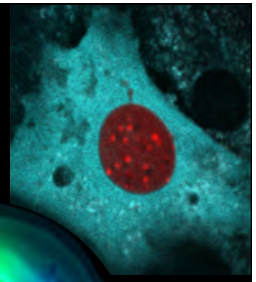
Genetically encoded fluorescent proteins



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O'Brien Center Workshop
IUSM
April 28, 2015

Overview:

The fluorescent proteins (FPs) for live cell imaging:

- Origins and Limitations:
  - General characteristics of the FPs
  - *Aequorea* color variants
  - New FPs from corals (and fish)
  - The (current) best FPs
  - Optical highlighters

Overview:

The fluorescent proteins (FPs) for live cell imaging:

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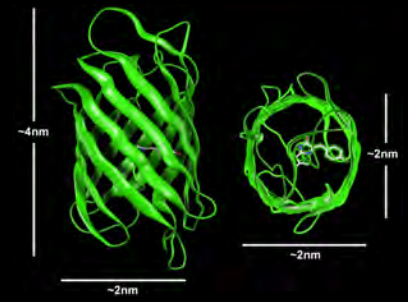
General characteristics of Aequorea GFP

- Cloned in 1992 by Prasher, the crystal structure of GFP was solved in 1996, showing the cyclic tripeptide buried in the center of an 11-strand β-barrel:



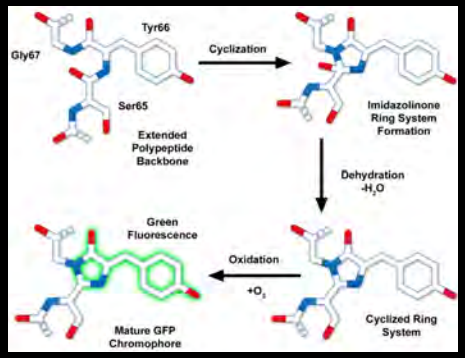
- This explained why the entire protein sequence was required for fluorescence.

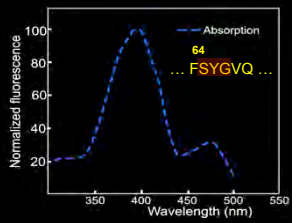
Prasher et al. (1992) Gene 111:229
Ormo et al. (1996) Science 273:1392

General characteristics of GFP

5

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





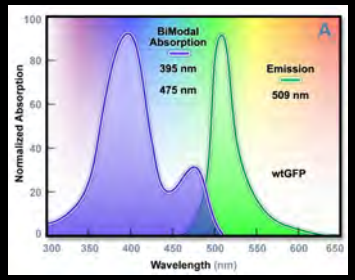
Cody et al. (1993) Biochemstry 32:1212

- 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 Aequorea GFP

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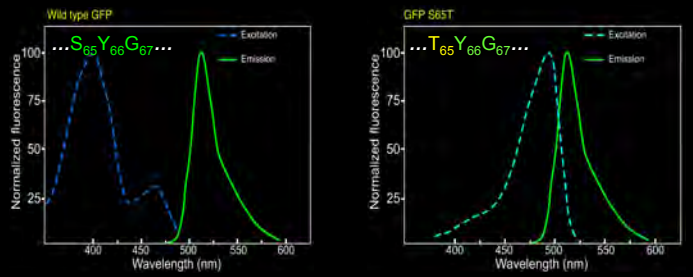
- Wild type GFP folds poorly at physiological temperature.
- "Humanized" codon usage, Kozak initiation codon.
- Mutations that improve efficiency of chromophore formation:
  - F64L dramatically improved maturation at 37°;
  - V68L enhances chromophore oxidation;
  - N149K improves folding rate;
  - M153T,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

Mutant variants of *Aequorea* GFP

8

- 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

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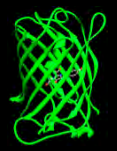
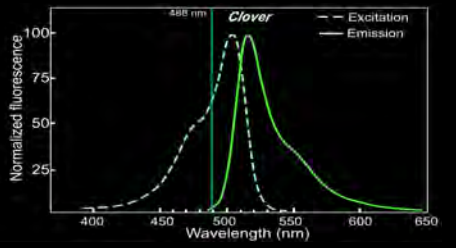
Spectral variants of GFP: a new GFP - Clover



Clover

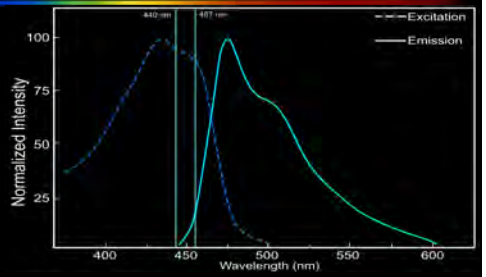


- Directed evolution of EGFP selecting for a brighter variant better suited as a FRET donor for orange and red FPs:
- Key mutations: EGFP + S30R, Y39N, S65G, Q69L, N105T, Y145F, M153T, V163A, I171V, T203H
  - Ex 505 nm, Em 515 nm,
  - intrinsic brightness of 84 (ε = 111, QY = 0.76),
  - maturation rapid, good photostability.

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

Spectral variants of GFP: Cerulean3 and Turquoise2

mCerulean3,
mTurquoise,
mTurquoise2

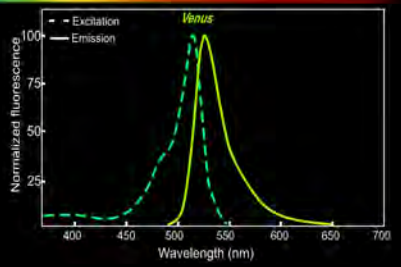


- Mutagenesis of ECFP > Cerulean > selection of a brighter variants; mTurquoise2: T65S/I146F allows better packed chromophore; QY 0.93!
- Key mutations: mCerulean + T65S/A145Y/I146F/S175G
  - Ex 434 nm, Em 475 nm,
  - intrinsic brightness of 28,
  - All have a single component lifetime.

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

Spectral variants of GFP: Venus and Citrine

mVenus
mCitrine



- Mutagenesis of EYFP selecting for a brighter yellowish FP with reduced halide and pH sensitivity:
- 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 photostable.*

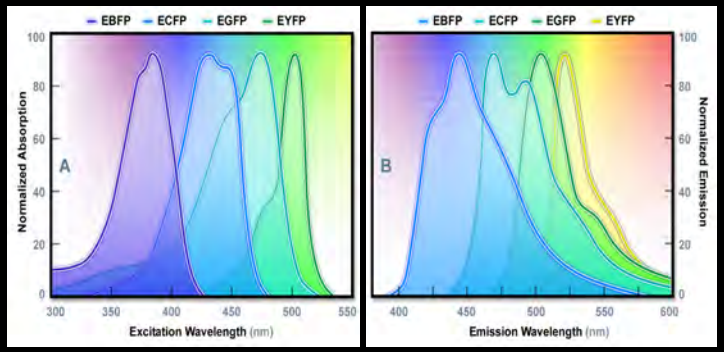
Nagai et al. (2002) Nature Biotech. 20:87;
Griesbeck et al. (2001) J Biol Chem 276:29188

* Useful for photobleaching techniques

Color variants derived from Aequorea GFP

13

- Mutagenesis of *Aequorea* GFP yielded color variants range from blue to yellow:



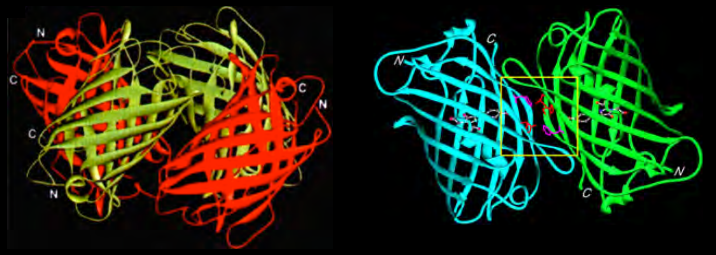
- The 530 nm emission of YFP was the most red-shifted of the GFP color variants.

Tsien (1998) Ann Rev Biochem 67:509

Aequorea FPs and dimer formation

14

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

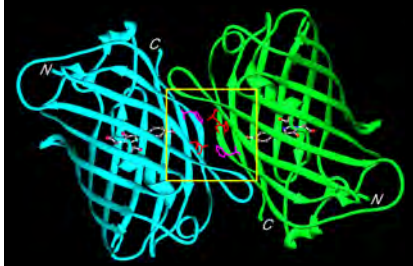


- GFP could be crystallized as a monomer, but the proteins can form dimers when highly concentrated.

Aequorea FPs and dimer formation

15

- 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 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.

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16

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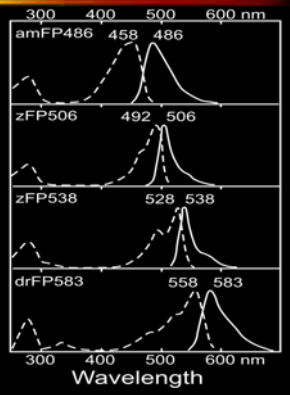
Fluorescent Proteins from other marine organisms

17

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

Mushroom anemone *Discosoma striata*

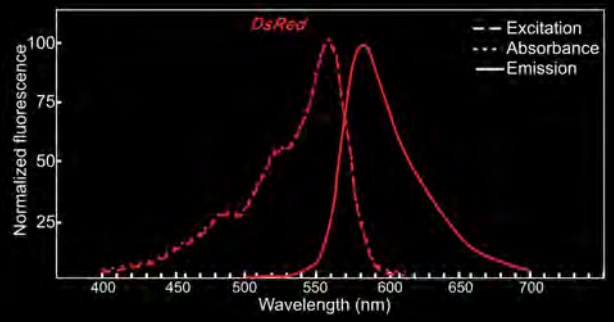


- DsRed from the mushroom anemone was the first of the red FPs, and enabled multicolor imaging in living cells.

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

Advantages of DsRed

18

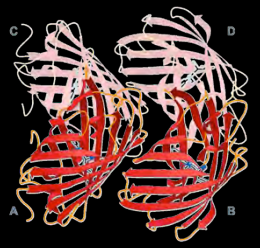


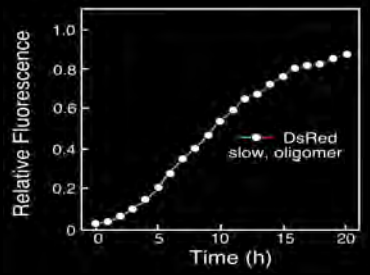
- DsRed is spectrally distinct from the *Aequorea* FPs;
- it is easily detected with standard optical filters;
- there is reduced cellular auto-fluorescence at longer wavelengths.

Problems with DsRed

19

- DsRed is an obligate tetramer in mammalian cells:



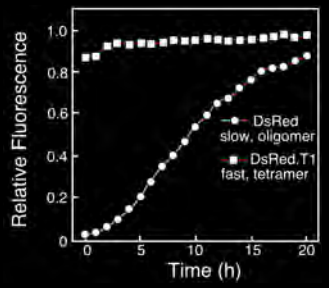


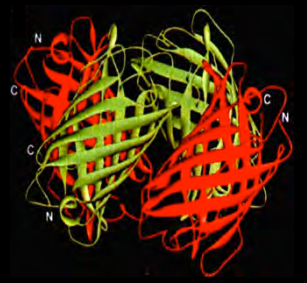
- DsRed requires nearly 20 h to fully mature, and there is a green intermediate form of the protein.
- DsRed tends to form oligomers, leading to misdirected fusion proteins.

New variants based on DsRed

20

- Mutagenesis to improve maturation: DsRed.T1



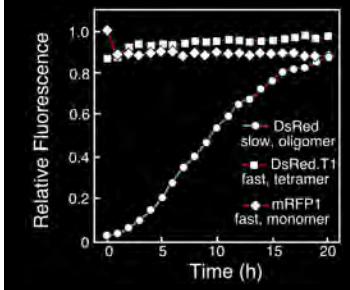


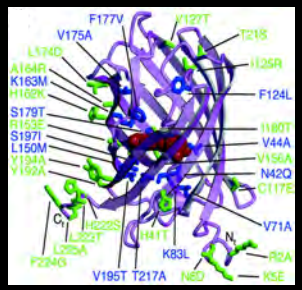
Bevis and Glick (2002) Nat. Biotech. 20:83

New variants based on DsRed

21

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



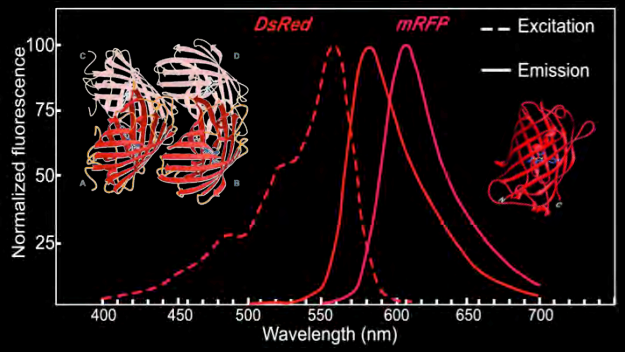


Campbell et al. (2002) PNAS 99:7877

mRFP1 was an improvement -

22

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



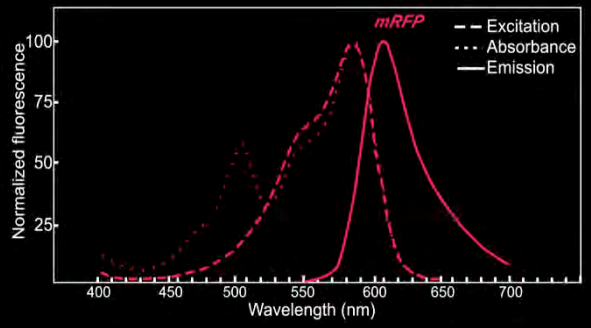
Campbell et al. (2002) PNAS 99:7877

but, mRFP was not optimal for some applications

23

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

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

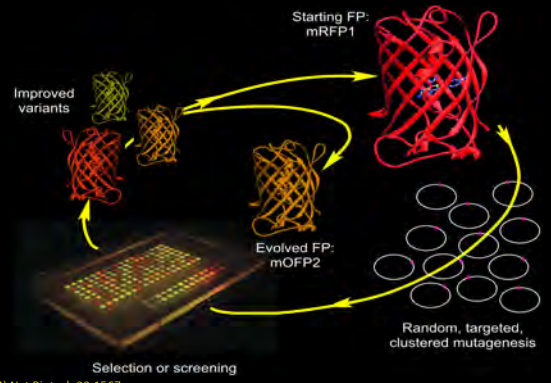


- New improved yellow, orange, red FPs were needed!

Directed evolution approaches were applied

24

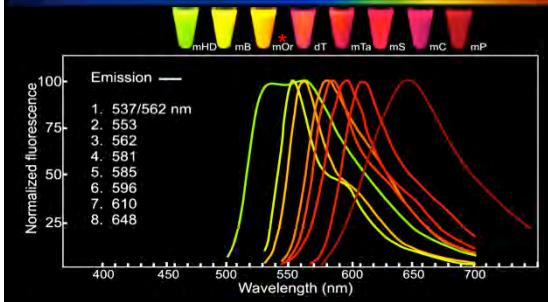
- Multiple rounds of mutagenesis, each followed by selection for FPs with desirable characteristics.



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

The next generation of FPs

25



* FPs based on mOrange photoconvert to a red form!

Kremers et al. (2009) Nat Methods 6:355

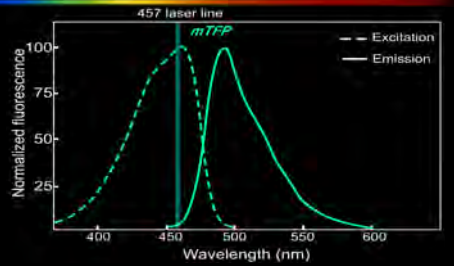
- Of the fruit series only mCherry and tdTomato are useful as probes.
- The other FPs in the series provided important information about structural contributions to photophysical properties.
- ❖ New FPs continue to be isolated from marine organisms – including fish.

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

The cloning of novel FPs from corals: mTFP1

26

Clavularia sp. "palm coral"



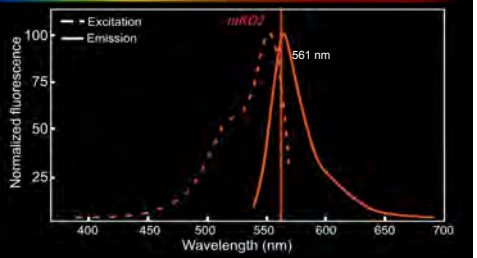
- Directed evolution of cFP484 from *Clavularia* selecting for a bright blue-green (Teal) FP:
- Key mutations mTFP1: Y67; N63T, Q66A, L72F, D125K, M127E, E144D::H163
  - Ex 462 nm, Em 492 nm, relatively narrow spectra,
  - intrinsic brightness of 54,
  - photostable; acid stable – accumulates in lysosomes.

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

The cloning of novel FPs from corals: mKO2

27

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,
  - maturation rapid, photostable - narrow Stokes shift.
  - mKO2 does not photoconvert to a red form

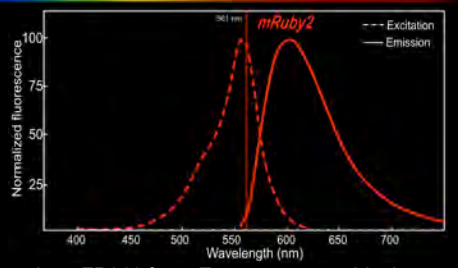
Sakaue-Sawano et al. (2008) Cell 132:487

Kremers et al. (2009) Nat Methods 6:355

The cloning of novel FPs from corals: mRuby2

28

Entacmaea quadricolor anemone



- Directed evolution of the dimeric eqFP611 from *Entacmaea quadricolor* selecting for a bright monomeric Red FP:
- Key mutations: mRuby + L15M, I102V, A119V, A131P
  - Ex 559 nm, Em 600 nm,
  - intrinsic brightness of 43,
  - maturation 2.5 h, photostable.

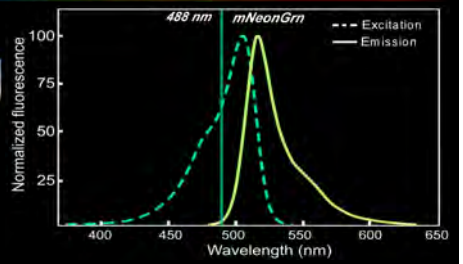
Lam et al. (2012) Nat Meth 9:1005

The cloning of novel FPs from fish: mNeon Green

29

Something New!

Branchiostoma lanceolatum
Lancelet Fish



- Directed evolution of a YFP from Lancelet fish for a bright, monomeric yellow FP, mNeon Green:
- Key mutations: Lan YFP + 21 mutations
  - Ex 506 nm, Em 517 nm,
  - intrinsic brightness of 92 (ε = 115, QY = 0.80),
  - maturation rapid, photostable – improvement over Venus?

Baumann et al. (2008) Bio Direct 3:28;
Shaner et al. (2013) Nat Meth 2413

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30

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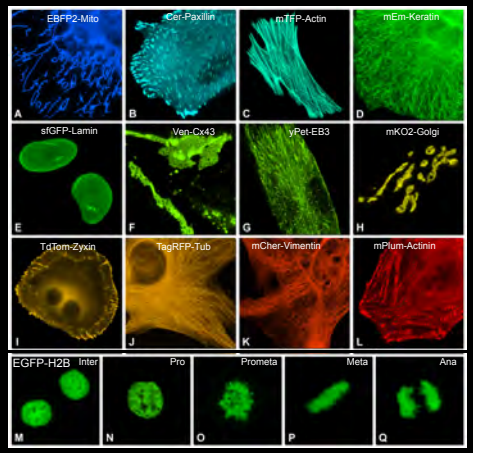
Subcellular distribution of FP-tagged proteins

31

- **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 (2015):

32

New	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	++++	Cabrit et al. 1999	Invitrogen
*	Clover	Green	505	515	84	++	Lam et al. 2012	Addgene
*	mNeonGrn	Green	506	517	92	++++	Shaner et al. 2013	Dr. Nathan Shaner
	Venus Citrine	Yellow/Grn	515 516	528 529	53 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	39	+++	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	Dr. Roger Tsien
*	mRuby2	Red	559	600	43	+++	Lam et al. 2012	Addgene
	mCherry	Red	587	610	17	+++	Shaner et al. 2004	Clontech
	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.

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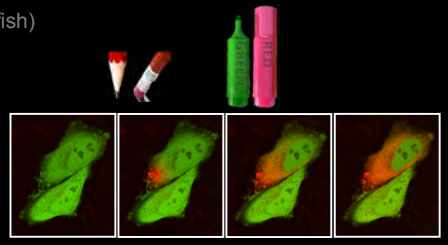
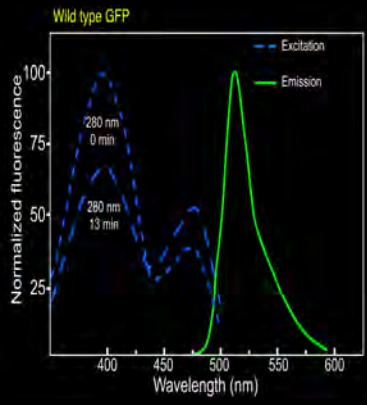


Photoswitching behavior of *Aequorea* GFP

34

- wtGFP has peak absorbance at 395 nm with a minor peak at 475 nm.
- Illumination with intense UV light resulted in decrease absorbance at 395 nm, and enhanced absorbance at 475 nm.
- This results from photoisomerization of the chromophore.

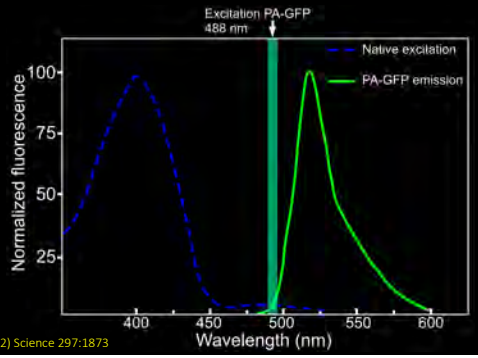


Cubitt et al. (1995) TIBS 20:448

PA-GFP: photoswitching variant of wtGFP

35

- PA-GFP is wtGFP with the T203H mutation. Optimal Ex is at 395 nm, with peak emission at 517 nm – there is little emission with Ex 488 nm:

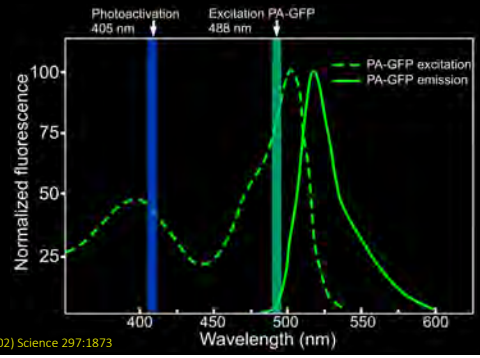


Patterson et al. (2002) Science 297:1873

PA-GFP: photoswitching variant of wtGFP

36

- PA-GFP is wtGFP with the T203H mutation. Optimal Ex is at 395 nm, with peak emission at 517 nm – there is little emission with Ex 488 nm:
- Intense 405 nm illumination switches excitation peak to 504 nm:

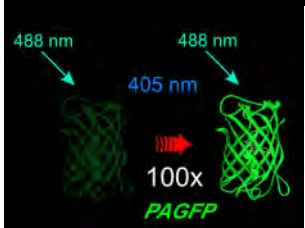


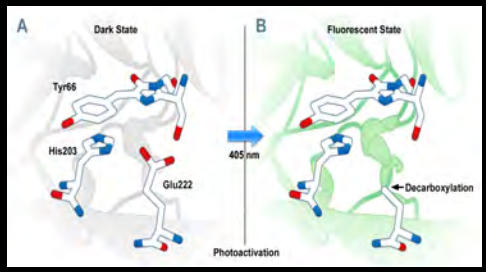
Patterson et al. (2002) Science 297:1873

PA-GFP: an optical marker

37

- Intense 405 nm illumination is thought to cause decarboxylation of the glutamic acid at position 222 near the chromophore;
- this converts the chromophore from a neutral to an anionic state:

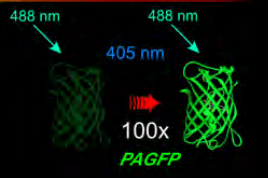


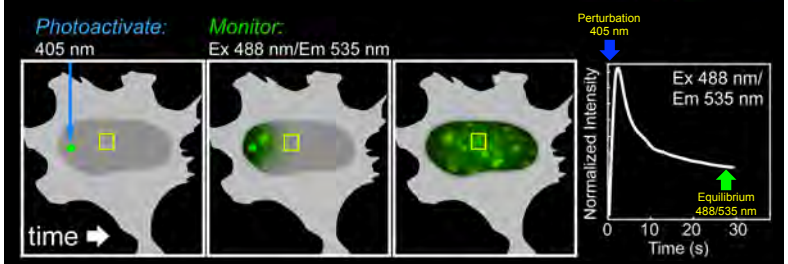


Imaging protein dynamics with PA-GFP

38

- Photoactivate PA-GFP with 405 nm laser in a region of interest in the cell.
- Monitor the diffusion of the newly fluorescent protein with 488 nm excitation over time.





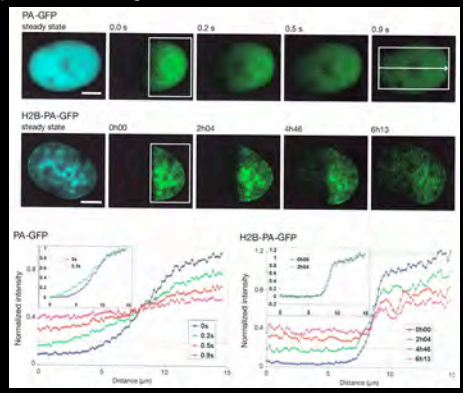
Advantages and limitations of photoactivation

39

- The redistribution of fluorescence over a dark background usually has better signal-to-noise compared to photobleaching.
- The photoactivation event is more rapid than photobleaching.
- The problem of probes in a reversible dark state is eliminated.

Limitations:

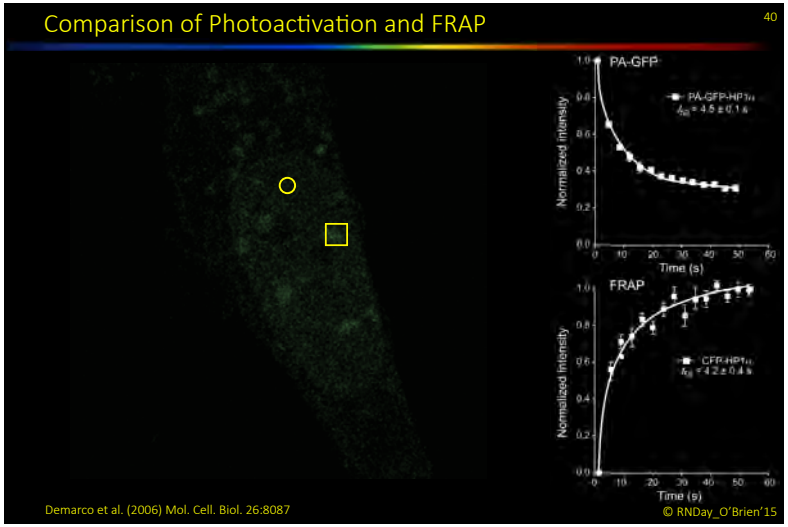
- Photoactivation requires irradiation at ~ 400 nm, which can be harmful.
- Can't localize the probe prior to activation.



Goldman et al. (2010) Live Cell Imaging 2nd edition

Comparison of Photoactivation and FRAP

40



Demarco et al. (2006) Mol. Cell. Biol. 26:8087

Dronpa: photoswitching variant from coral

41

Pectinia sp.

- Dronpa, a photo-switchable FP, was cloned from a coral.
- Native Dronpa was isolated as a oligomeric complex;
- and then engineered to a monomeric form.
- Dronpa is excited at 490 nm, very bright green emission (IB=80).
- Continued excitation drives Dronpa into a dark state.
- It can be reactivated by illumination at 405 nm.

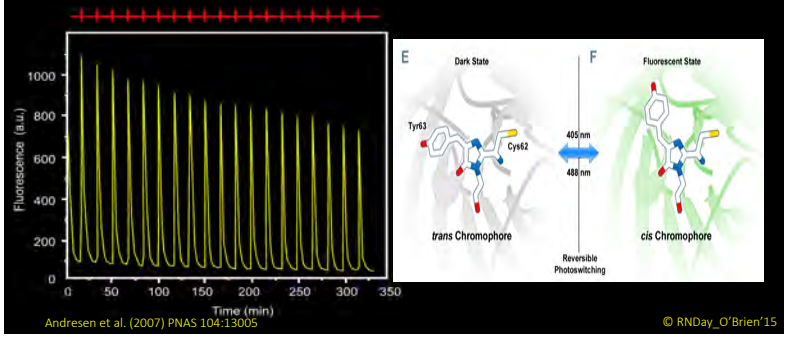
(Dronpa: Disappearance of a ninja)

Ando et al. (2004) Science 104:13005

Photoswitching behavior of Dronpa

42

- The protein is capable of repeated switching between the fluorescent and dark states;
- light-activated cis-trans photoisomerization of the chromophore generating the different dark and fluorescent states.

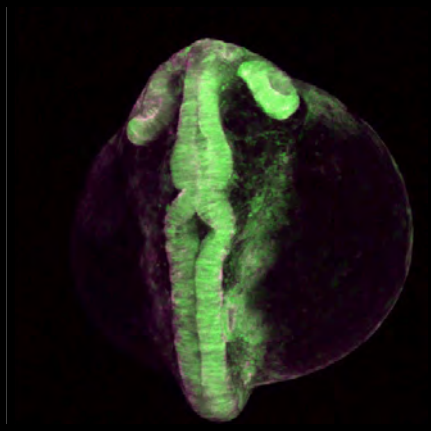


Andresen et al. (2007) PNAS 104:13005

Photoswitching behavior of Dronpa

43

- Dronpa was expressed in the developing zebra fish embryo.
- Fluorescence was detected and switched off at 488 nm, and then reactivated with a 405 nm pulse.
- Sequential regions are marked and erased over time.



Aramaki and Hatta (2006) Dev. Dynamics 235:2192

Photo-convertible FPs: optical highlighters

44

- Some recently discovered FPs can change their color.
- Light-induced photo-conversion of FPs may serve as a protection mechanism to absorb high-energy sunlight - sunscreen.
- Kaede was the first photo-convertible FP, isolated from the stony coral Trachyphyllia.

(Kaede: Japanese maple leaf)

Trachyphyllia geoffroyi

Photo-convertible FPs: optical highlighters

45

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

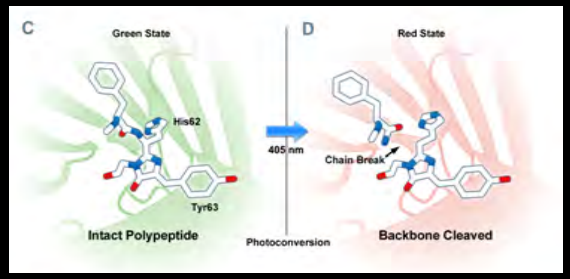


Photo-convertible FPs: optical highlighters

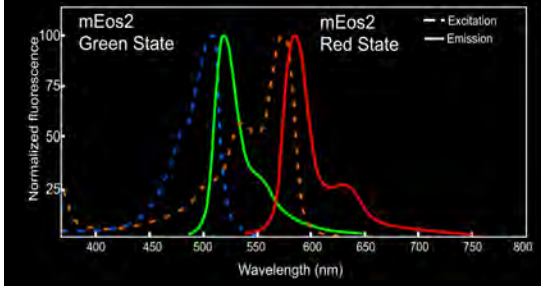
46

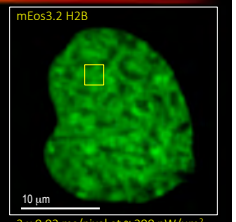
- mEos2 is the best performing monomeric green-to-red photo-convertible FP. (Eos is the Greek goddess of dawn)

McKinney (2009) Nat Meth 6:131
Day and Davidson (2009) Chem. Soc. Rev. 38:2887

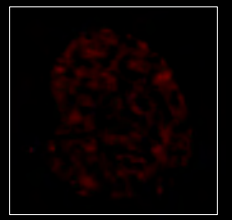
- Directed evolution yielded mEos3.2 with improved brightness, maturation, photostability.

Zhang et al. (2012) Nat Meth 9:727





3 x 0.02 ms/pixel at ~ 200 nW/µm²



Photoconversion of mEos3.2 H2B: Time lapse

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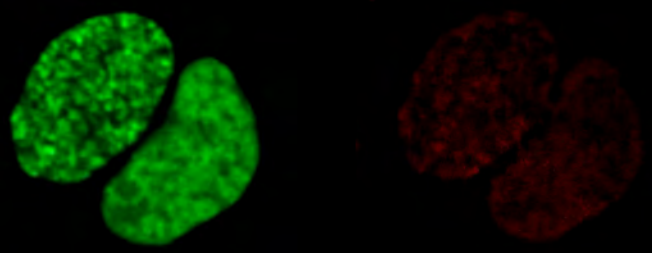
Photoconversion: 405 nm ex 75%; 4 X 0.02 ms/pixel
Time series: 512x512, 0.1 ms/pixel, 2 min interval, 25 images



Advantages and limitations of FPs

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Advantages:

- Visualizing the behavior of FP-tagged proteins in the natural context of living cells, tissues, organisms.
  - Marking cell populations in TG animals (Brainbow mice);
  - following proteins in specific organelles, multi-color labeling, dynamics (FCS);
  - Super-resolution (PALM), tumor progression, protein-protein interactions (FRET).

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

Limitations:

- Early variants of FPs should be avoided: ECFP, EYFP, DsRed...
- Potential for overexpression artifacts in transient transfections (stables?)
  - Verify localization with immunostaining of endogenous proteins (which is correct);
  - Verify interactions with co-IP – both require specific Abs;
  - Verify behavior with mutagenesis for loss of function (endogenous background).
  - Knockin to replace endogenous protein!

Gibson et al (2013) Nat Methods 10:715

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