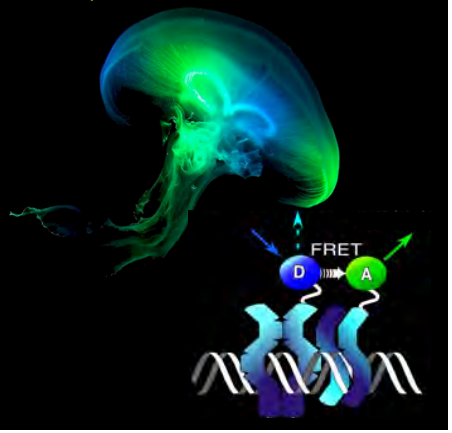
Visualizing protein interactions in living cells: FRET with the fluorescent proteins

Richard N. Day. Ph.D.
Indiana University
School of Medicine
Department of Cellular & Integrative Physiology



George M. O'Brien Center
Workshop on Applied Microscopy
in Kidney Research - April 2011

Overview

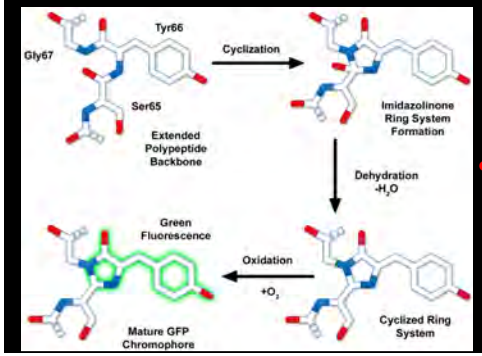
- The genetically encoded fluorescent proteins (FPs):
  - General characteristics of the FPs.
  - Mutant color variants based on *A.v.* GFP.
  - FPs derived from *Discosoma striata* - mRFP and the fruits.
  - New FPs derived from corals.
  - The (current) best FPs.
- Förster (Fluorescence) resonance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

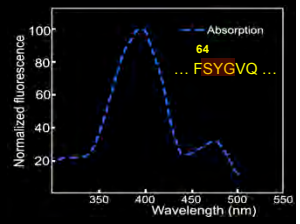
Aequorea victoria Green Fluorescent Protein (GFP)

- *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.

General characteristics of GFP

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



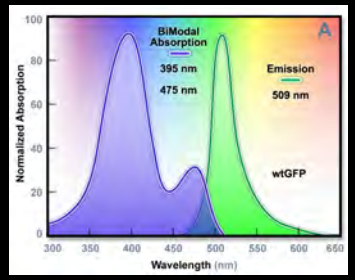


Cody et al. (1993) *Biochemistry* **32**:1212

- 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:



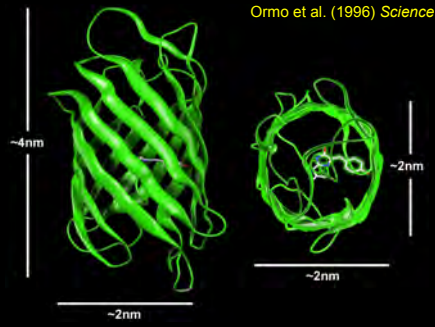
M_1….VTTF-$S_{65}Y_{66}G_{67}$-VQCFS…K_{238}

- 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.



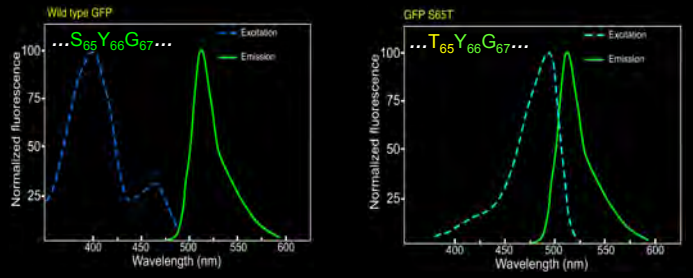
- This explained why the entire protein sequence was required for fluorescence.

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 37°;
  - 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

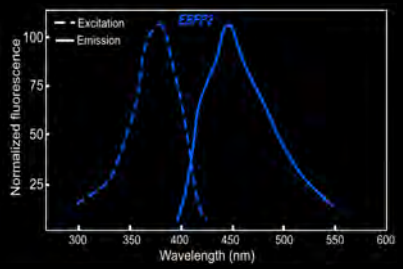
Mutant color variants of *A.v.* GFP

	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

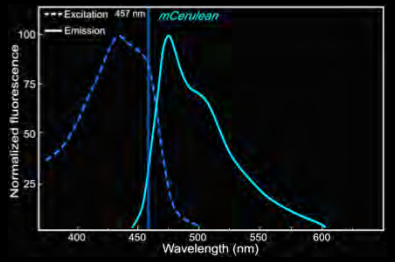


- Directed evolution of EBFP from *Aequorea* for selection of a brighter, more stable blue FP; incorporates superfolder mutations:
- Key mutations: EBFP + S30R, Y39N, T65S, S72A, I128V, F145H, M153A, D155V, A206V, V224R
  - Ex 383 nm, Em 448 nm;
  - intrinsic brightness of 18;
  - photostable - *especially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

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

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

mCerulean3

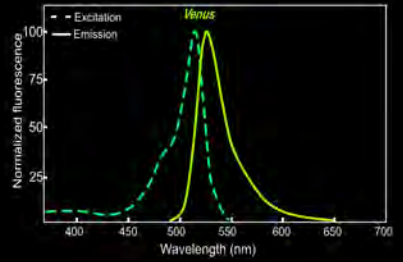


- Mutagenesis of ECFP - selection of a brighter, more stable Cerulean; optimization of β-strand 7 & 8, plus T65S > mCerulean3
- Key mutations: mCerulean + T65S/S147H/D148G/K166G/I167L/R168N/H169
  - Ex 334 nm, Em 475 nm;
  - intrinsic brightness of 34 (similar to EGFP);
  - very photostable, decrease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

Markwardt et al. (2011) *PLoS ONE 6(3):e17896*

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

mVenus

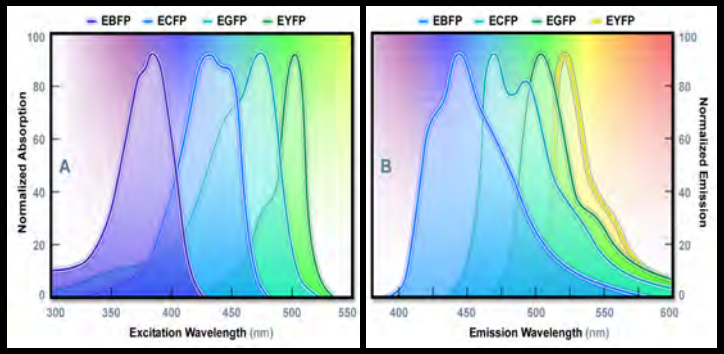


- Mutagenesis of EYFP from *Aequorea* for selection of a brighter Yellowish FP with reduced halide and pH sensitivity:
- Key mutations: F46L, F64L, S65G, S72A, M153T, V163A, T203Y, A206K
  - Ex 515 nm, Em 528 nm;
  - intrinsic brightness of 54;
  - Maturation rapid - *NOT very photostable*.

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

Mutant color variants of *A.v.* GFP

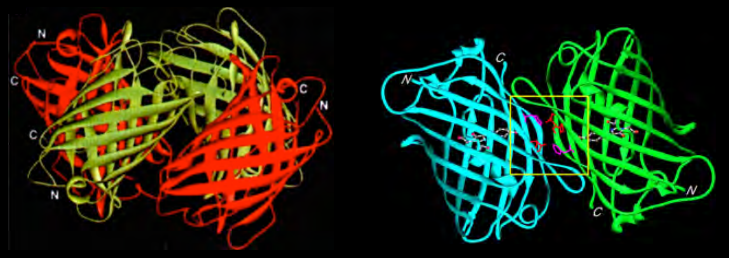
- *A.v.*-base FP color variants from blue to yellow:



- 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

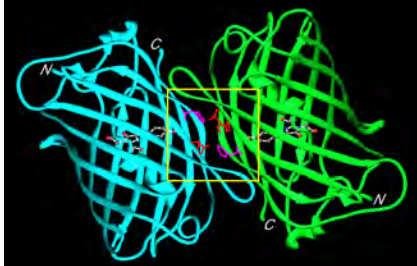
- Most of the natural FPs that have been characterized are either dimers, tetramers, or higher-order complexes.



- 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.

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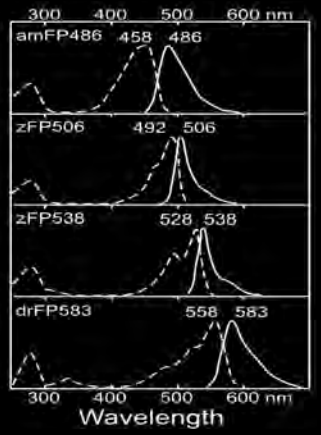
Fluorescent Proteins from other marine organisms

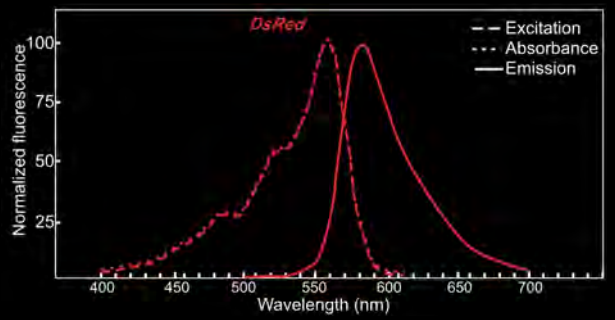
- Most of the colors in reef corals result from GFP-like proteins.

Mushroom anemone *Discosoma striata*



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

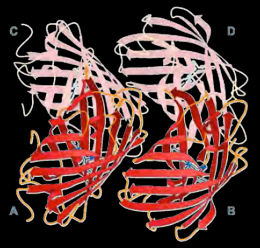
Advantages of DsRed

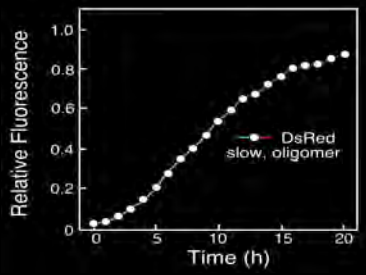


- Very bright and spectrally distinct from the *Aequorea* FPs;
- easily detected with standard optical filters;
- reduced cellular auto-fluorescence at longer wavelengths.

Problems with DsRed

- DsRed is an obligate tetramer in mammalian cells:

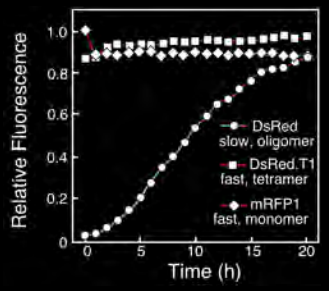


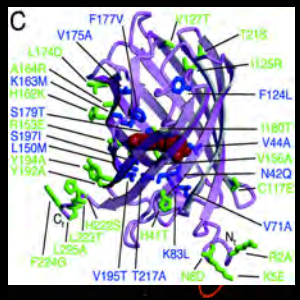


- DsRed requires nearly 20 h to fully mature, and there is a green intermediate form of the protein.
- DsRed tends to form oligomers, leading to misdirected fusion proteins.

New variants based on DsRed

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

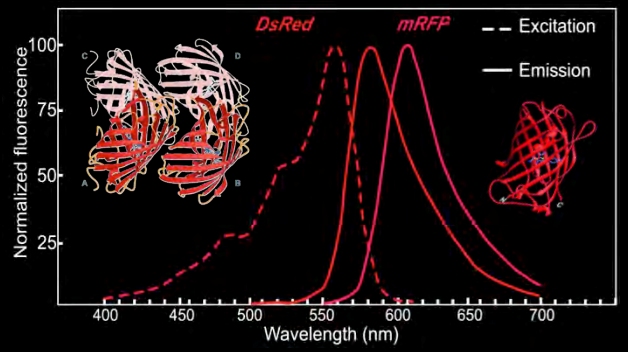




Campbell et al. (2002) *PNAS* **99**:7877

mRFP1 was an improvement-

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

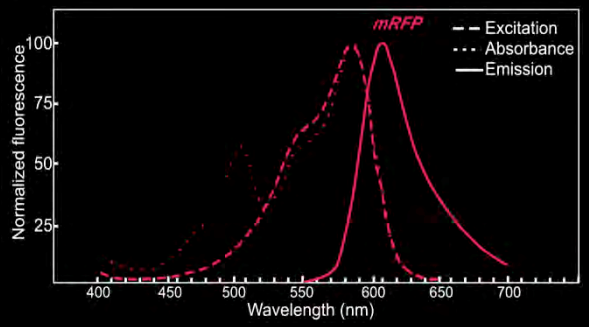


Campbell et al. (2002) *PNAS* **99**:7877

but, mRFP was not optimal

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

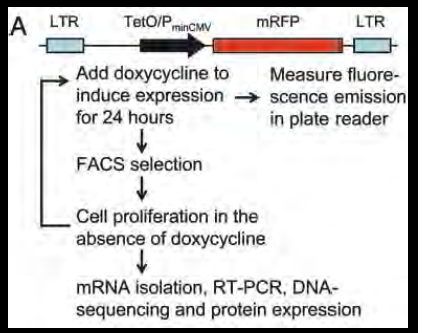
Hillesheim et al. (2006) *Biophys J* **91**:4273



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

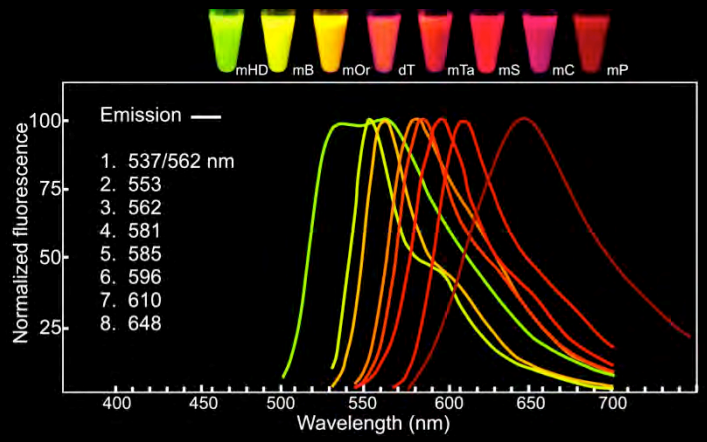
Directed evolution yields new FPs

- 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

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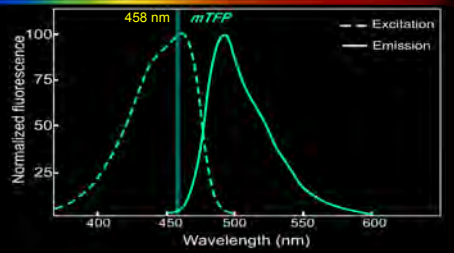
The cloning of novel FPs from corals: mTFP1

Clavularia sp. "palm coral"

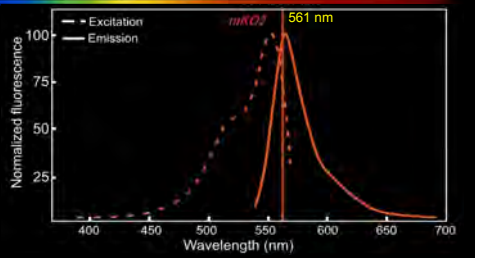


- Directed evolution of cFP484 from *Clavularia* sp. for selection of a bright blue-green (Teal) FP:
- Key mutations mTFP1: Y67; N63T, Q66A, L72F, D125K, M127E, E144D::H163
  - Ex 462 nm, Em 492 nm, relatively narrow spectra;
  - intrinsic brightness of 54;
  - photostable - *acid stable*.

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

The cloning of novel FPs from corals: mKO2

Fungia concinna "mushroom coral"

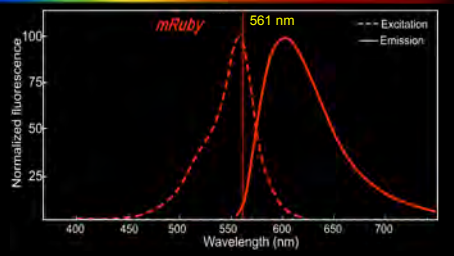


- Directed evolution of Kusabira orange from *Fungia concinna* for selection of a bright monomeric Orange FP:
- Key mutations: Kusabira + K49E, P70V, F176M, K185E, K188E, S192G, L210Q
  - Ex 551 nm, Em 565 nm;
  - intrinsic brightness of 36;
  - Maturation rapid, photostable - *narrow Stokes shift*.

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

The cloning of novel FPs from corals: mRuby

Entacmaea quadricolor anemone



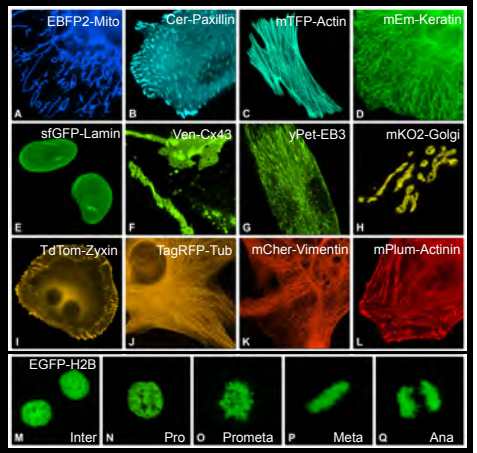
- Directed evolution of a dimeric eqFP611 from *Entacmaea quadricolor* for selection of a bright monomeric Red FP:
- Key mutations: eqFP611 F102I + 29 mutations.
  - Ex 558 nm, Em 605 nm;
  - intrinsic brightness of 39;
  - Maturation 2.8 h, photostable.

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

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	++++	Markwardt et al. 2011	Dr. Mark Rizzo
mTFP	Teal	462	492	54	+++	Ai et al. 2006	Allele Biotech
EmGFP	Green	487	509	39	++++	Cubitt et al. 1995	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.

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  - The (current) best FPs.
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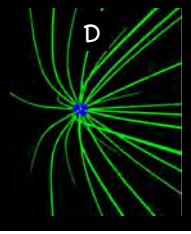
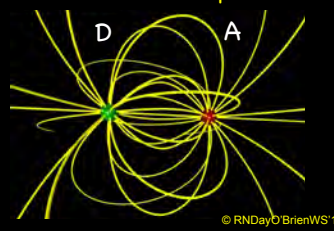
  - Methods used to measure FRET - strengths and weaknesses.
  - Summary.

Förster (Fluorescence) resonance energy transfer (FRET)

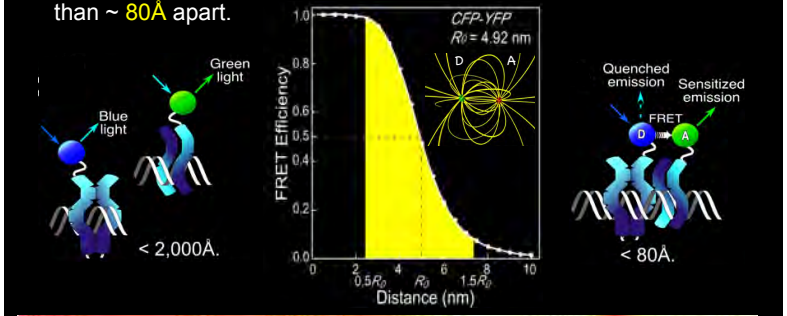
- FRET is the direct transfer of excited state energy from a donor fluorophore to a nearby acceptor.
- A fluorophore in the excited-state is an oscillating dipole that creates an electric field (the donor - D).
- If another fluorophore enters the electric field, energy can be transferred directly to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





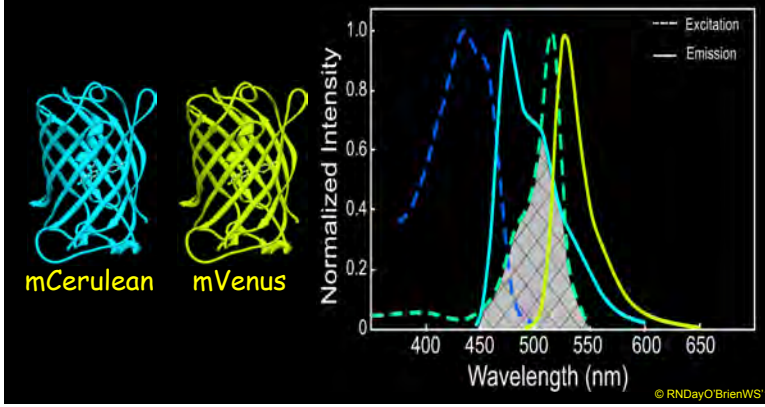
FRET measures the spatial relationship between the FPs

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



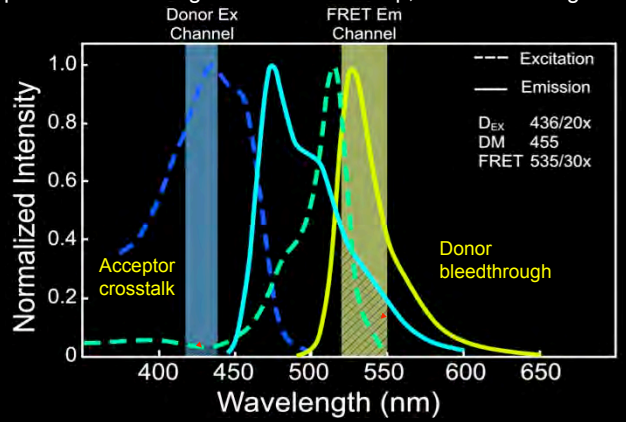
The spectral overlap requirement

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



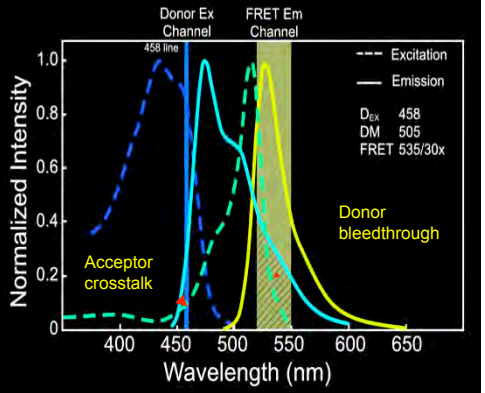
Spectral bleedthrough background signals

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



Spectral bleedthrough background signals

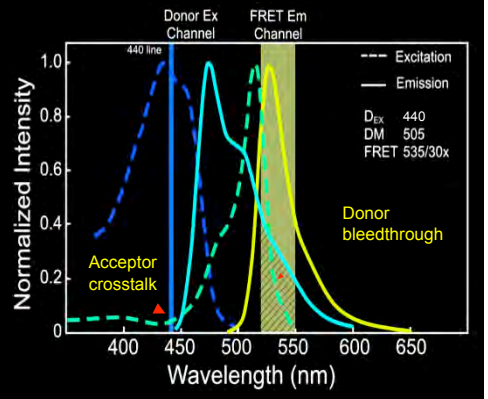
- This is still a problem when using LSCM –



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!

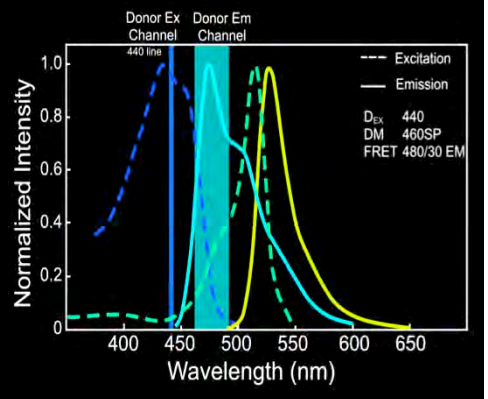


Spectral bleedthrough background signals

- 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!

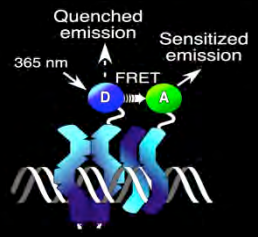
❖ Alternatively, methods that detect changes in donor fluorescence are typically not affected by SBT, and can be most accurate.



Some 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!*

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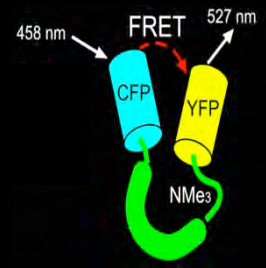
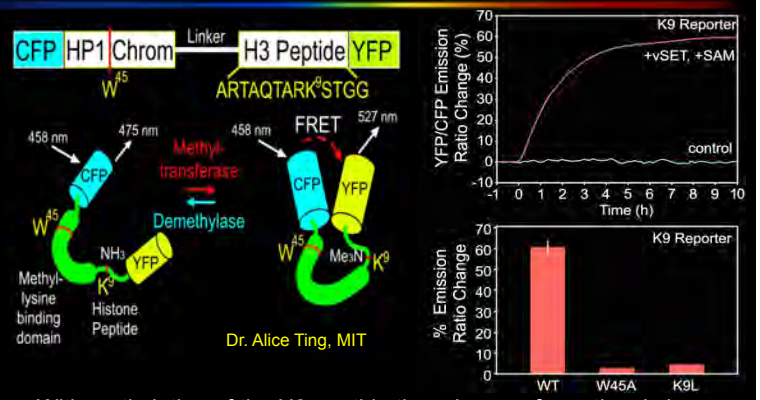


Ratio imaging of biosensor probes

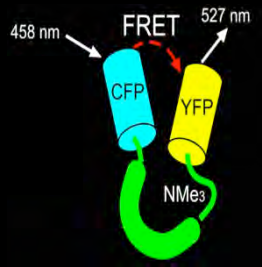


- With methylation of the H3 peptide there is a conformational change, allowing a intramolecular complex to form with chromodomain.

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

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.



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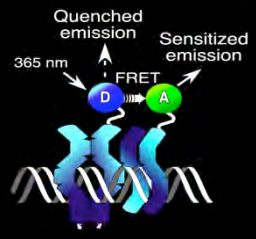
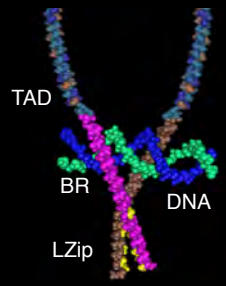
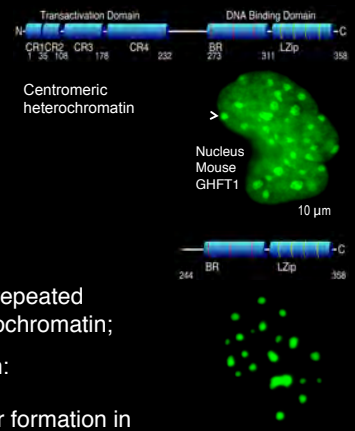


The model: C/EBPα dimer formation in the cell nucleus





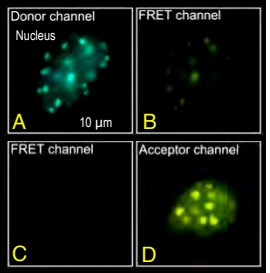
- Binds as an obligate dimer to repeated elements in centromeric heterochromatin;
- requires only the B-Zip domain:
- We used FRET to detect dimer formation in regions of heterochromatin.

Sensitized emission measurements

pFRET Algorithm requires 7 different images:

- Control images:
  - ECFP-C/EBP

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

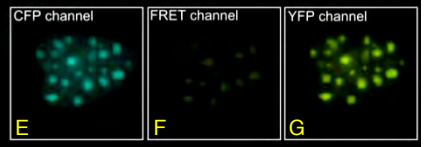


  - EYFP-C/EBP

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

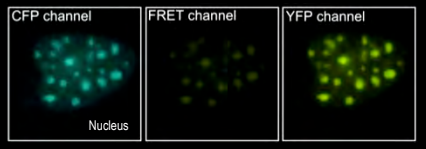
- Experimental images:
  - ECFP-C/EBP + EYFP-C/EBP

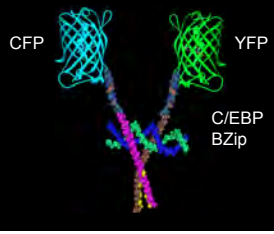
E. Don Channel
F. FRET Channel
G. Acc Channel

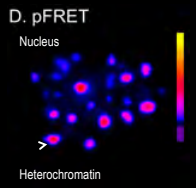


Sensitized emission: C/EBPα dimer formation

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

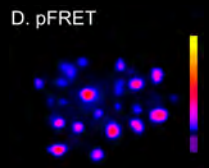






Sensitized emission measurements

- 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.

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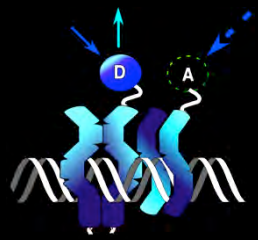
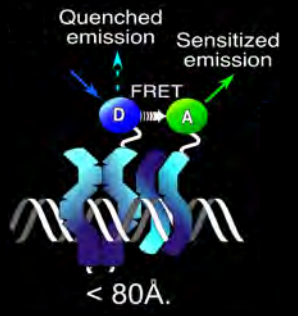


Acceptor photobleaching

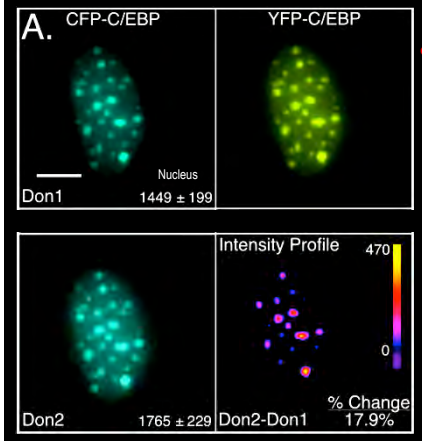
- Energy transfer results in quenching of D emission and sensitized emission from the A.



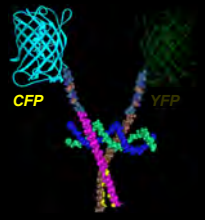
Acceptor photobleaching

- 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



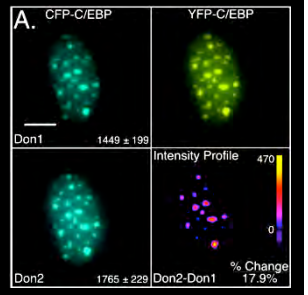
- C/EBPα dimers in regions of heterochromatin.



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.



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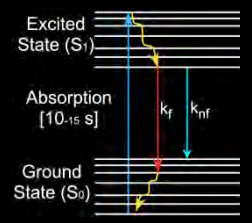
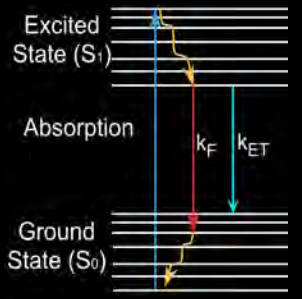


Fluorescence Lifetime

- 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.

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



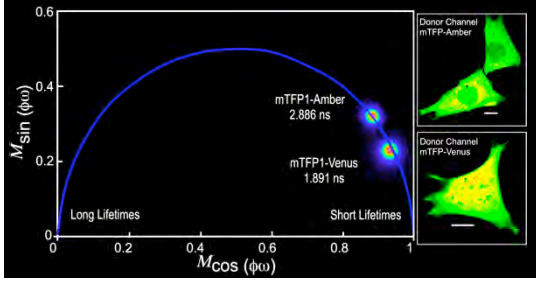
❖ Quenching events cause the fluorescence lifetime to shorten - this can be accurately measured microscopically.

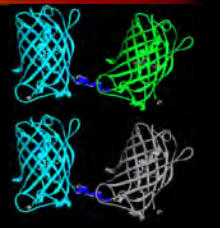
FLIM measurements of "FRET standards"

- The monomeric Teal FP (mTFP1) linked directly to Venus.

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

- Amber is a non-absorbing mutant of Venus (Y66C) that folds properly; an important control for the donor environment.



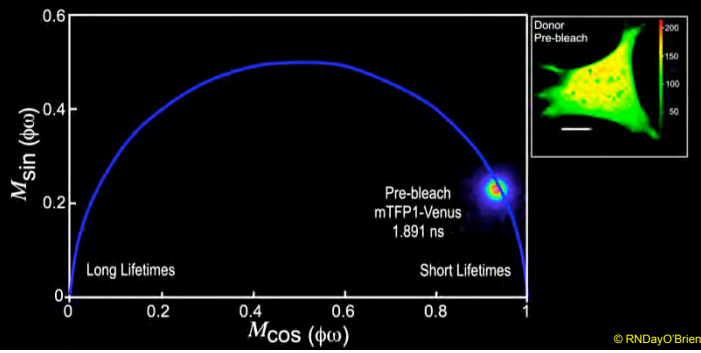


- FLIM detects the shorter lifetime of the quenched donor:

$E\% = 1 - \tau_{DA}/\tau_D = 35\%$

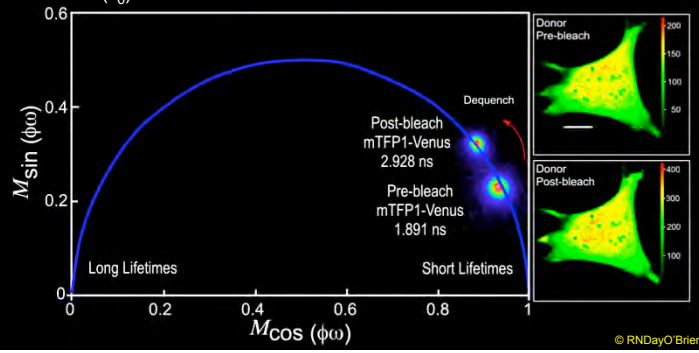
Verifying results with pbFRET

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



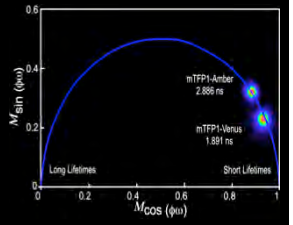
Verifying results with pbFRET

- 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.



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

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	++++	Markwardt et al. 2011	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.