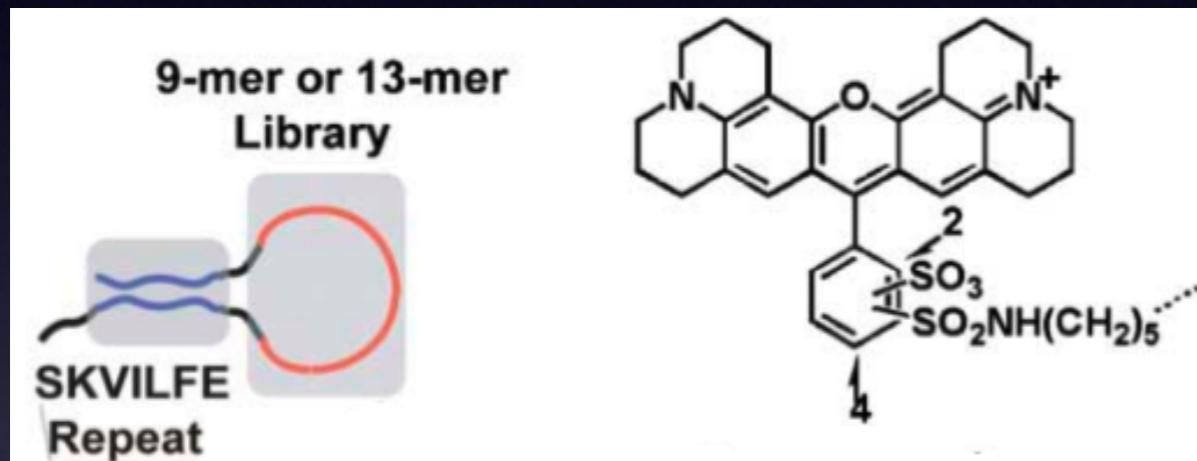
- **HaloTag (Promega) – Dehalogenase**
- **SNAP Tag (Covalys) - O₆-alkylguanine-DNA alkyltransferase (AGT)**



- labels with any (biotin/Cy5)
- Multiple colors
- organic dyes can be brighter
- Stoichiometry
- pulse chase possible

Non-Covalent Labeling

Peptide sequences evolved to bind fluorophores

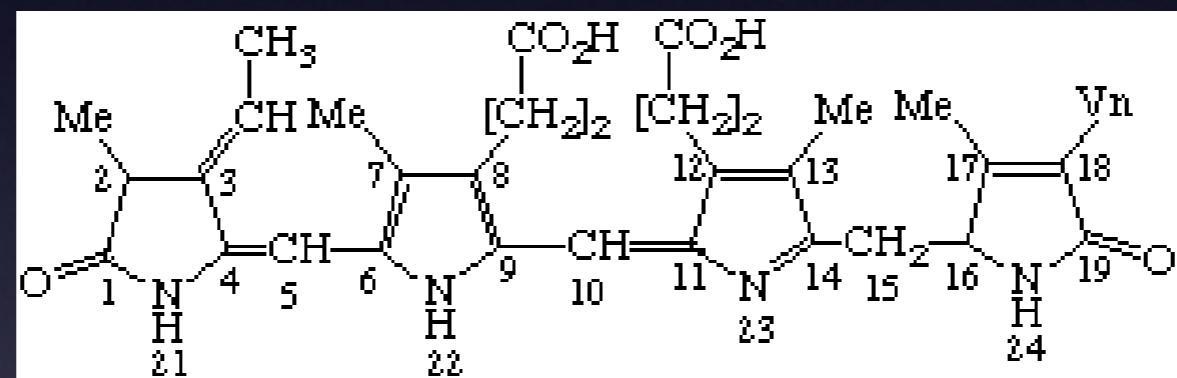


Tris or Tetra-NTA Affinities down to the low pM

High-affinity drug-binders FKPB12(F36V) - 12 kDa tag - SLF' binds at 94 pM

Phycobiliproteins

- Proteins present in cyanobacteria and certain algae
- Chromophore: covalently bound phycobilins



- High QY and Absorption coefficient, long lifetime
- Large -> Antibody conjugate

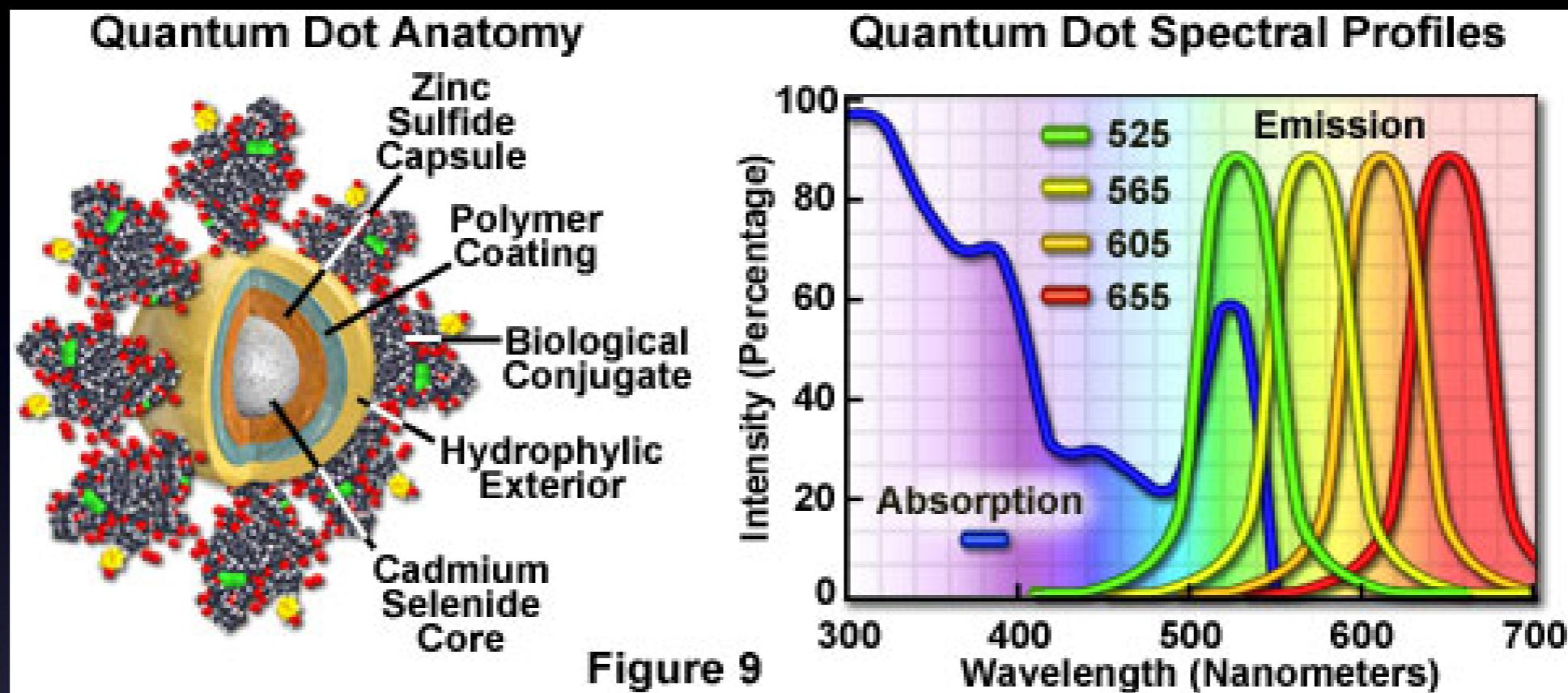
Lanthanide Chelates

- Chelated rare earth ions
- Large Stokes shift, long lifetime

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Chelate	Excitation (nm)	Emission (nm)
Europium (Eu)	340	615
Samarium (Sm)	340	642
Terbium (Tb)	300	545

Quantum-dots



nanometre-scale crystals composed of atoms of an inorganic semiconductor material

Advantages

- Very bright
- Very photostable
- Excitation possible at a single wavelength
- Visible in electron microscope

Disadvantages

- Large size
- Multivalent linkage

Quantum dot labeling

- Biotin/Streptavidin Linkage
- Biotin maleimide (in vitro)
- Biotin HaloTag/SNAP
- Biotin carrier protein
- BiotinLigase/AP1

- Antibody Conjugates – immunohistochemistry
- Direct linkage to proteins/peptides – targeting to cell compartments

QuickTime™ and a
decompressor
are needed to see this picture.

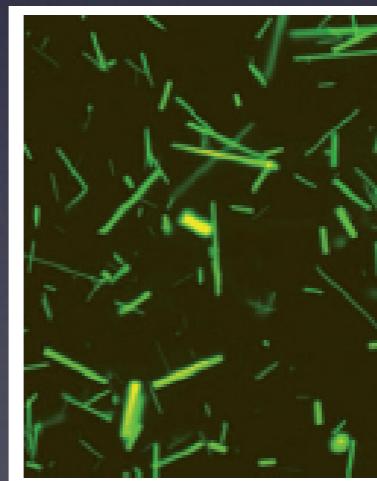
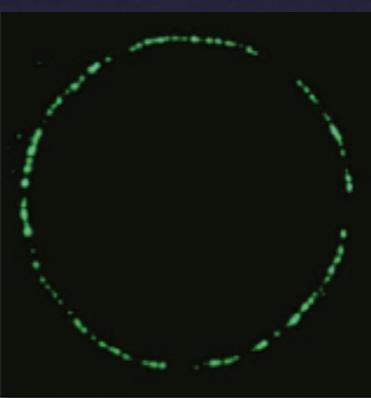
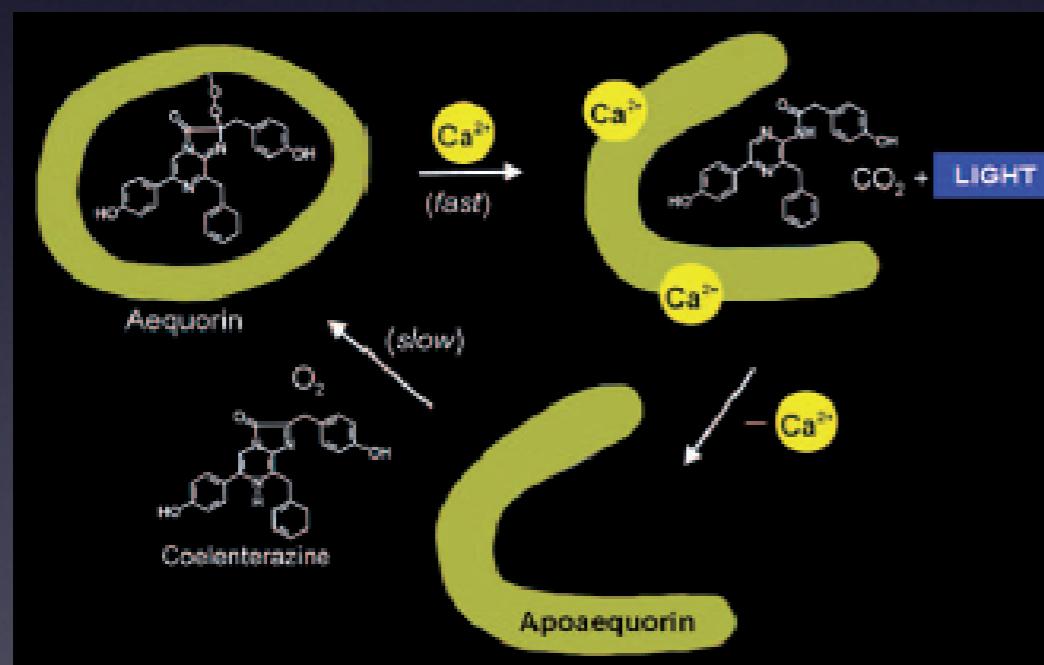
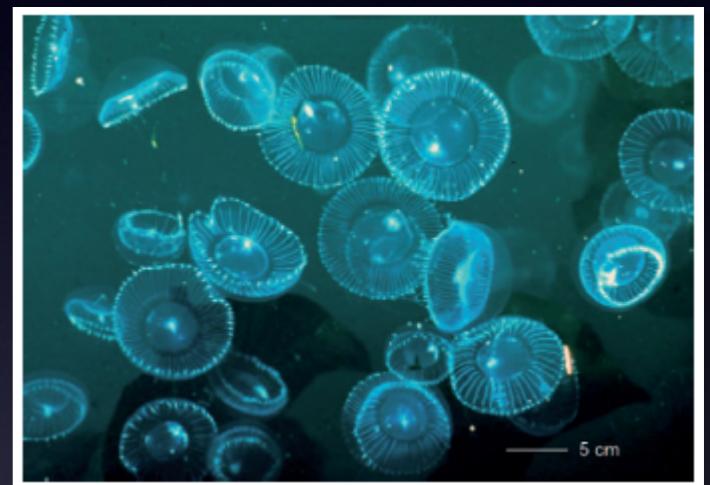
Qdot labelled dynein via
HaloTag:Biotin:Streptavidin
linkage moving on axonemes

proteins

Discovery



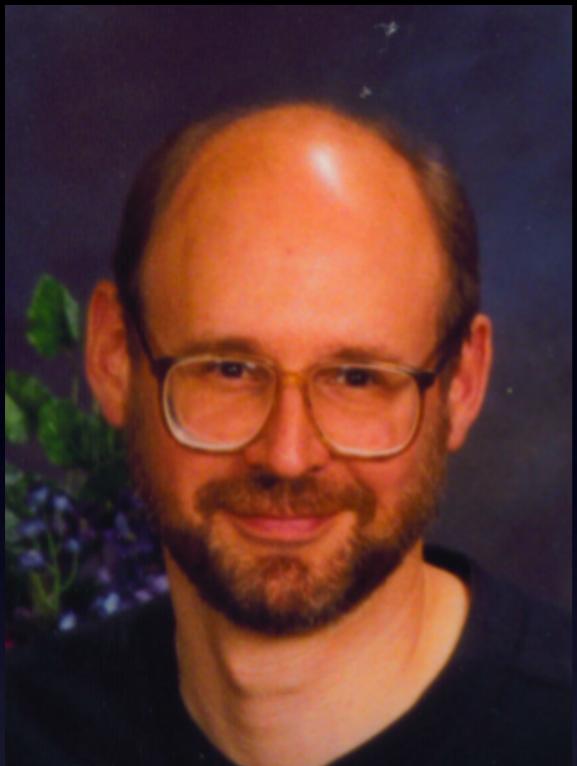
Discovery



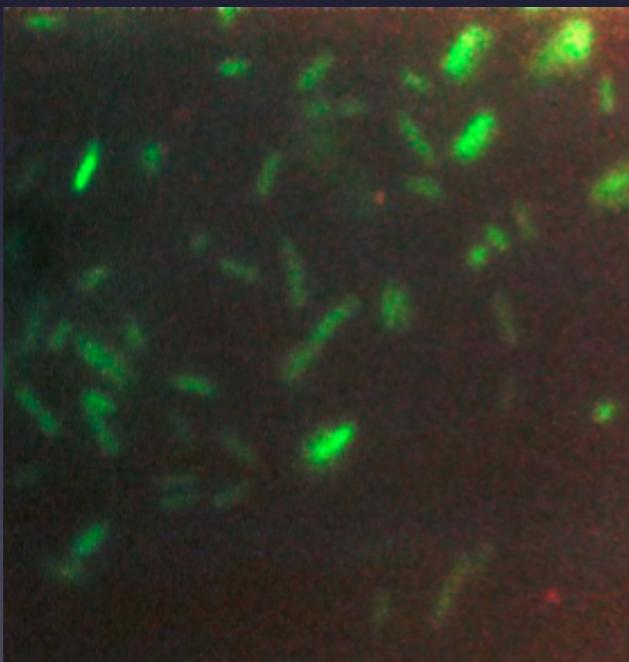
GFP (100 mg)
↓
Denature at 90 °C
Digest with papain
Extraction with butanol at pH 1
TLC purification
↓
Isolated chromophore (0.1 mg)

Images from Osamu Shimomura

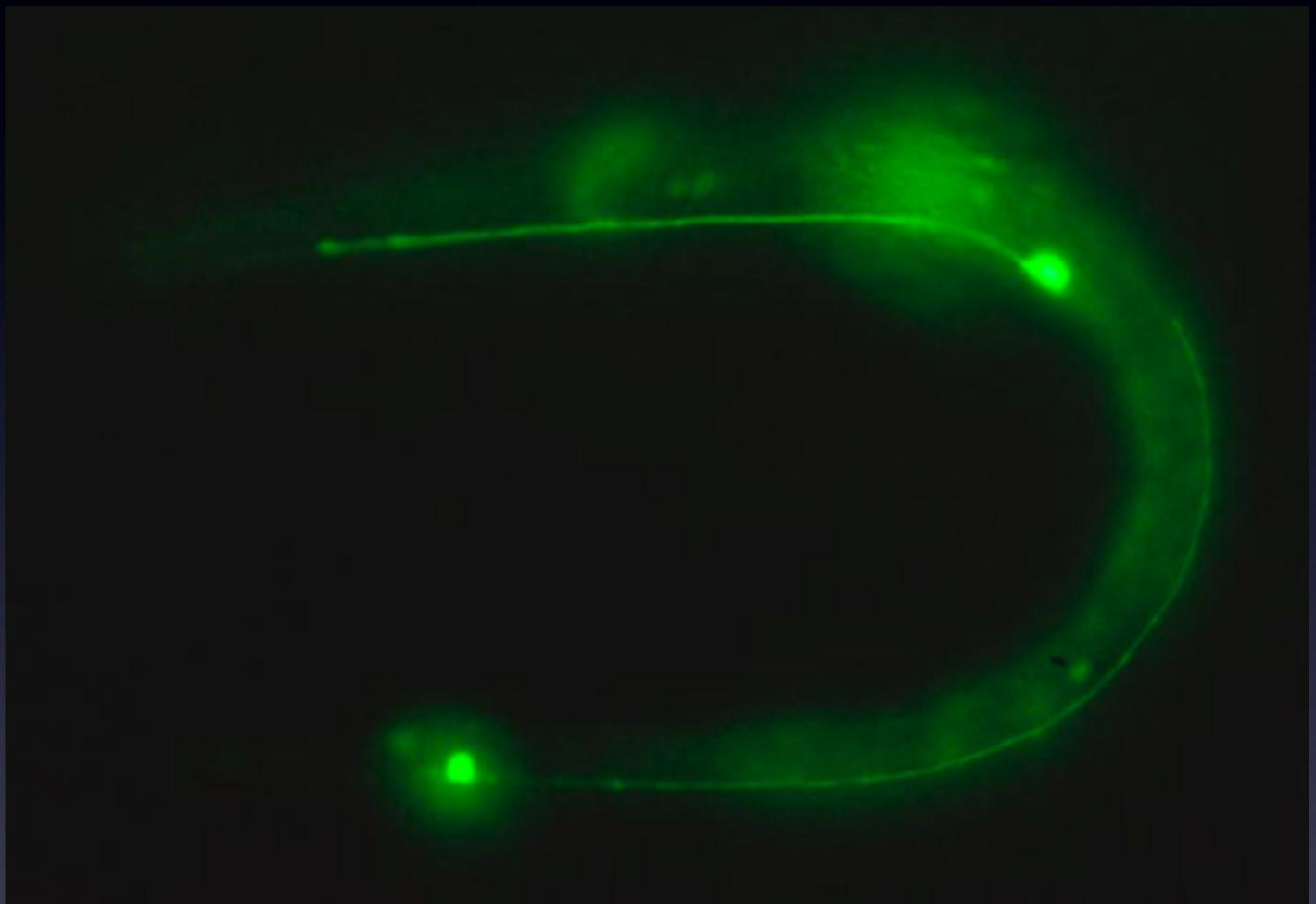
No co-factors needed!



Douglass Prasher



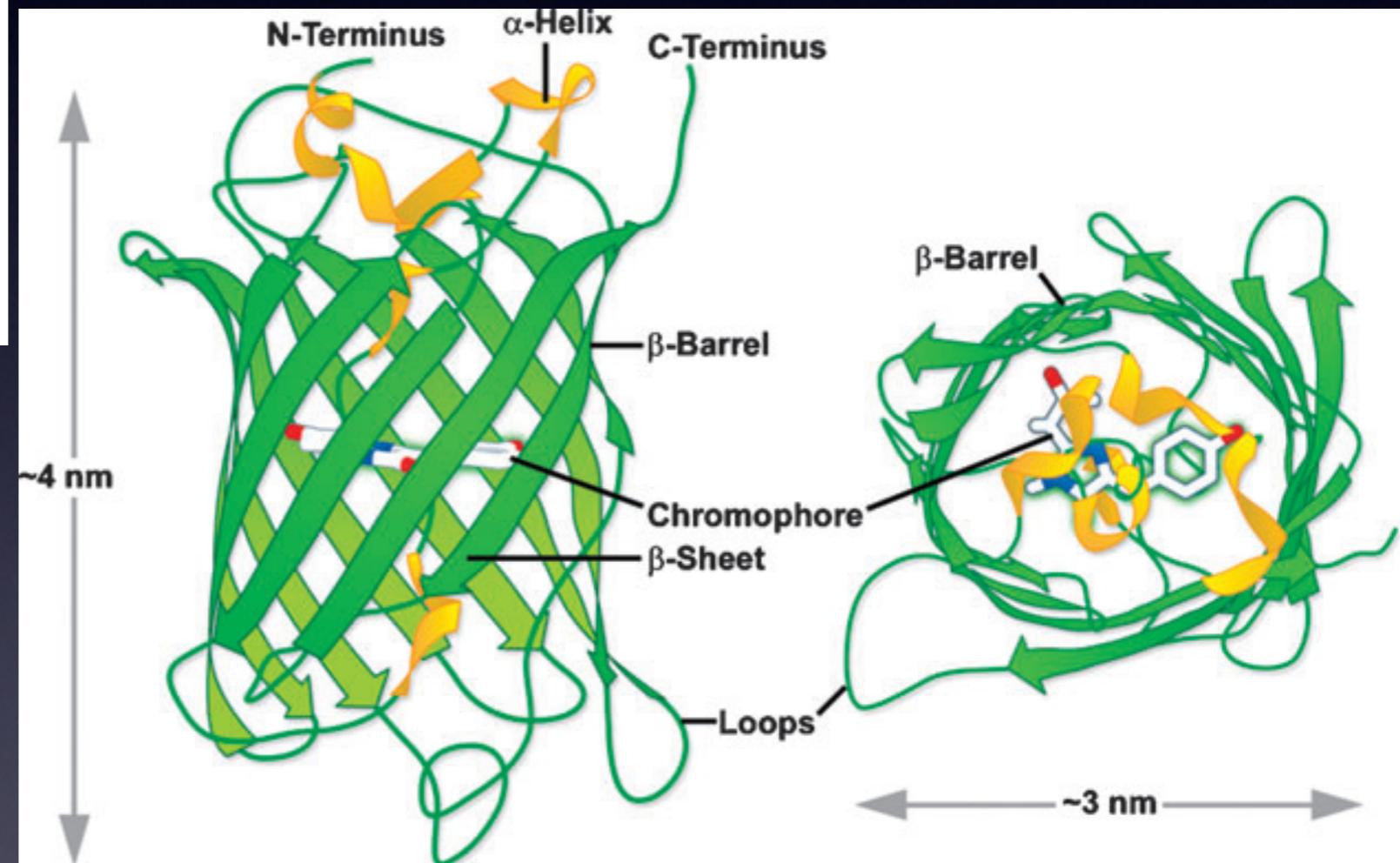
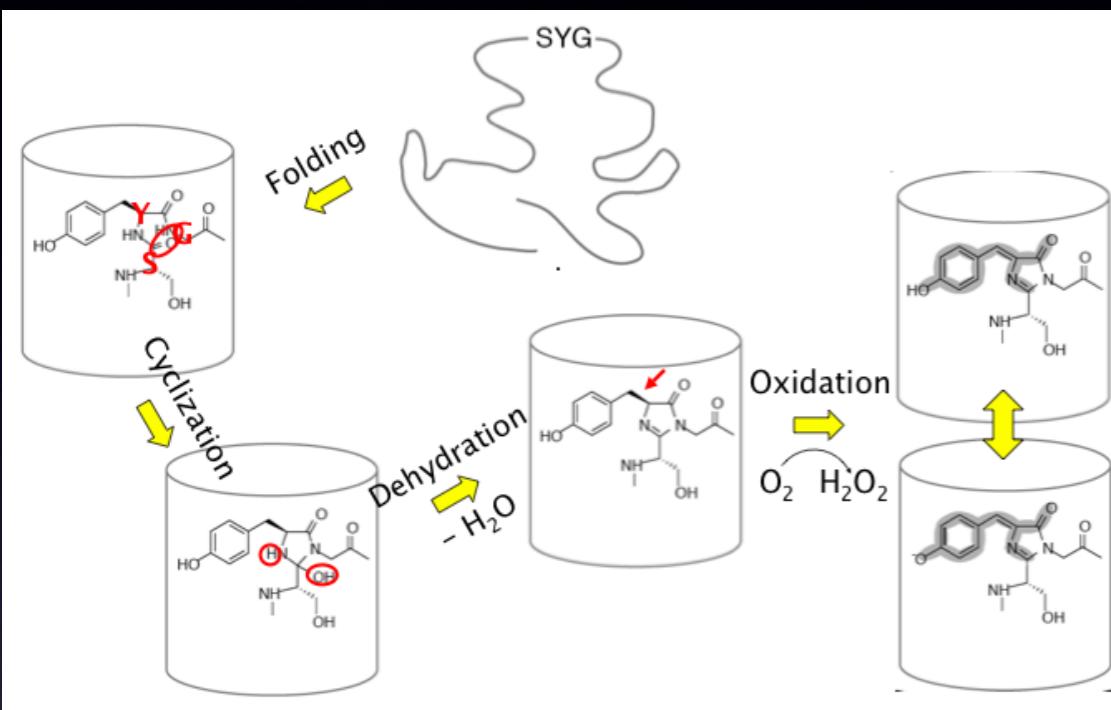
First GFP expression
in *E. coli*



and *C. elegans*

Images from Martin Chalfie

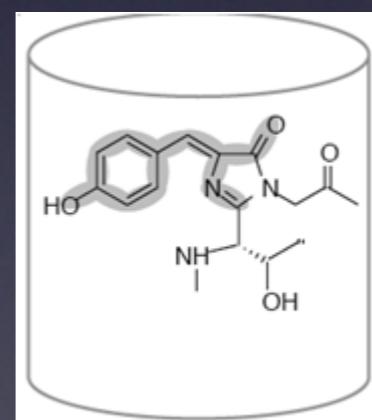
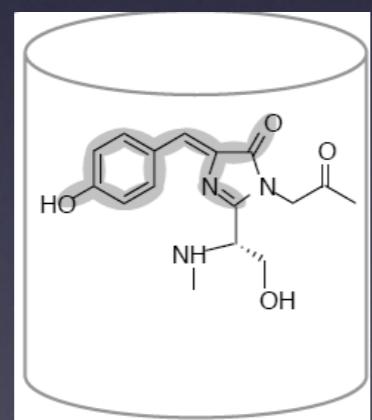
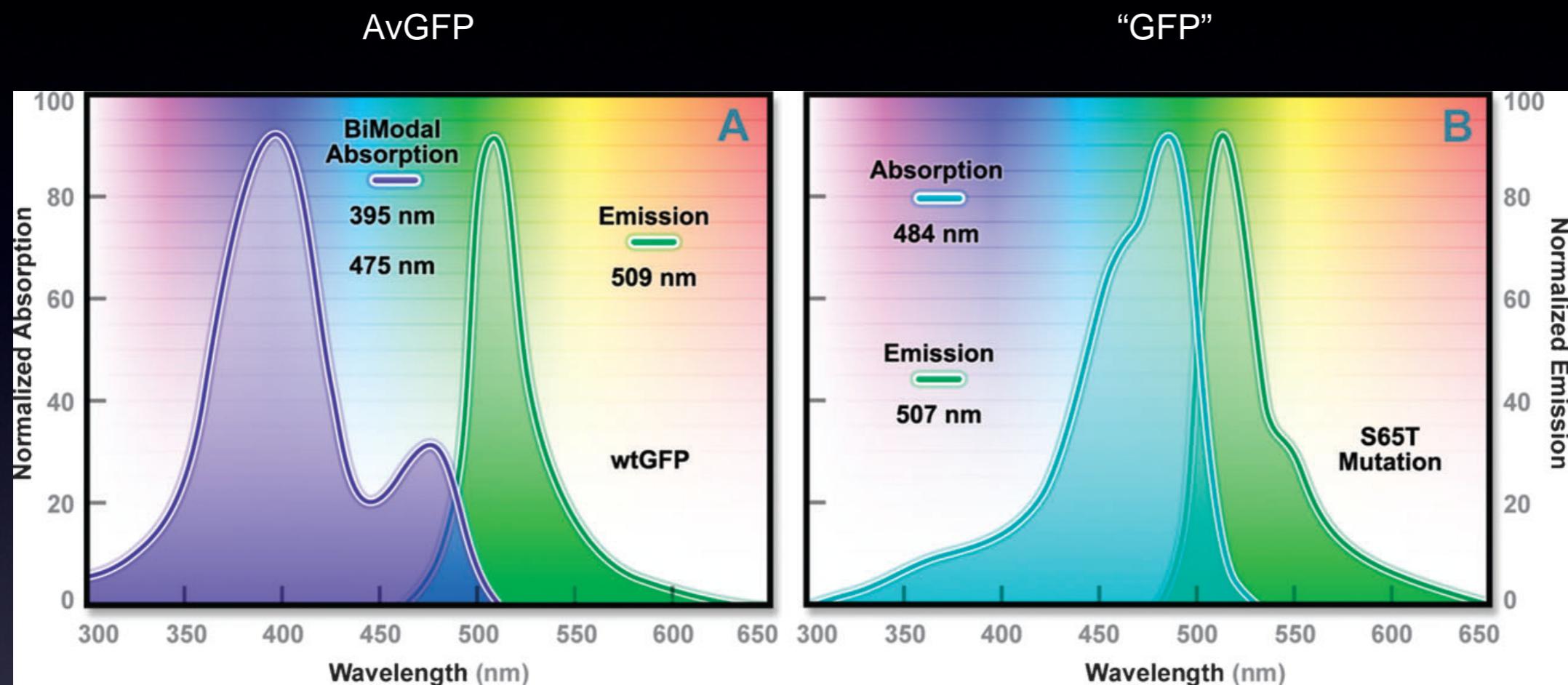
GFP



Day and Davidson, *Chem Soc Rev*, 2009(38) 2887

~240 Amino acids, 27 kDa

Improving the wild type GFP



S65T

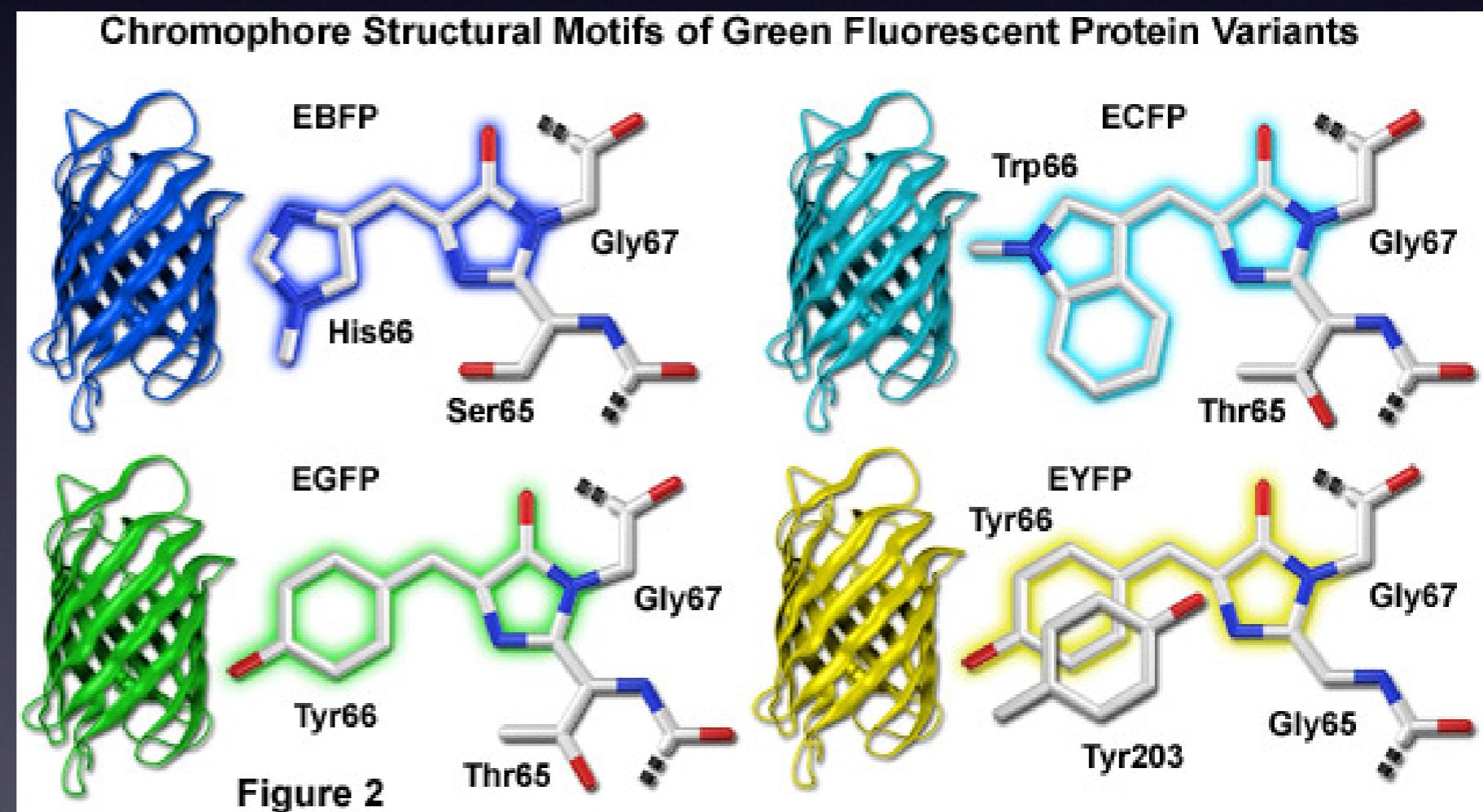
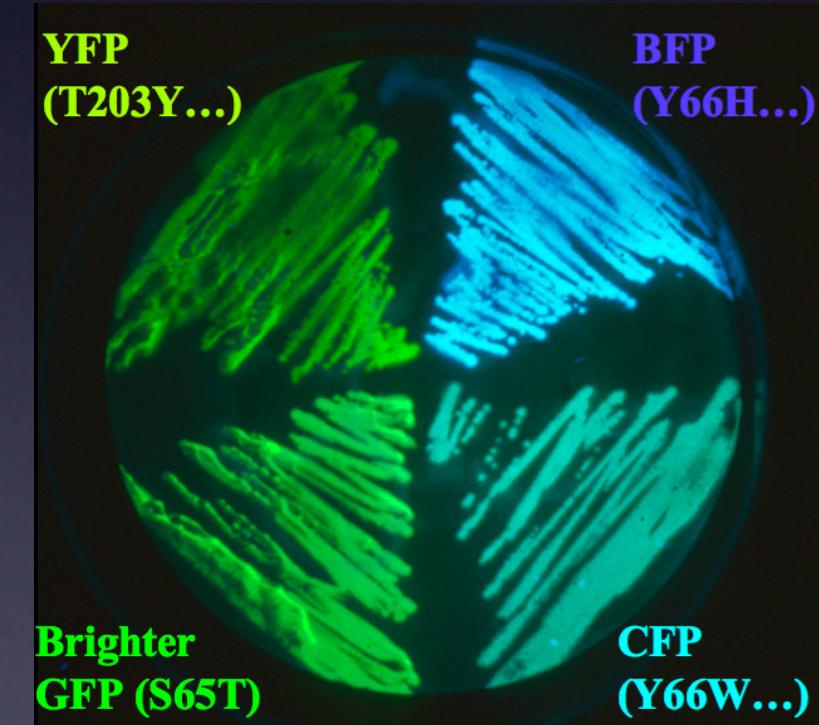
GFP-Actin in Drososophila S2 cells

QuickTime™ and a
Graphics decompressor
are needed to see this picture.

Steve Rogers, 2002

Jellyfish (*Aequorea*) fluorescent protein family

AvGFP
↓
GFP (S65T), EGFP (S65T, F64L)
↓
BFP, CFP, YFP
↓
Cerulean , CyPet, Sapphire, Venus, Citrine ,
Ypet...

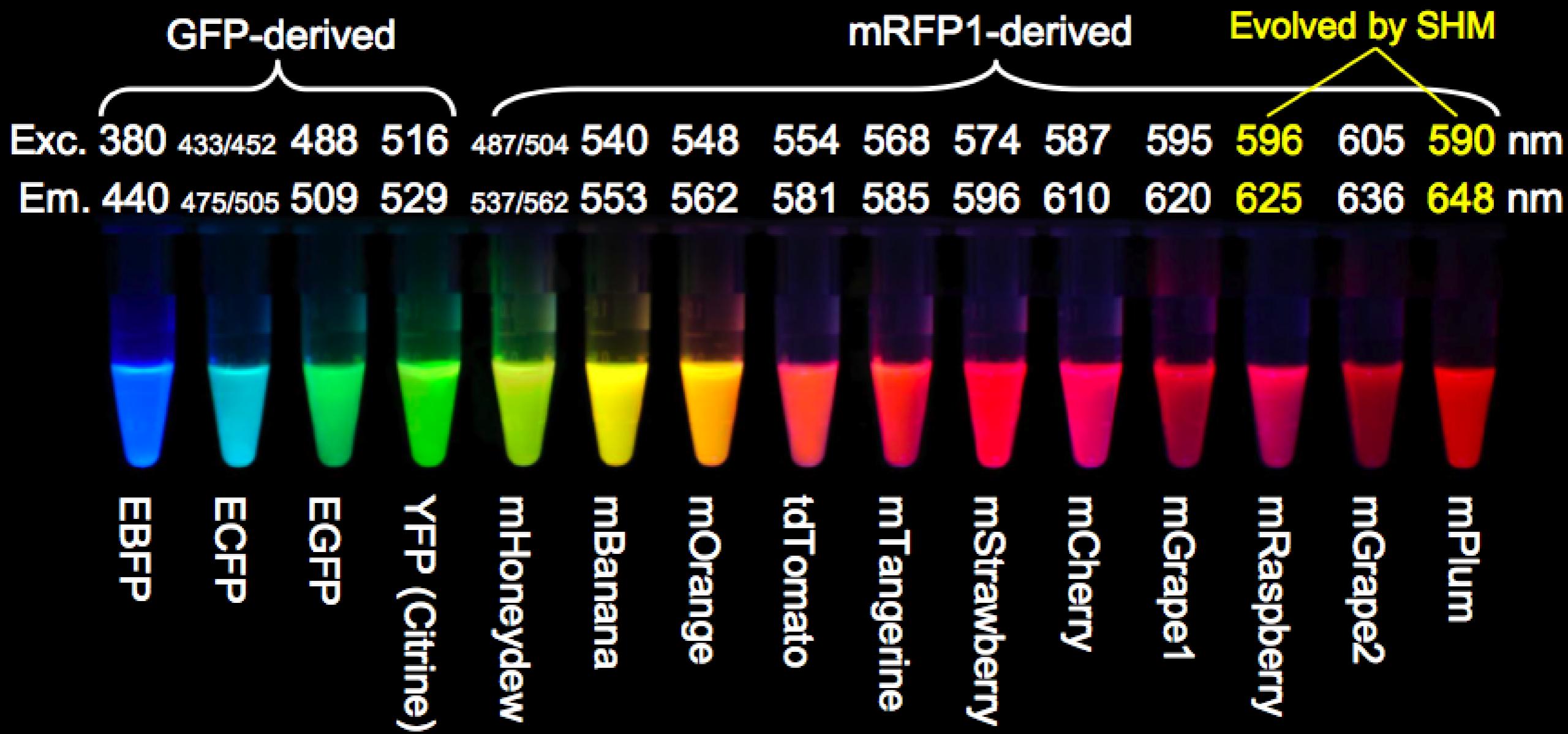


Seeking red colors...

Lukyanov lab:
PCR with
degenerate primers
on tropical corals
(from Moskow pet
shop)



The 2004 palette of nonoligomerizing fluorescent proteins

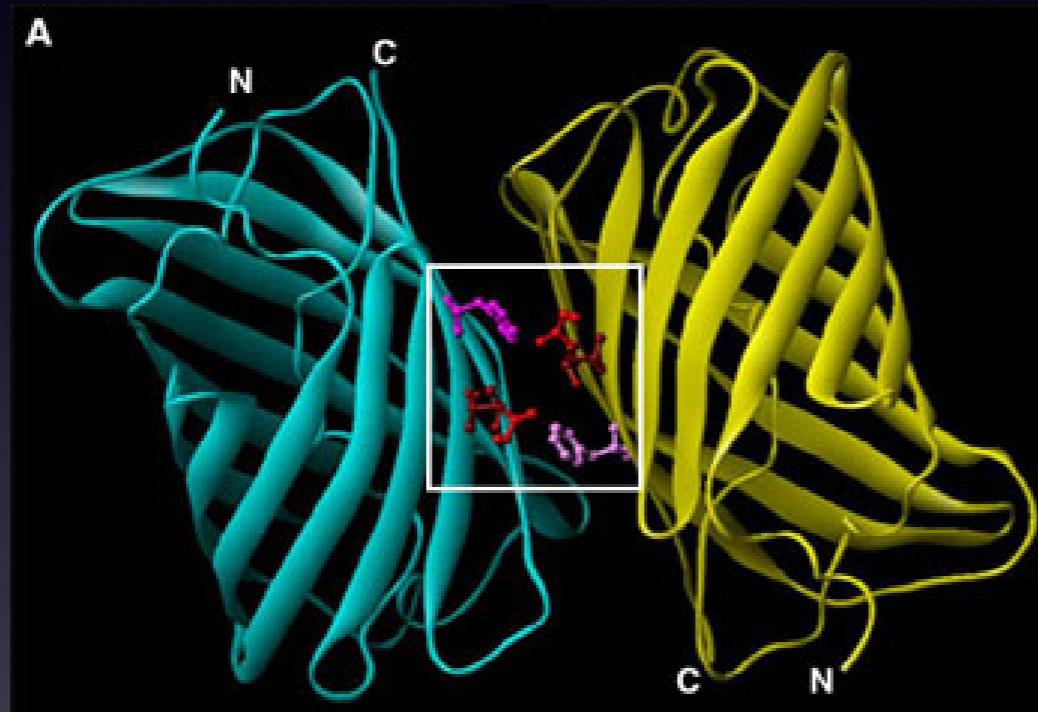


Nathan Shaner et al (2004) *Nature Biotech.* 22: 1567-1572

Lei Wang et al (2004) *Proc. Natl. Acad. Sci. USA* 101: 16745-16749

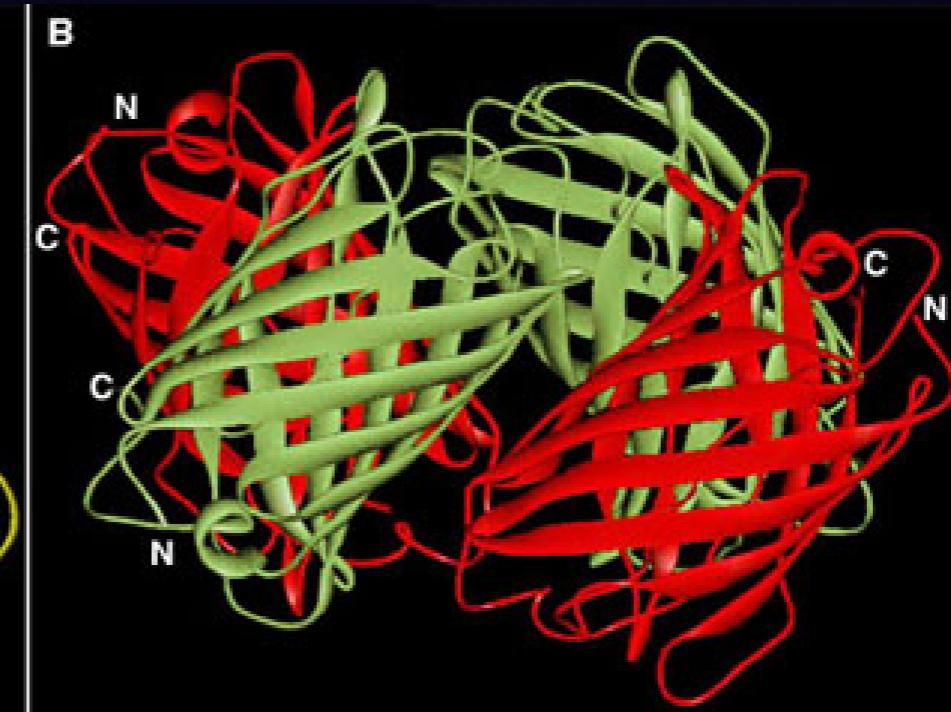
Oligomerization

GFP



Monomer / weak
dimer

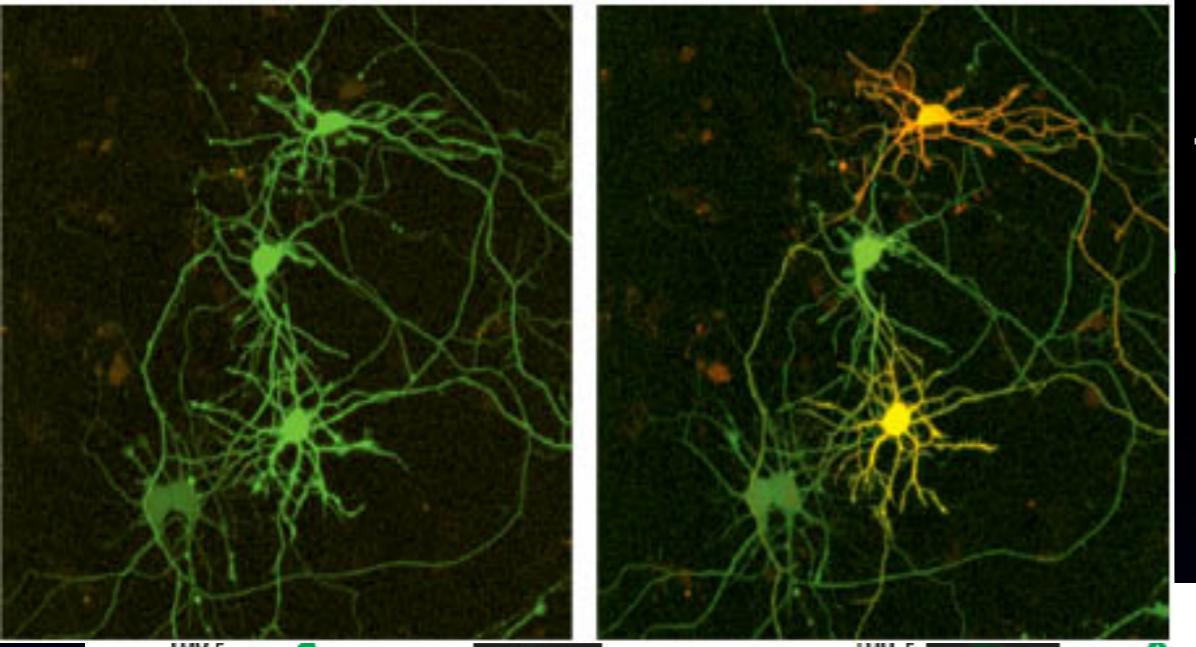
dsRed



Tetramer

Fluorescent proteins

Protein	λ_{ex}	λ_{em}	ϵ	QY	Brightness	Source
CFP	433	475	32500	0.4	13.0	Tsien
GFP	488	507	56000	0.6	33.6	Tsien
Citrine	516	529	77000	0.76	58.5	Tsien
PhiYFP	525	537	130000	0.4	52.0	Evrogen
MkOrange	548	559	51600	0.6	31.0	Miyawaki
tdimer2	552	579	120000	0.68	81.6	Tsien
tdtomato	554	581	138000	0.69	95.2	Tsien
DsRed-monomer	556	586				Clontech
mRFP1	584	607	44000	0.25	11.0	Tsien
mCherry	587	610	72000	0.22	15.8	Tsien
tHcRed	590	637	160000	0.04	6.4	Clontech



fluorescent proteins be activated or altered by light

Color-changing

Green-red:

Kaede, EosFP, KikGR,...

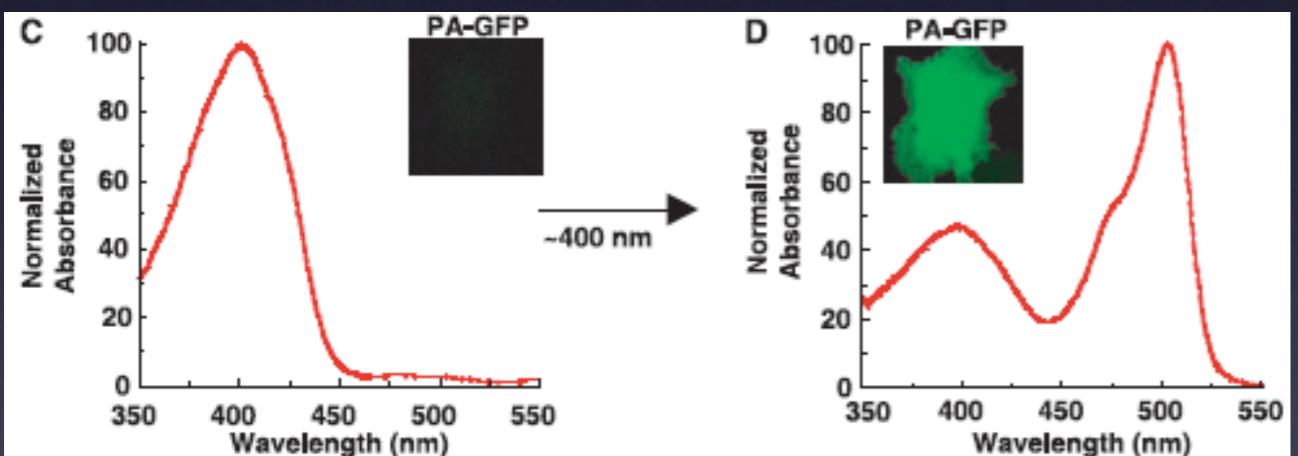
Cyan-green:

PS-CFP

An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein

Ryoko Ando*, Hiroshi Hama*, Miki Yamamoto-Hino*, Hideaki Mizuno*, and Atsushi Miyawaki**

We happened to leave one of the protein aliquots on the laboratory bench overnight. The next day, we found that the protein sample on the bench had turned red, whereas the others that were kept in a paper box remained green. Although the sky had been partly cloudy, the red sample had been exposed to sunlight through the south-facing windows.



George H. Patterson and Jennifer Lippincott-Schwartz,
2002

Reversibly switchable
KFP, Dronpa

Acknowledgements and Resources

- Kurt Thorn
- Bo Huang
- Mats Gustaffson
- Andrew Carter

Lakowicz - Principles of Fluorescence Spectroscopy

QuickTime™ and a decompressor are needed to see this picture.

Goldman et al. - Live Cell Imaging: A Laboratory Manual

Day and Davidson, Chem Soc Rev, 2009(38) 2887