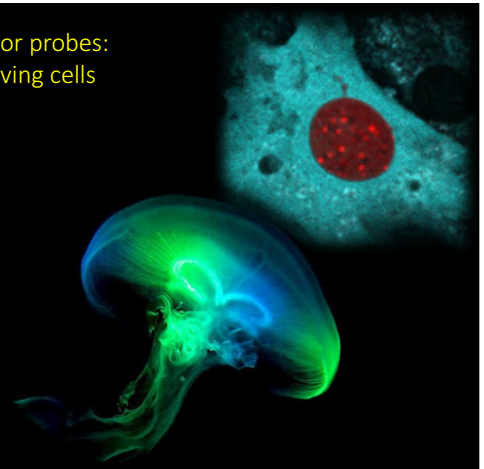
Fluorescent proteins and biosensor probes: monitoring protein dynamics in living cells



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

O'Brien Center Workshop
IUSM
April 11, 2017

Overview

- The fluorescent proteins (FPs) for live cell imaging:
  - General characteristics of the FPs.
  - *Aequorea* color variants.
  - New FPs from corals (and fish).
  - The (current) best FPs.
- Applications: Biosensor probes
  - General characteristics of biosensor probes.
  - Standards for biosensors.
  - Examples of biosensors.

Overview

- The fluorescent proteins (FPs) for live cell imaging:
  - General characteristics of the FPs.

Aequorea victoria Green Fluorescent Protein (GFP)

- *Aequorea victoria* makes the chemiluminescent protein aequorin, which emits blue light.
  Aequorin is a calcium-activated photoprotein that uses coelenterazine to generate light.
- GFP is an auto-fluorescent protein that absorbs the blue light energy and shifts the emission to green light.
  This is a FRET process.

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

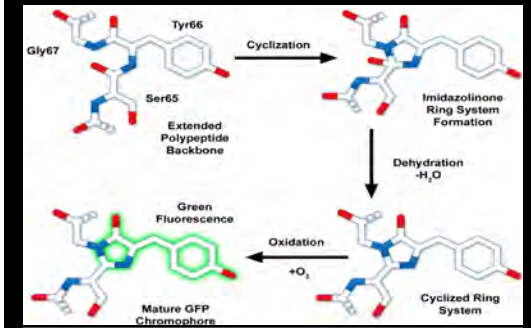
Day and Davidson (2014) The Fluorescent Protein Revolution (CRC Press)

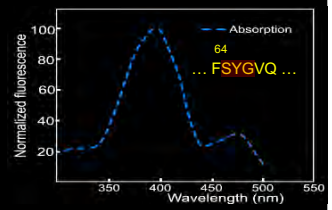
General characteristics of GFP

5

- Shimomura purified GFP and showed that a 6 aa fragment was responsible for the light absorption properties.

Cody et al. (1993) Biochemstry 32:1212



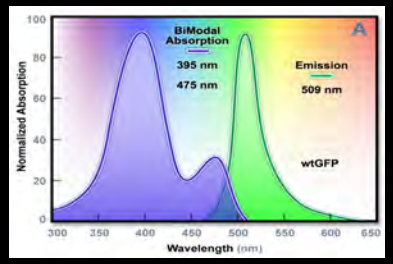


- This led to definition of the chromophore formed by the cyclization of the -SYG-:

General characteristics of GFP

6

- The wild type GFP displays a complex absorption spectrum:



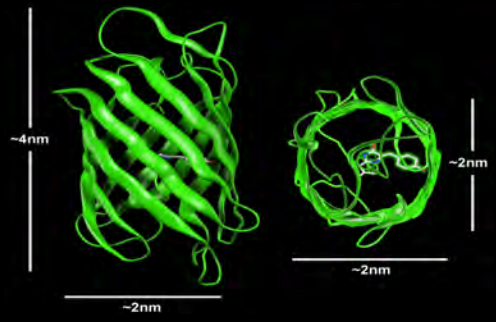
M_1….VTTF-$S_{65}Y_{66}G_{67}$-VQCFS…K_{238}

- The Tyr_{66} is protonated, and absorbs strongly at 397 nm.
- A charged intermediate accounts for the secondary absorption at 476 nm.

General characteristics of GFP

7

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



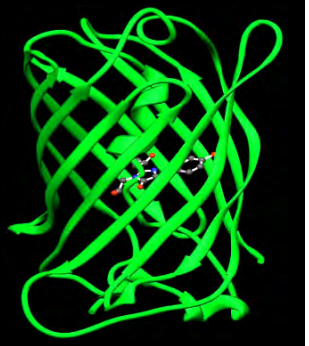
- This explained why the entire protein sequence was required for fluorescence.

Ormo et al. (1996) Science 273:1392

General characteristics of GFP

8

- 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)



Overview

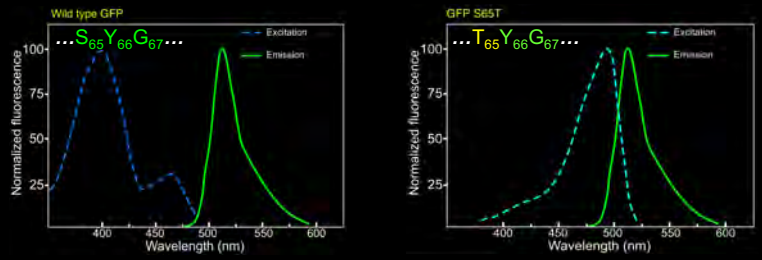
9

- The fluorescent proteins (FPs) for live cell imaging:
  - General characteristics of the FPs.
  - *Aequorea* color variants.

Mutant variants of Aequorea GFP

10

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



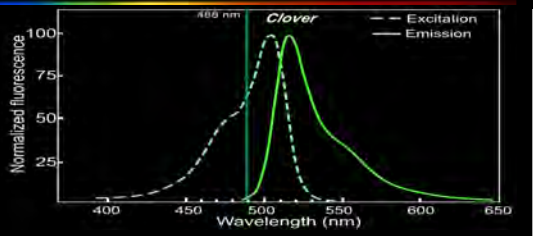
- The shifted single-peak absorption and improved brightness made GFP S65T more useful for live-cell imaging.
- Other chromophore mutations shifted the emission spectrum.

Tsien (1998) Ann Rev Biochem 67:509

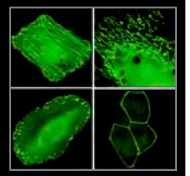
Spectral variants of GFP: mClover3

11

mClover3



- Directed evolution of EGFP selecting for a brighter variant better suited as a FRET donor for orange and red FPs: Clover → Clover2
- Key mutations: Clover2 + N149Y, S160C, A206K
  - Ex 505 nm, Em 515 nm,
  - intrinsic brightness of 85 (ε = 109, QY = 0.78),
  - maturation rapid, improved photo-stability – FRET donor

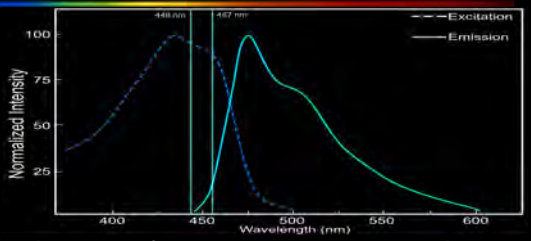


Bajar et al. (2016) Sci Rep 6:20889

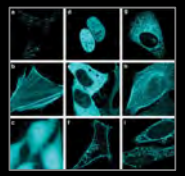
Spectral variants of GFP: Cerulean3 and Turquoise

12

mCerulean3, mTurquoise, mTurquoise2



- Mutagenesis of ECFP → Cerulean → selection of a brighter, more stable variants → mTurquoise2 T65S/I146F allows better packed chromophore QY 0.93!
- Key mutations: mCerulean + T65S/A145Y/I146F/S175G
  - Ex 434 nm, Em 475 nm,
  - intrinsic brightness of 28, improved photo-stability.
  - All have single component lifetimes.

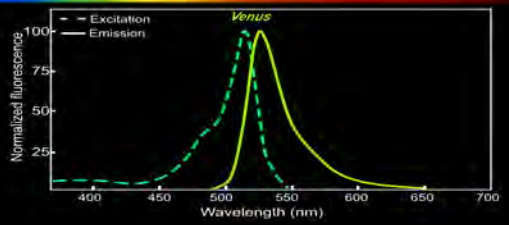


Goedhardt et al. (2012) Nat. Comm. 3:751; Markwardt et al. (2011) PLoS One 6:e17896

Spectral variants of GFP: improved yellow FPs

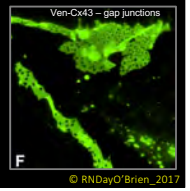
13

mVenus
mCitrine



- Mutagenesis of EYFP selecting for a brighter FPs with reduced halide and pH sensitivity (F46L) and improved photo-stability (Q69L):
- Key mutations: F46L, F64L, S65G, Q69L, S72A, M153T, V163A, T203Y, A206K
  - Ex 515 nm, Em 528 nm,
  - intrinsic brightness of 54,
  - maturation rapid – Venus NOT very photo-stable.*

* Useful for photo-bleaching techniques

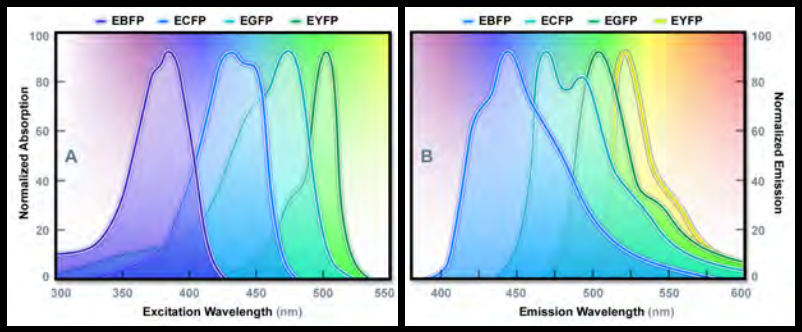


Nagai et al. (2002) Nature Biotech. 20:87; Griesbeck et al. (2001) J Biol Chem 276:29188

Mutant variants of Aequorea GFP

14

- Aequorea GFP-based color variants range from blue to yellow fluorescence:

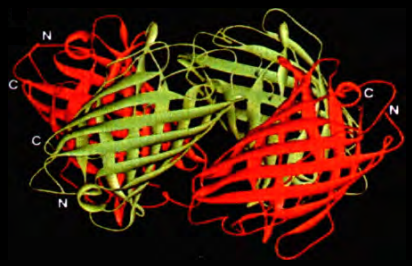


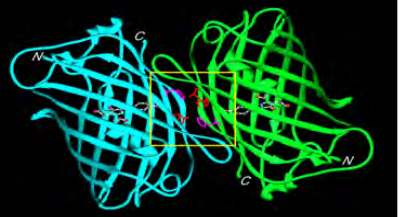
- The 530 nm emission of YFP was the most red-shifted of the color variants derived from Aequorea GFP.

Aequorea FPs and dimer formation

15

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



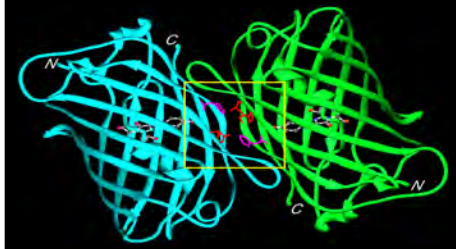


- Aequorea GFP could be crystallized as a monomer, but the proteins can form dimers when highly concentrated with limited mobility.

Aequorea FPs and dimer formation

16

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



- The substitution of alanine at position 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

17

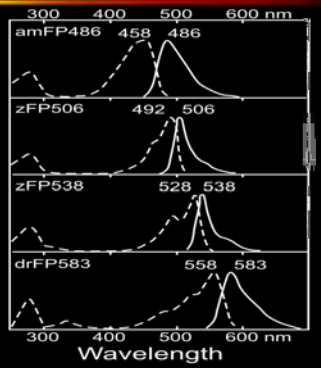
- The fluorescent proteins (FPs) for live cell imaging:
  - General characteristics of the FPs.
  - Aequorea color variants.
  - New FPs from corals (and fish).

Fluorescent Proteins from other marine organisms

18

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

Mushroom anemone Discosoma striata

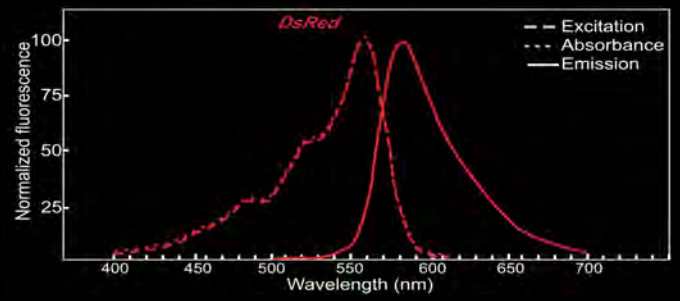


- DsRed from the mushroom anemone was the first red FP to be cloned, allowing genetically encoded multicolor imaging in living cells.

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

Advantages of DsRed

19

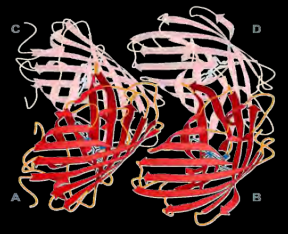


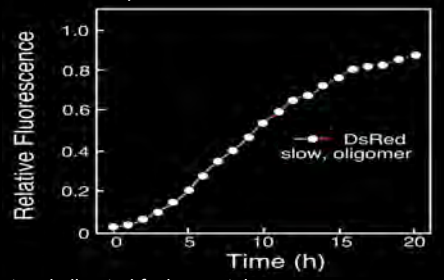
- DsRed is spectrally distinct from the Aequorea-derived FPs;
- it is easily detected with standard optical filters;
- there is reduced cellular auto-fluorescence at longer wavelengths.

Limitations of DsRed

20

- DsRed is an obligate tetramer in mammalian cells that requires nearly 20 h to fully mature, and there is a green intermediate form of the protein.





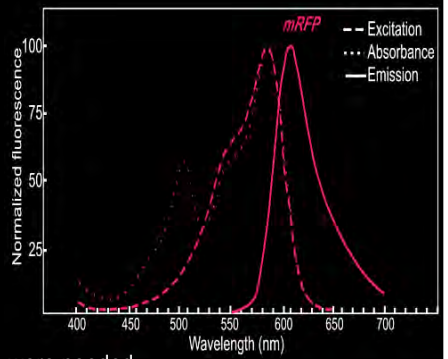
- DsRed tends to form oligomers, leading to misdirected fusion proteins.
- Engineered by directed mutagenesis to break the tetramer, followed by random mutagenesis to recover a monomeric red FP – mRFP1.

Campbell et al. (2002) PNAS 99:7877

mRFP1 was an improvement – but was not optimal

21

- Advantages: mRFP1 overcame tetramer and slow maturation, and shifted the spectra by 25 nm.
- Limitations: mRFP1 has decreased quantum yield and photo-stability compared to DsRed.
- There is a non-fluorescent form absorbs at 503 nm - 60% is in this dark state.
  Hillesheim et al. (2006) Biophys J 91:4273

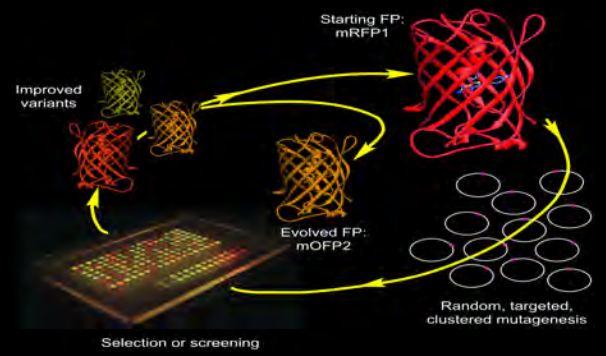


- New improved yellow, orange, red FPs were needed.

Directed evolution approaches were applied

22

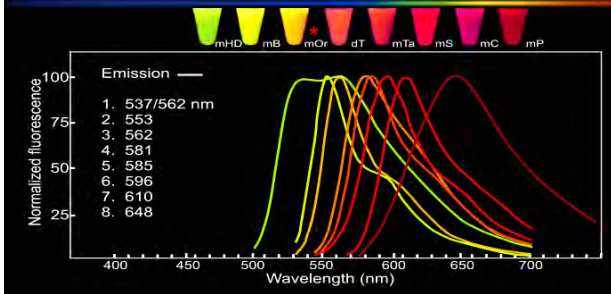
- mRFP1 was subjected to multiple rounds of mutagenesis, each followed by selection for FPs with desirable characteristics (directed evolution).



Shaner et al. (2004) Nat Biotech 22:1567; Shaner et al. (2007) J Cell Sci 120:4247

The second generation of FPs

23



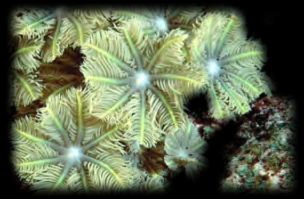
* FPs based on mOrange photo-convert to a red form!
Kremers et al. (2009) Nat Methods 6:355

- Of the fruit series only mCherry and tdTomato are broadly used.
- The other FPs in the series provided important information about structural contributions to photo-physical properties.
- New FPs continue to be isolated from marine organisms – including fish.

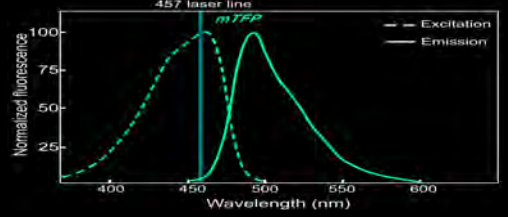
Shaner et al. (2004) Nat Biotech 22:1567; Shaner et al. (2007) J Cell Sci 120:4247

Engineering novel FPs from corals: mTFP1

24



Clavularia sp. "palm coral"

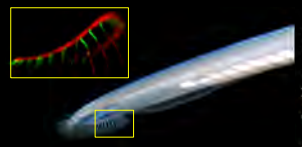


- Directed evolution of cFP484 from *Clavularia* selecting for a bright blue-green (Teal) FP (chromophore Y67 from GFPs):
- Key mutations mTFP1: Y67; N63T, Q66A, L72F, D125K, M127E, E144D::H163A
  - Ex 462 nm, Em 492 nm, relatively narrow spectra,
  - intrinsic brightness of 54,
  - photo-stable; acid stable – accumulates in lysosomes.

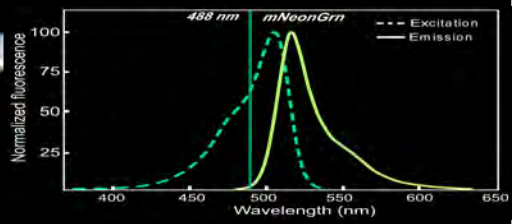
Ai et al. (2006) Biochem. J 400:531

Engineering novel FPs from fish: mNeon Green

25



Branchiostoma lanceolatum
Lancelet Fish



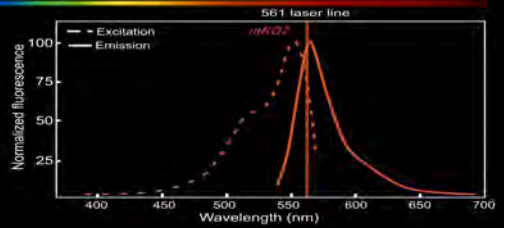
- Directed evolution of dLan YFP from Lancelet fish for a bright, monomeric yellow FP called mNeon Green:
- Key mutations: dLan YFP + 21 mutations
  - Ex 506 nm, Em 517 nm,
  - intrinsic brightness of 92 (ε = 115, QY = 0.80), brightest monomeric FP!
  - Rapid maturation, photo-stable – improvement over Venus.

Baumann et al. (2008) Bio Direct 3:28; Shaner et al. (2013) Nat Meth 10: 407

Engineering novel FPs from corals: mKO2

26

Fungia concinna mushroom coral



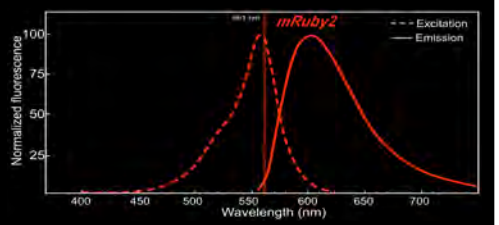
- Directed evolution of Kusabira orange from *Fungia concinna* selecting for a bright, monomeric orange FP:
- Key mutations: Kusabira + K49E, P70V, F176M, K185E, K188E, S192G, L210Q
  - Ex 551 nm, Em 565 nm,
  - intrinsic brightness of 36, no photo-conversion,
  - rapid maturation, photo-stable - narrow Stokes shift.

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

Engineering novel FPs from corals: mRuby3

27

Entacmaea quadricolor anemone



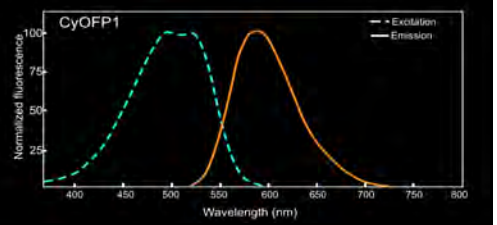
- Directed evolution of the dimeric eqFP611 from *Entacmaea quadricolor* selecting for a bright monomeric Red FP: Ruby → Ruby2
- Key mutations: mRuby2 + 21 substitutions
  - Ex 558 nm, Em 592 nm,
  - intrinsic brightness of 58 (ε = 128, QY = 0.45); very bright RFP,
  - rapid maturation, highly photo-stable – FRET acceptor.

Lam et al. (2012) Nat Meth 9:1005

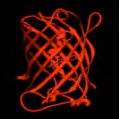
Re-engineering novel FPs: CyOFP1

28

Cell expressing CyOFP1



- Directed evolution of the far-red FP mNeptune2 to a bright cyan-excitable orange-red FP:
- Key mutations: mNeptune2 + M160K + 32 mutations, 2 deletions
  - Ex 497-523 nm, Em 589 nm
  - intrinsic brightness of 31 (ε = 40, QY = 0.76),
  - rapid maturation, highly photo-stable, large Stokes shift.
  - Multicolor imaging with single excitation – FRET applications?



Chu et al. (2016) Nat Biotech 10.1038

Optical highlighters: green to red photo-conversion

29

- Illumination of some green optical highlighter proteins with blue light (405 nm) results in a rapid shift to red emission.
- The color change is stable and irreversible;
- and is driven by a light-catalyzed cleavage in the chromophore:

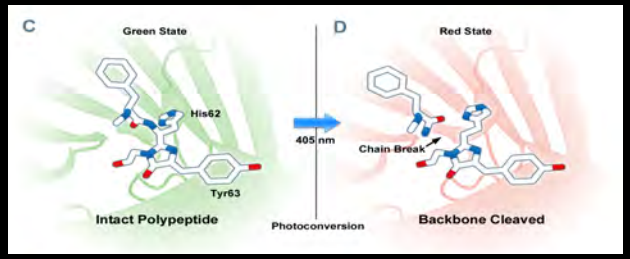
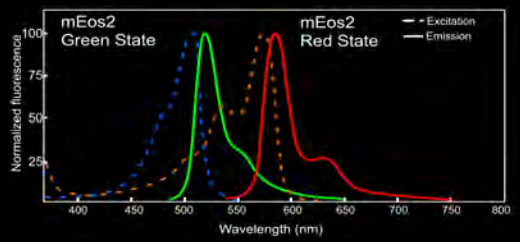


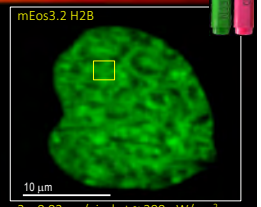
Photo-convertible FPs: optical highlighters

30

- mEos2 is a bright monomeric photo-convertible FP.
  (Eos is the Greek goddess of dawn)

  McKinney (2009) Nat Meth 6:131
  Day and Davidson (2009) Chem. Soc. Rev. 38:2887
- Directed evolution yielded mEos3.2 with improved brightness, maturation, and photo-stability.
  Zhang et al. (2012) Nat Meth 9:727





3 x 0.02 ms/pixel at ~ 200 nW/µm²

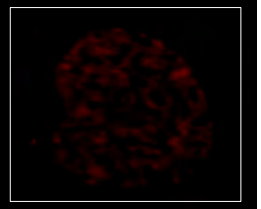


Photo-conversion of mEos3.2 H2B: Time lapse

31

Green Channel

Red Channel

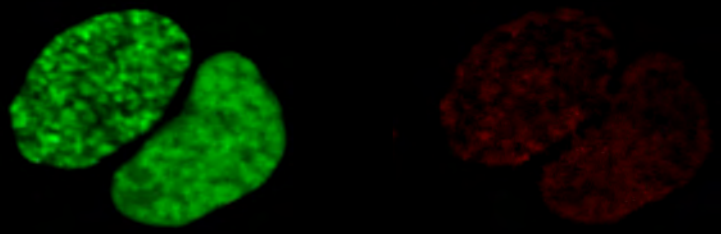


Photo-conversion: 405 nm ex 75%; 4 X 0.02 ms/pixel
Time series: 512x512, 0.1 ms/pixel, 2 min interval, 25 images

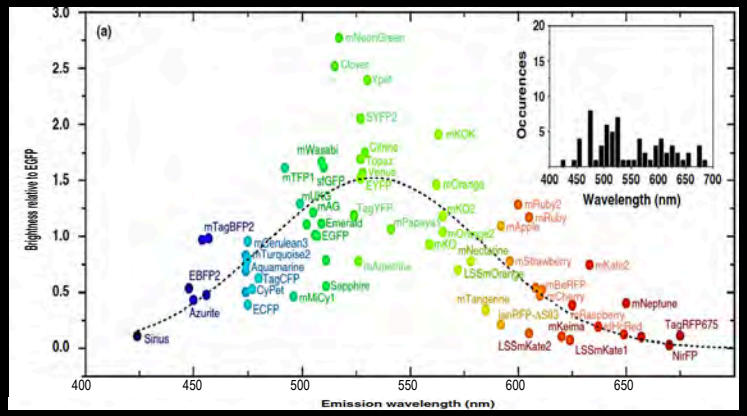
Overview

32

- The fluorescent proteins (FPs) for live cell imaging:
  - General characteristics of the FPs.
  - Aequorea color variants.
  - New FPs from corals (and fish).
  - The (current) best FPs.

Summary of the current FPs

33



Adam et al. (2014) COCB 20:92

Useful FP tool box (2016):

34

Next Gen	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	24	+++ *	Markwardt et al. 2011	Dr. Mark Rizzo
*	mTurquoise2	Cyan	433-445	475-503	28	+++ *	Goedhart et al. 2012	Dr. Theo Gadella
	mTFP	Teal	462	492	54	+++ *	Ai et al. 2006	Allele Biotech
	EmGFP	Green	487	509	39	++++	Cubitt et al. 1999	Invitrogen
*	Clover3	Green	505	515	85	++++	Bajar et al. 2016	Addgene (Clover2)
*	mNeonGrn	Green	506	517	92	++++	Shaner et al. 2013	Dr. Nathan Shaner
	Venus Citrine	Yellow/Grn	515 516	528 529	54 58	+ +++	Nagai et al. 2002 Griesbeck et al. 2001	Dr. Atsushi Miyawaki Dr. Roger Tsien
	Amber†	None			0		Koushik et al. 2006	Addgene
	mKO2 (Kusabira)	Orange	551	565	36	+++	Karasawa et al. 2004; Sakaue-Sawano 2008	MBL International
	mTagRFP-T	Orange	555	584	33	++++	Merzlyak et al. 2007; Shaner et al. 2008	Evrogen
	tdTomato	Orange	554	581	95	+++	Shaner et al. 2004	Addgene
*	CyOFP1	Red	497-523	569	31	+++	(Chu et al. 2016)	(Released Now)
	mCherry	Red	587	610	17	+++	Shaner et al. 2004	Clontech
*	mRuby3	Red	559	600	58	++++	Bajar et al. 2016	Addgene (RubyZ)
	mKate2 (Katushka)	Deep Red	588	633	25	++++	Shcherbo et al. 2009	Evrogen

* Depends on illumination source;
† Y66C mutant folds, but does not absorb or emit - important control for FRET-FLIM.

Overview

35

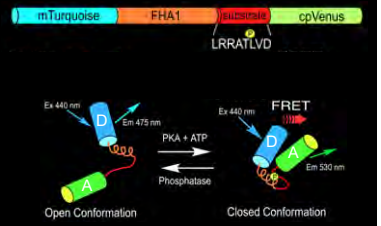
- The fluorescent proteins (FPs) for live cell imaging:
  - General characteristics of the FPs.
  - *Aequorea* color variants.
  - New FPs from corals (and fish).
  - The (current) best FPs.
- Applications: Biosensor probes
  - General characteristics of biosensor probes.
  - Standards for biosensors.
  - Examples of biosensors.

Why biosensor probes?

36

- The genetically encoded biosensor probes enable noninvasive detection of spatial and temporal characteristics of *specific* cell signaling or metabolic events.
- Biosensors typically contain a reporter module consisting of donor and acceptor FPs directly linked by a sensing unit that detects a specific cellular event.
- If the biosensor conformation places the acceptor (A) fluorophore close (<80Å) to the donor (D),

FRET-based Biosensor probe

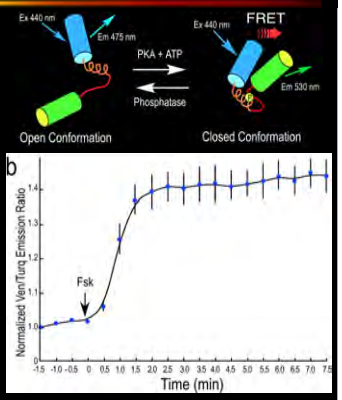


- then energy can be transferred directly by Förster resonance energy transfer (FRET).

Why biosensor probes?

37

- Energy transfer quenches the donor signal, while causing increased emission from the acceptor.
- The change in FRET ratio (Ven/Turq emission) is a sensitive and quantitative read-out of the probe response to the cell signaling event.
- Generally, the loss of signal in highly scattering biological tissues prevents comparison of intensity measurements obtained from different depths – but, ratio images obtained from biosensors will be minimally affected by depth.



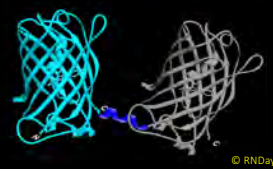
Intramolecular FRET: The FRET standards

38

- To validate measurements of biosensors, FRET standards were generated using Turquoise coupled to Venus through progressively longer linkers.

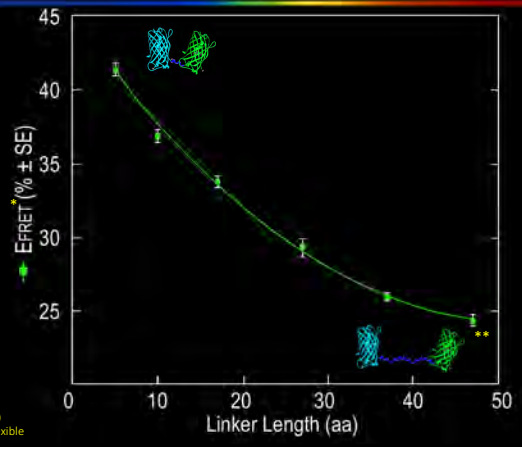
FRET Standard	Linker composition
Turquoise- 5 aa - Amber	-SGLRS-
Turquoise- 5 aa - Venus	-SGLRS-
Turquoise- 10 aa - Venus	-SGLRSPPVAT-
Turquoise- 17 aa - Venus	-SGLRSRAQASNAAVDGT-
Turquoise- 27 aa - Venus	-SGLRSENLYFQGPREFPGGTAGPVATV-
Turquoise- 36 aa - Venus	-SGLRSENLYFQGPREFPGGTGSGRGSGTGTAGPVAT-
Turquoise- 46 aa - Venus	-SGLRSENLYFQGPREFPGGTGSGRGSGTGTGSGRGSGTGTAGPVAT-

- Fusions were also created with Amber - the Y66C mutant of Venus that folds correctly, but does not act as a FRET acceptor.



Measurements of FRET standards in living cells

39



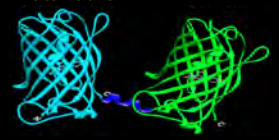
* $E_{FRET} = 1 - (\tau_{DA}/\tau_D)$
** Ser/Gly linkers are flexible

Characterized FRET standards for biosensor development

40

- The FRET standards serve as starting point for the development of FRET-based biosensor probes.
- Insertion of the chosen biosensor sensing units for specific cellular events into the FRET standards is relatively straight-forward.
- A kinase activity reporter (AKAR) is a reporter of intracellular PKA activity.

FRET Standard



FRET-based Biosensor probe

Overview

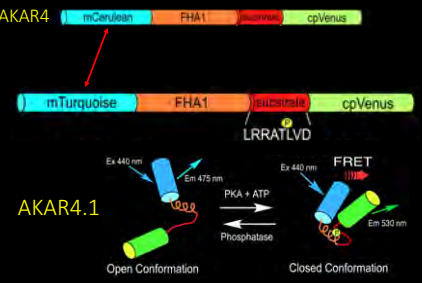
41

- The fluorescent proteins (FPs) for live cell imaging:
  - General characteristics of the FPs.
  - *Aequorea* color variants.
  - New FPs from corals (and fish).
  - The (current) best FPs.
- Applications: Biosensor probes
  - General characteristics of biosensor probes.
  - Standards for biosensors.
  - Examples of biosensors.

Improving FRET-based biosensor probes

42

- AKAR4 consists of Cerulean and circular permuted (cp) Venus coupled by a PKA specific substrate and the low affinity phospho-substrate binding domain, FHA1.
- For use in intravital microscopy (IVM, more later) we replaced Cerulean with mTurquoise.
- The phosphorylation of the PKA-specific substrate allows binding of FHA1, altering the distance between the FRET pair.



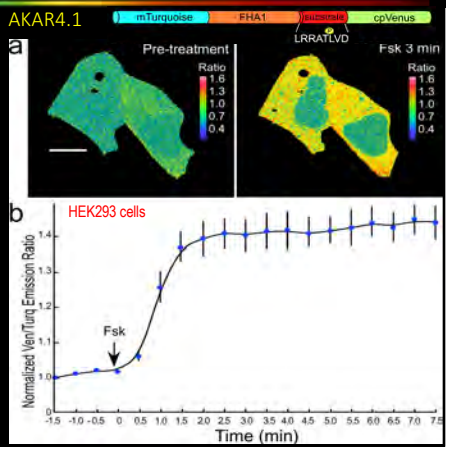
- The changing intramolecular FRET signal reports the spatiotemporal dynamics of PKA activity inside living cells.

Modification of AKAR4 from Zhou et al. (2012) Methods in Enzymol 504:317

Demonstration of ratiometric biosensor measurements: PKA

43

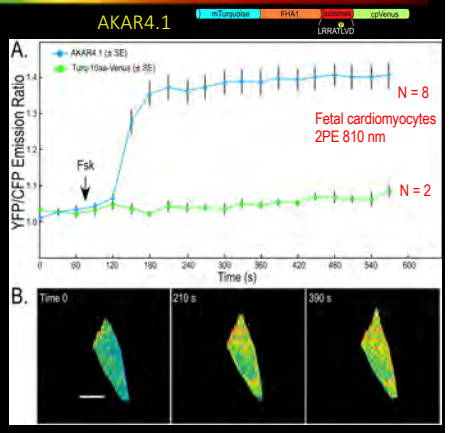
- Biosensor activity was evaluated in HEK293 cells.
- The emission signals from the cells were simultaneously monitored in the cyan (454-494 nm) and yellow (520-580 nm) channels.
- After collecting baseline measurements, the cells were treated with the PKA agonist forskolin (Fsk).
- There was a rapid and pronounced (1.4-fold) increase in the Venus to Turquoise emission ratio.



Demonstration of ratiometric biosensor measurements: PKA

44

- The FRET ratiometric imaging of isolated primary mouse fetal cardiomyocytes expressing either the AKAR4.1 biosensor or the mTurquoise-10AA-Venus FRET standard.
- The addition of Fsk results in a rapid increase in the YFP/CFP emission ratio for AKAR4.1, but not the FRET standard.

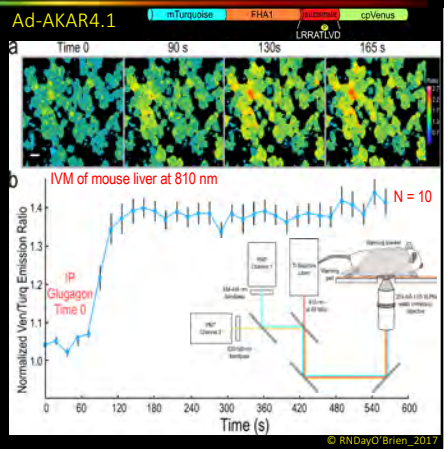


Tao et al. (2015) Am J Physiol Cell Physiol 309: C724

Monitoring PKA activity mouse hepatocytes with IVM

45

- The AKAR4.1 adenoviral vector was introduced by tail vein injection and imaged by IVM after 7 days.
- The mice were fasted for 3 h, and baseline images were collected.
- Imaging was continued after IP injection of glucagon (200 µg/kg).
- The Ven/Turq emission ratio changed within ~1 min of glucagon administration (time 0).

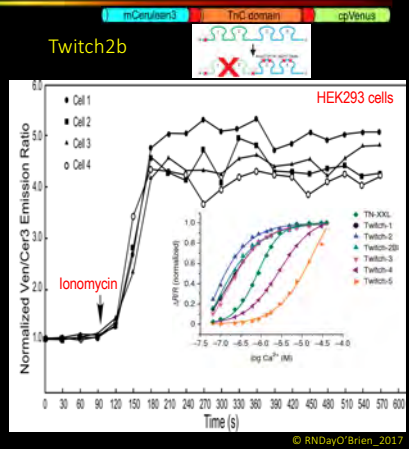


Day et al. (2016) Nat Protocols 11:2066

Improved calcium sensing probes

46

- The calcium biosensor, Twitch2b, is among the best probes for sensing changes in intracellular calcium.
- The reporter domain is optimized for photo-stability and brightness, and has a robust dynamic range (5-fold).
- The sensor (TnC) contains four minimal EF hand domains, each with high-affinity calcium binding.
- Deletion and mutagenesis of the TnC domain is used to tune the affinity over the range of 100 nm to 200 mM.

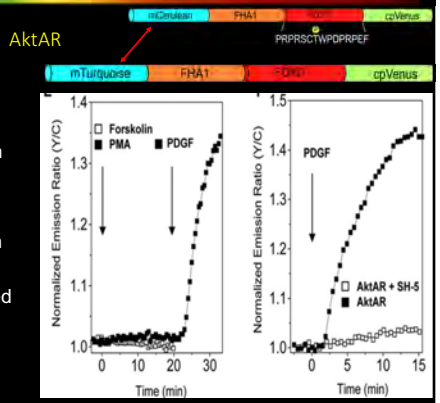


Thestrup, T. et al. (2014) Nat Methods 11, 175-182.

Improved Akt biosensor

47

- The PI3K/Akt (PKB) pathway is a major hub in the signal transduction network.
- We obtained the AktAR plasmid (Addgene 61624) and have exchanged Cerulean with mTurquoise.
- Gao and Zhang reported a strong emission ratio change in response to PDGF (but not Fsk or PMA), which was specifically blocked by SH-5.
- We're testing this probe for intravital microscopy.



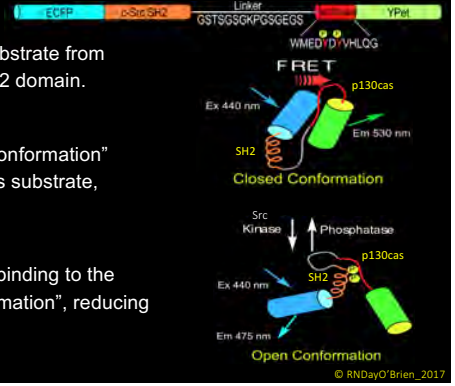
Gao and Zhang demonstrated specificity of AktAR response to PDGF and blockade by inhibitor SH-5.

Gao and Zhang (2008) Mol Biol Cell 19, 4366.

Src-kinase biosensor probe

48

- The the non-receptor tyrosine kinase Src plays a central role in a variety of cellular functions.
  - The biosensor consists of the c-Src substrate from p130 cas that is linked to the c-Src SH2 domain.
  - The biosensor protein is in a "closed conformation" before phosphorylation of the p130 cas substrate, yielding a high FRET efficiency.
  - The phosphorylation allows substrate binding to the SH2 domain, causing an "open conformation", reducing the FRET signal.

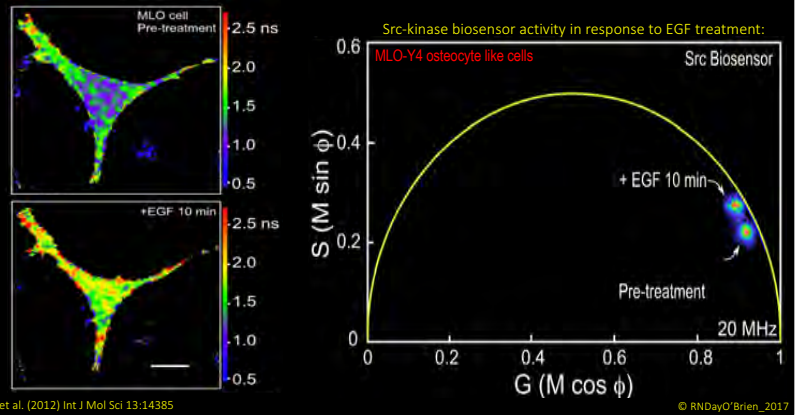


Wang et al. (2005) Nature 434:1040

Measuring Src-induced conformational changes with FRET-FLIM

49

- Lifetime imaging (more later) maps the subcellular distribution of Src kinase activity:

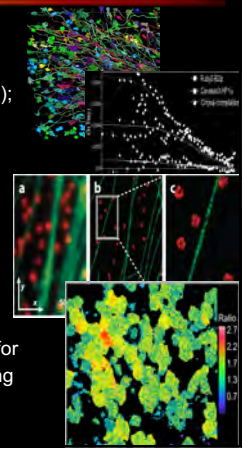


Hum et al. (2012) Int J Mol Sci 13:14385

Fluorescent proteins and biosensor probes

50

- Advantages: visualizing the behavior of proteins in the natural context of living cells, tissues, or organisms:
  - Marking cell populations in transgenic animals (Brainbow mice);
  - following proteins in specific organelles, multi-color labeling, dynamics (FRAP, FCS);
  - Super-resolution (PALM), tumor progression (IVM), protein-protein interactions (FRET).
  - Ratiometric imaging of genetically encoded biosensor probes for quantitative measurements of dynamic changes in cell signaling pathways in living animals.



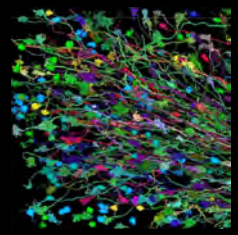
Day and Davidson (2014) The Fluorescent Protein Revolution (CRC Press)

Fluorescent proteins and biosensor probes

51

- Limitations: Heisenberg uncertainty principle - every measurement perturbs the system being measured - light perturbs the system.
  - The expression of any amount of exogenous protein is "over-expression" - how this influences regulatory pathways in living cells is difficult to determine.
  - There is the potential that exogenous probes may perturb the underlying cellular mechanisms they are designed to detect.

Lock et al. (2015) Cell Calcium 6:638



- The ultimate goal is to develop methods that allow us to answer specific questions. If the technique provides an accurate answer to your question, it is a valid approach.

Some reviewers may (will) disagree...