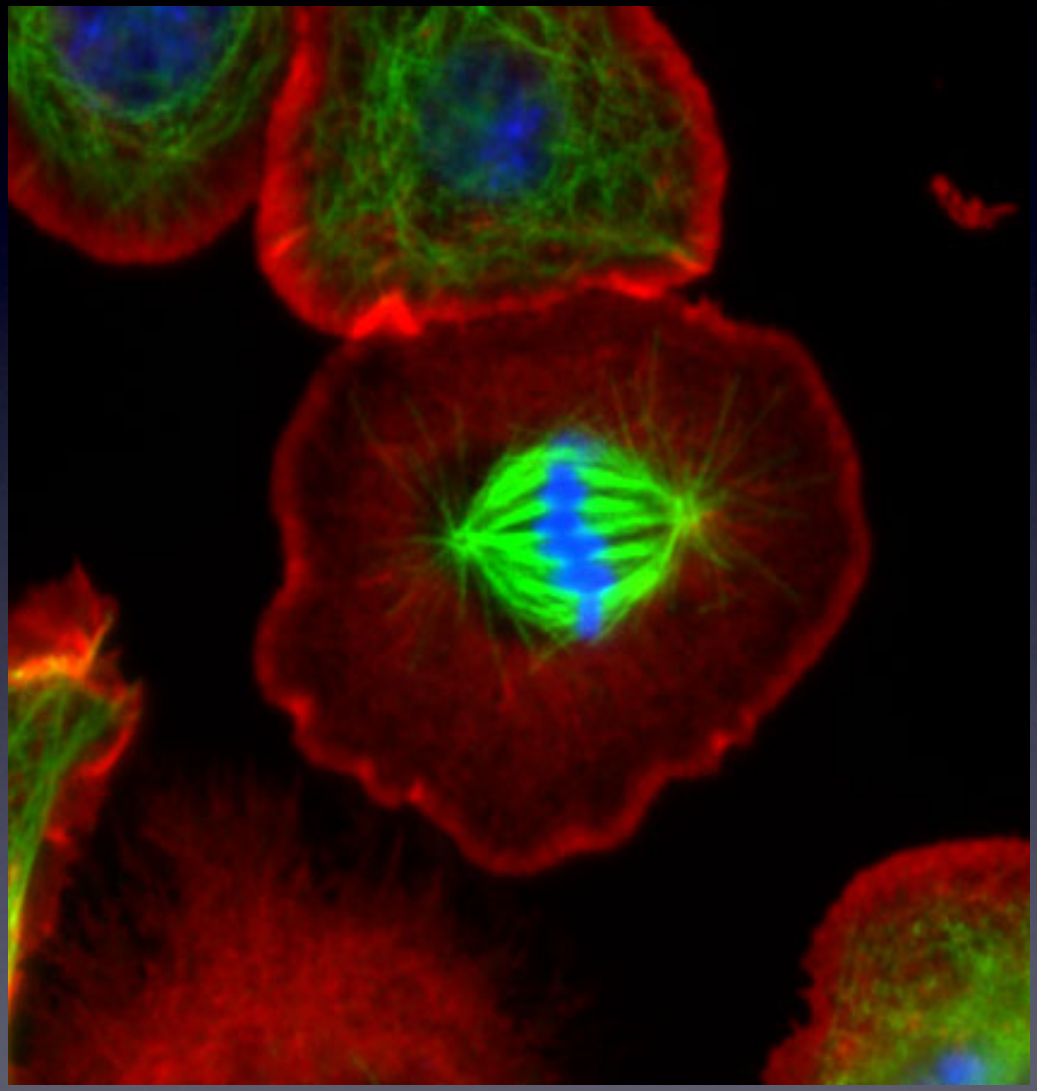
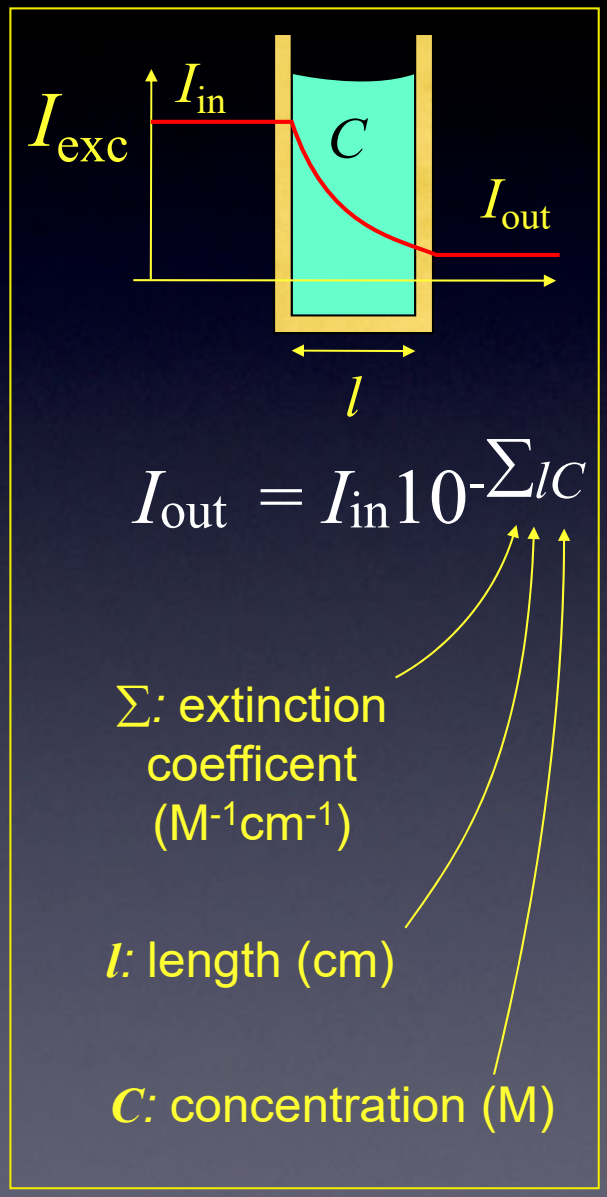
Fluorescent dyes and their use as biological probes



Nico Stuurman
Microscopy Course UCSF
April 19, 2010

Parameters of fluorescent dyes

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

332/456
QY 0.77

Fluorescein

490/520
QY 0.925

Rhodamine

554/573
QY 0.28

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

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

554/568
QY 0.14

652/672
QY 0.18

Also, Cy2, Cy5.5

Alexa dye series

499/517
QY 0.60

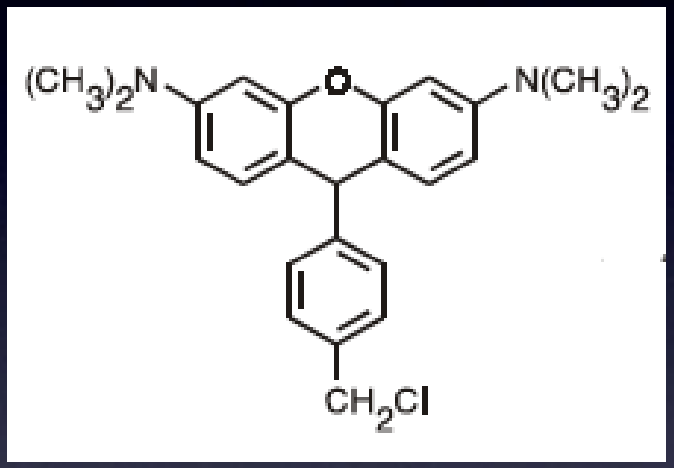
Organic Dyes

Alexa series (a commercial, not chemical) family from Molecular Probes

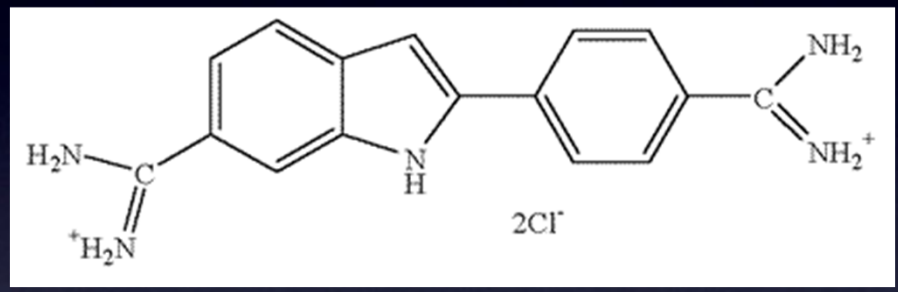
Parameters of some fluorophores

Dye	l_{ex}	l_{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



Mitotracker
Oxidized in mitochondria in fluorescent compound

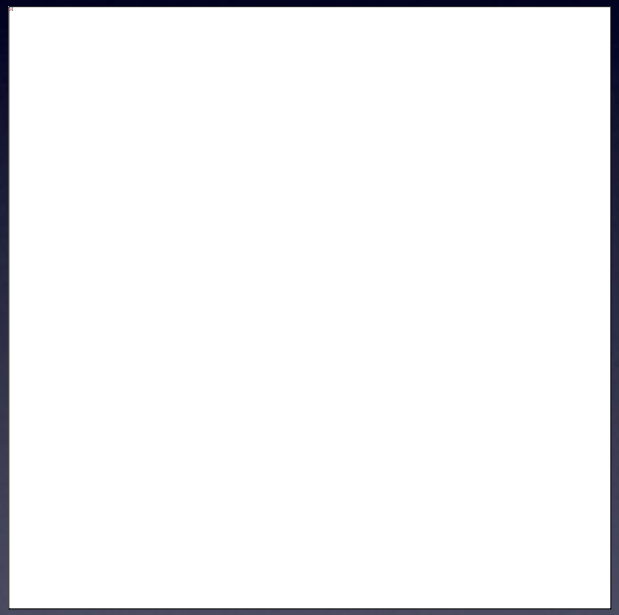


DAPI
Hoechst33258
Hoechst 33342
~20 fold enhancement
TOTO, YOYO

Conjugation of organic dyes

Chemistry/Method
Amino groups (lysine): succinimidyl ester or isothocyanate

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

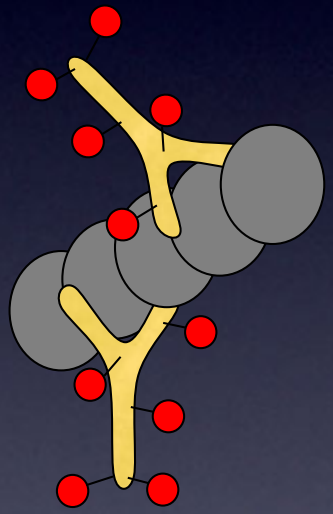


Targets

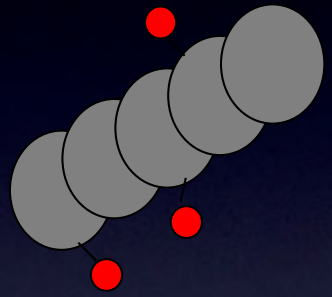
- Small molecules, i.e. phalloidin, taxol
- Proteins: labeling site unspecific
- Antibodies: direct/indirect labeling (Label density)

Fluorescent labeling

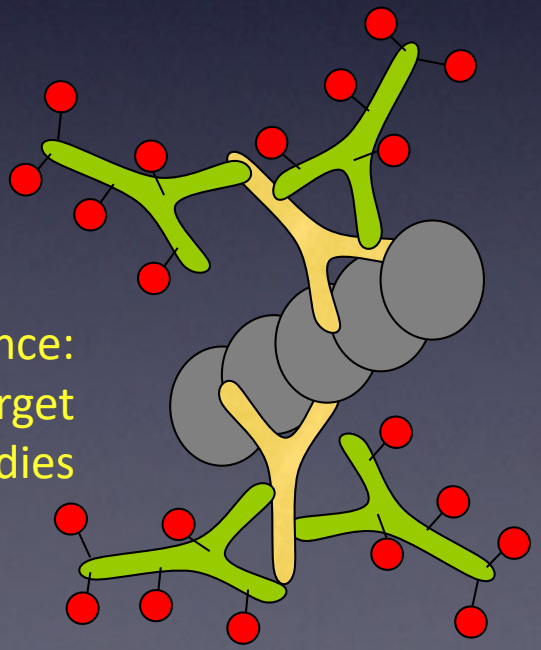
Direct immunofluorescence:
labeled antibodies against target



Direct labeling (& microinjection)
of target molecules



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

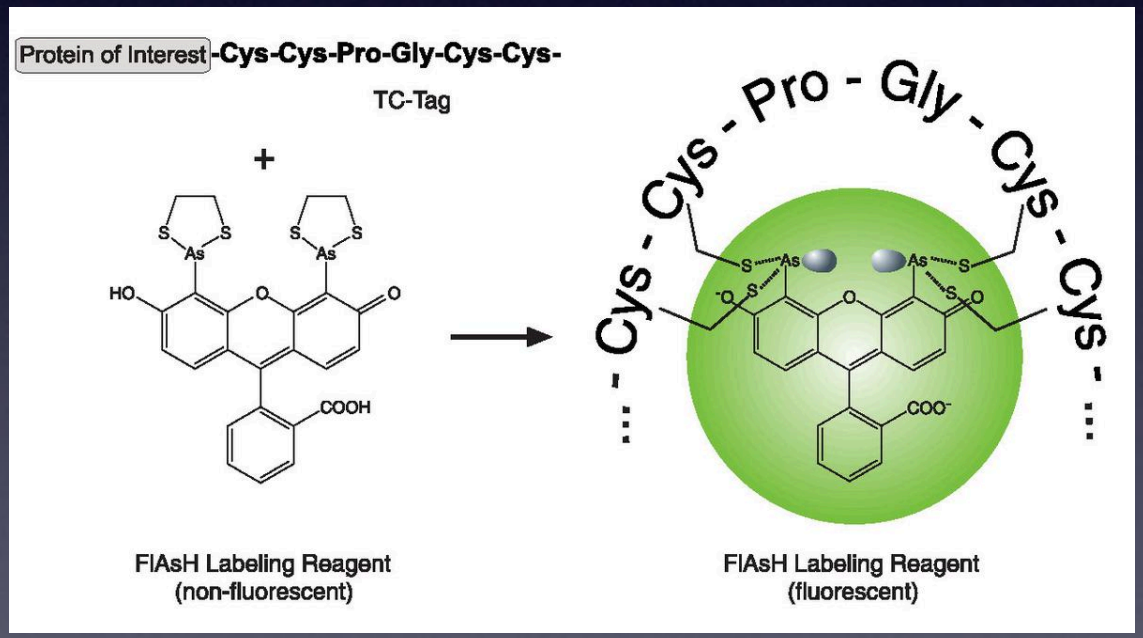


Site-Specific Labeling

Chemistry/Method

Sulfhydryl groups (cystein): maleimide

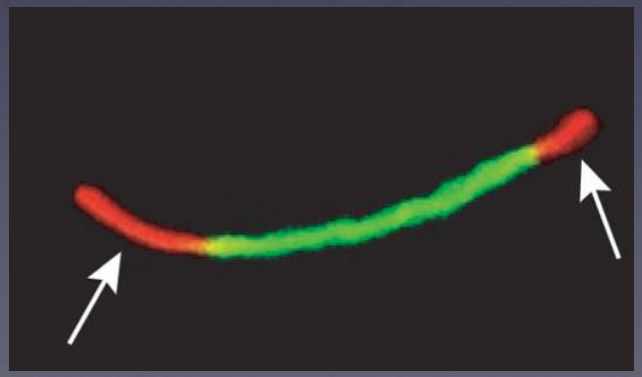
Engineer Cys-light version of target protein



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

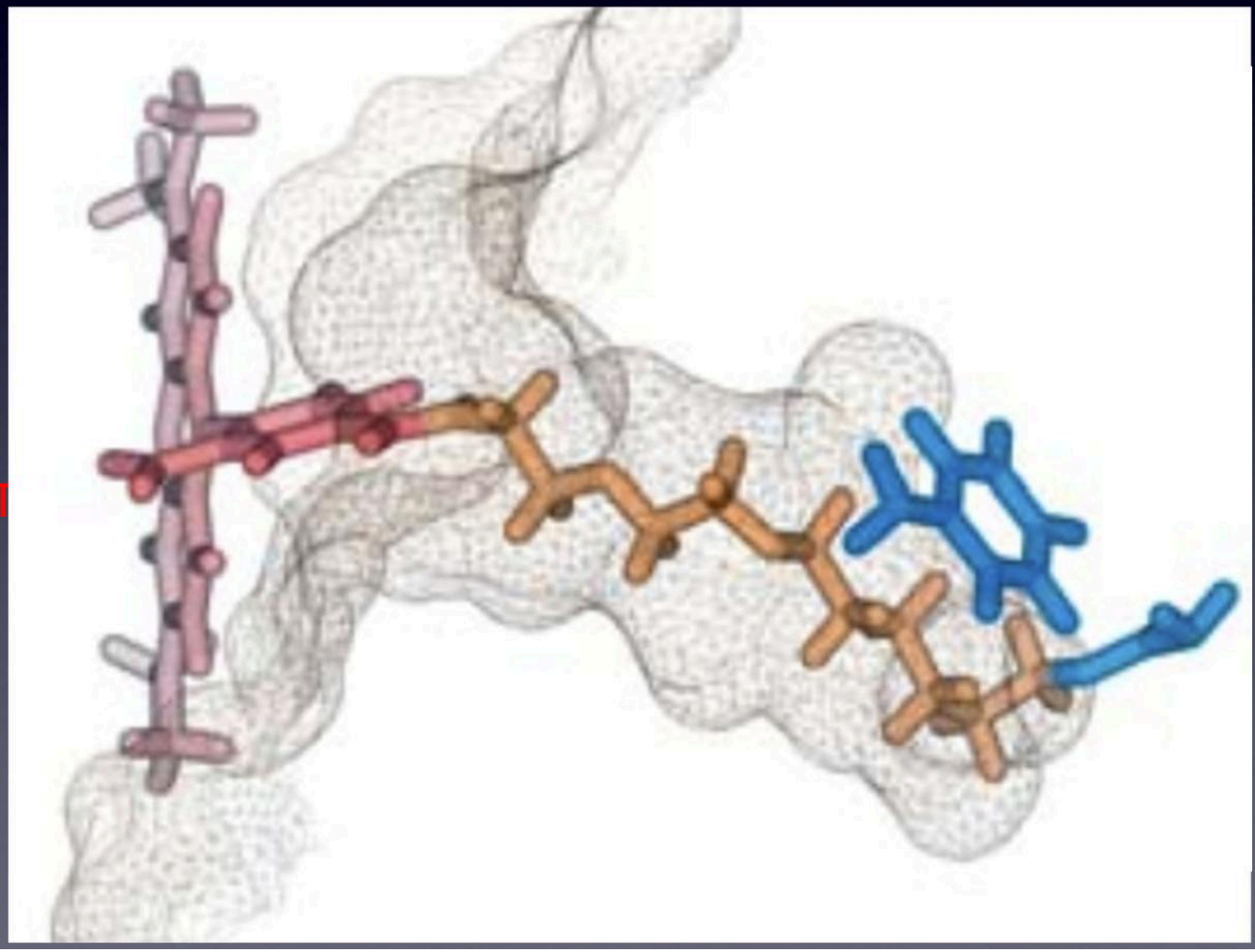
FlAsH/ReAsH

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



Covalent attachment of dyes to genetic tags

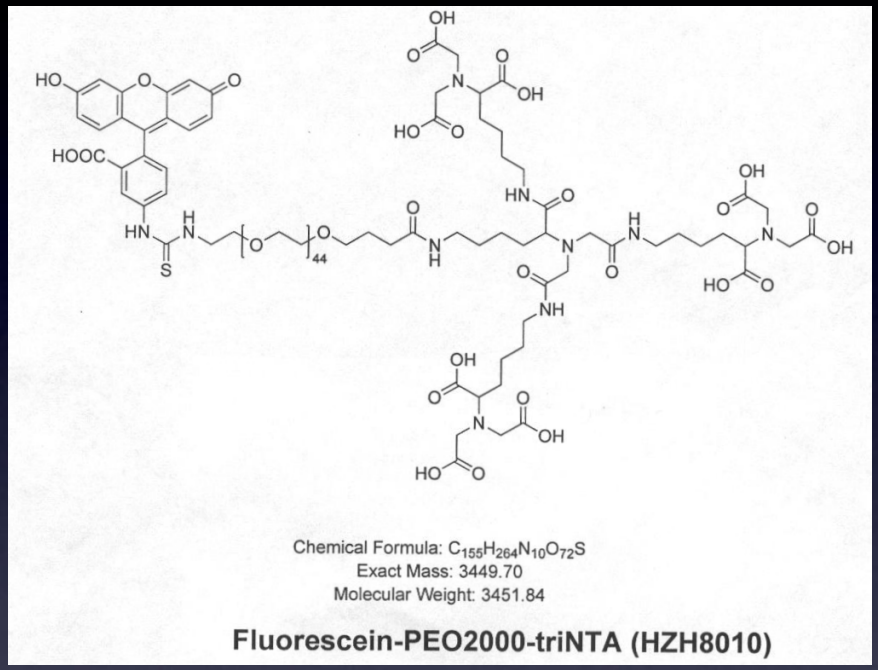
- **HaloTag (Promega) – Dehalogenase**
- **SNAP Tag (Covalys) - O6-alkylguanine-DNA alkyltransferase (AGT)**



- labels with anythin
(biotin/Cy5)
- Multiple colors
- organic dyes can b
- Stoichiometry cont
- pulse chase possib

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

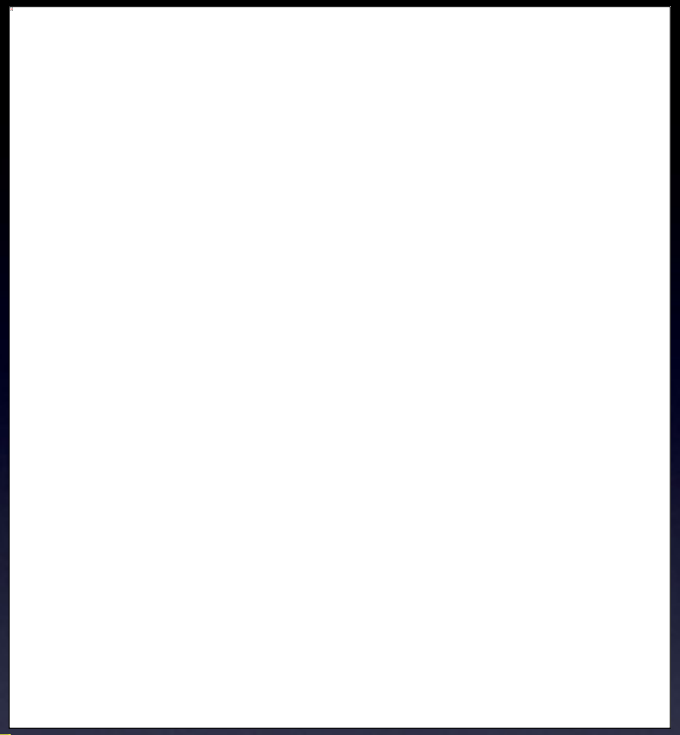
Phycobiliproteins

- 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



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

Quantum-dots

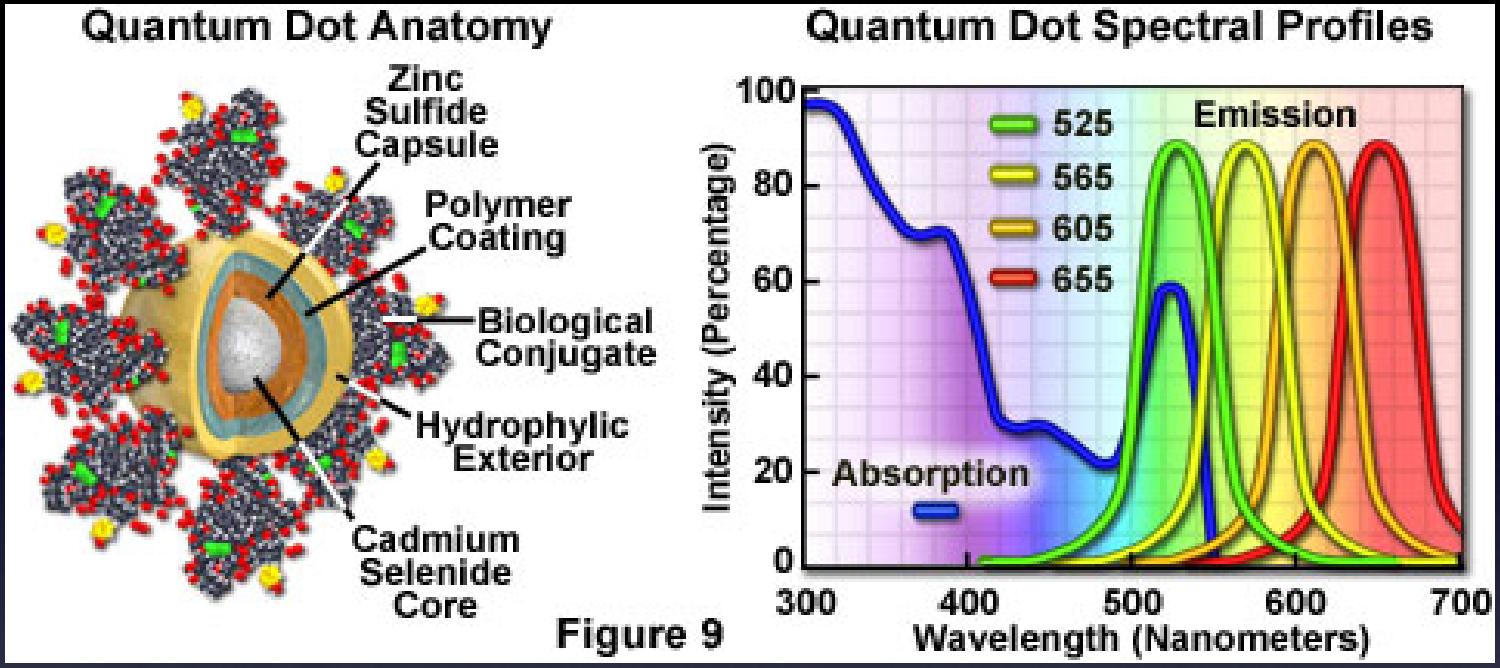


Figure 9

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

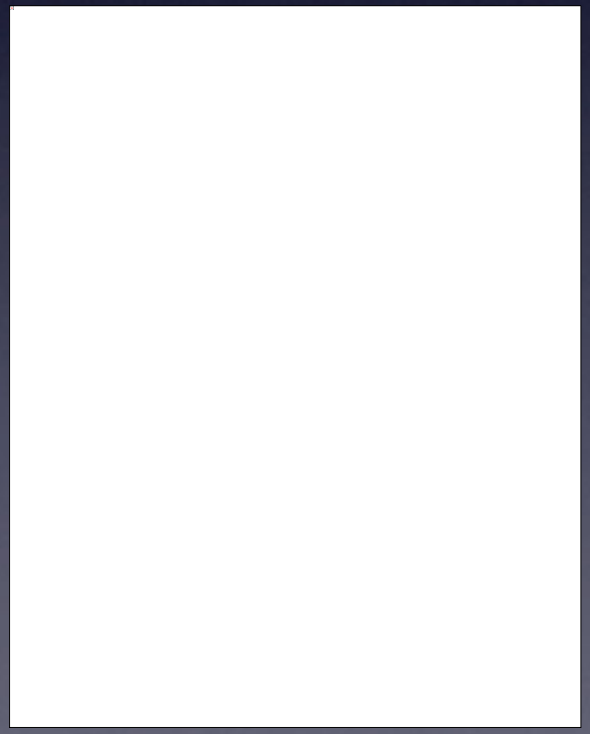
Advantages
- Very bright
- Very photostable
- Excitation possible at a single wavelength
- Visible in electron microscope

Disadvantages
- Large size
- Multivalent linkage

Quantum dot labeling

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

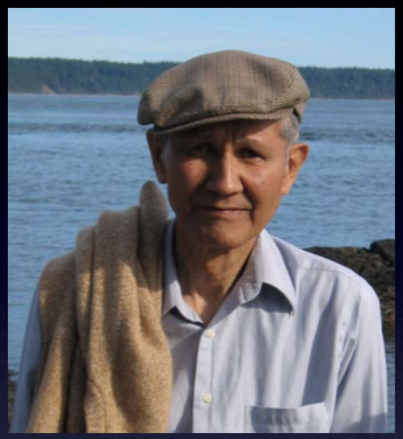
- Antibody Conjugates – immunohistochemistry
- Direct linkage to proteins/peptides – targeting to cell compartments

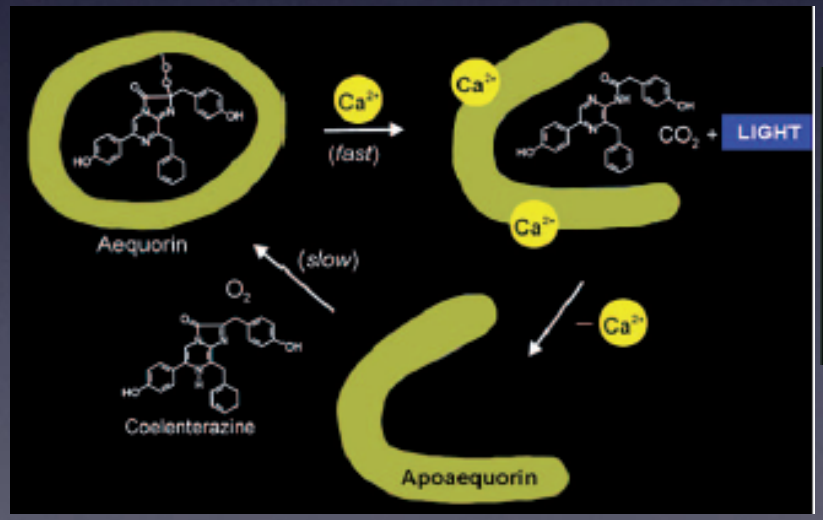


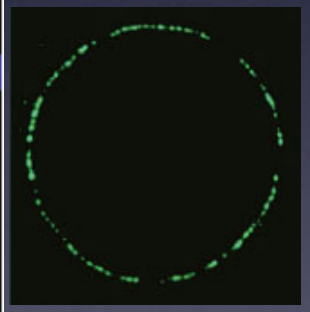
Qdot labelled dynein via HaloTag:Biotin:Streptavidin linkage moving on axonemes

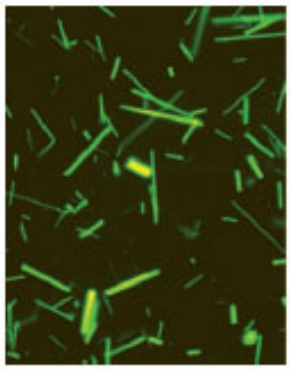
Fluorescent proteins

Discovery









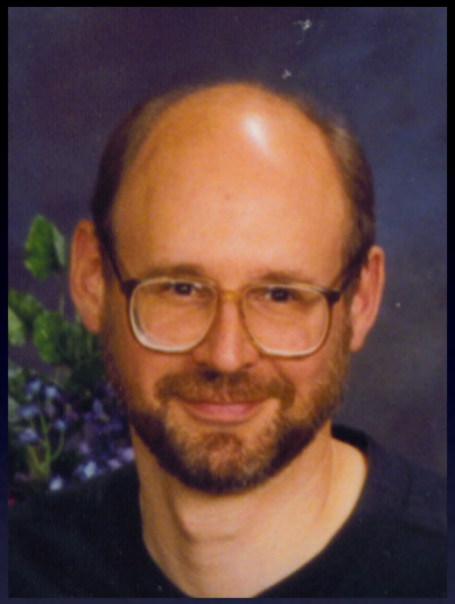
GFP (100 mg)

Denature at 90 °C
Digest with papain
Extraction with butanol at pH 1
TLC purification

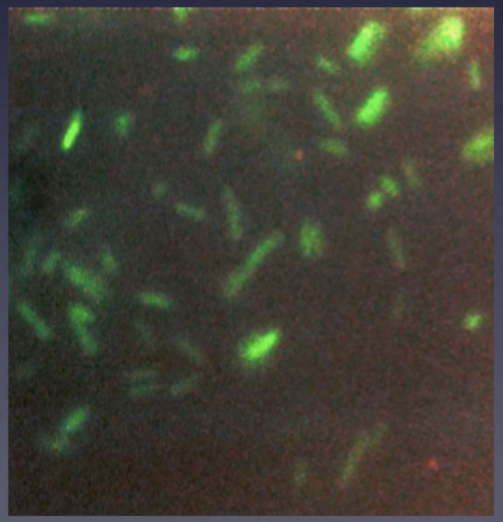
Isolated chromophore (0.1 mg)

Images from Osamu Shimomura

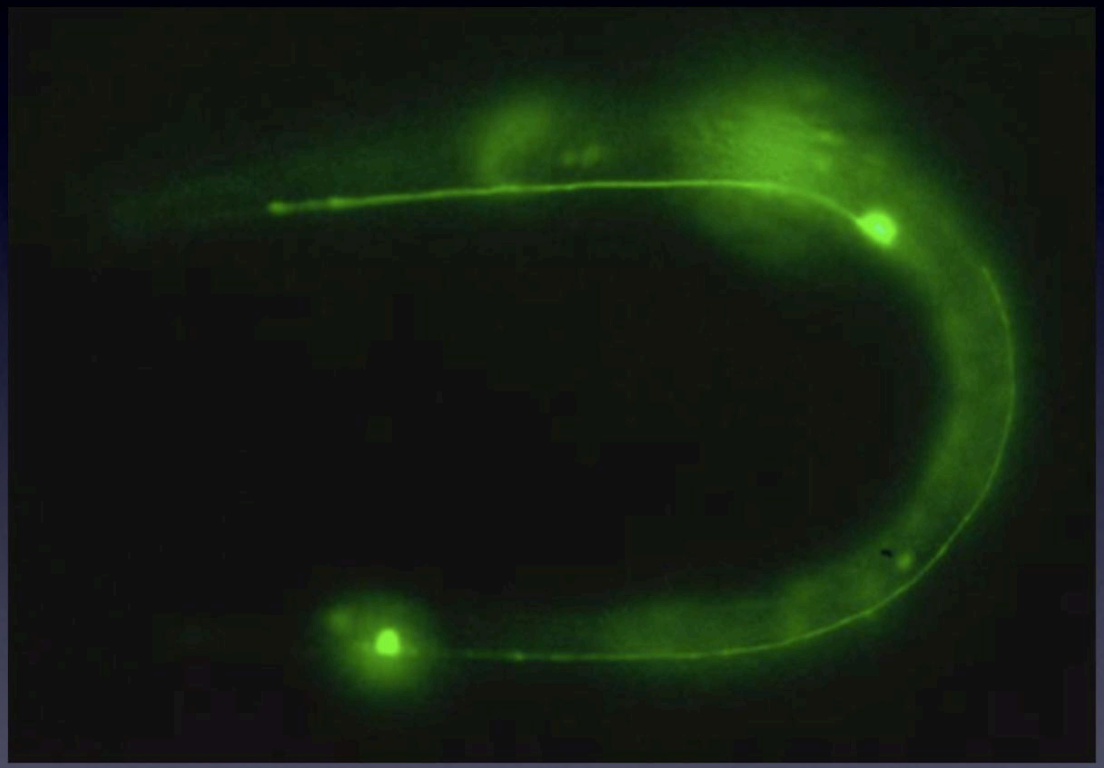
No co-factors needed!



Douglash Prasher



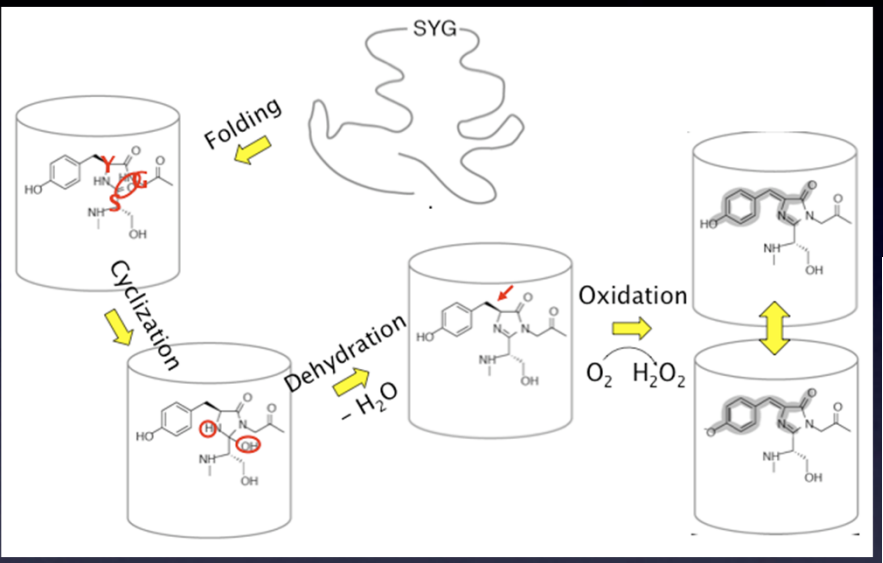
First GFP expression in E. coli

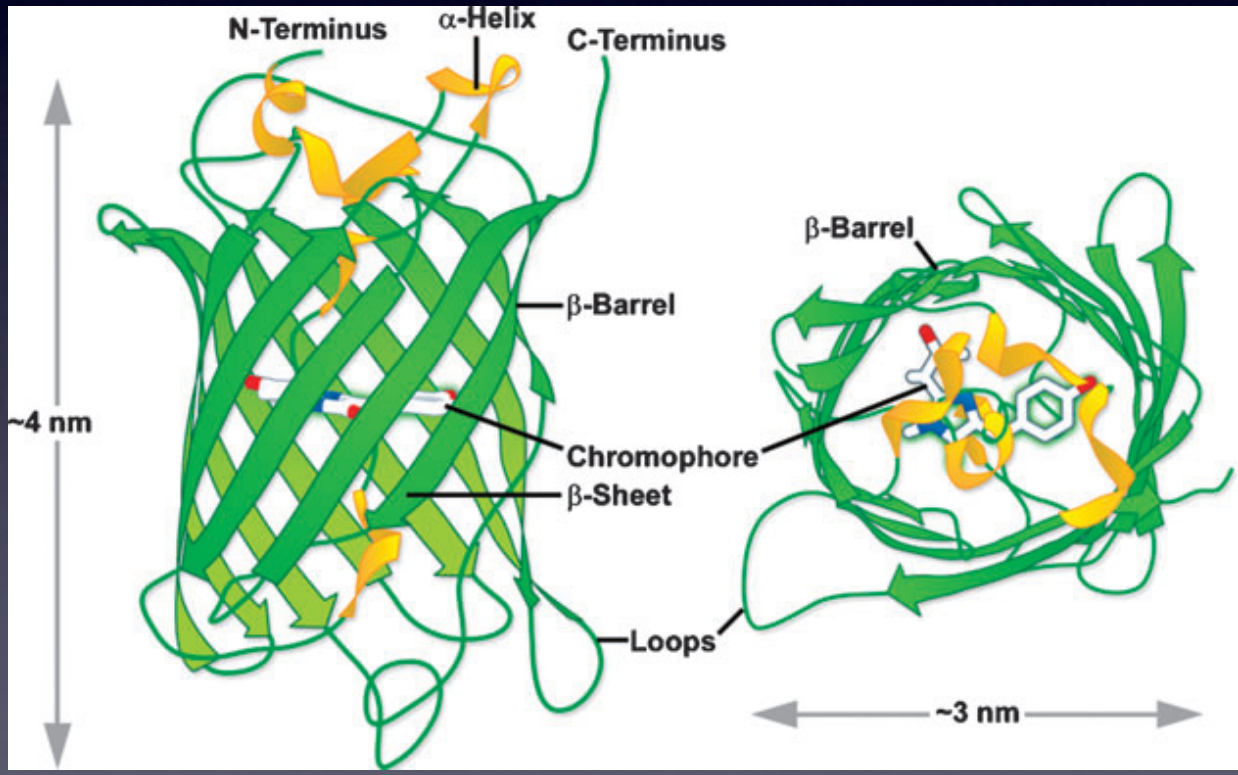


and C. elegans

Images from Martin Chalfie

GFP





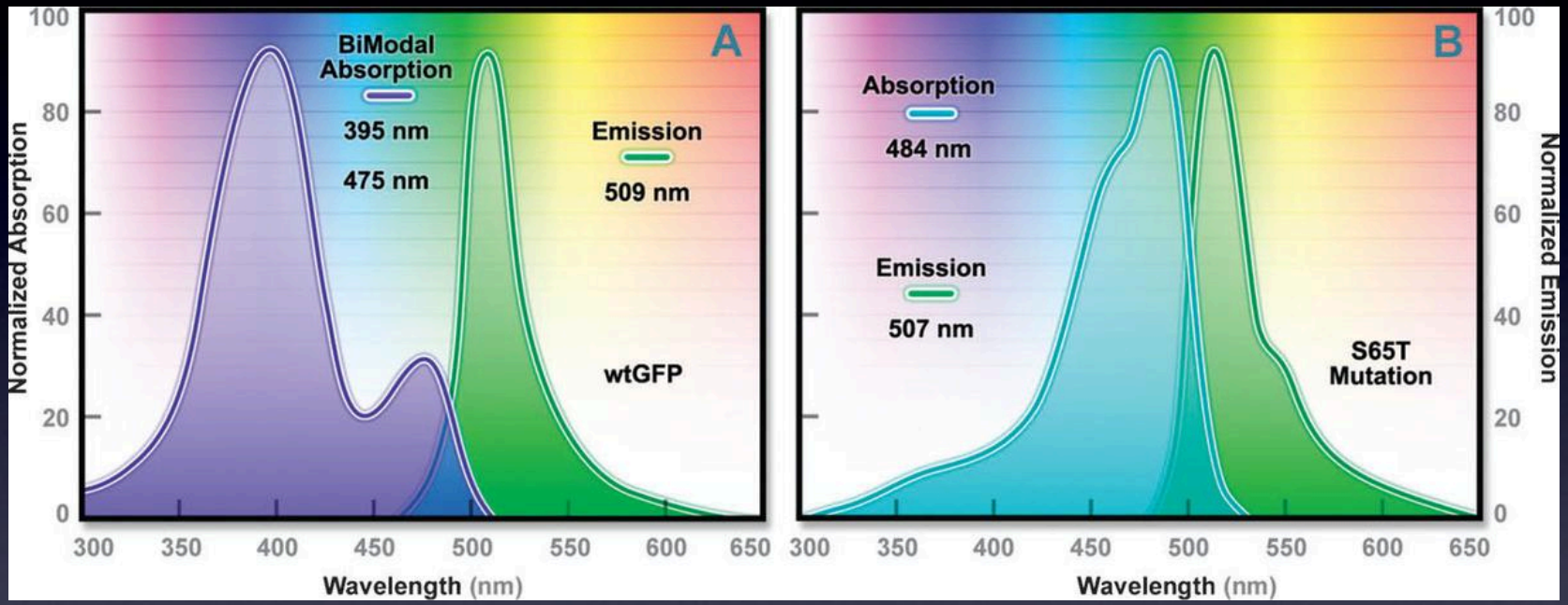
Day and Davidson, *Chem Soc Rev*, 2009(38) 2887

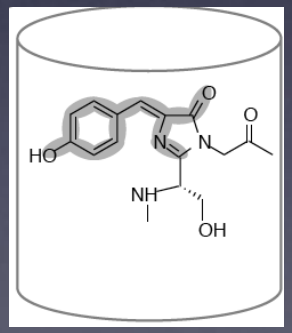
~240 Amino acids, 27 kD

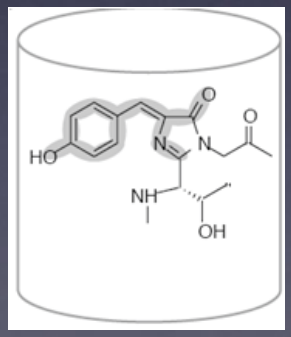
Improving the wild type GFP

AvGFP

"GFP"



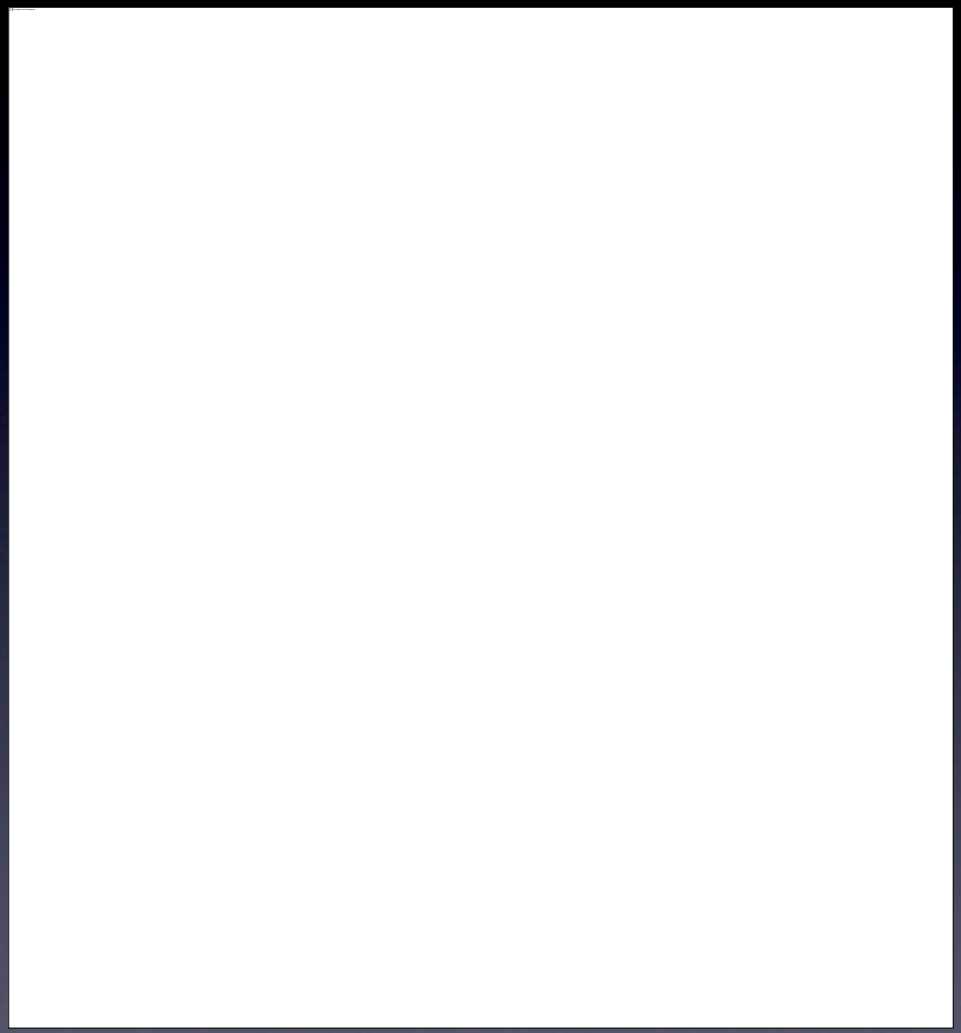




S65T

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

GFP-Actin in Drososophila S2 cells



Steve Rogers, 2002

Jellyfish (*Aequorea*) fluorescent protein family

AvGFP

↓

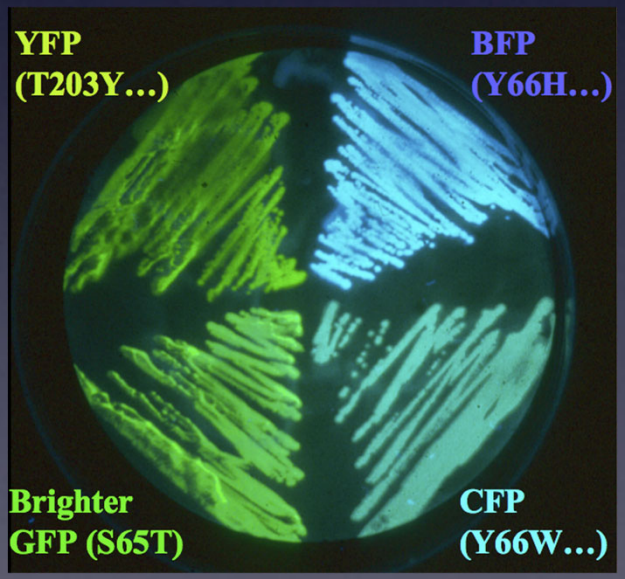
GFP (S65T), EGFP (S65T, F64L)

↓

BFP, CFP, YFP

↓

Cerulean , CyPet, Sapphire, Venus, Citrine , Ypet…



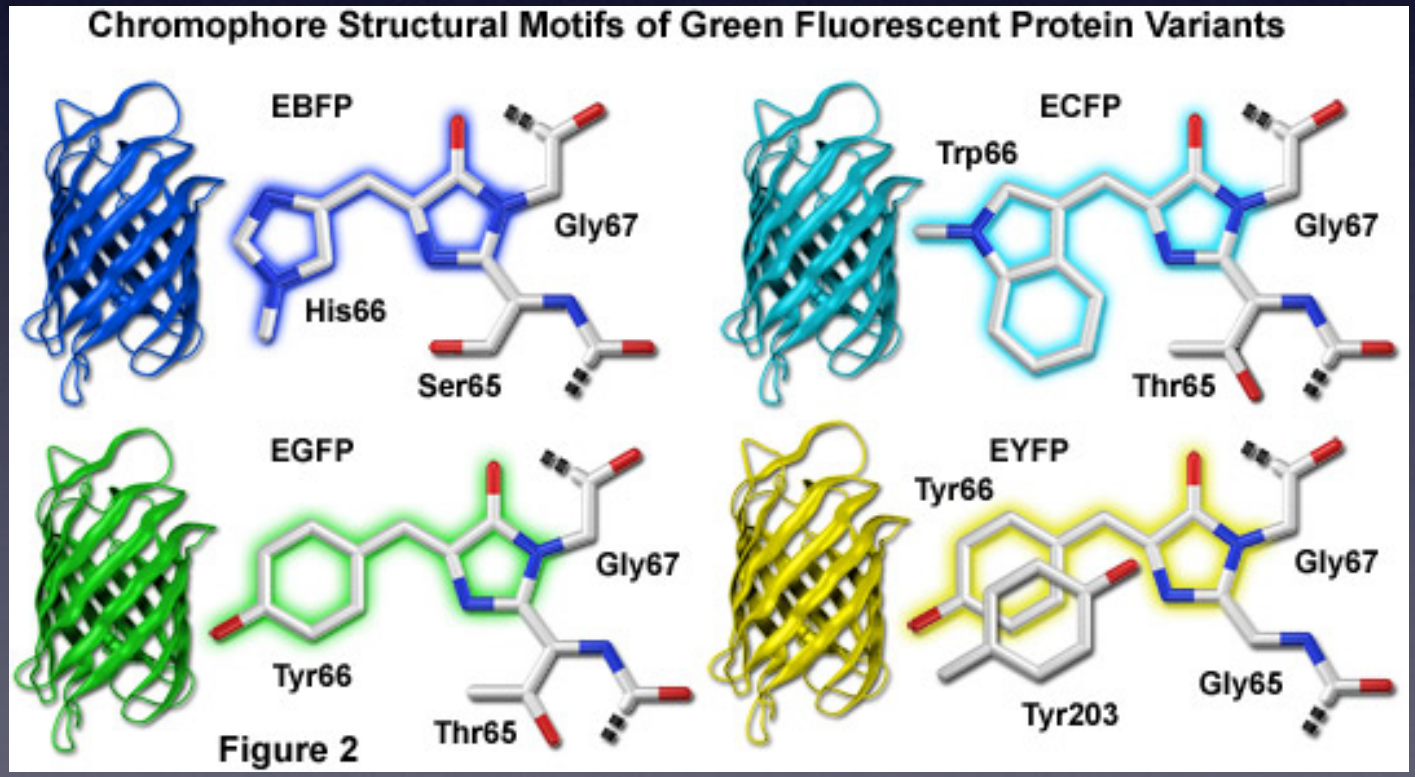
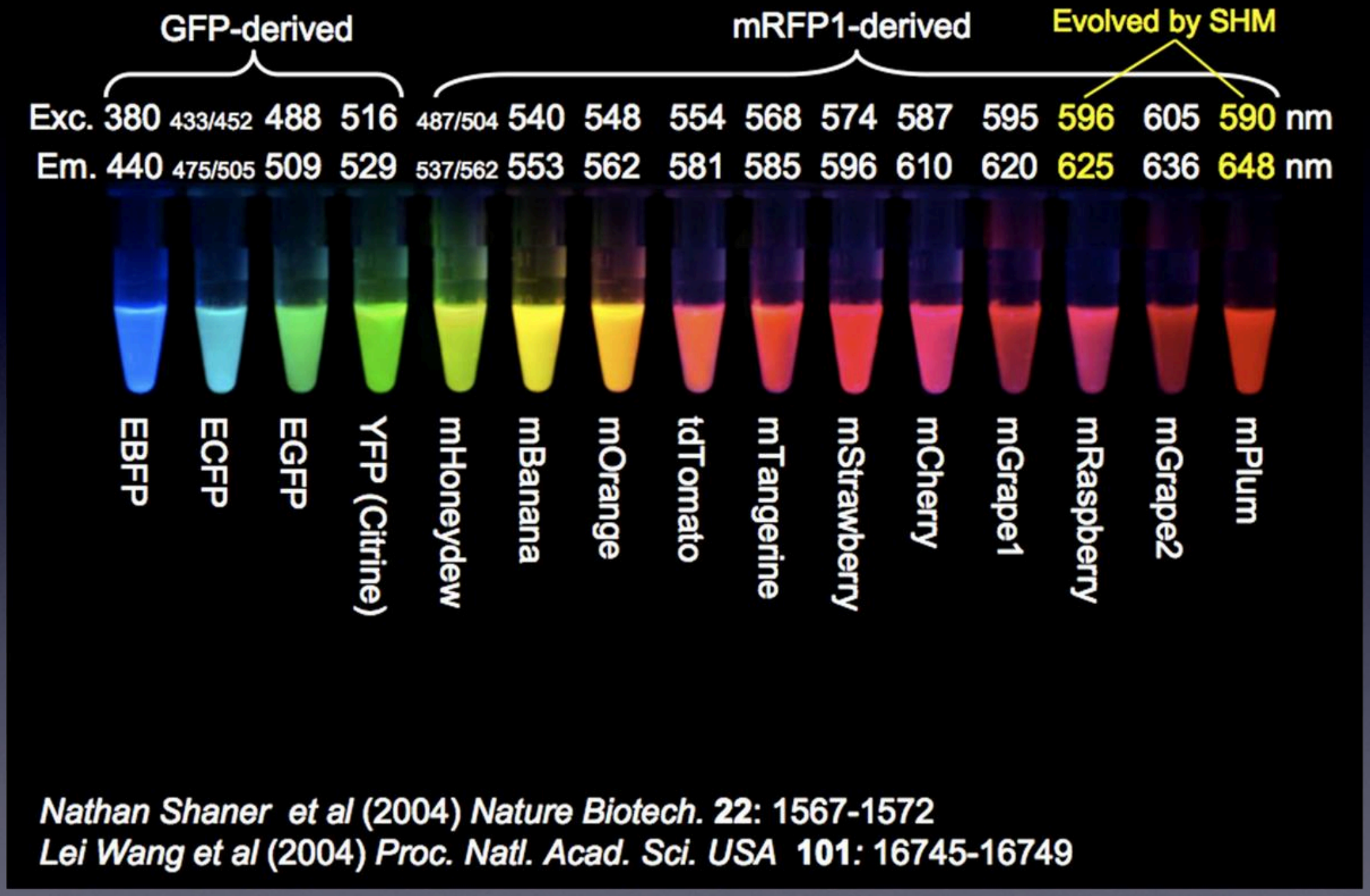


Figure 2

Seeking red colors…

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

The 2004 palette of nonoligomerizing fluorescent proteins

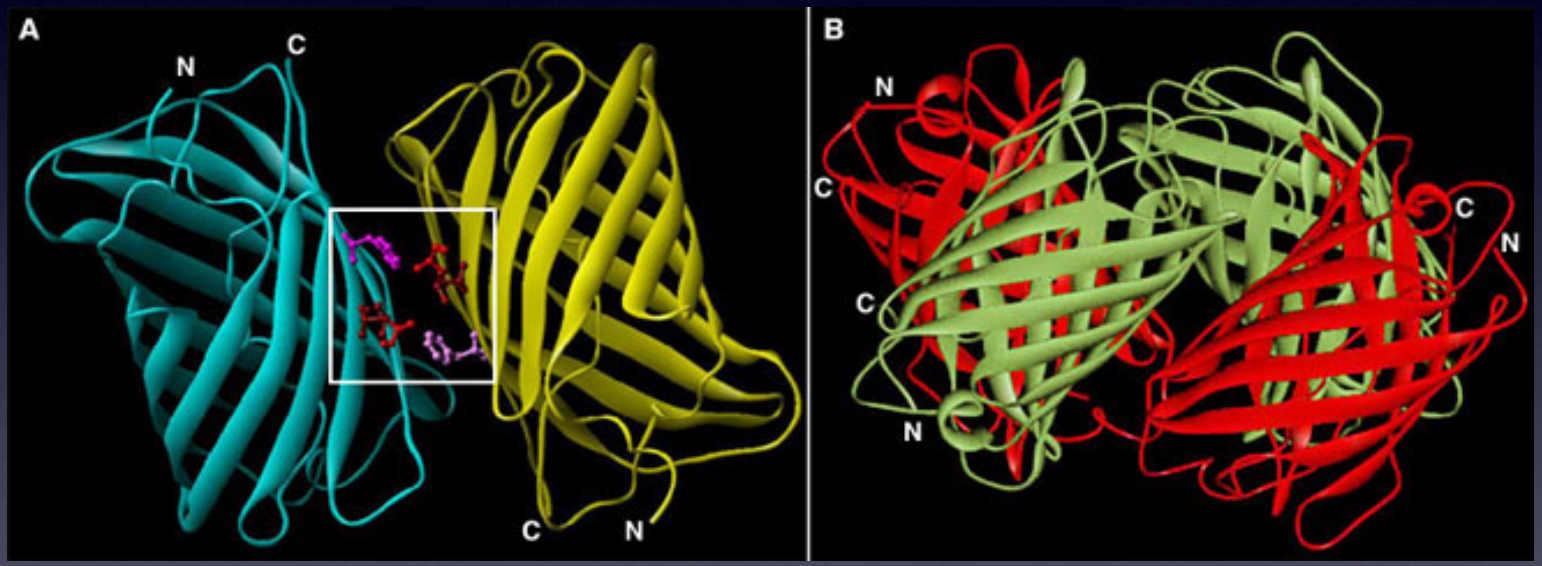


Nathan Shaner et al (2004) *Nature Biotech.* **22**: 1567-1572
Lei Wang et al (2004) *Proc. Natl. Acad. Sci. USA* **101**: 16745-16749

Oligomerization

GFP

dsRed



Monomer / weak dimer

Tetramer

Fluorescent proteins

Protein	⌊ex	⌊em	∑	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

Acknowledgements and Resources

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

Lakowicz - Principles of Fluorescence Spectroscopy

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

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