Genetically encoded fluorescent proteins and FRET imaging in living cells

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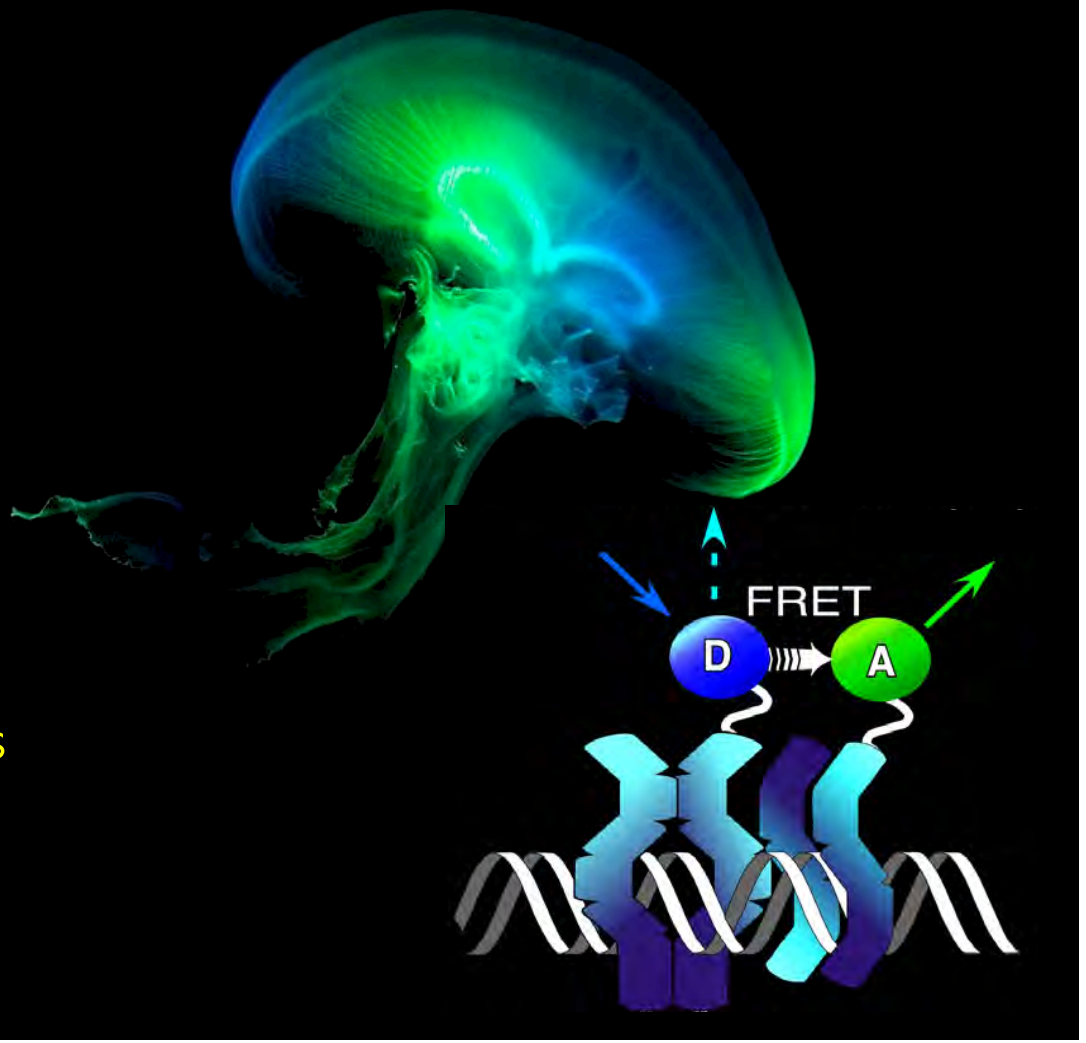


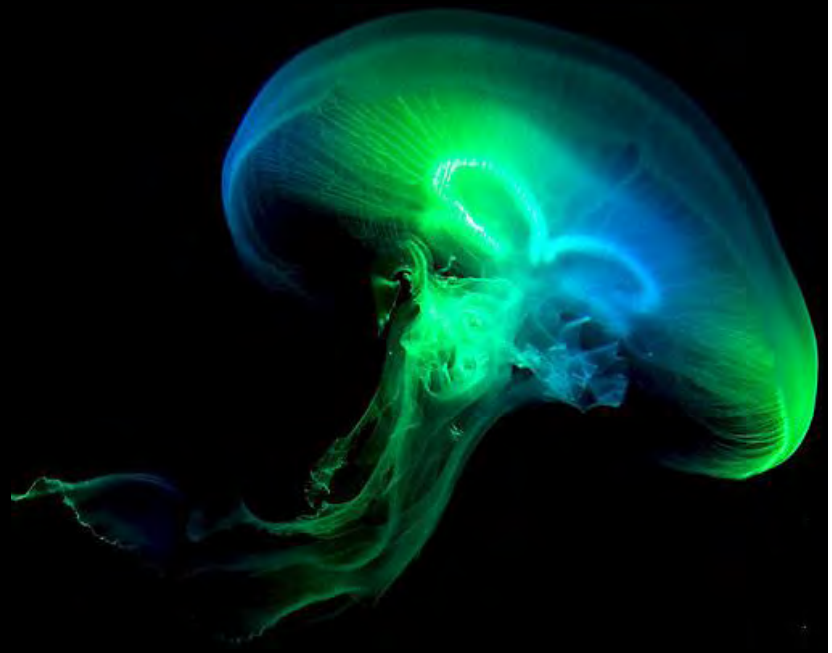
Principles of Fluorescence Techniques
The University of Chicago
April 6-8, 2011

Genetically encoded fluorescent proteins

- The genetically encoded fluorescent proteins (FPs):
  - General characteristics of the FPs.
  - Mutant color variants based on *A.v.* GFP.
  - FPs derived from *Discosoma striata* - mRFP and the fruits.
  - New FPs derived from corals.
  - The (current) best FPs.
- Förster (Fluorescence) resonance energy transfer (FRET):
  - General requirements for FRET.
  - Spectral bleedthrough background.
  - Methods used to measure FRET - strengths and weaknesses.
  - FPs for FRET – optimal pairs.
  - Summary.

Aequorea victoria Green Fluorescent Protein (GFP)

- *Aequorea victoria* makes the chemiluminescent protein aequorin, which emits blue light.

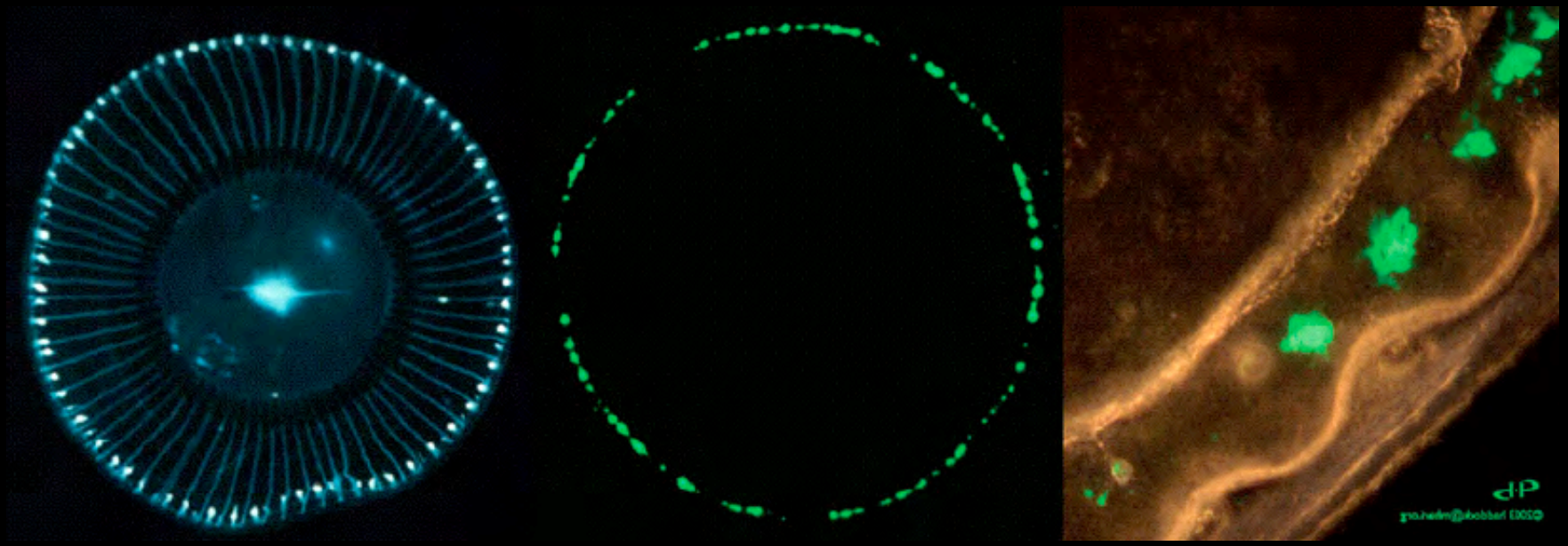


- GFP absorbs the blue light and shifts the emission to green light.

- The cloning of GFP caused a *revolution* in cell biology - allowing genetically encoded fluorescence labeling.

A brief history of GFP

- The isolation and cloning of *Aequorea victoria* GFP.

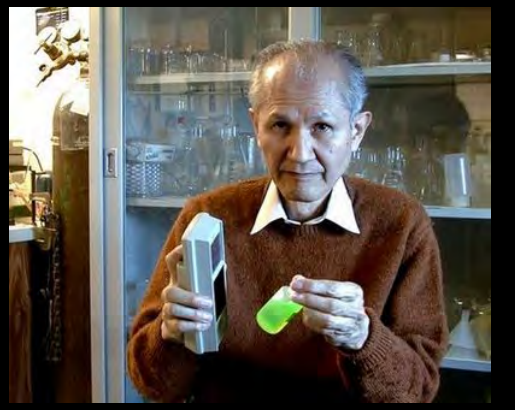


Zimmer (2009) *Chem. Soc. Rev.* **38:**2823

A brief history of GFP

- Shimomura *et al.* (1962) isolated the jellyfish chemiluminescent protein aequorin.

  Shimomura et al. (1962) *J. Cell. Comp. Physiol.* **59:**223



- Aequorin is a luciferase that uses coelenterazine in a calcium-dependent reaction that yields blue light.

- Shimomura also observed the jellyfish extracts exhibited very bright greenish fluorescence under UV illumination.

- In the 1970's, Shimomura and colleagues purified the autofluorescent protein - the jellyfish GFP.

- They showed that GFP absorbed the excited state energy from aequorin via an energy transfer process, and emitted green light.

  Morise et al. (1974) *Biochemistry* **13**:2656

A brief history of GFP

- While in Milton Cormier's laboratory, Douglas Prasher cloned the gene encoding aequorin.

  Prasher et al. (1985) *Biochem. Biophys. Res. Commun.* **126**:1259.

- Using the same cDNA library, Prasher later cloned a cDNA (*gfp10*) encoding the *Aequorea* GFP.

  Prasher et al. (1992) *Gene* **111**:229

- He recognized the potential of GFP as a tool for cell biologists, but was cautious of the difficulties in producing the fluorescent protein in other biological systems.

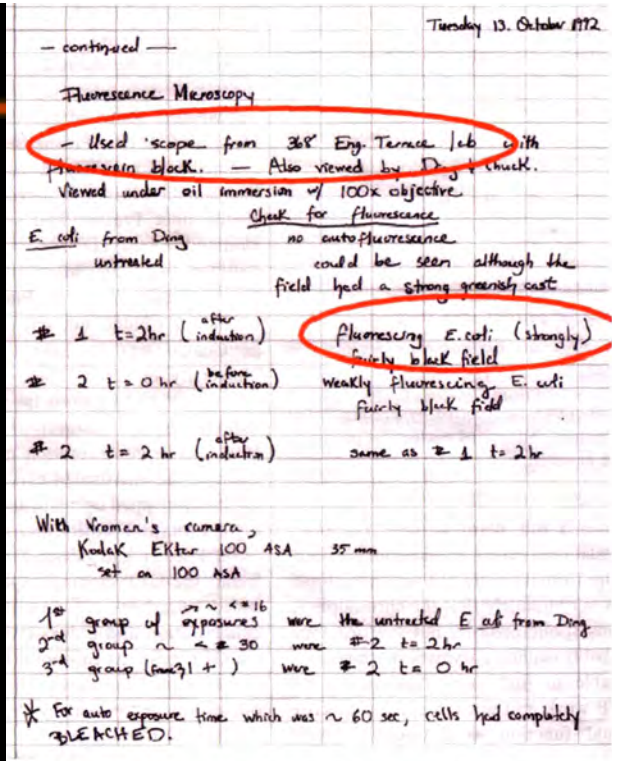
A brief history of GFP

- In September, 1992, Martin Chalfie obtained the GFP gene from Prasher, and PCR cloned it into expression plasmids.

- In October, 1992, Chalfie's student first expressed GFP in bacteria.

- Chalfie *et al.* (1994) demonstrated that GFP could be expressed in bacteria and worms.

  Chalfie et al. (1994) *Science* **263**:802


Tuesday 13. October 1992

– continued –

Fluorescence Microscopy

– Used 'scope from 368 Eng. Terrace lab with fluorescein block. – Also viewed by Dr. Chuck. Viewed under oil immersion w/ 100x objective.

E. coli from Ding untreated — Check for fluorescence: no autofluorescence could be seen although the field had a strong greenish cast

1 t=2hr (after induction) — fluorescing E. coli (strongly) faintly black field

2 t=0 hr (before induction) — weakly fluorescing E. coli faintly black field

2 t= 2 hr (after induction) — same as # 1 t= 2hr

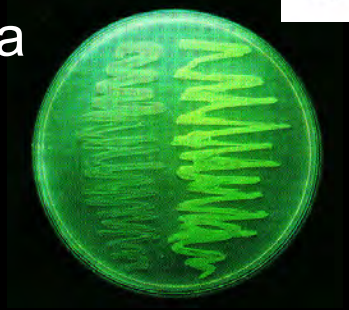
With Vromen's camera, Kodak Ektar 100 ASA 35 mm set on 100 ASA

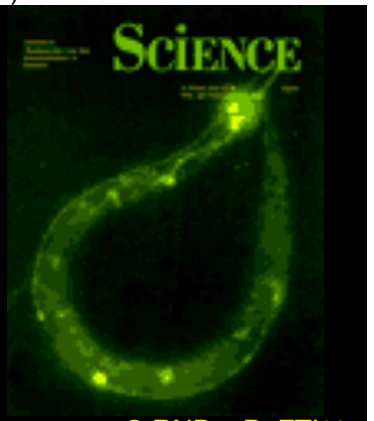
1st group of ~ exposures ~ < # 16 were the untreated E coli from Ding
2nd group ~ ~ # 30 were # 2 t= 2hr
3rd group (empty +) were # 2 t= 0 hr

* For auto exposure time which was ~ 60 sec, cells had completely BLEACHED.

Fig. 4 Ghia Euskirchen's lab notebook for October 13, 1992.

Zimmer (2009) *Chem. Soc. Rev.* **38**:2823.

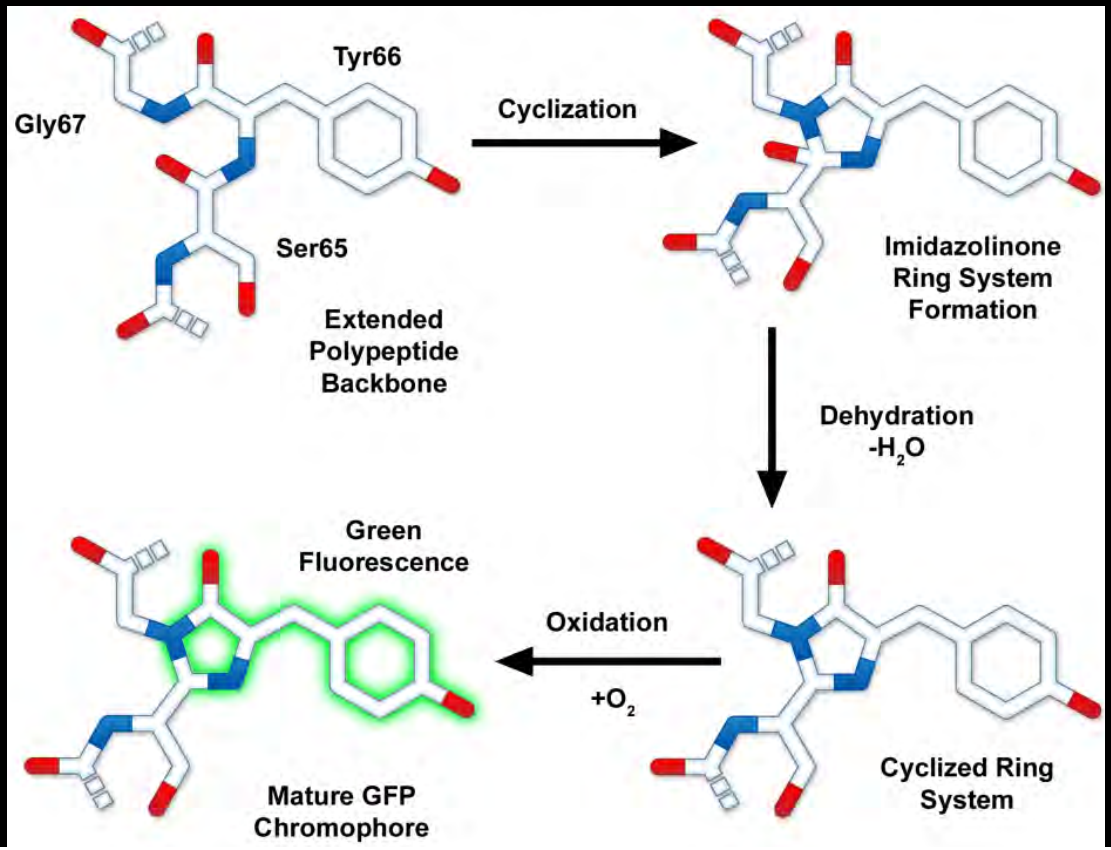


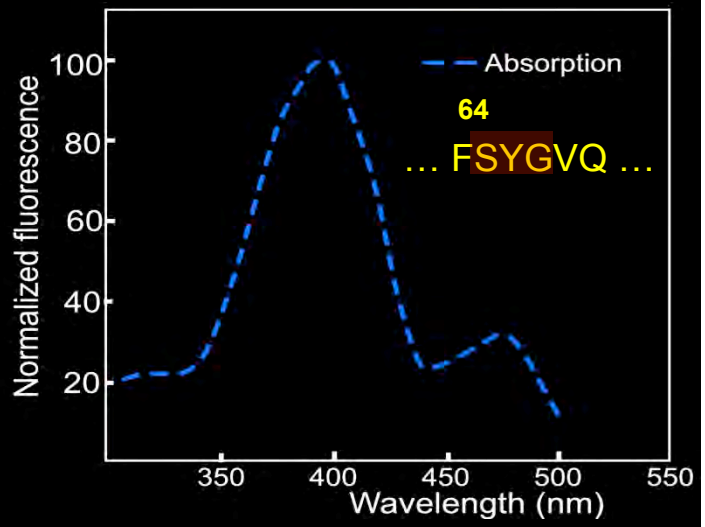


- This showed that no additional factors were required for complete chromophore formation.

General characteristics of GFP

- Using purified GFP, Shimomura showed that a 6 AA fragment was responsible for all light absorption properties.



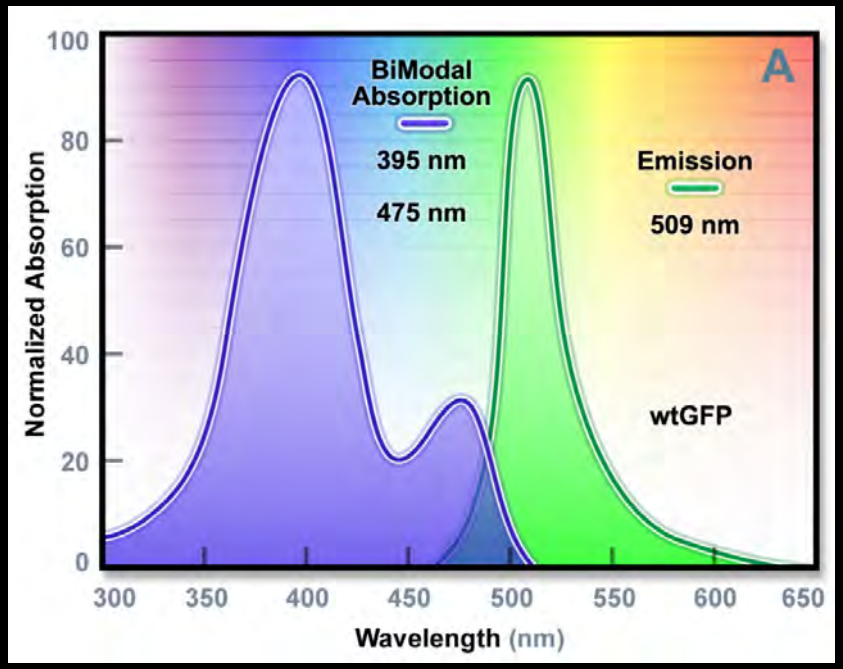


Cody et al. (1993) *Biochemistry* **32**:1212

- This led to definition of the chromophore formed by the cyclization of the -SYG-:

General characteristics of GFP

- The wild type GFP displays a complex absorption spectrum:

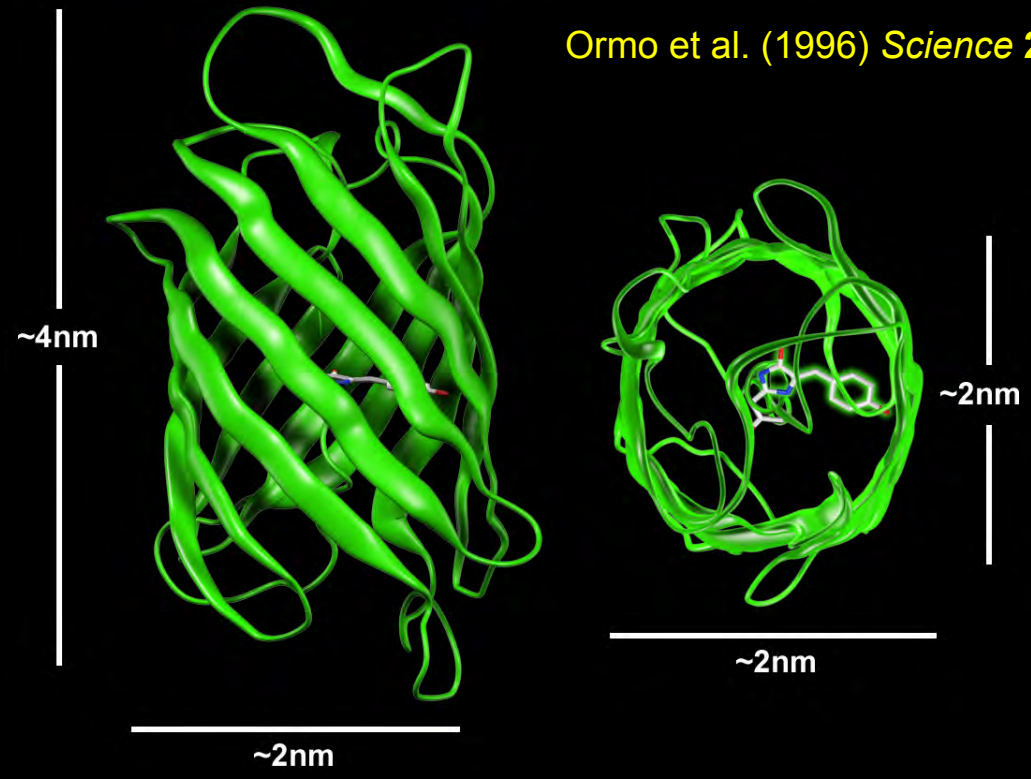


M_1….VTTF-$S_{65}Y_{66}G_{67}$-VQCFS…K_{238}

- The Tyr66 is protonated, and absorbs strongly at 397 nm.
- A charged intermediate accounts for the secondary absorption at 476 nm.

General characteristics of GFP

- In 1996, the crystal structure of GFP was solved, showing the cyclic tripeptide buried in the center of an 11-strand β-barrel:

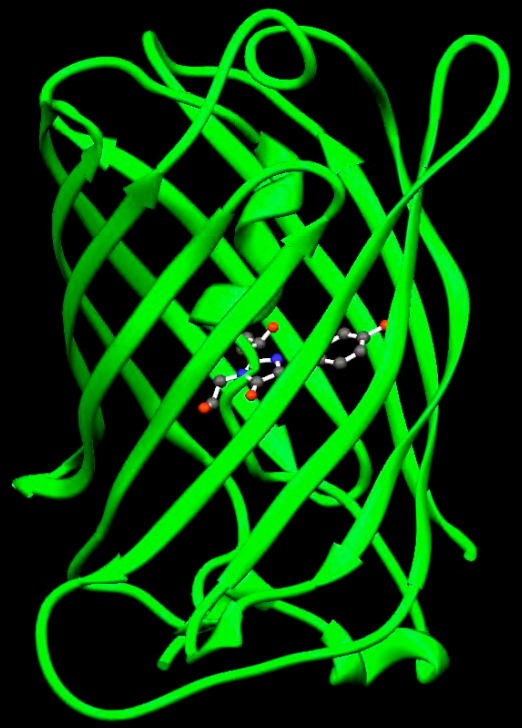


Ormo et al. (1996) *Science* **273**, 1392.

- This explained why the entire protein sequence was required for fluorescence.

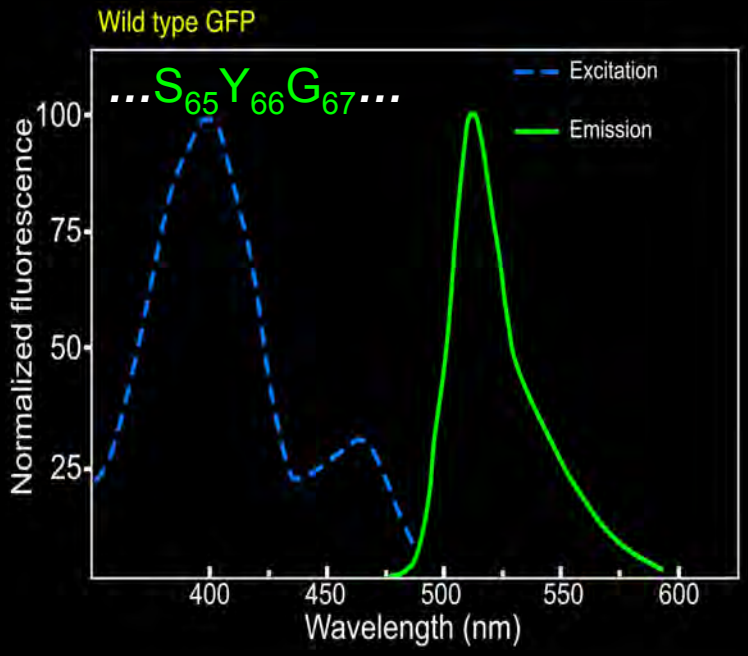
General characteristics of GFP

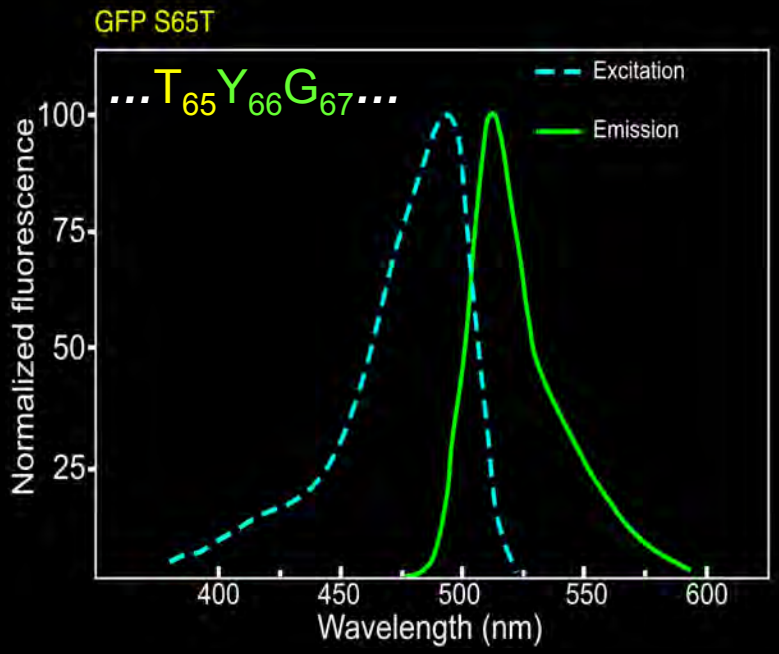
- Wild type GFP folds poorly at physiological temperature.
- "Humanized" codon usage, Kozak initiation codon.
- Mutations that improve efficiency of chromophore formation:
  - F64L dramatically improved maturation at 37º;
  - V68L enhances chromophore oxidation;
  - N149K improves folding rate;
  - M153T, V163A enhances folding.
- The *enhanced* FPs (e.g., EGFP)



Mutant variants of *A.v.* GFP

- Mutation of the chromophore position Ser 65 > Thr stabilized the chromophore, yielding a single absorption peak at 489 nm.

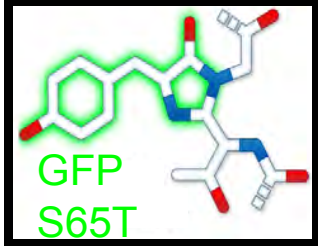
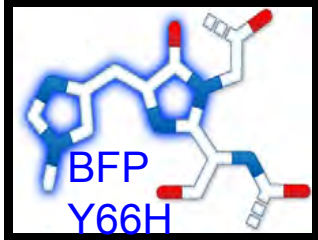
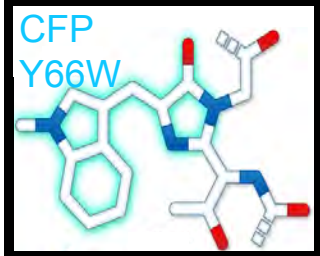
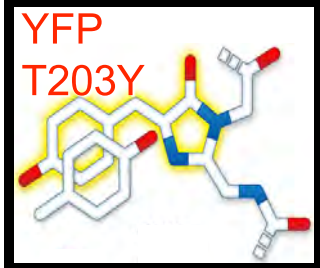




- The shifted single peak absorption and improved brightness made GFP^{S65T} more useful for live-cell imaging.
- Other chromophore mutations shifted the emission spectrum:

Tsien (1998) *Ann Rev Biochem.* **67**:509

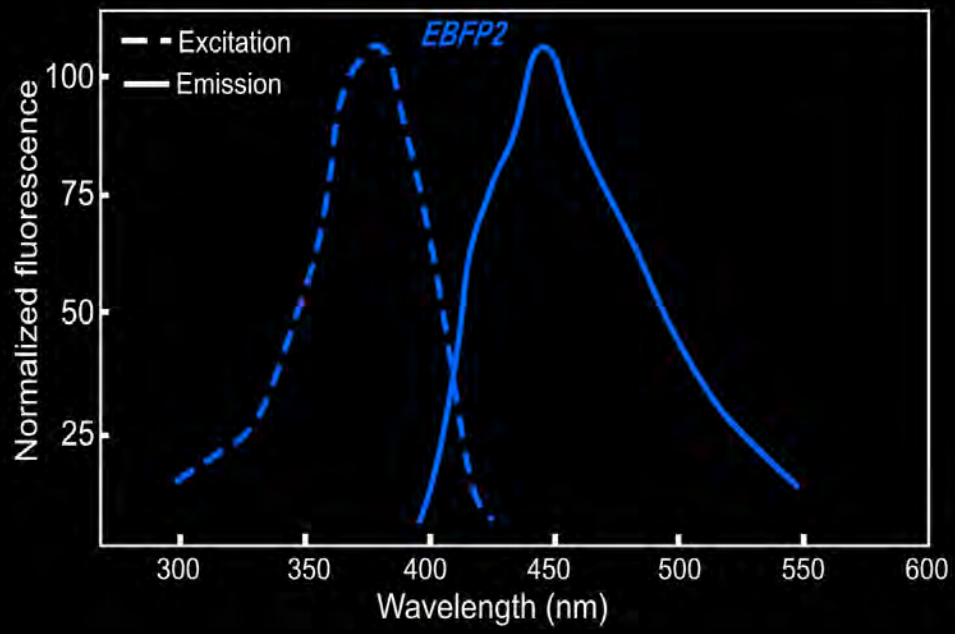
Mutant color variants of *A.v.* GFP

	Ex (nm)	Em (nm)	EC	QY	IB	Stability
 GFP S65T	488	507	56	0.6	34	+++
 BFP Y66H	383	445	29	0.3	9	+
 CFP Y66W	439	476	33	0.4	13	+++
 YFP T203Y	514	527	83	0.6	49	++

Tsien (1998) *Ann Rev Biochem.* **67**:509

New and improved *A.v.* color variants:

EBFP2

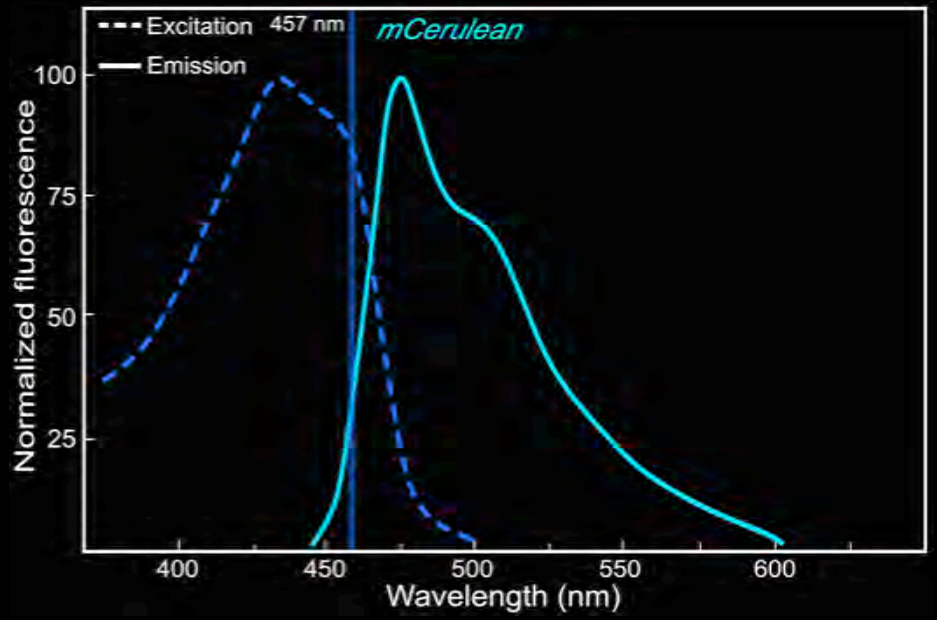


- Directed evolution of EBFP from *Aequorea* for selection of a brighter, more stable blue FP; incorporates superfolder mutations:
- Key mutations: EBFP + S30R, Y39N, T65S, S72A, I128V, F145H, M153A, D155V, A206V, V224R
  - Ex 383 nm, Em 448 nm;
  - intrinsic brightness of 18;
  - photostable - *especially useful as 2-photon ex probe*.

Ai *et al.* (2007) *Biochem.* **46**:5904

New and improved *A.v.* color variants:

mCerulean3

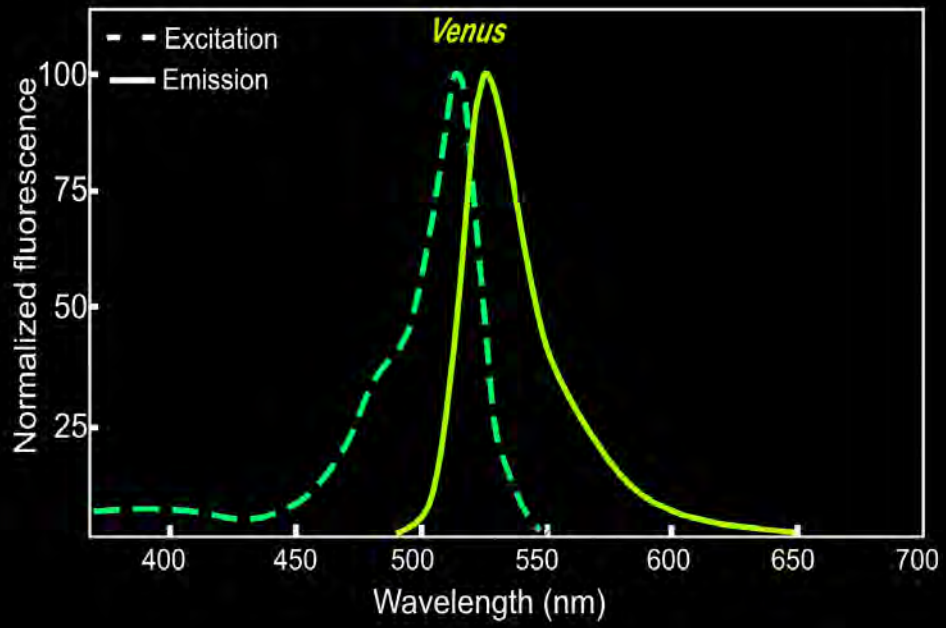


- Mutagenesis of ECFP - selection of a brighter, more stable Cerulean; optimization of β-strand 7 & 8, plus T65S > mCerulean3
- Key mutations: mCerulean + T65S/S147H/D148G/K166G/I167L/R168N/H169
  - Ex 334 nm, Em 475 nm;
  - intrinsic brightness of 34 (similar to EGFP);
  - very photostable, decrease photoswitching, single lifetime.

Markwardt et al. (2011) *PLoS One, In Press*

New and improved *A.v.* color variants:

mVenus

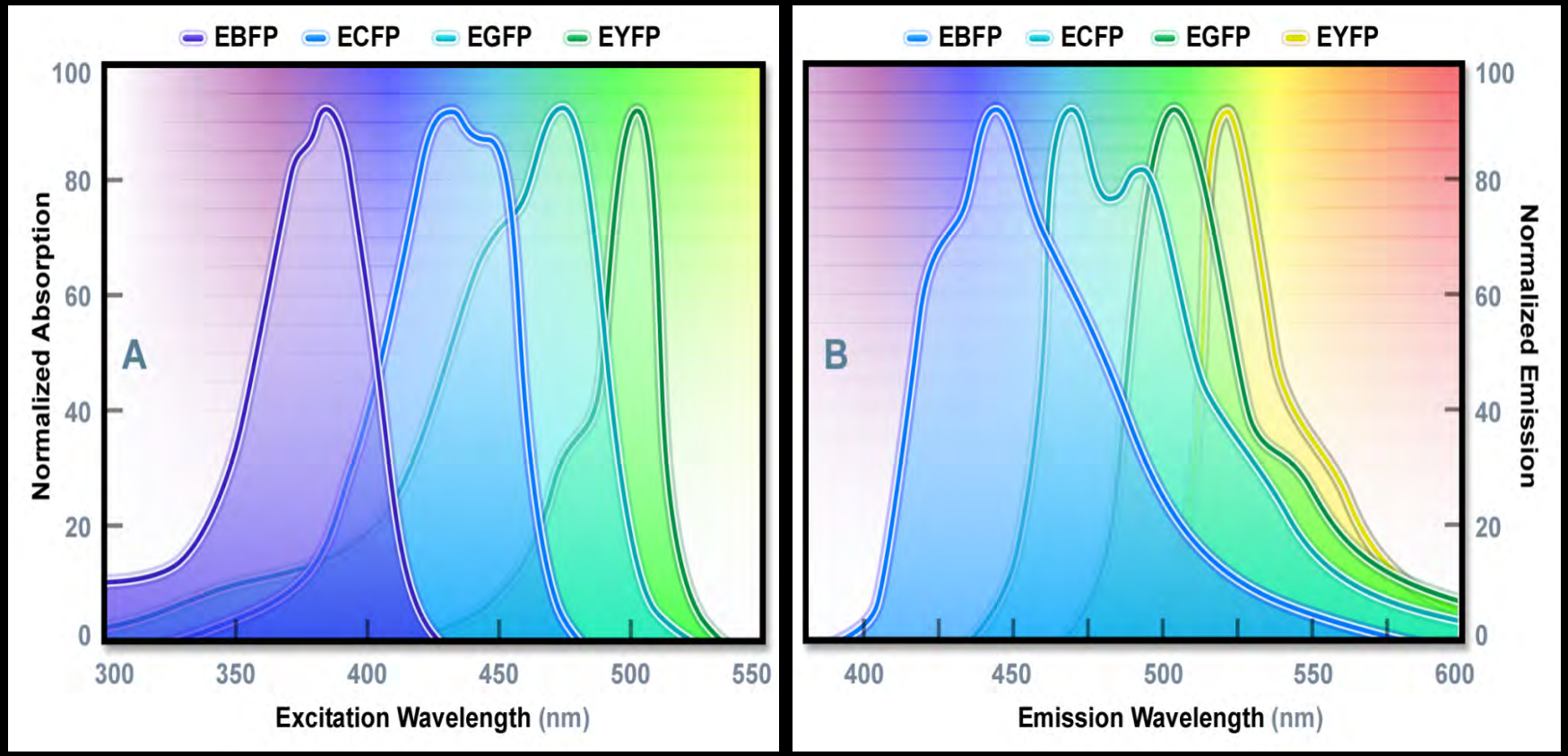


- Mutagenesis of EYFP from *Aequorea* for selection of a brighter Yellowish FP with reduced halide and pH sensitivity:
- Key mutations: F46L, F64L, S65G, S72A, M153T, V163A, T203Y, A206K
  - Ex 515 nm, Em 528 nm;
  - intrinsic brightness of 54;
  - Maturation rapid - *NOT very photostable*.

Nagai et al. (2002) *Nature Biotech.* **20**:87

Mutant color variants of *A.v.* GFP

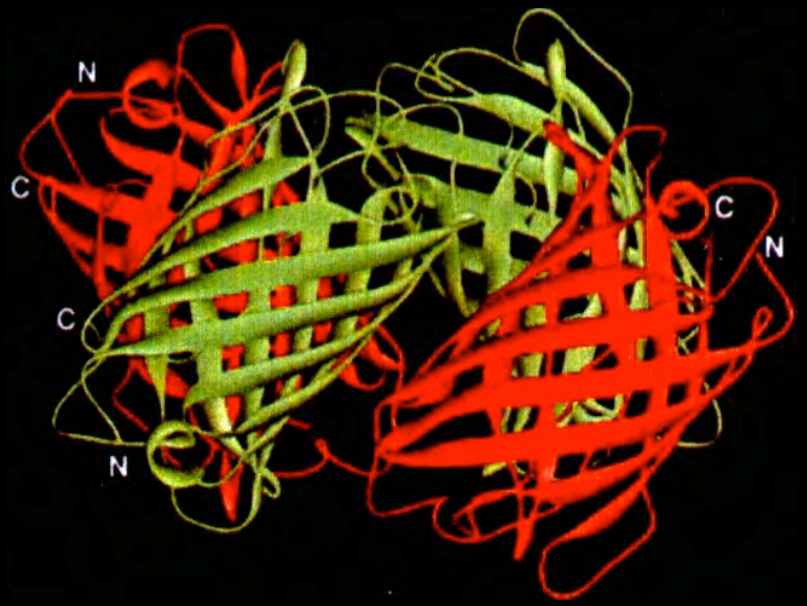
- *A.v.*-base FP color variants from blue to yellow:

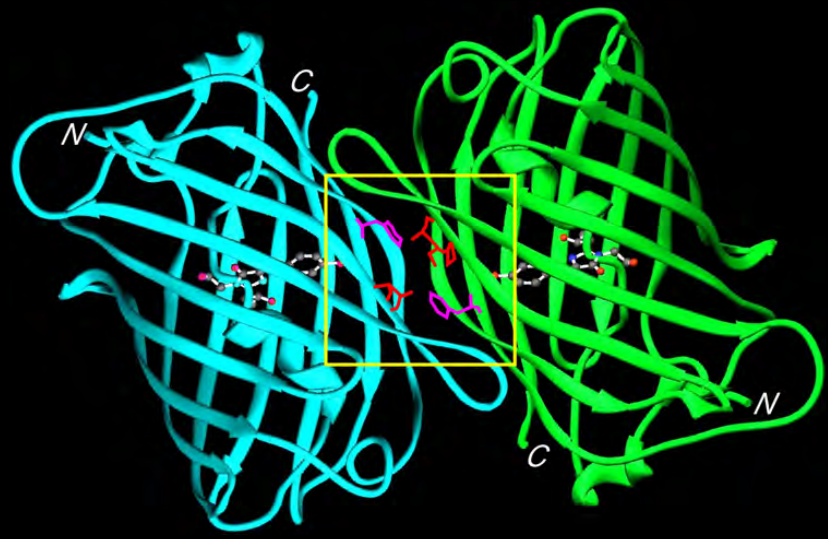


- The 530 nm emission of YFP was the most red-shifted of the color variants derived from *A.v.* GFP.

Aequorea FPs and dimer formation

- Most of the natural FPs that have been characterized are either dimers, tetramers, or higher-order complexes.

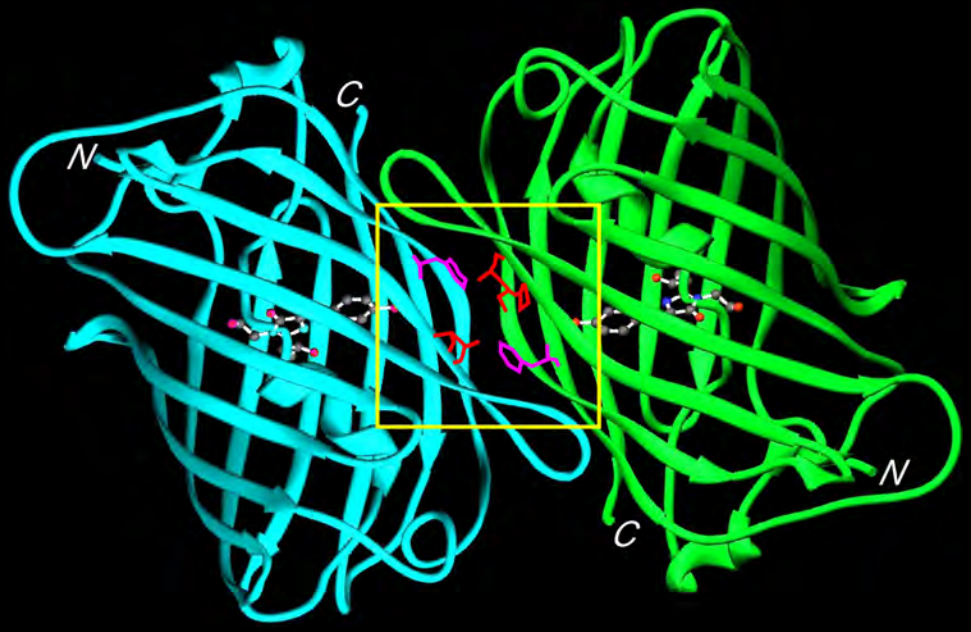




- GFP could be crystallized as a monomer, but the proteins can form dimers when highly concentrated.

Aequorea FPs and dimer formation

- Dimerization is not typically observed when the proteins are free to diffuse within the cell;
- but, the expression of FPs at high concentrations in a diffusion limited volume can lead to the formation of dimers.



- The substitution of alanine^{206} with lysine (*A206K*) prevents dimer formation.

  Zacharias et al (2002) *Science* **296**:913; Kenworthy (2002) *TBCS* **27**:435

- This is *especially* important for FRET-based imaging methods.

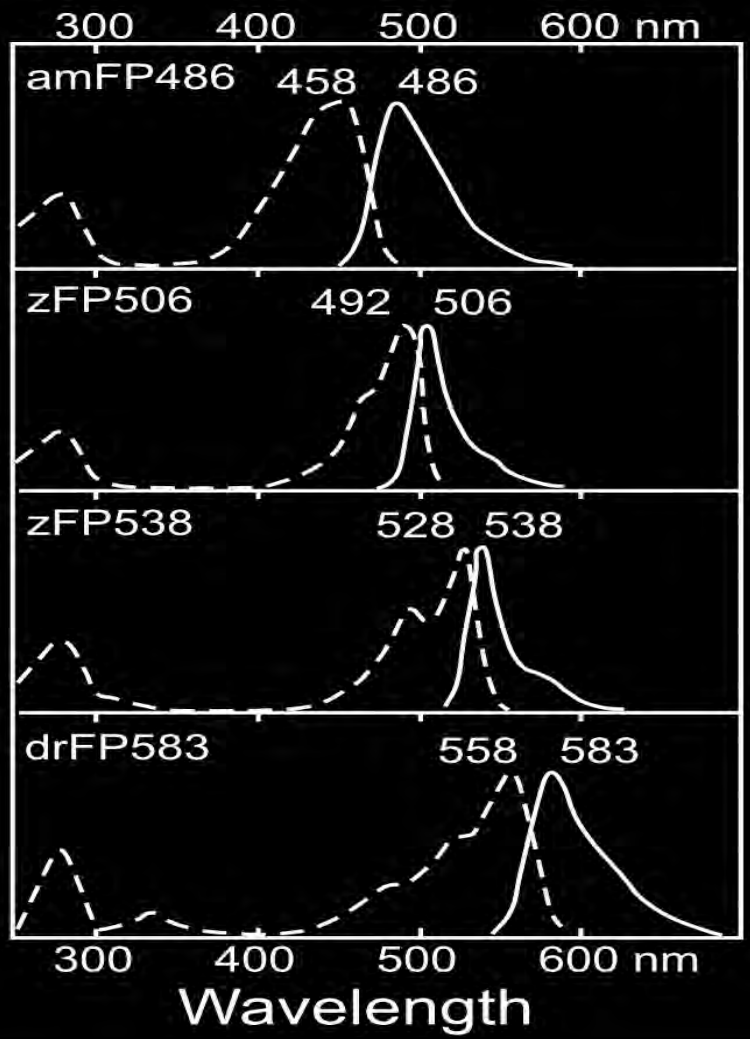
Overview

- The genetically encoded fluorescent proteins (FPs):
  - General characteristics of the FPs.
  - Mutant color variants based on *A.v.* GFP.
  - FPs derived from *Discosoma striata* - mRFP and the fruits.

Fluorescent Proteins from other marine organisms

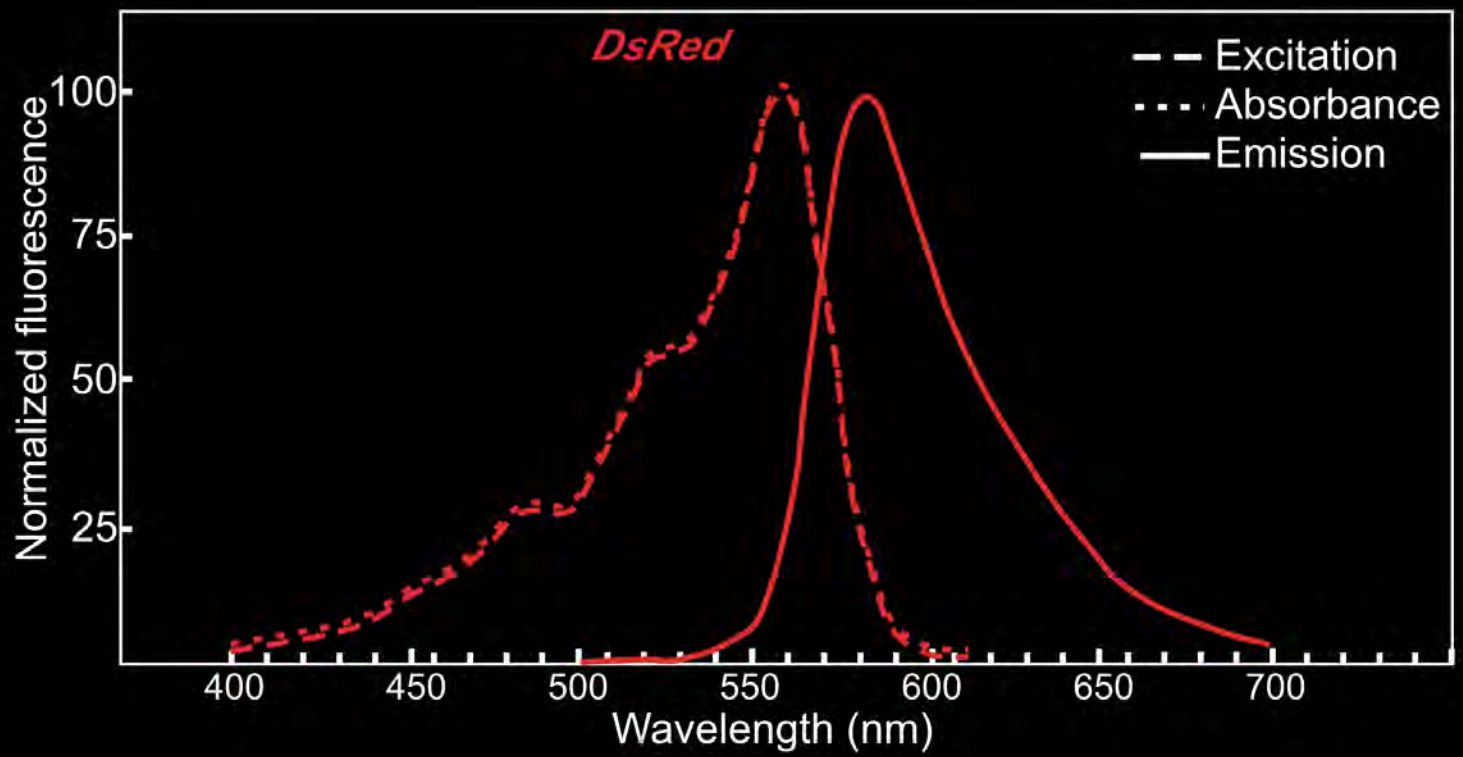
- Most of the colors in reef corals result from GFP-like proteins.

Mushroom anemone *Discosoma striata*



Matz et al. (1999) *Nat. Biotech.* **17**:996

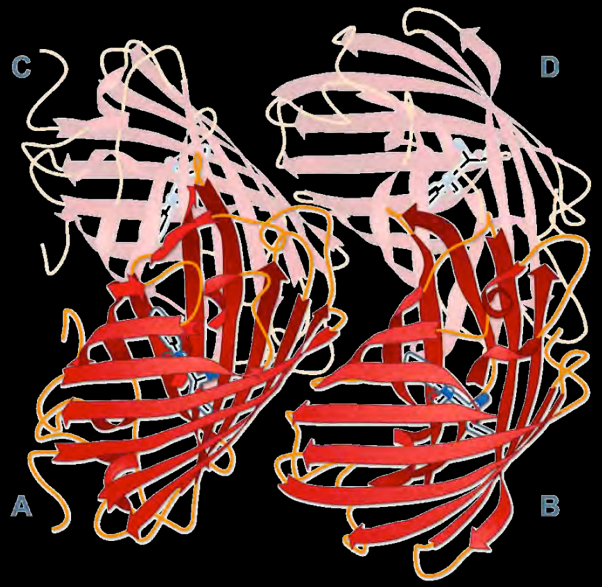
Advantages of DsRed

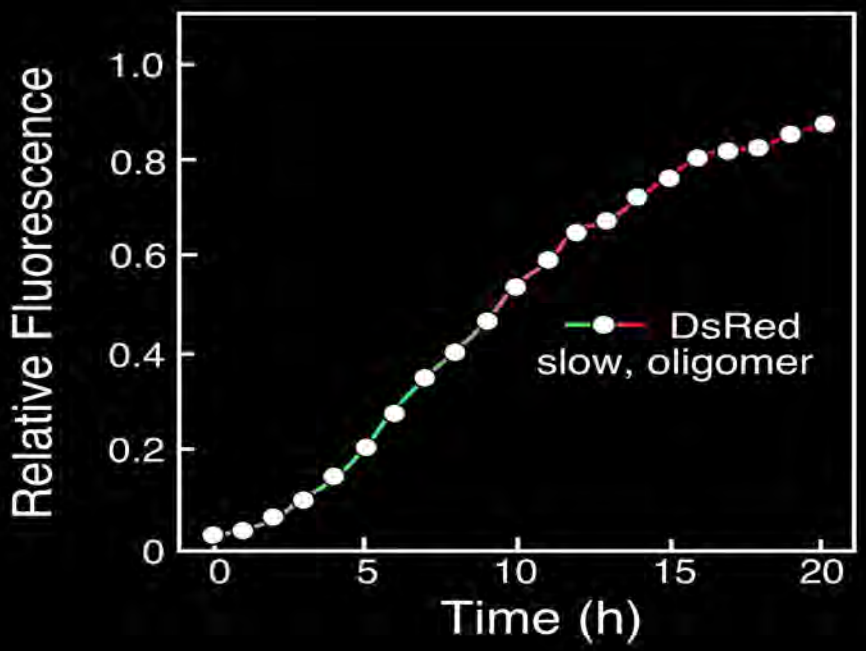


- DsRed is spectrally distinct from the *Aequorea* FPs.
  - It is easily detected with standard optical filters;
  - there is reduced cellular auto-fluorescence at longer wavelengths.
  - Cells are more tolerant of illumination at longer wavelengths.

Problems with DsRed

- DsRed is an obligate tetramer in mammalian cells:

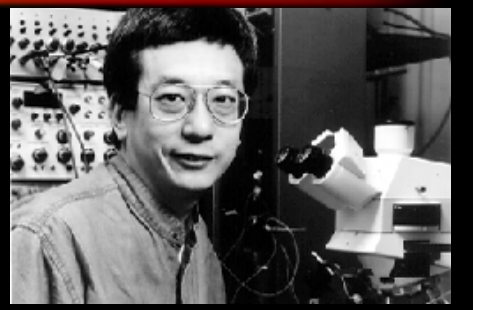


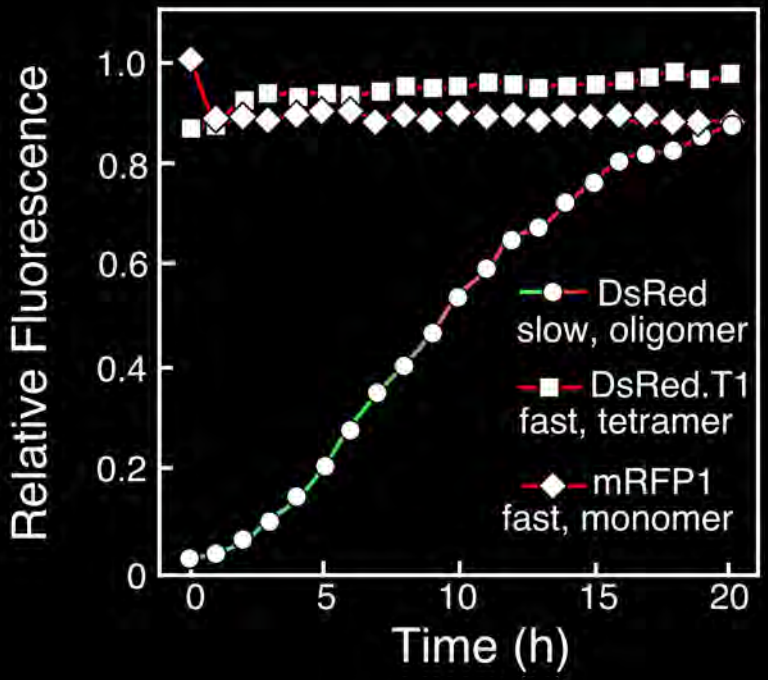


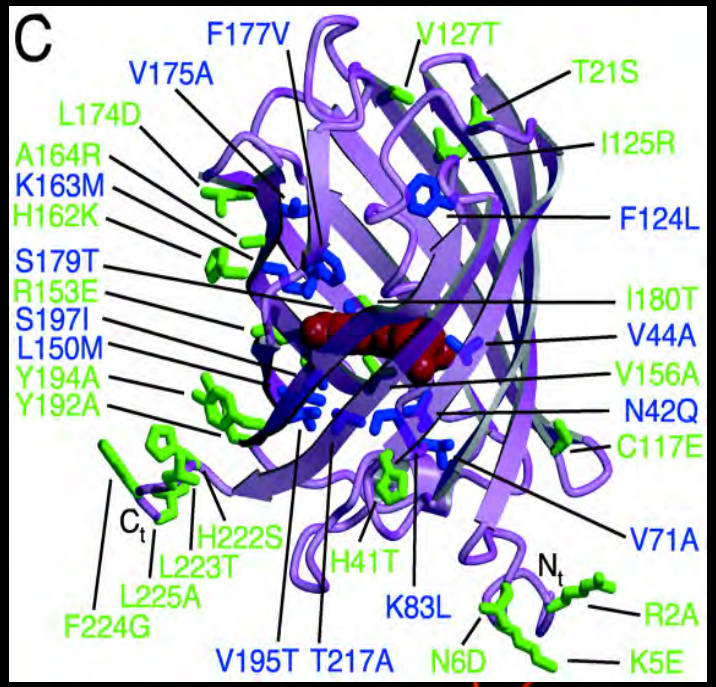
- DsRed tends to form oligomers, leading to misdirected fusion proteins.
- DsRed requires nearly 20 h to fully mature, and there is a green intermediate form of the protein.

New variants of DsRed

- Mutagenesis to improve maturation: DsRed.T1
- Site-directed mutagenesis to break the tetramer;
- Random mutagenesis to recover red fluorescence.



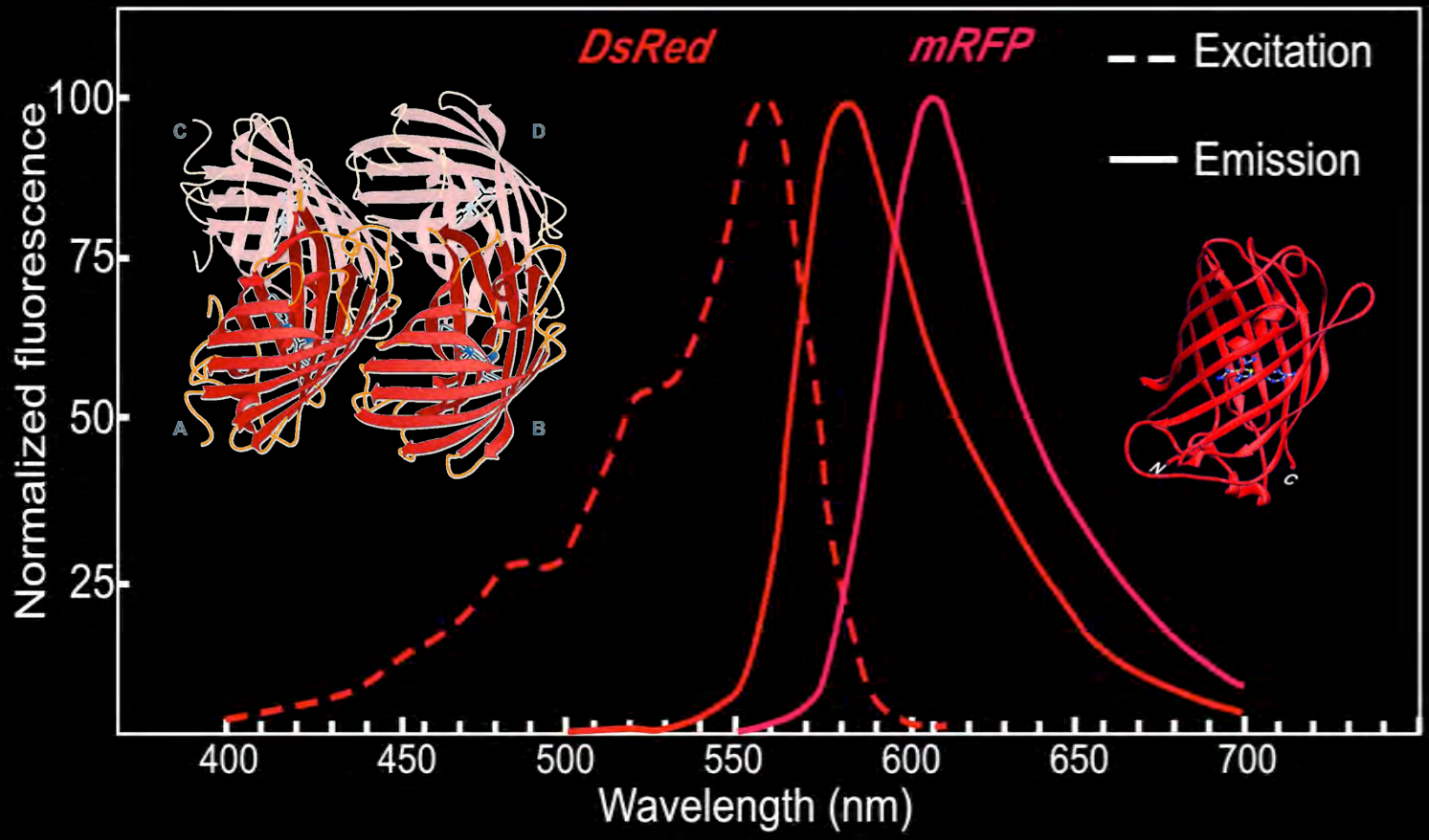




Campbell et al. (2002) *PNAS* **99**:7877

mRFP1 was an improvement-

- mRFP1 overcame tetramer and slow maturation;
- and shifted excitation and emission by 25 nm.

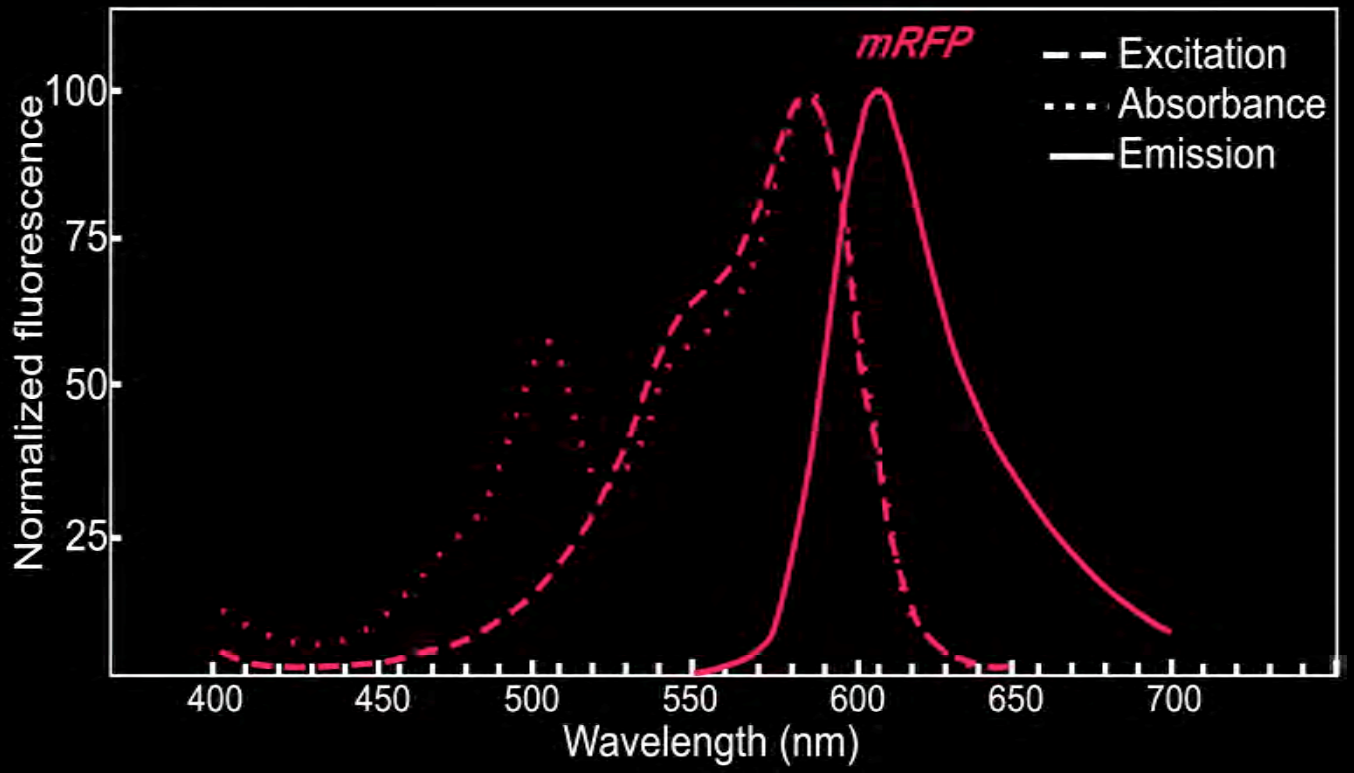


Campbell et al. (2002) *PNAS* **99**:7877

but, mRFP was not optimal for some applications

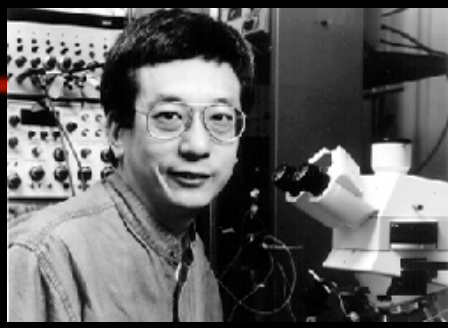
- mRFP1 has decreased quantum yield and photostability;
  a non-fluorescent form absorbs at 503 nm - 60% in a dark state.

Hillesheim et al. (2006) *Biophys J* **91**:4273

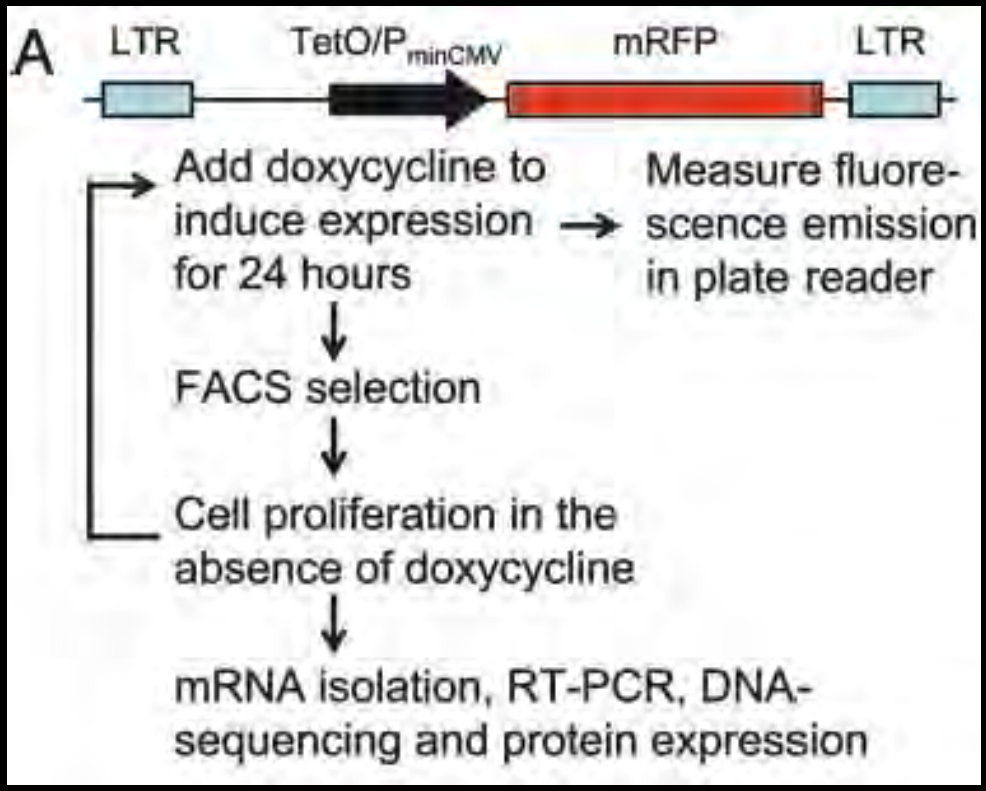


- New improved yellow, orange, red FPs were needed:

Directed evolution yields new FPs

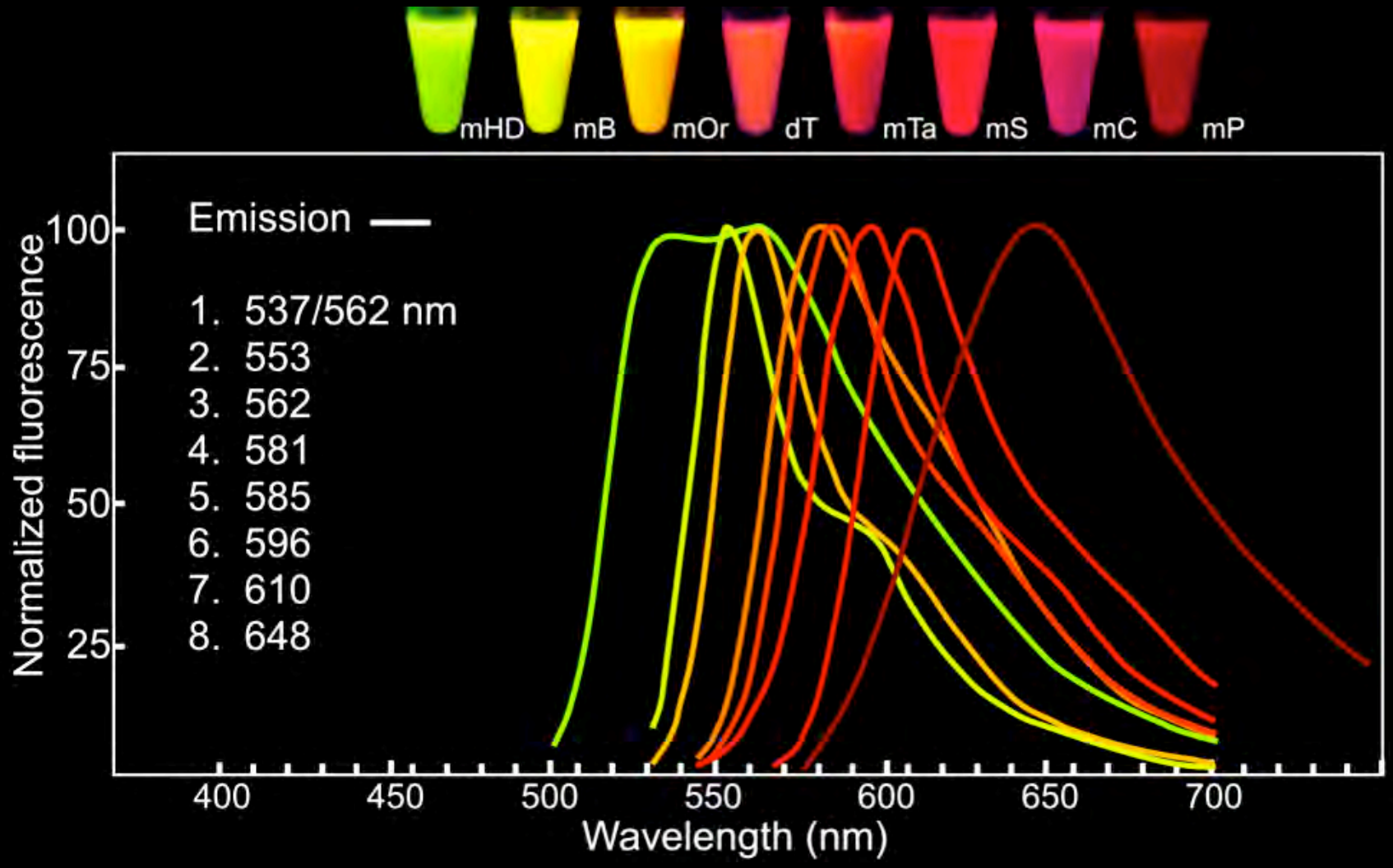


- Human B cells generate antibody diversity by somatic hypermutation.
- Transfect B cells with Tet-inducible mRFP1 and induce expression.
- FACS to select cells producing spectral variants.
- Each round took only a few days.



Wang *et al.* (2004) *PNAS* **101**:16745

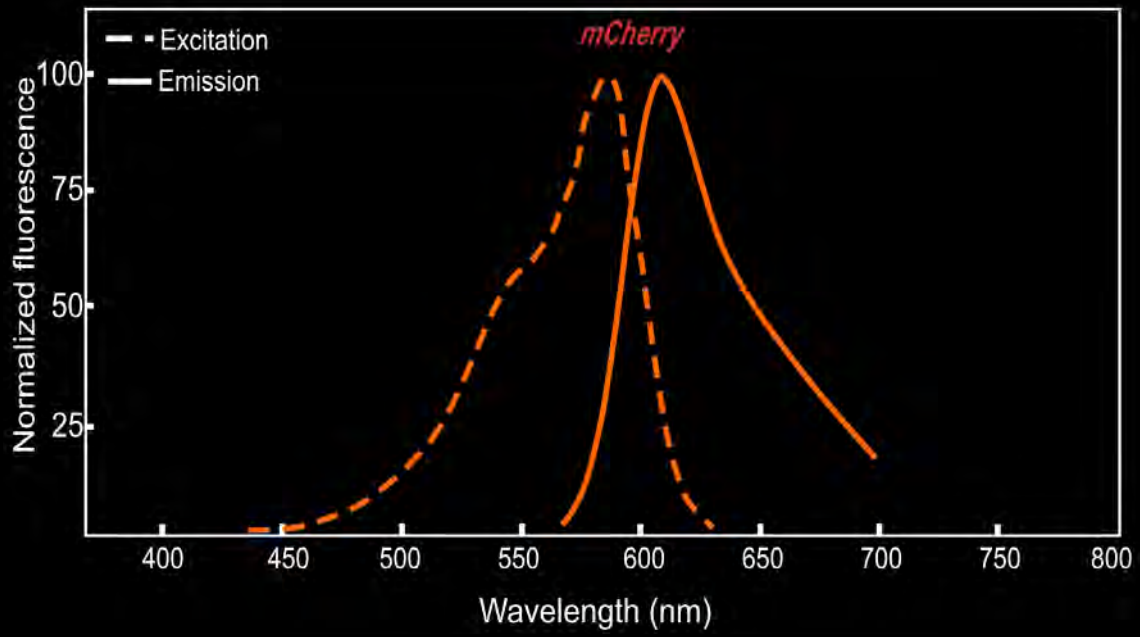
The next generation of FPs



Shaner *et al.* (2004) *Nat. Biotech.* **22**:1567

mCherry - Fruit series optimized RFP

Discosoma striata

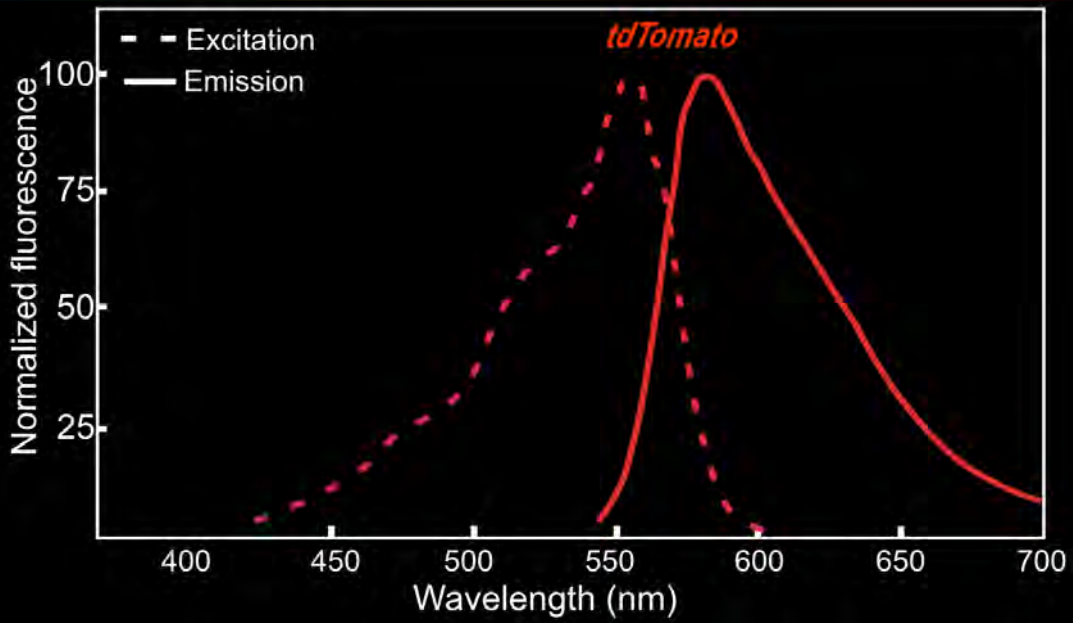


- Directed evolution of mRFP1 from DsRed with selection for a bright, photostable, monomeric red FP:
- Key mutations: Q66M, M163Q, M182K, T195V
  - Ex 587 nm, Em 610 nm;
  - intrinsic brightness of 17;
  - Maturation 15 min, photostable.

Shaner et al. (2004) *Nat. Biotech.* **22**:1567

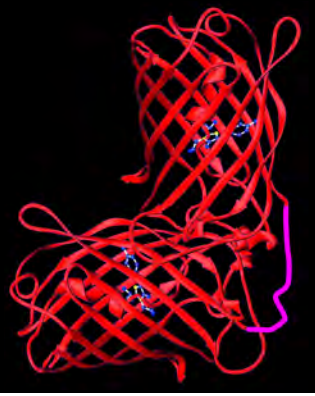
tdTomato - Fruit series dimeric FP

Discosoma striata



- Directed evolution of mRFP1 from DsRed with selection for a very bright, photostable, tandem dimer Orange FP:

- Key Feature: 12 aa linker "GHGTGSTGSTSS"
  - Ex 554 nm, Em 581 nm;
  - intrinsic brightness of 95;
  - Maturation 60 min - *tandem dimer*.



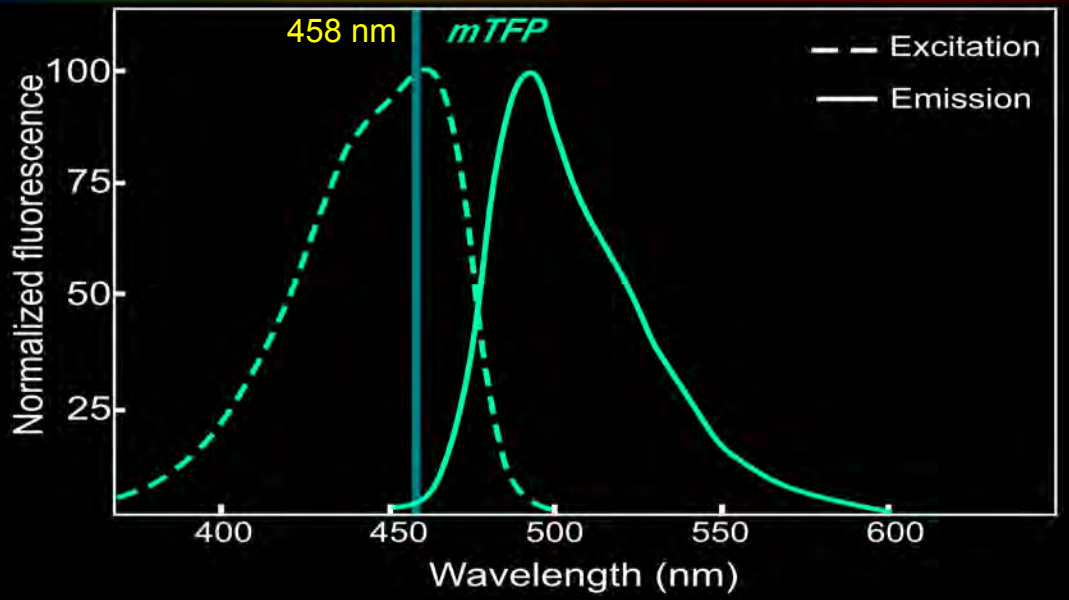
Shaner et al. (2004) *Nat. Biotech.* **22**:1567

Genetically encoded fluorescent proteins

- The genetically encoded fluorescent proteins (FPs):
  - General characteristics of the FPs.
  - Mutant color variants based on *A.v.* GFP.
  - FPs derived from *Discosoma striata* - mRFP and the fruits.
  - New FPs derived from corals.

The cloning of novel FPs from corals: mTFP1

Clavularia sp. “palm coral”

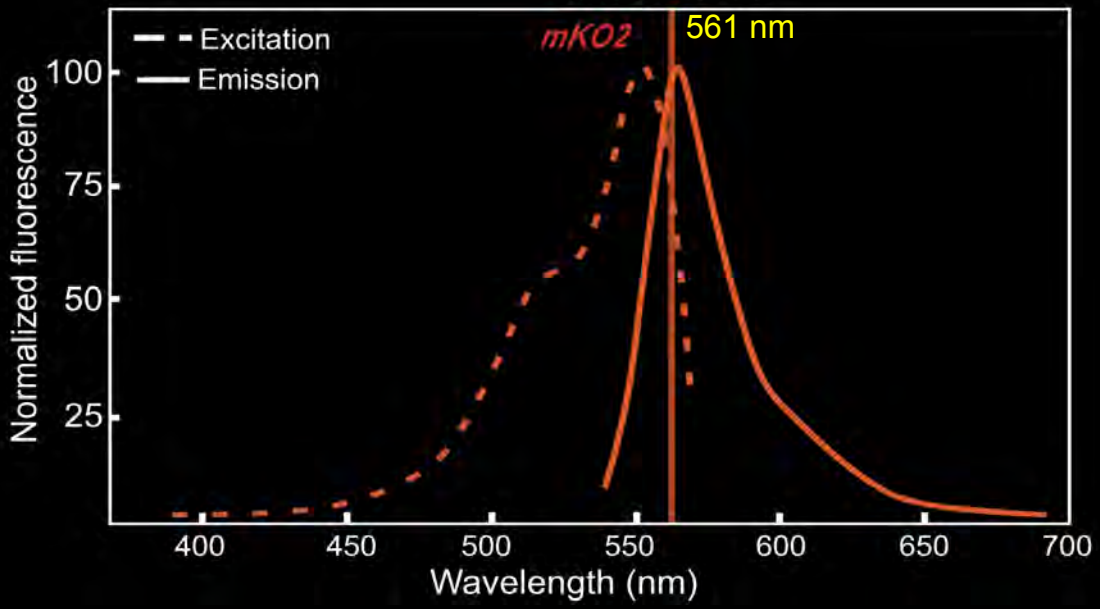


- Directed evolution of cFP484 from *Clavularia* sp. for selection of a bright blue-green (Teal) FP:
- Key mutations mTFP1: Y67; N63T, Q66A, L72F, D125K, M127E, E144D::H163
  - Ex 462 nm, Em 492 nm, relatively narrow spectra;
  - intrinsic brightness of 54;
  - photostable - *acid stable*.

Ai *et al.* (2006) *Biochem. J* **400**:531

The cloning of novel FPs from corals: mKO2

Fungia concinna "mushroom coral"



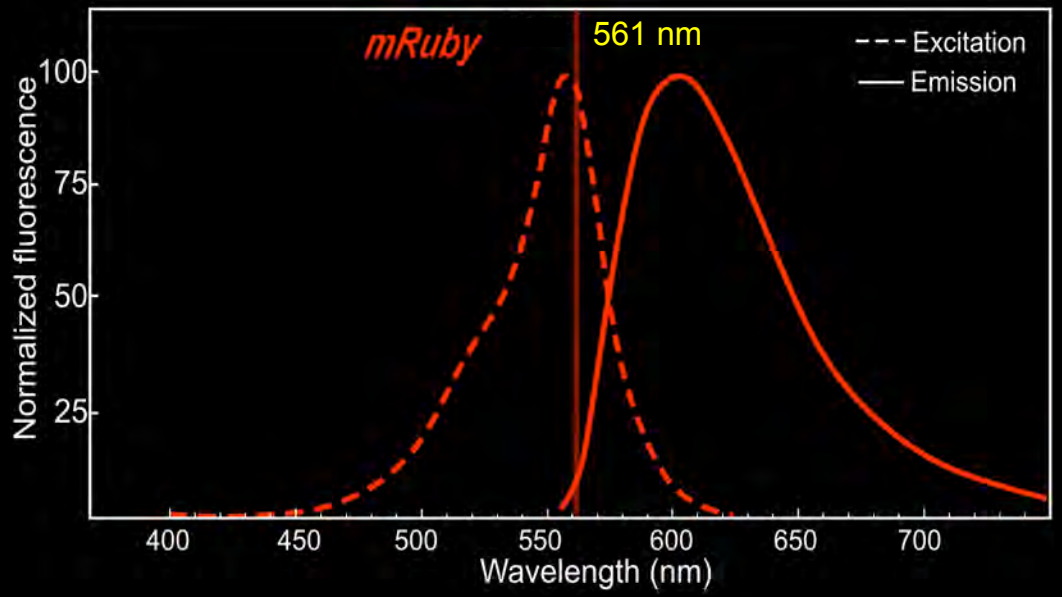
- Directed evolution of Kusabira orange from *Fungia concinna* for selection of a bright monomeric Orange FP:

- Key mutations: Kusabira + K49E, P70V, F176M, K185E, K188E, S192G, L210Q
  - Ex 551 nm, Em 565 nm;
  - intrinsic brightness of 36;
  - Maturation rapid, photostable - *narrow Stokes shift*.

Sakaue-Sawano et al. (2008) *Cell* **132**:487

The cloning of novel FPs from corals: mRuby

Entacmaea quadricolor anemone



- Directed evolution of a dimeric eqFP611 from *Entacmaea quadricolor* for selection of a bright monomeric Red FP:

- Key mutations: eqFP611 F102I + 29 mutations.
  - Ex 558 nm, Em 605 nm;
  - intrinsic brightness of 39;
  - Maturation 2.8 h, photostable.

Kredel et al. (2009) *PLoS One* **4**:e4391

Genetically encoded fluorescent proteins

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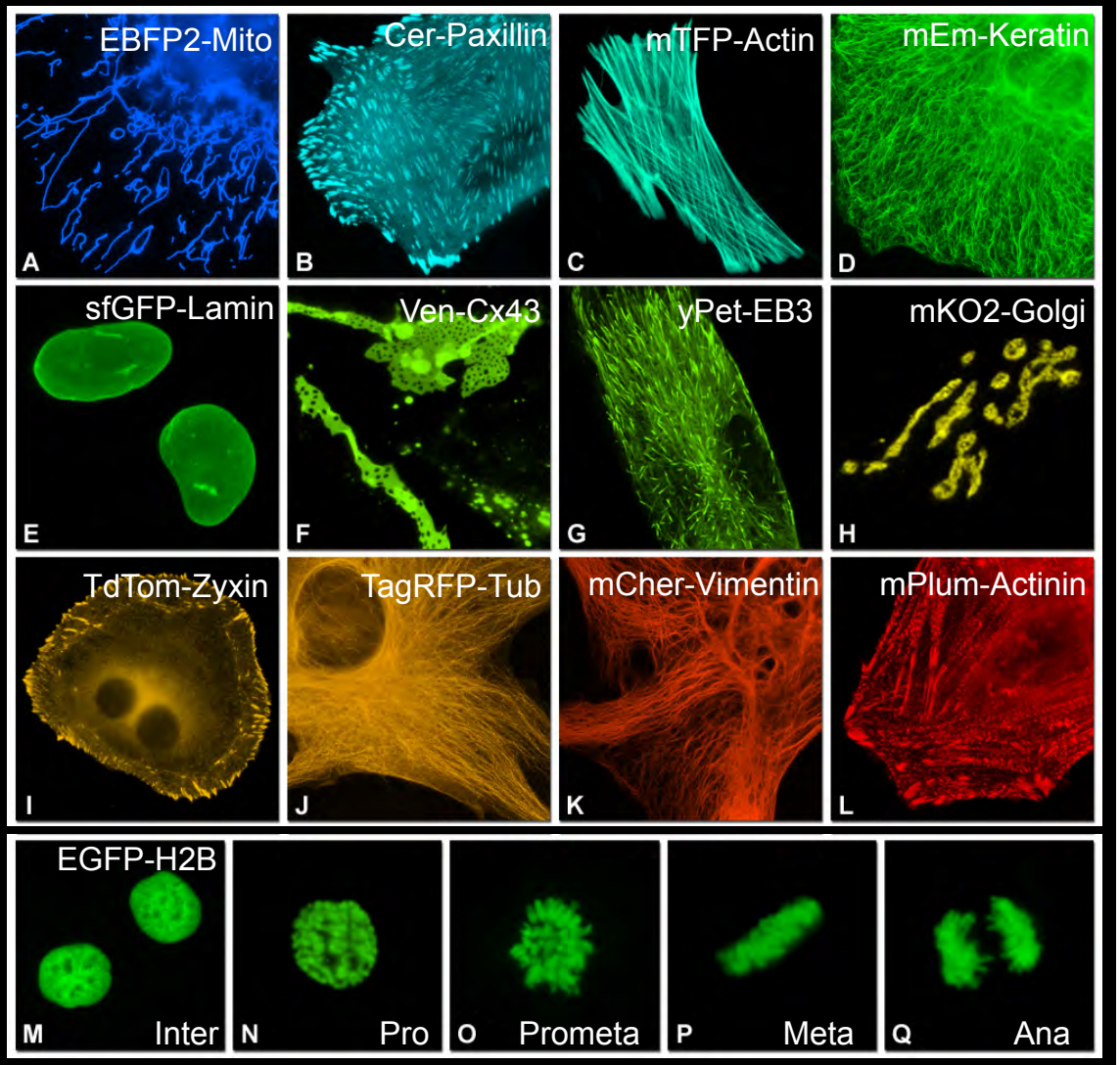
  - Mutant color variants based on *A.v.* GFP.
  - FPs derived from *Discosoma striata* - mRFP and the fruits.
  - New FPs derived from corals.
  - The (current) best FPs.

Subcellular distribution of FP-tagged proteins

- **Distribution:**
  Does the fusion protein replicate the localization of the endogenous protein?

- **Function:**
  Does the fusion protein have *all* of the functions of the endogenous protein?
  (rapid, efficient maturation, monomer help)



Shaner et al. (2007) *J Cell Sci* 120:4247

Useful FP tool box (2011):

Protein	Color	Peak Ex	Peak Em	Brightness	Photo-Stability	Reference	Source
EBFP2	Blue	383	448	18	++	Ai et al. 2007	Dr. Robert Campbell
Cerulean3	Cyan	433-445	475-503	35	++++	Rizzo et al. 2004	Dr. Mark Rizzo
mTFP	Teal	462	492	54	+++	Ai et al. 2006	Allele Biotech
EmGFP	Green	487	509	39	++++	Cubitt et al. 1999	Invitrogen
Venus	Yellow/Grn	515	528	53	+	Nagai et al. 2002	Dr. Atsushi Miyawaki
REACh	Yellow/Grn	515	528	1*	+	Ganesan et al. 2006	Dr. Sundar Ganesan
Amber	None	ND	ND	0**		Koushik et al. 2006	Dr. Steven Vogel
mKO2 (Kusabira)	Orange	551	565	36	+++	Karasawa et al. 2004; Sakaue-Sawano 2008	MBL International
mTagRFP-T	Orange	555	584	31	++++	Merzlyak et al. 2007; Shaner et al. 2008	Evrogen
tdTomato	Orange	554	581	95	+++	Shaner et al. 2004	Dr. Roger Tsien
mRuby	Red	558	605	39	++++	Kredel et al. 2009	Dr. Jorg Wiedenmann
mCherry	Red	587	610	17	+++	Shaner et al. 2004	Clontech
mKate (Katushka)	Deep Red	588	635	15	++++	Shcherbo et al. 2007	Evrogen

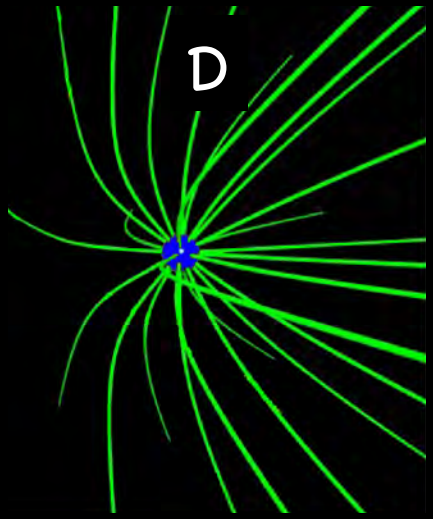
* Dark probe useful for FRET-FLIM; ** Y66C mutant folds but does not absorb or emit - important control for FRET-FLIM. Adapted from Day and Davidson, 2009.

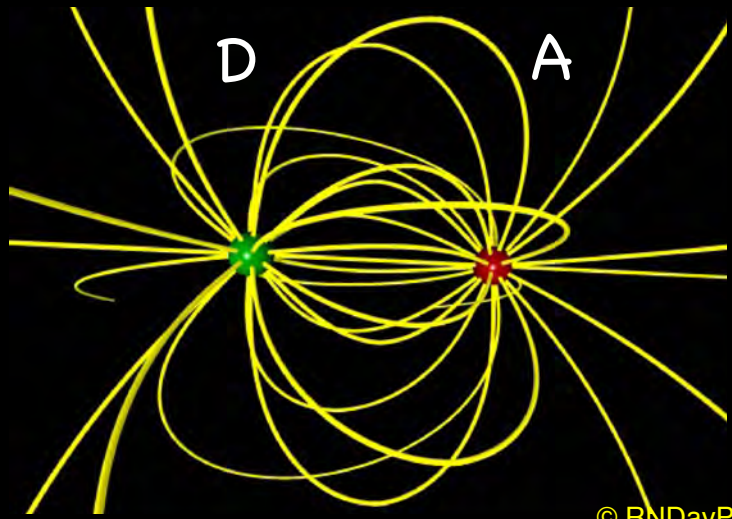
Genetically encoded fluorescent proteins

- The genetically encoded fluorescent proteins (FPs):
  - General characteristics of the FPs.
  - Mutant color variants based on *A.v.* GFP.
  - FPs derived from *Discosoma striata* - mRFP and the fruits.
  - New FPs derived from corals.
  - The (current) best FPs.
- Förster (Fluorescence) resonance energy transfer (FRET):
  - General requirements for FRET.
  - Spectral bleedthrough background.
  - Methods used to measure FRET - strengths and weaknesses.
  - FPs for FRET – optimal pairs.
  - Summary.

What is FRET?

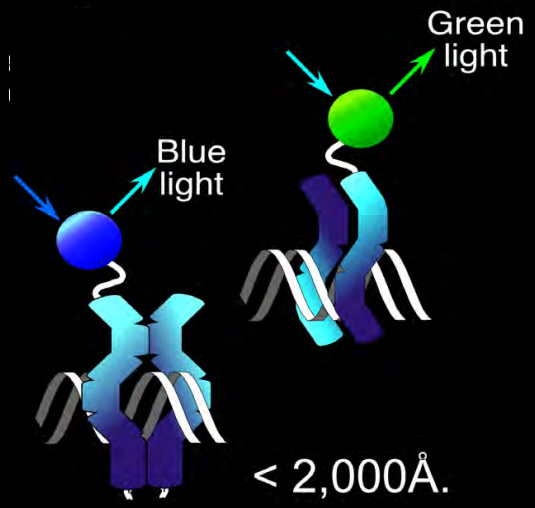
- FRET is the direct transfer of excited state energy from a donor fluorophore to a nearby acceptor.
- A fluorophore in the excited-state is an oscillating dipole that creates an electric field (the donor - D).
- If another fluorophore enters the electric field, energy can be transferred directly to that fluorophore (the acceptor - A).
- No intermediate photon!

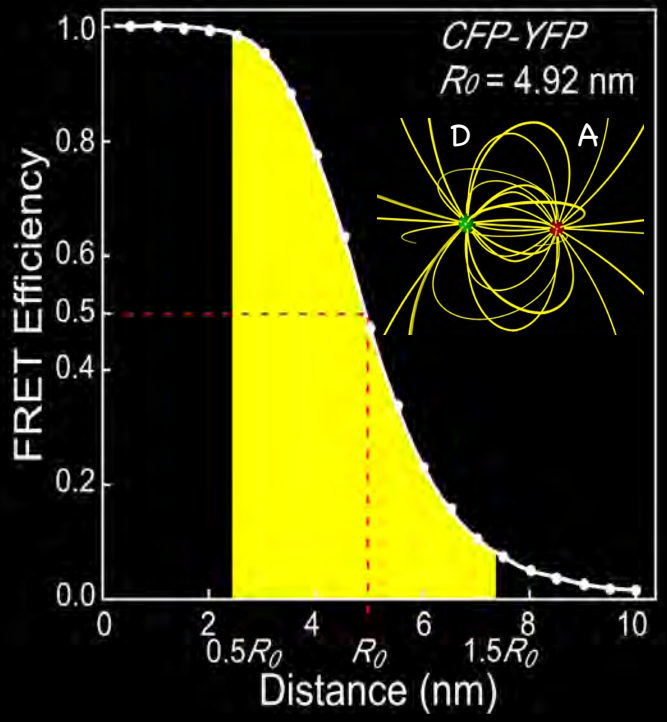


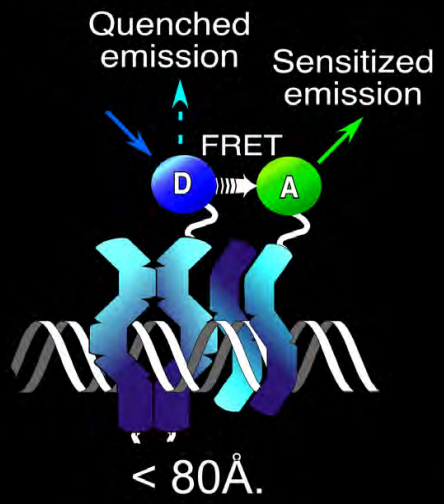


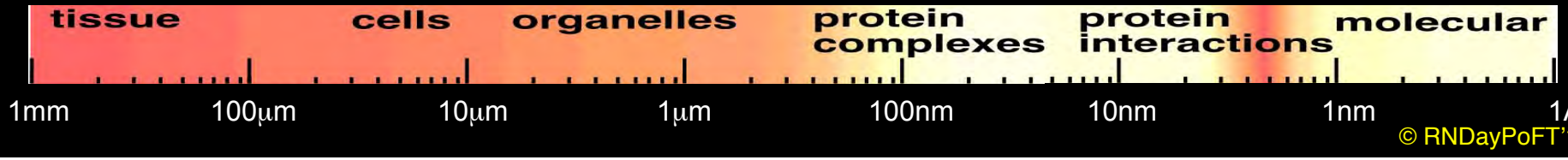
FRET measures the spatial relationship between the FPs

- The optical resolution of the light microscope is limited to 200 nm.
- The detection of FRET indicates the fluorophores are less than ~ 80Å apart:







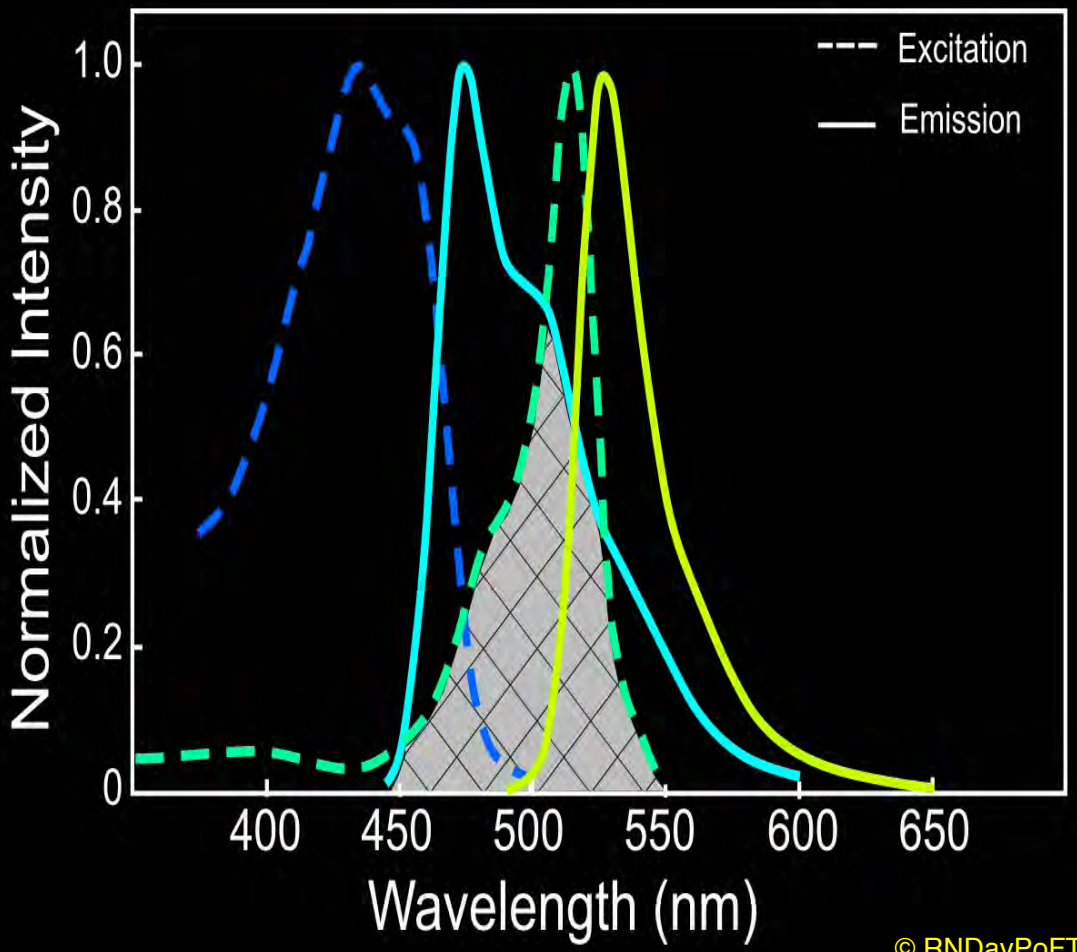


The spectral overlap requirement

- The donor emission spectrum must significantly overlap the absorption spectrum of the acceptor.

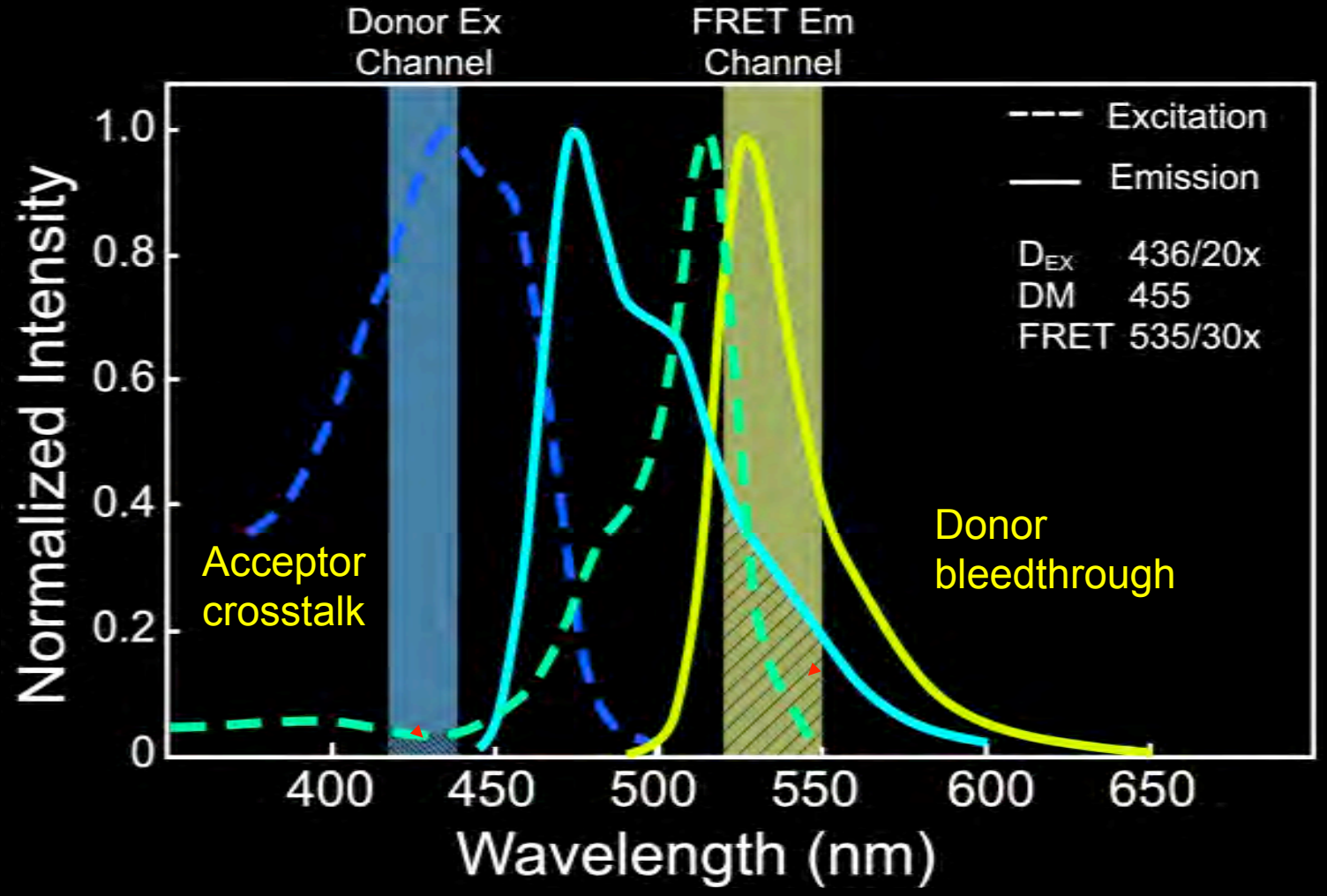
mCerulean

mVenus



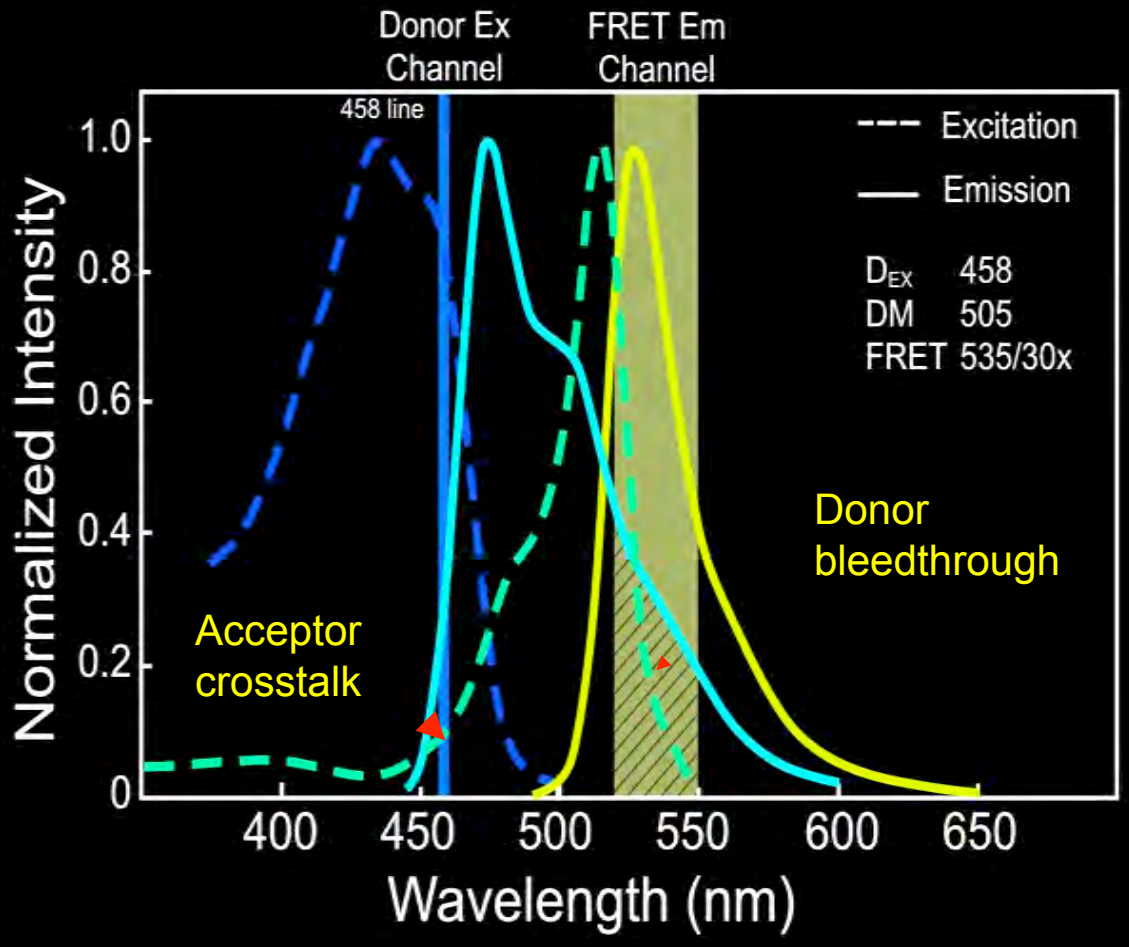
Spectral bleedthrough background signals

- Spectral bleedthrough - the more overlap, the more background.



Spectral bleedthrough background signals

- This is still a problem when using LSCM –

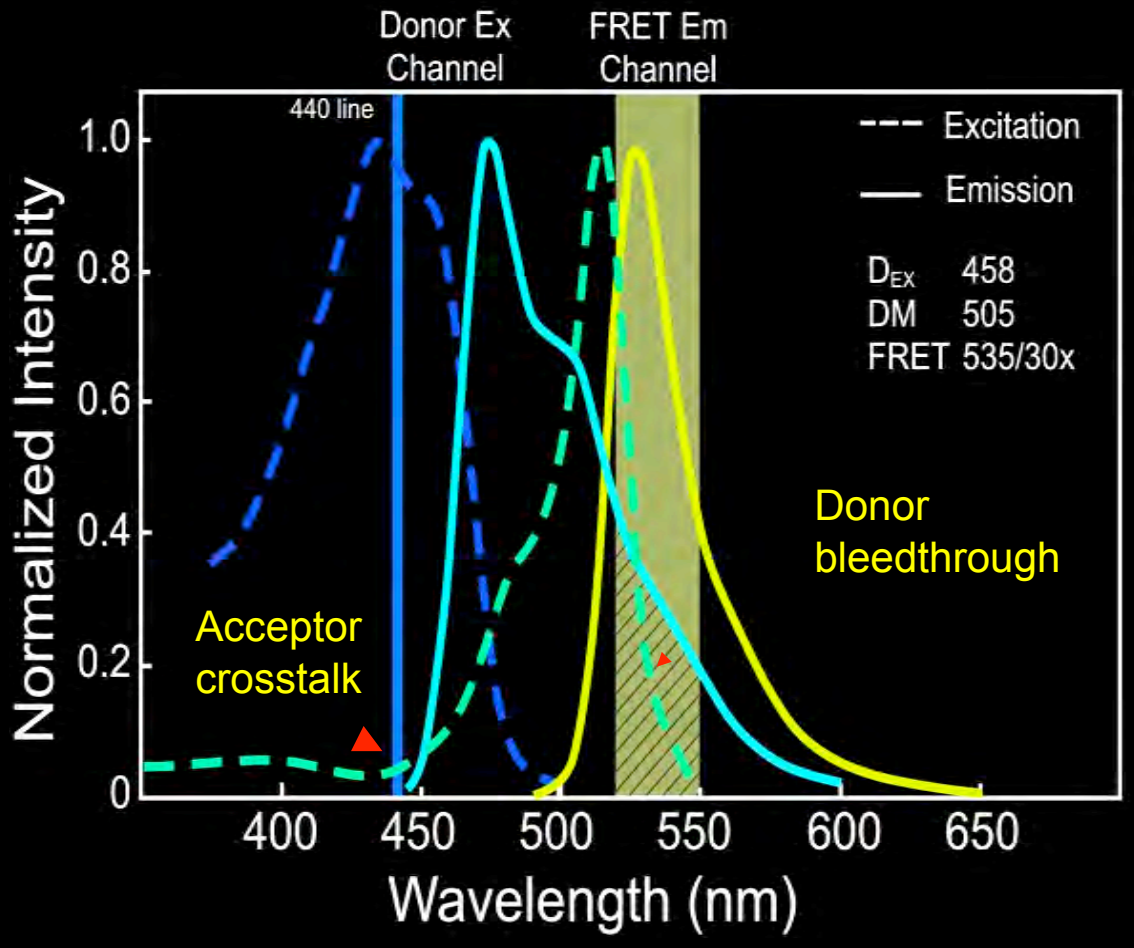


Spectral bleedthrough background signals

- This is still a problem when using LSCM –

  even with diode lasers:

  - The accurate measurement of FRET by sensitized acceptor emission requires removal of SBT!

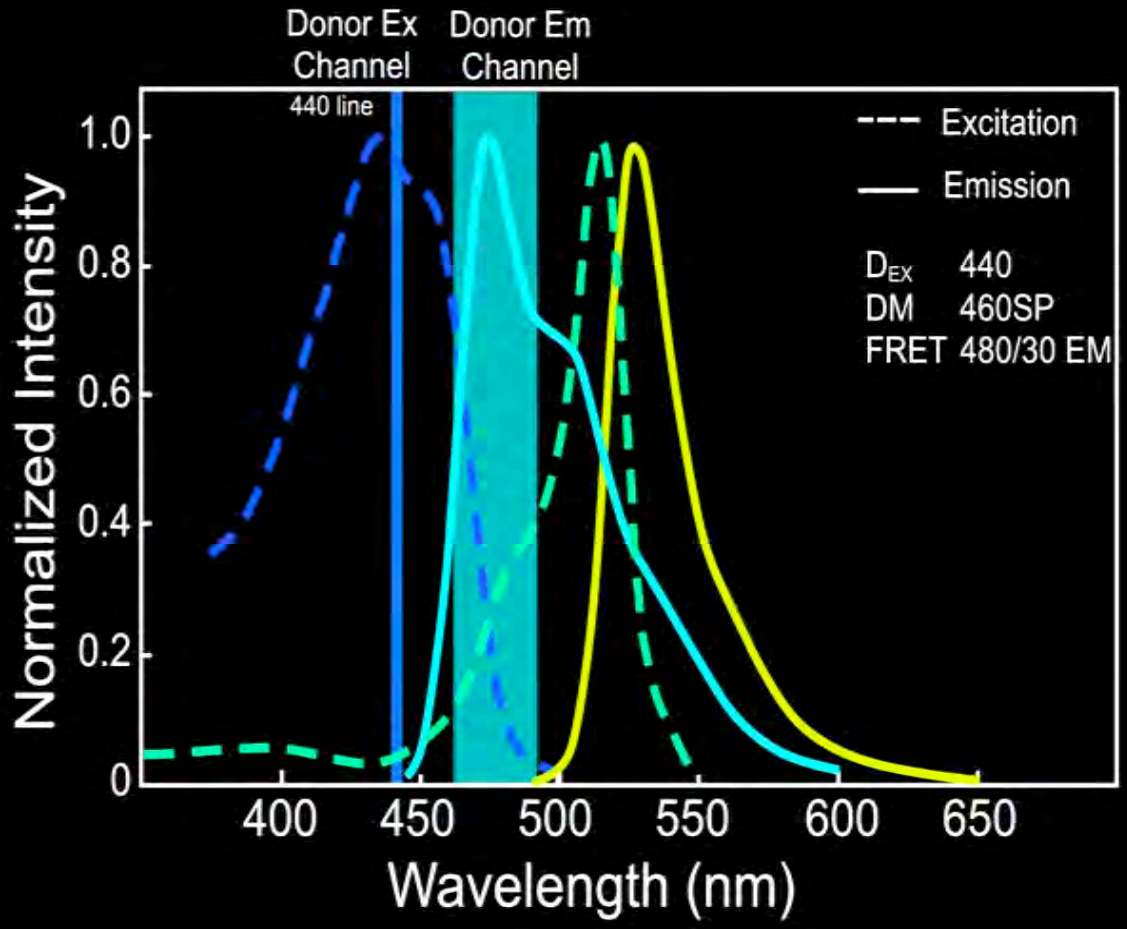


Spectral bleedthrough background signals

- This is still a problem when using LSCM – even with diode lasers:

  - The accurate measurement of FRET by sensitized acceptor emission requires removal of SBT!

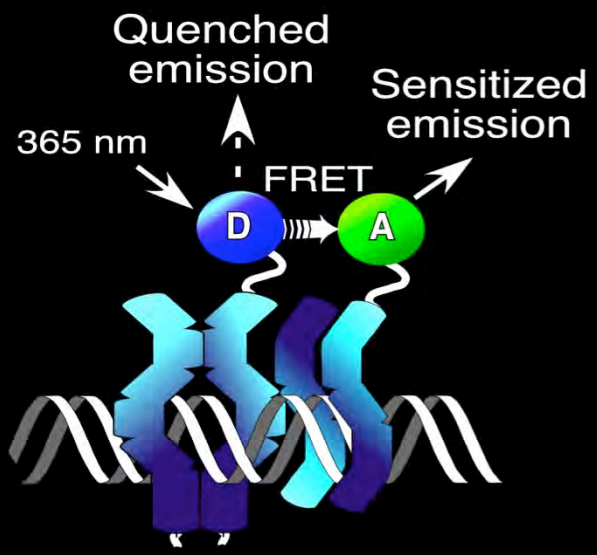
  - Alternatively, methods that detect changes in donor fluorescence are typically not affected by SBT, and can be most accurate.



Methods used to measure FRET

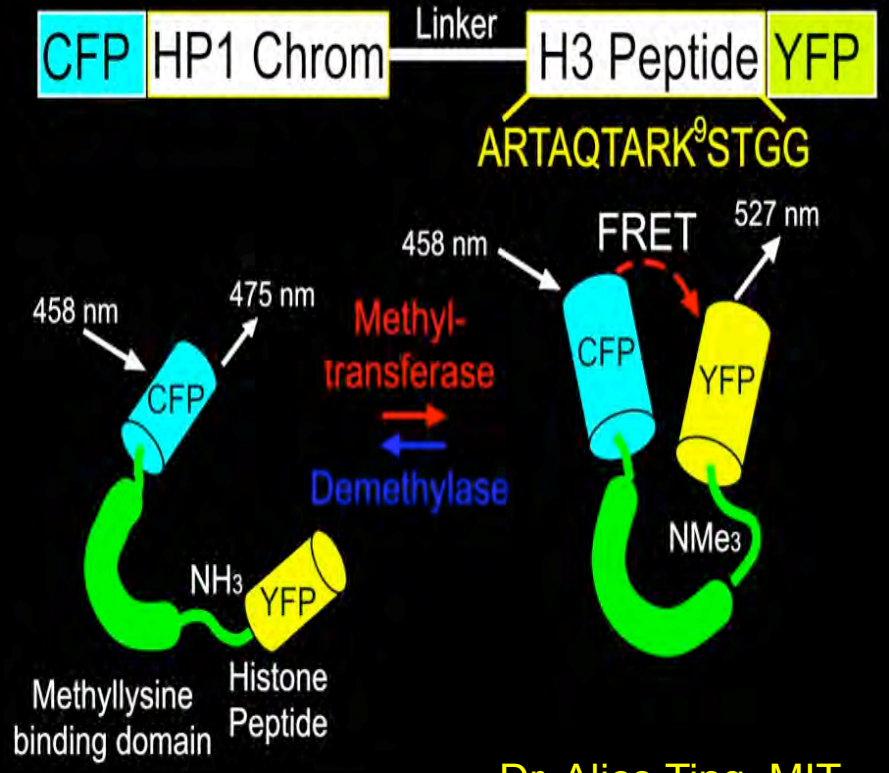
- There are many different ways to measure FRET:

1. Ratio Imaging - Biosensor proteins
   - Requires D:A be fixed at 1:1
2. Sensitized acceptor emission:
   - E = FRET - [Spectral cross-talk]
3. Acceptor photobleaching:
   - $E = 1 - (I_{DA}/I_D)$
4. Donor lifetime measurements:
   - $E = 1 - (\tau_{DA}/\tau_D)$

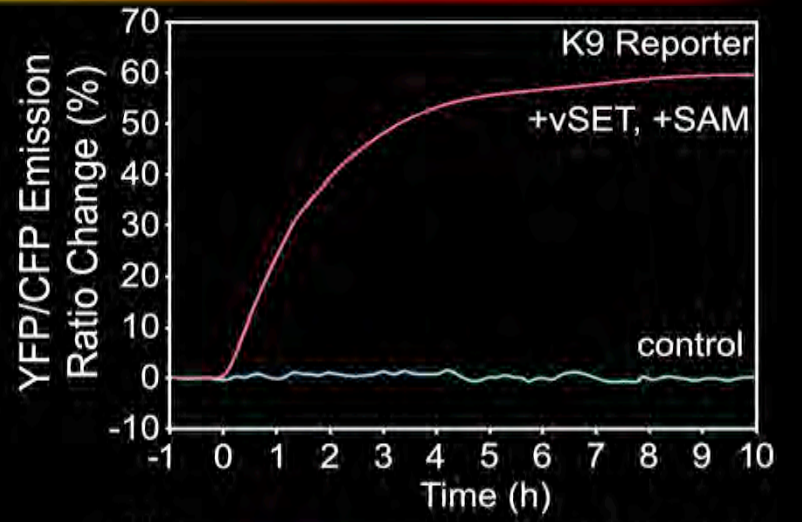


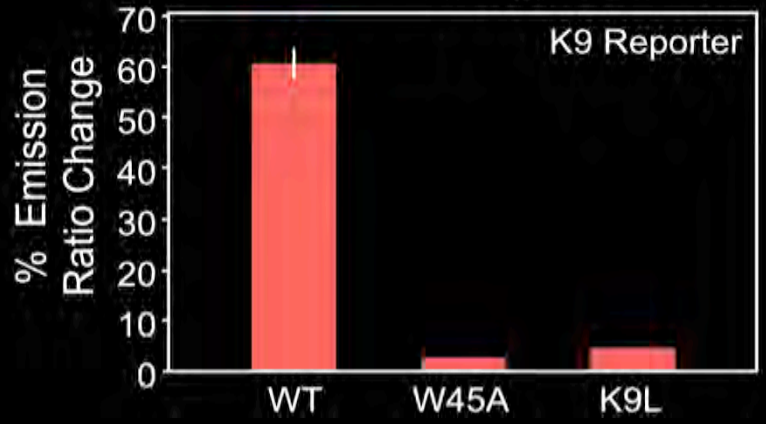
❖ *Most reviewers will ask for at least two different methods!*

Ratio imaging of biosensor probes



Dr. Alice Ting, MIT





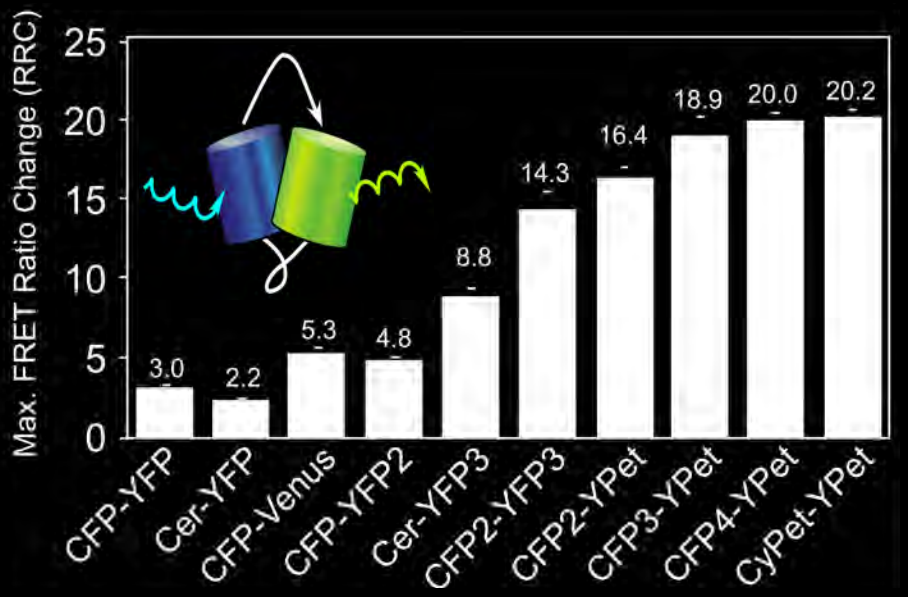
- With methylation of the H3 peptide there is a conformational change, allowing a intramolecular complex to form with chromodomain.

Lin *et al.* (2004) *JACS* 126:5982

FPs optimized for biosensors: CyPet & YPet

- Library screening method to co-evolve linked cyan and yellow FPs.
- Select for mutants that enhance FRET.

Nguyen and Daugherty (2005) *Nat. Biotech.* 23:355



- Mutations reduced the Förster distance - why enhanced FRET?

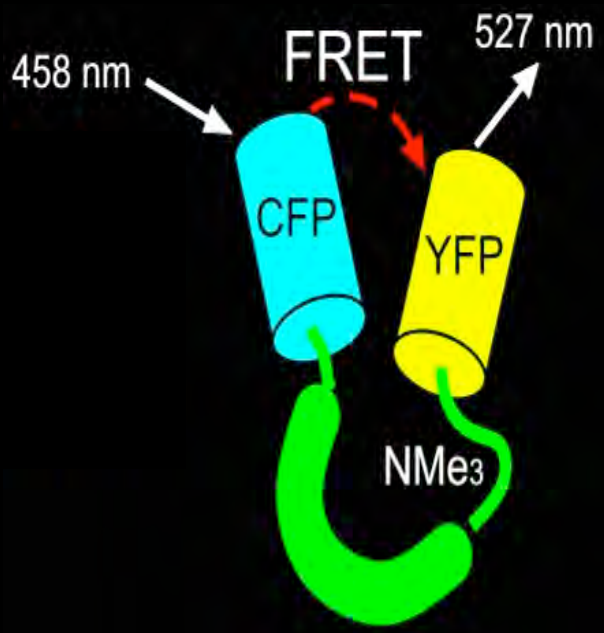
- Changes promote weak interactions between the linked FPs.

Vinkenborg (2007) *Chem.Biochem.* 8:1119

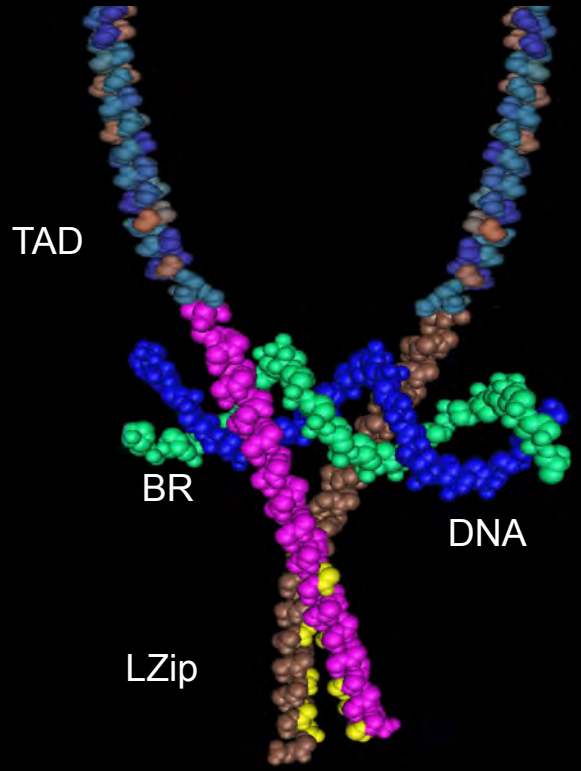
Ratio imaging of biosensor probes

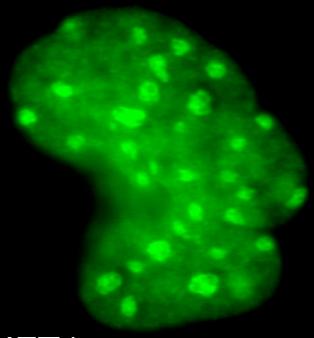
- Strength -
  - Simple approach - bleed-through background is constant (1:1).
  - Large scale screening applications.

- Weakness -
  - Limited to linked probes;
  - Limited dynamic range.
  - Function difficult to predict.

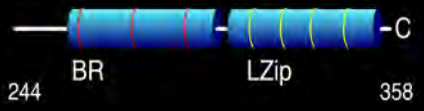


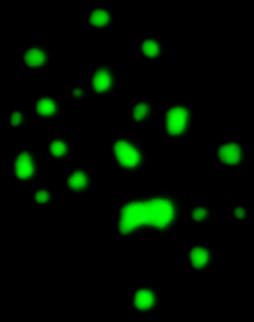
C/EBPα localizes to heterochromatin





GHFT1





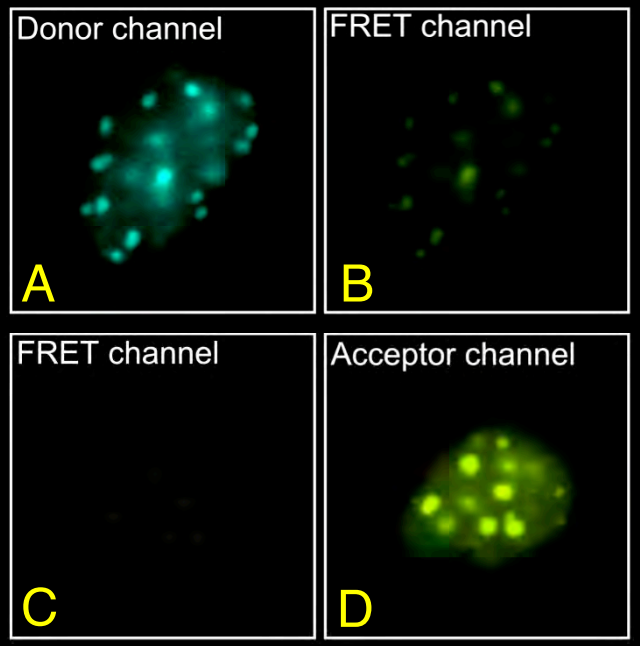
- binds to repeated elements in centromeric heterochromatin as an obligate dimer.

Sensitized emission measurements

pFRET Algorithm requires 7 different images:

- Control images:
  - ECFP-C/EBP

A. Donor alone - Don Channel
B. Donor alone - FRET Channel

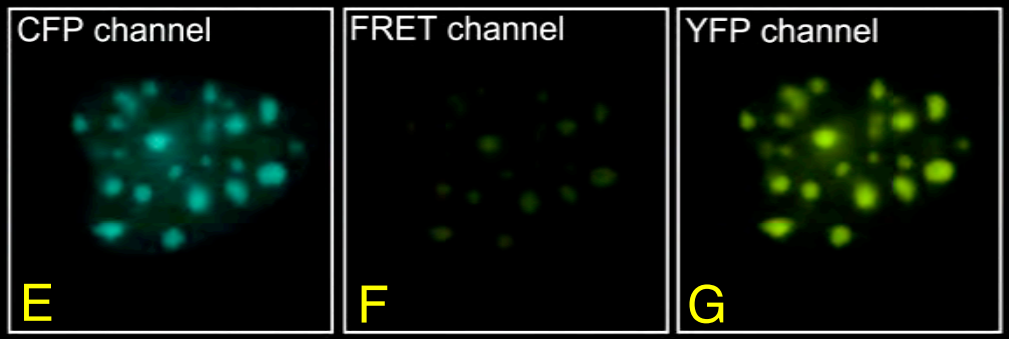


  - EYFP-C/EBP

C. Acceptor alone - FRET Channel
D. Acceptor alone - Acc Channel

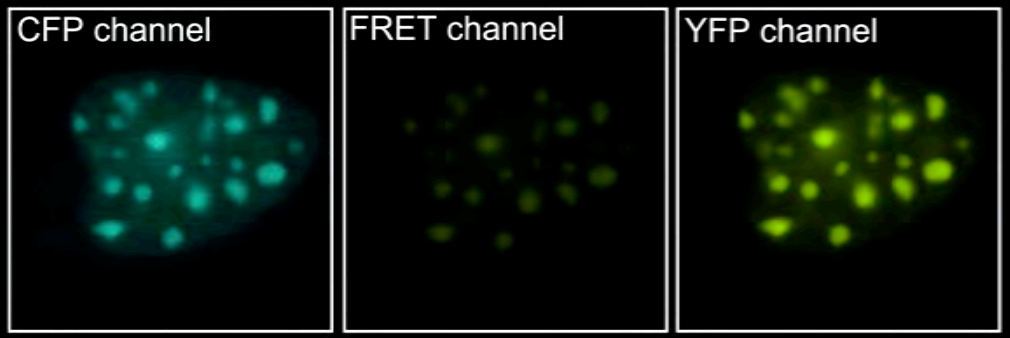
- Experimental images:
  - ECFP-C/EBP + EYFP-C/EBP

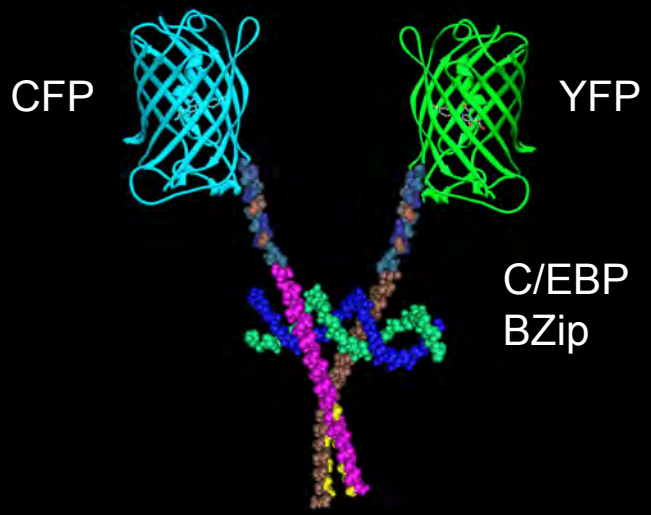
E. Don Channel
F. FRET Channel
G. Acc Channel

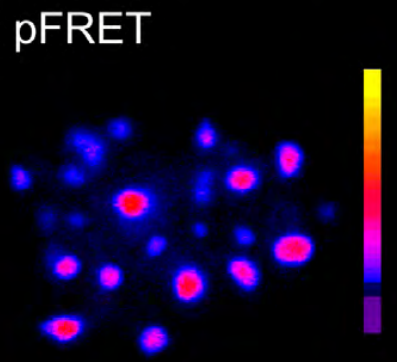


Sensitized emission: C/EBPα dimer formation

- The two spectral crosstalk components, determined from the control cell measurements, are removed from the FRET image.

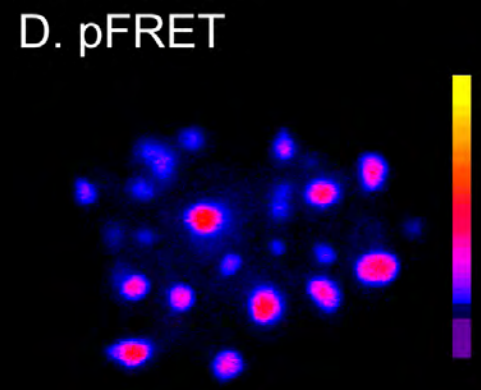






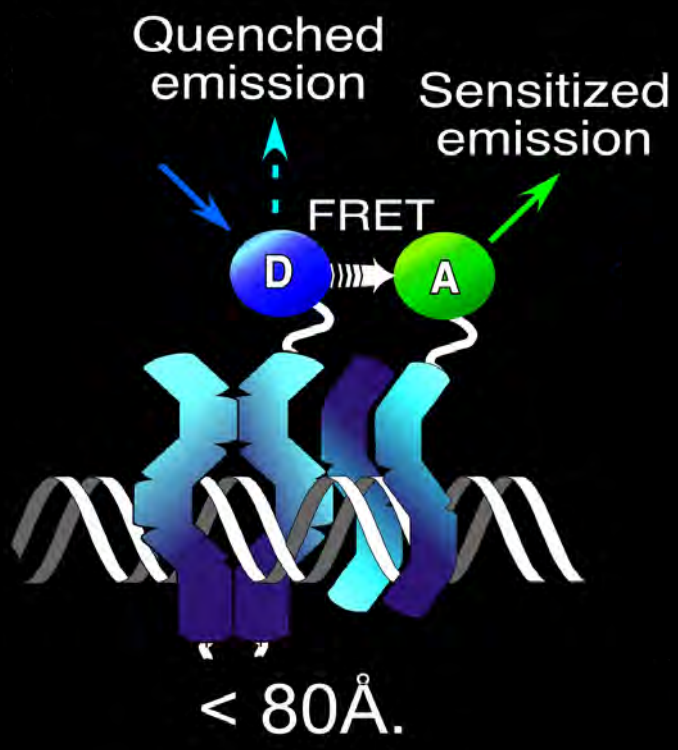
Sensitized emission measurements

- Strength -
  - Simple algorithms available on most imaging systems;
  - Compatible with most types of imaging (except 2-photon).
- Weakness -
  - Very sensitive to quality of the control data;
  - Subject to artifacts of cell movement.



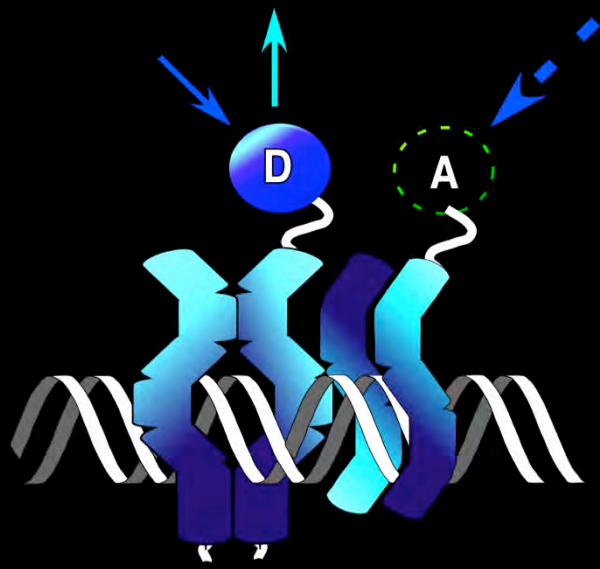
Acceptor photobleaching

- Energy transfer results in quenching of D emission and sensitized emission from the A.

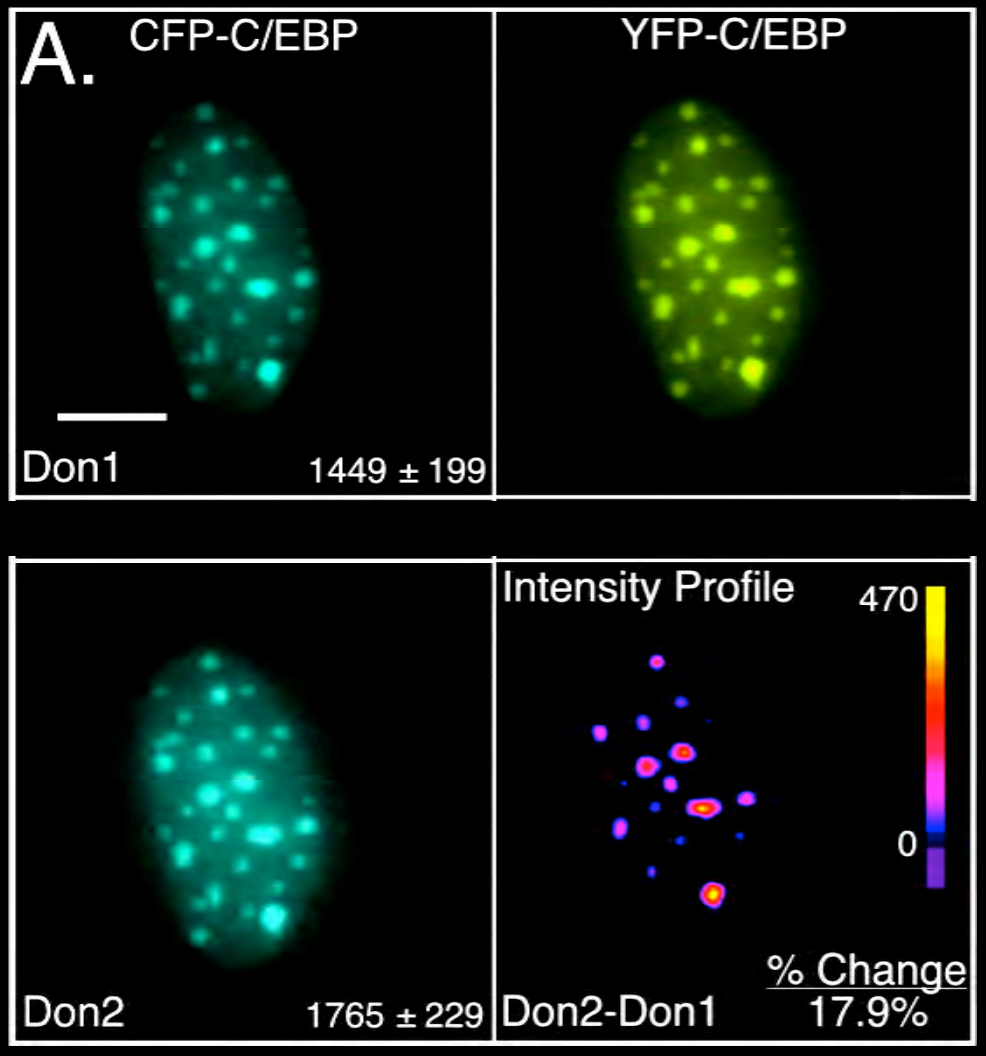


Acceptor photobleaching

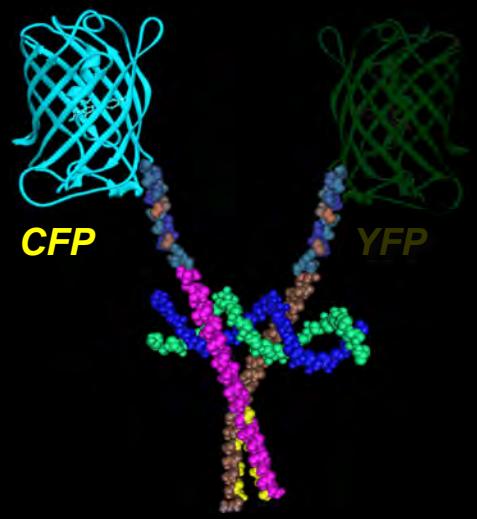
- Energy transfer results in quenching of D emission and sensitized emission from the A.
- Photobleaching the acceptor relieves donor quenching.
- De-quenching is detected in the donor channel - less prone to spectral bleedthrough.



Acceptor photobleaching: C/EBPα dimer formation



- C/EBPα dimers in regions of heterochromatin.

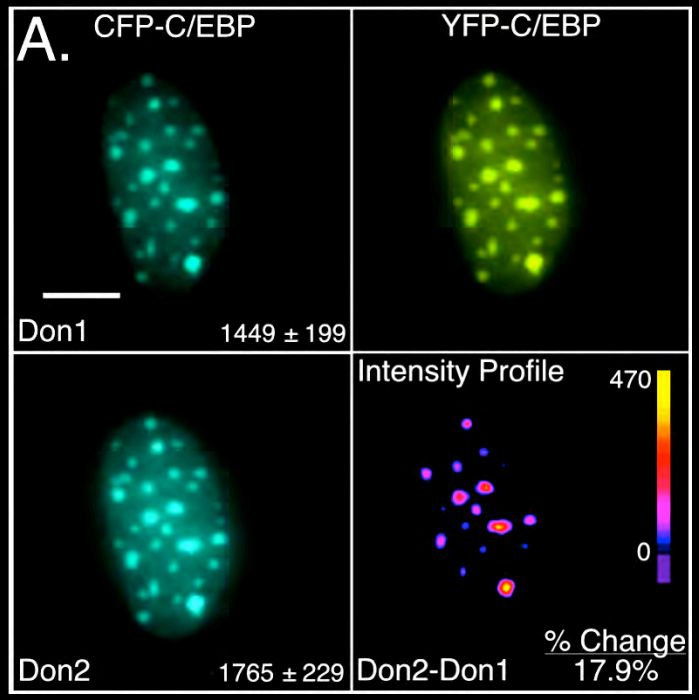


Acceptor photobleaching

Acceptor photobleaching

- Strength -
  - Simple approach that uses each cell as its own control - can be very accurate.
  - Commonly used to verify results from other methods.

- Weakness -
  - Requires selective bleaching;
  - Subject to artifacts of cell movement.
  - Endpoint assay - no dynamics.

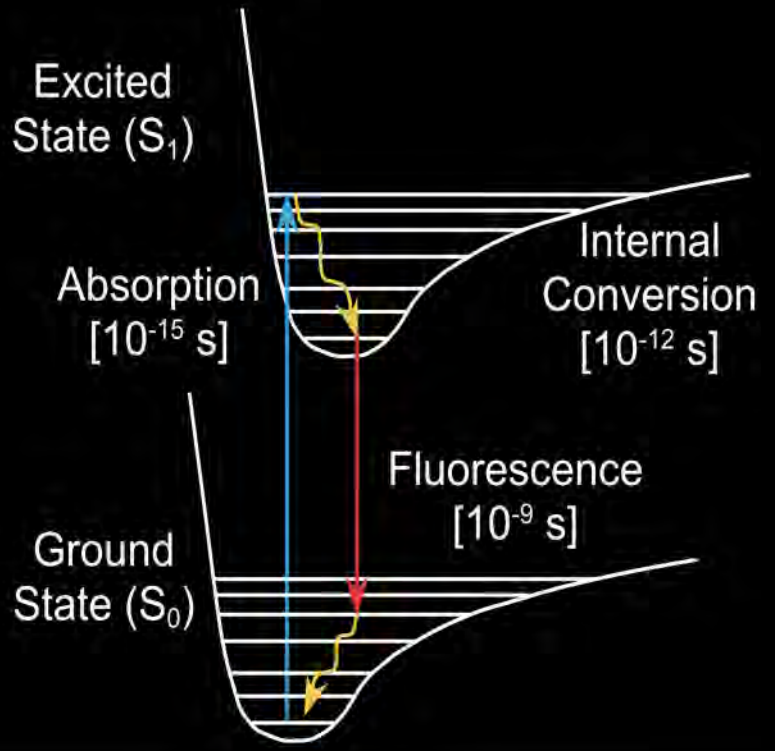


Fluorescence Lifetime

- Fluorescence lifetime (τ): the average time a population of fluorophores spend in the excited state before returning to the ground state.

- Every fluorophore has a characteristic lifetime, typically on the order of a few nanoseconds:

Probe	τ_m
Coumarin6	2.5 ns
Cerulean FP	3.1
mEGFP	2.9
mCherry FP	1.5
mRuby FP	2.7

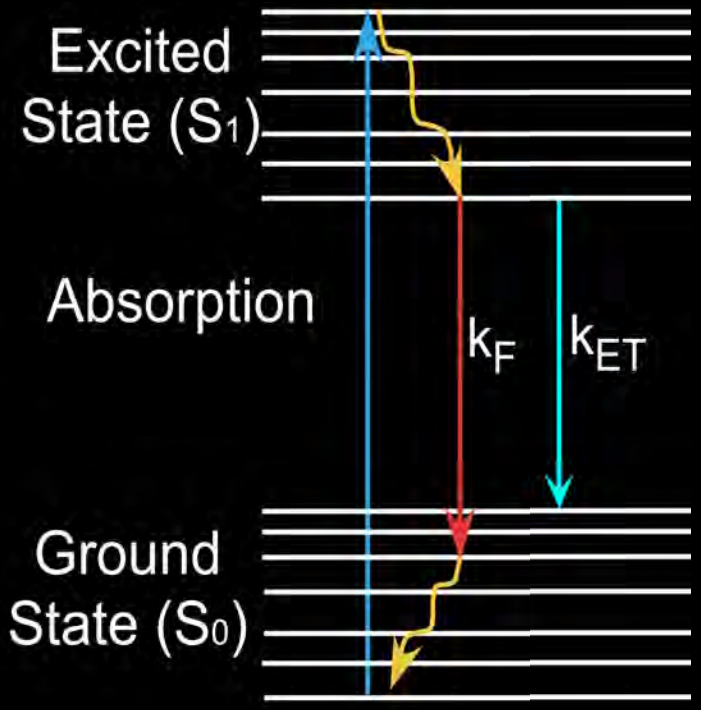


Fluorescence Lifetime

- Förster (Fluorescence) resonance energy transfer (FRET) is a quenching pathway that directly influences the excited state.

- Quenching: nonradiative energy transfer (k_{ET}) allowing transition to the ground state without fluorescence emission.

$$1/\tau = k_F + k_{ET}$$



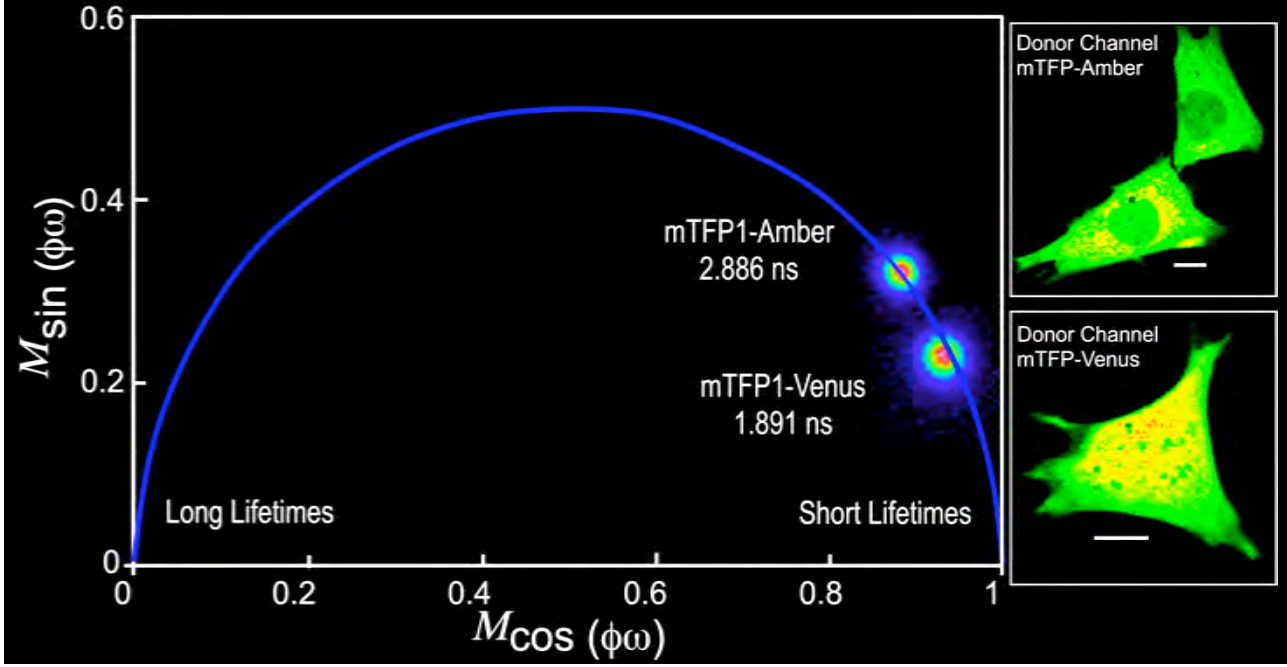
❖ Quenching events cause the fluorescence lifetime to shorten - this can be accurately measured microscopically.

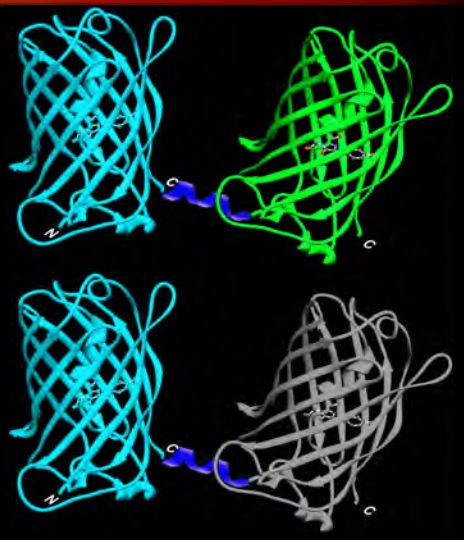
FRET standards with mTFP1-mVenus

- The monomeric Teal FP (mTFP1) is a very bright, photostable FP with excellent spectral overlap with Venus.

  Day *et al.* (2008) J Biomed Opt **13**:031203

- Amber is a non-absorbing mutant of Venus (Y66C) that folds properly – donor environment control for FRET-FLIM.

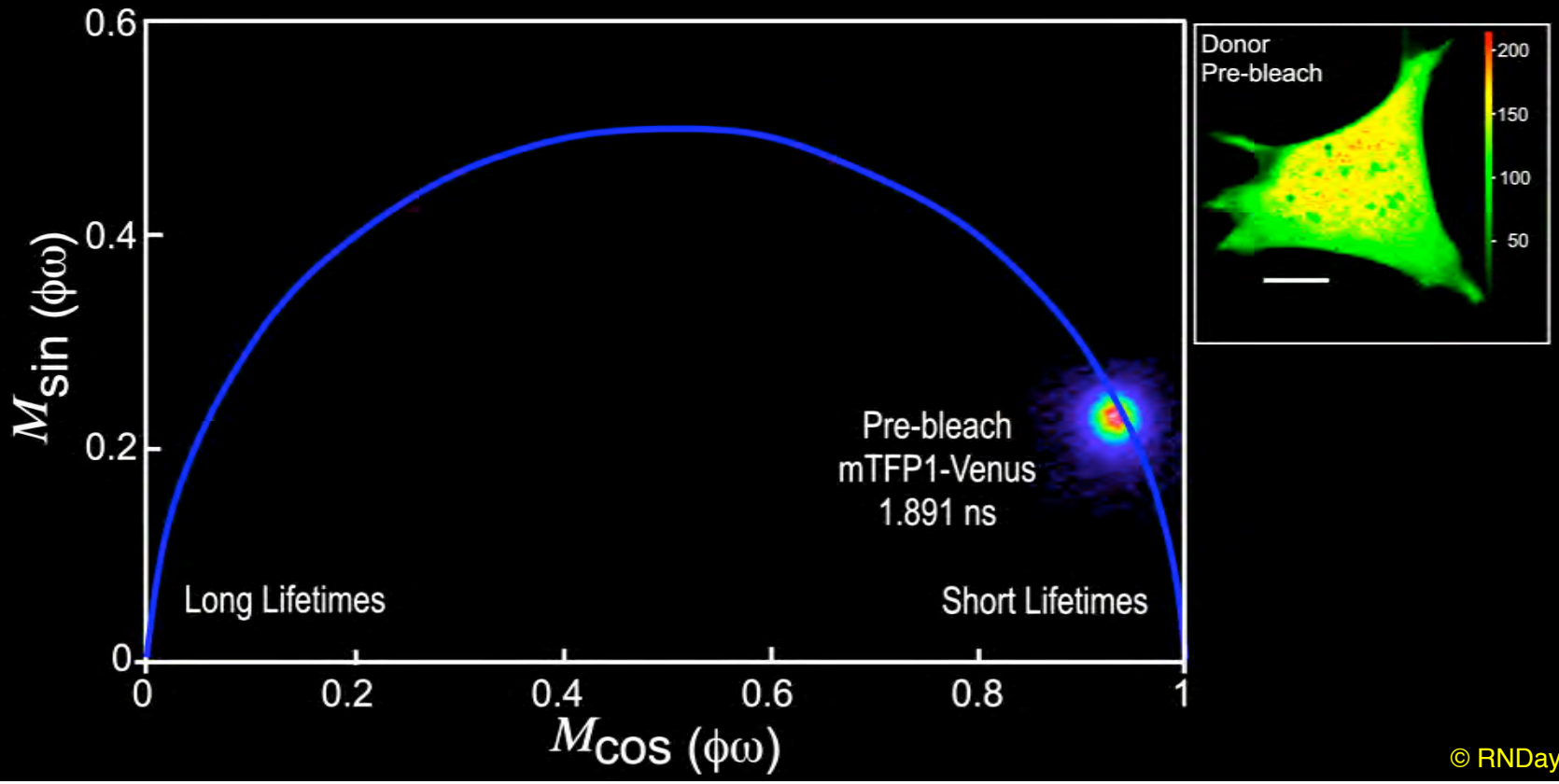




- FLIM detects the shorter lifetime of the quenched donor.

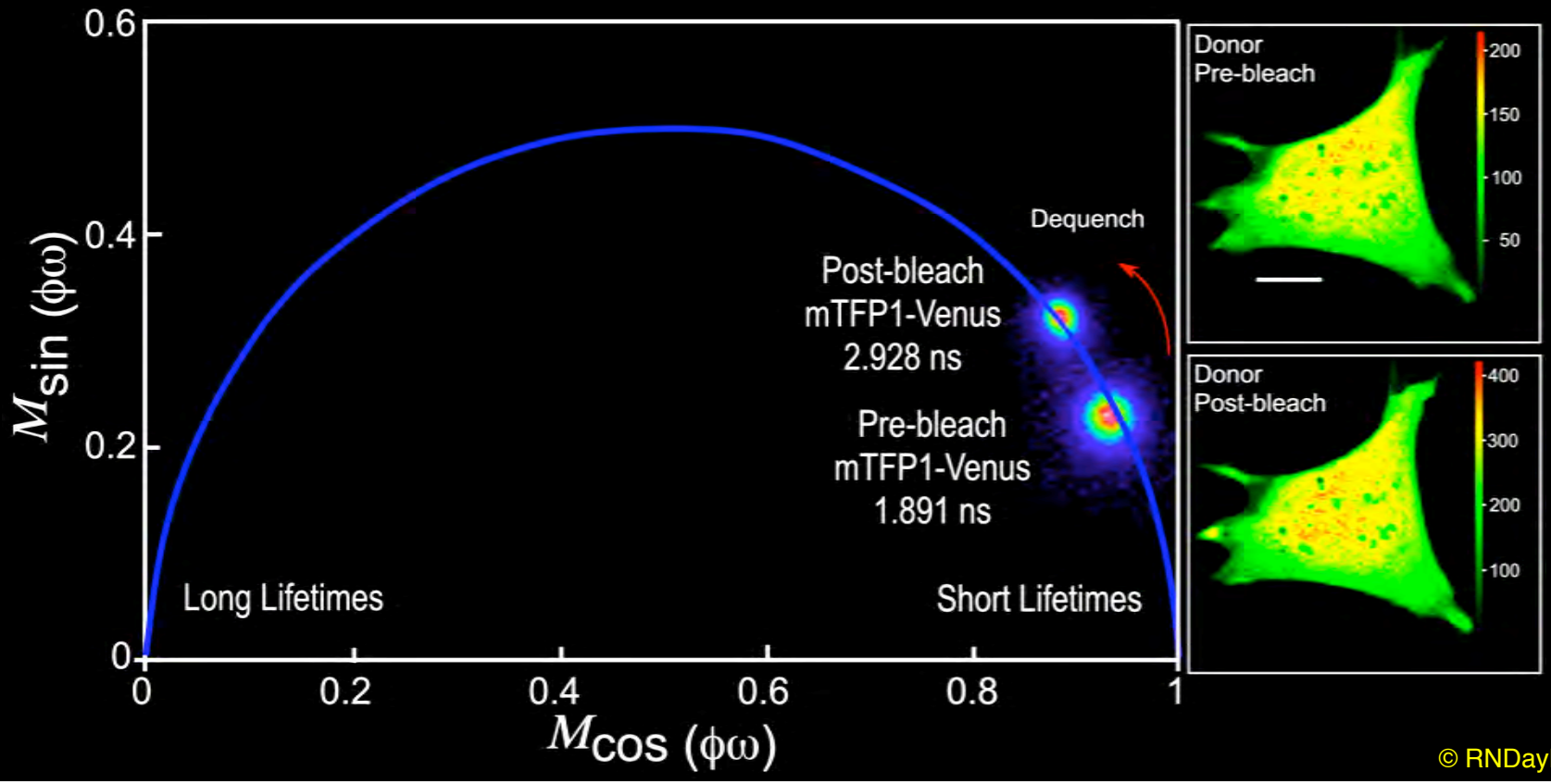
Verifying results with pbFRET

- The quenched state of the donor can be verified by acceptor photobleaching.



Verifying results with pbFRET

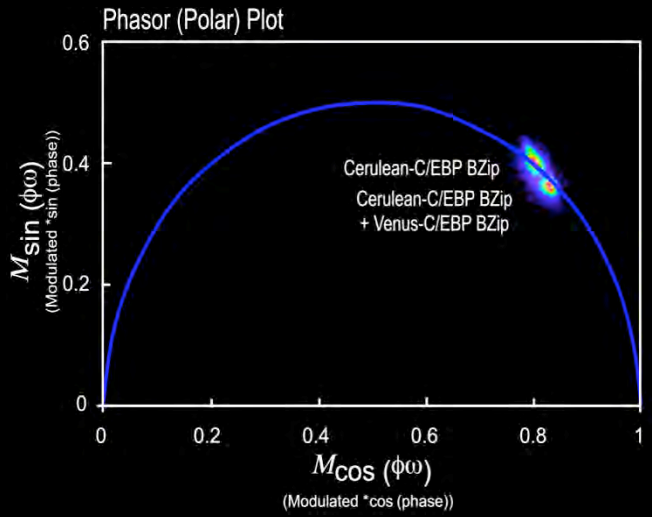
- The quenched state of the donor can be verified by acceptor photobleaching.
- Photobleaching Venus results in dequenching and a return to the radiative (τ_0) lifetime of mTFP1.



Fluorescence Lifetime

- Strength -
  - Measurements are not influenced by intensity or probe concentration;
  - Quenched (bound) and unquenched donor populations quantified.
  - Independent method to verify intensity measurements.

- Weakness -
  - System and analysis are complex;
  - Photon-intensive - measurements can take many seconds to acquire.



Requirements for GOOD FP FRET pairs

- Strong spectral overlap:
  - Narrow acceptor excitation and donor emission spectra.
    Spectral bleedthrough is the major issue.
- Donor with high QY and acceptor with high EC:
  - Current FPs cover a range of ~2000%.
    YPet = 80; mPlum = 4
- Reduced spectral bandwidth (primarily donor emission):
  - Difficult to engineer - new FPs from corals.
    mTFP1 good example, reduced crosstalk.
- Matched photostability:
  - Directed evolution to select stable variants.
    Cerulean3, TagRFP-T.
- Matched donor and acceptor maturation:
  - Difficult to achieve when mixing reef coral and jellyfish proteins
    Reef coral FPs often mature slower than *Aequorea* FPs

Useful FP FRET tool box (2011):

Protein (Acronym)	Ex (nm)	Em (nm)	EC x 10^{-3} $M^{-1}cm^{-1}$	QY	Relative Brightness (% of EGFP)[a]	Use as FRET probe	Reference
Aequorea-based FPs							
EBFP2	383	448	32.0	0.56	53	Donor to GFP/YFP	Ai et al. 2007
mCerulean3	433	475	40.0	0.87	103	Donor to YFP	Markwardt et al. 2011
mTurquoise	435	477	35.0	0.51	53	Donor to YFP	Goedhart et al. 2010
EGFP	488	507	56.0	0.60	100	Donor to OFP, RFP	Ai et al. 2007
Venus	515	528	92.2	0.57	156	Acceptor for CFP, Donor to RFP	Nagai et al. 2002
Citrine	516	529	77.0	0.76	174	Acceptor for CFP	Griesbeck et al. 2001
T-Sapphire	399	511	44.0	0.60	79	Long Stokes shift, donor to OFP	Zapata-Hommer and Griesbeck 2003
mAmetrine	406	526	45.0	0.58	78	Long Stokes shift, donor to OFP	Ai et al. 2008
REACh	515	528	92.2	0.04	1	Strong absorber, weak emitter Acceptor for FLIM studies	Ganesan et al. 2006; Murakoshi et al. 2008
Amber	None	None	0	0	0	Non-absorbing, non-fluorescent, probe environment control	Koushik et al. 2006
Coral FPs							
Midoriishi Cyan	472	495	27.3	0.90	73	Donor to mKO	Karasawa et al. 2004
mTFP1	462	492	64.0	0.85	162	Donor to YFP, OFP	Day et al. 2008
Kusabira Orange2	551	565	63.8	0.62	118	Acceptor for CFP	Karasawa et al. 2004
dTomato	554	581	69.0	0.69	142	Acceptor for mTFP1, YFP Acceptor for mAmetrine	Sun et al. 2010 Ai et al. 2008
mCherry	587	610	72.0	0.22	47	Acceptor for GFP	Yasuda et al. 2006
TagRFP-T	555	584	81.0	0.41	99	Acceptor for GFP	Shcherbo et al. 2009
mRuby	558	605	112.0	0.35	117	Acceptor for GFP	Kredel et al. 2009

From: Day and Davidson (2011) *In preparation*

FRET Summary

- The *absence* of FRET does not mean that two proteins do not interact!
- FRET signals do not *prove* a direct interaction between two proteins - they define the spatial relationship of the fluorophores.
- Spectral bleedthrough limits the detection of FRET signals:
  - ratio imaging is straightforward - but only applies to the biosensor proteins with linked FPs (fixed 1:1);
  - computer algorithms estimate and remove the SBT - but rely on data from different control cells;
  - acceptor photobleaching FRET overcomes this limitation, but is an end-point assay;
  - fluorescence lifetime methods provide independent verification, but measurements take time, and the analysis is complex.

FRET Summary

- Use FRET standards to characterize the experimental model, and check the imaging system.
- FRET measurements don't replace biochemical approaches - both are necessary.
- FRET measurements can provide evidence for protein interactions in the context of the living cell, *but….*
- it is critical to verify FRET measurements!
  - Sensitize acceptor measurements > acceptor photobleaching
  - Donor lifetime measurements > acceptor photobleaching