Fundamentals in Biophotonics

Chromophores – Extrinsic Fluorophores – GFP-like Fluorophores

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Biological intrinsic fluorophores

- the extracellular matrix can also contribute to autofluorescence because of the intrinsic properties of collagen and elastin.

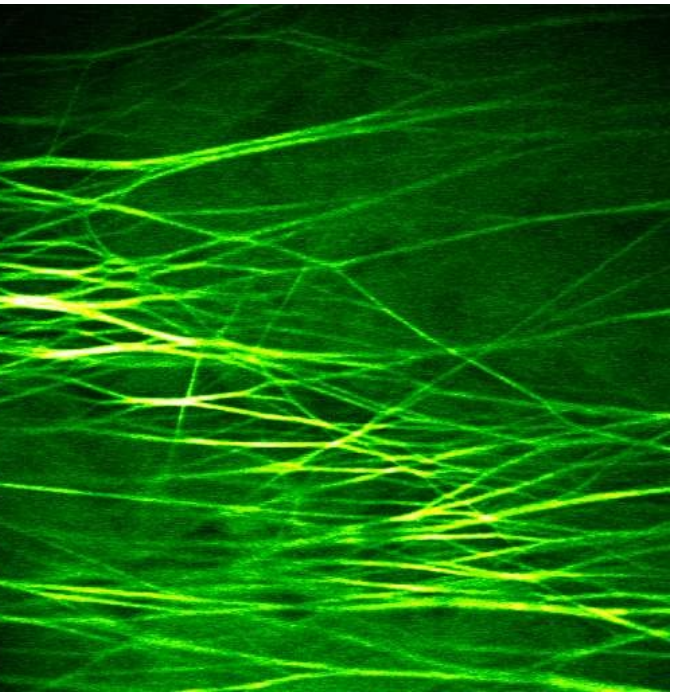
- **Collagen**
- It is the major extracellular matrix component, which is present to some extent in nearly all organs and serves to hold cells together in discrete units

- Collagen fluorescence in load-bearing tissues is associated with cross-links, hydroxylysyl pyridoline (HP) and lysyl pyridinoline (LP).

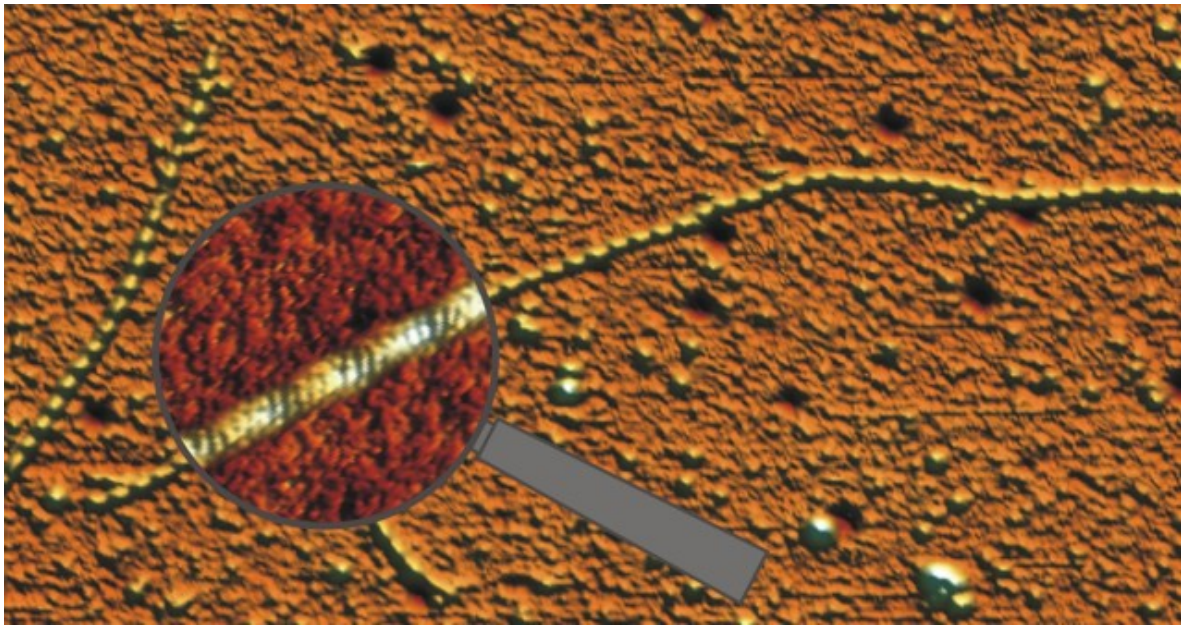
- Collagen crosslinks are altered with age and with invasion of cancer into the extracellular matrix

Collagen and elastin

- 2-photon auto-fluorescence image (right) of a bovine mesenteric collecting lymphatic vessel

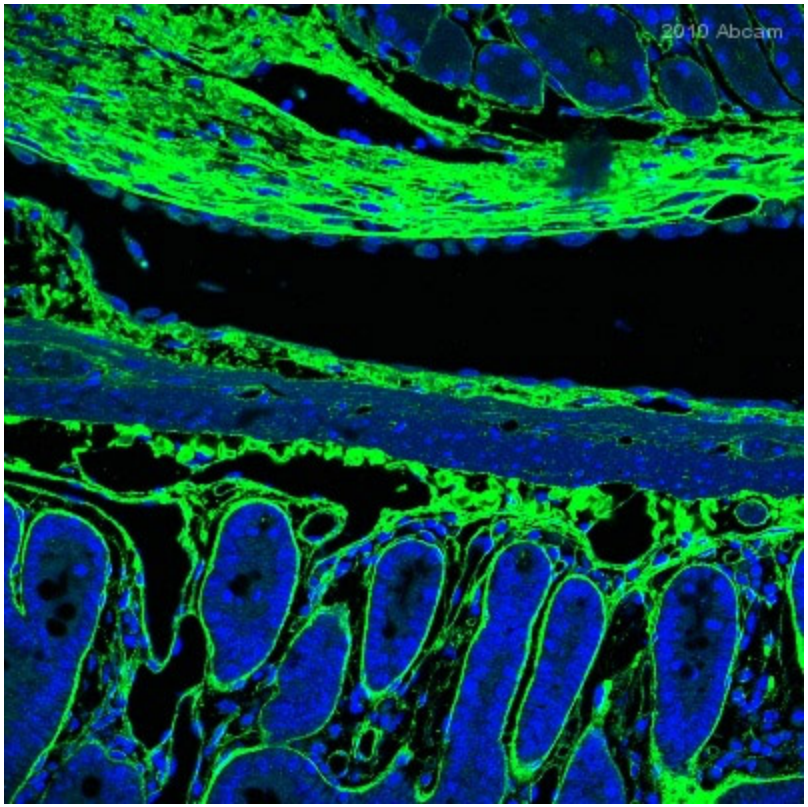


The stretched elastin fibers are clearly visible as bright straight lines, mostly in the direction of the vessel (left/right). There is a dimmer green background which is the spill over of the collagen fluorescence



Extrinsic Fluorophores

- Frequently molecules of interest are non-fluorescent or intrinsic florescence is not adequate



Collagen I antibody (ab21286) =secondary Antibody labeled with FITC

Number of extrinsic Fluorophores increased dramatically over the past decade

Nice list of the current **available** fluorophores can be found on the Molecular probes handbook

http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook.html

Lipids

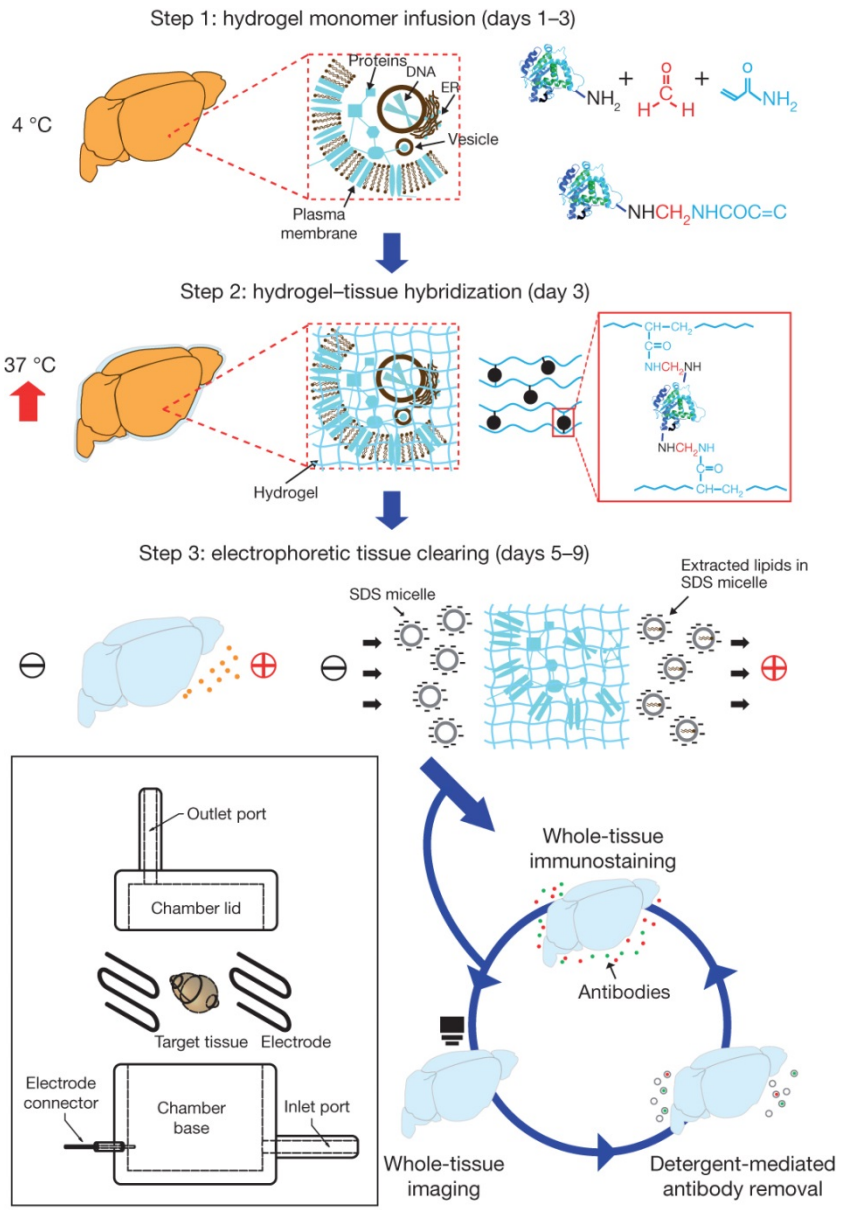
- Lipids are non-polar (hydrophobic) compounds, soluble in organic solvents.
- Most membrane lipids are amphipathic, having a non-polar end and a polar end.
- Fatty acids consist of a hydrocarbon chain with a carboxylic acid at one end.
- A 16-C fatty acid: **CH3(CH2)14-COO-**
- **Non-polar** **polar**

- A 16-C fatty acid with one cis double bond between C atoms 9-10 may be represented as 16:1 cis D9.

Lipids		
Phospholipids	436	540, 560
Lipofuscin	340–395	540, 430–460
Ceroid	340–395	430–460, 540
Porphyrins	400–450	630, 690

FAD, flavin adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; AND(P)H, reduced nicotinamide adenine dinucleotide phosphate.

CLARITY method



Tissue is crosslinked with formaldehyde (red) in the presence of hydrogel monomers (blue), covalently linking tissue elements to monomers that are then polymerized into a hydrogel mesh (followed by a day-4 wash step; Methods). Electric fields applied across the sample in ionic detergent actively transport micelles into, and lipids out of, the tissue, leaving fine-structure and crosslinked biomolecules in place. The ETC chamber is depicted in the boxed region

K Chung *et al. Nature* **000**, 1-6 (2013) doi:10.1038/nature12107

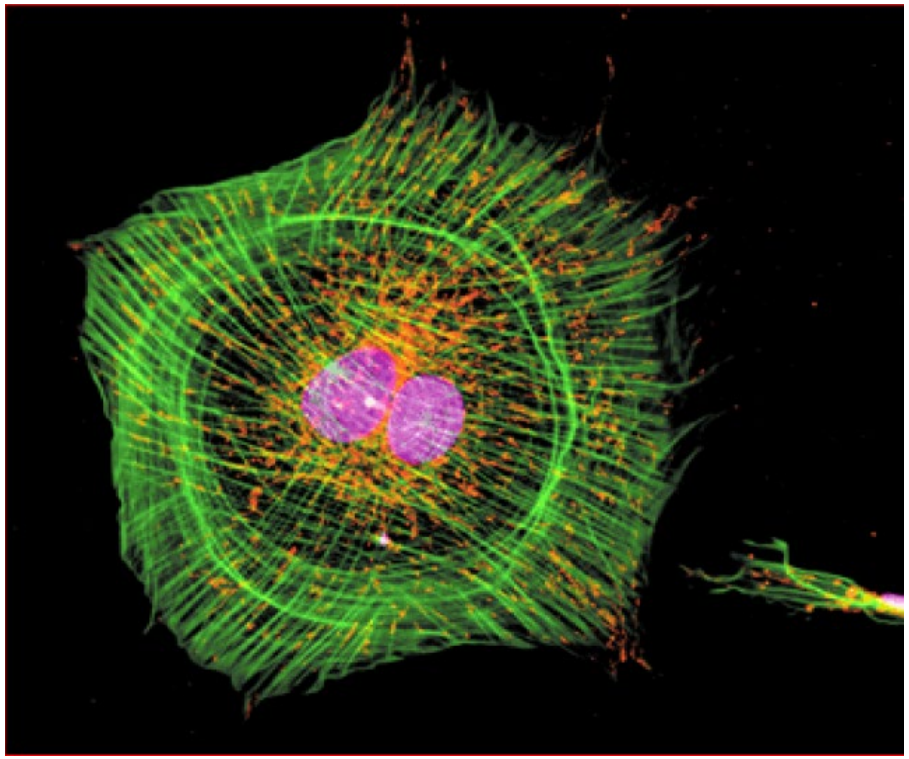
MOVIES

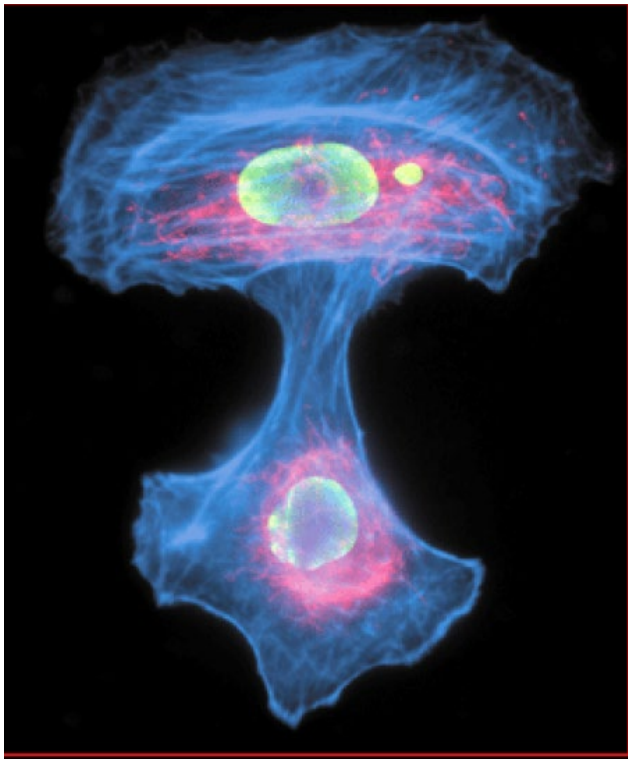
Outline

- GFP
  - Discovery
  - Mechanism
  - Limitations
  - Applications
- How to measure gene expression / Translation / Transcription
- Single Fluorophore Detection by Localization
- Stroboscopic excitation
- Experimental setup considerations
- Examples

Historical - histochemistry with fluorochromes

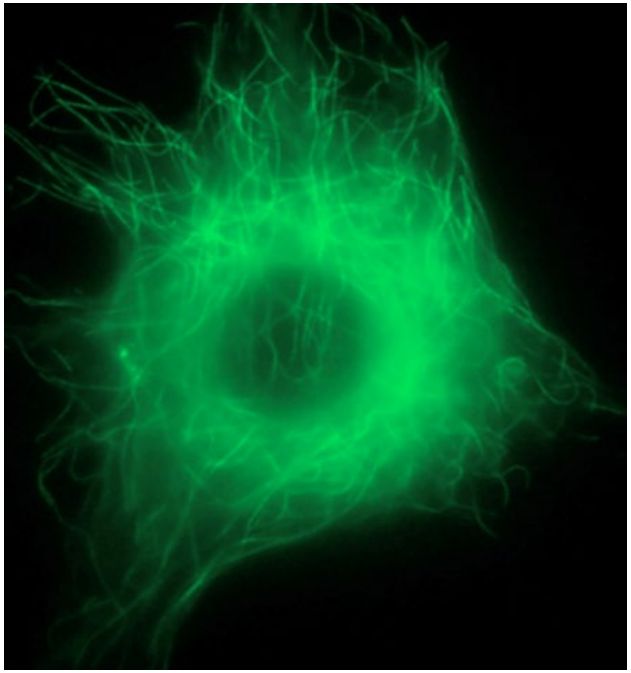
- Fluorochromes were generally used to study preserved cells and tissues.
- Microtechnical preparation of cells can alter their chemical composition and morphology.
- Developmental processes were difficult to study in fixed and killed organisms.





Trends in cytochemistry

- Fluorochromes that stain living cells and tissues are becoming widely used.
- In particular, many of the membrane staining fluorochromes available from Molecular Probes can diffuse throughout cells, e.g. Alexa Fluors, cy-series, FM stains, Di-series.
- They are for the most part, however, only short term indicators of developmental processes.

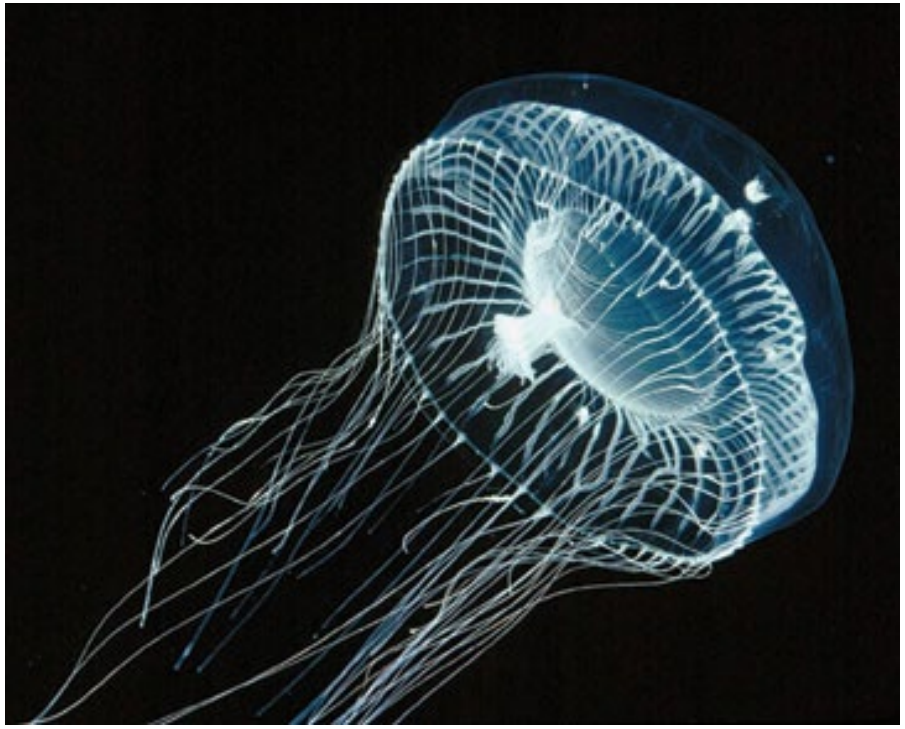


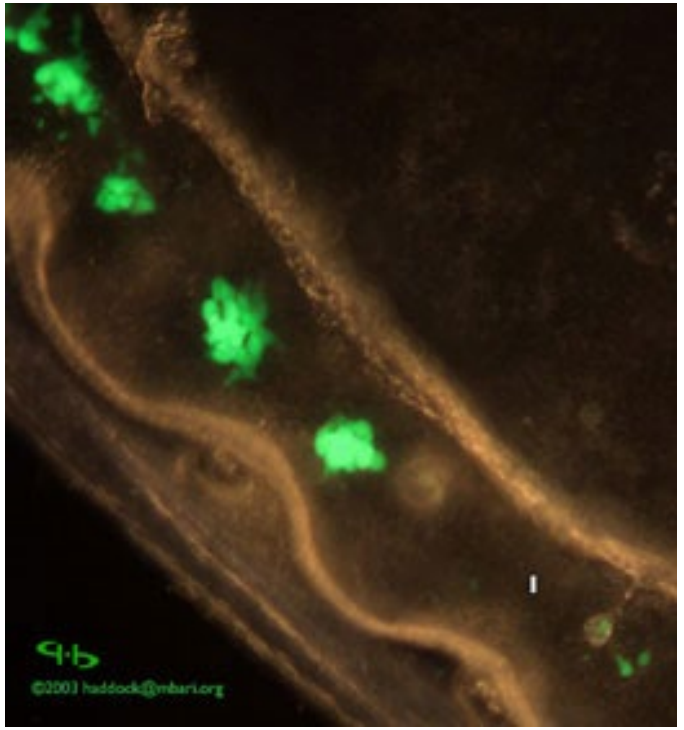
Fluorescent proteins

- Continually produced within living cells and subject to cellular targeting, partitioning, and turnover processes as with all other proteins.
- These proteins are very bright and non-toxic which means that cell and tissue development can be monitored over the long term.
- Importantly, fluorescent protein expression and sub-cellular localisation can be controlled using molecular biological techniques.
- Enabled super resolution
- Optogenteics

First…what are fluorescent proteins?

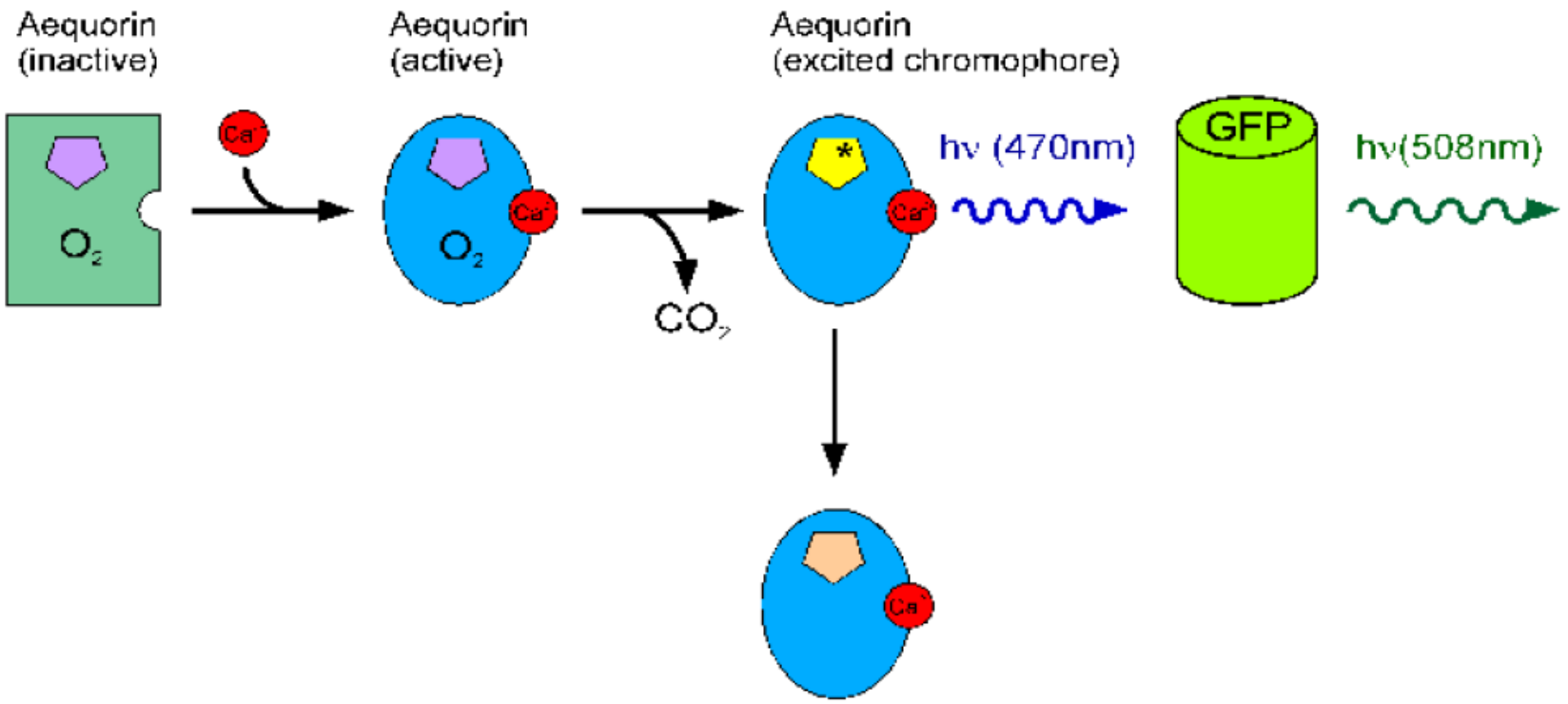
- The most widely used fluorescent proteins are derivatives of a native protein from the jellyfish *Aequorea victoria*.
- Many wild type fluorescent proteins are known from coelenterates.
- Fluorescent proteins serve as energy-transfer acceptors, receiving energy from a Ca2+-activated chemiluminescent protein called aequorin.





First…what are fluorescent proteins?

- Aequorin binds Ca2+ and emits blue chemiluminescence.
- energy is transferred to GFP which fluoresces in green



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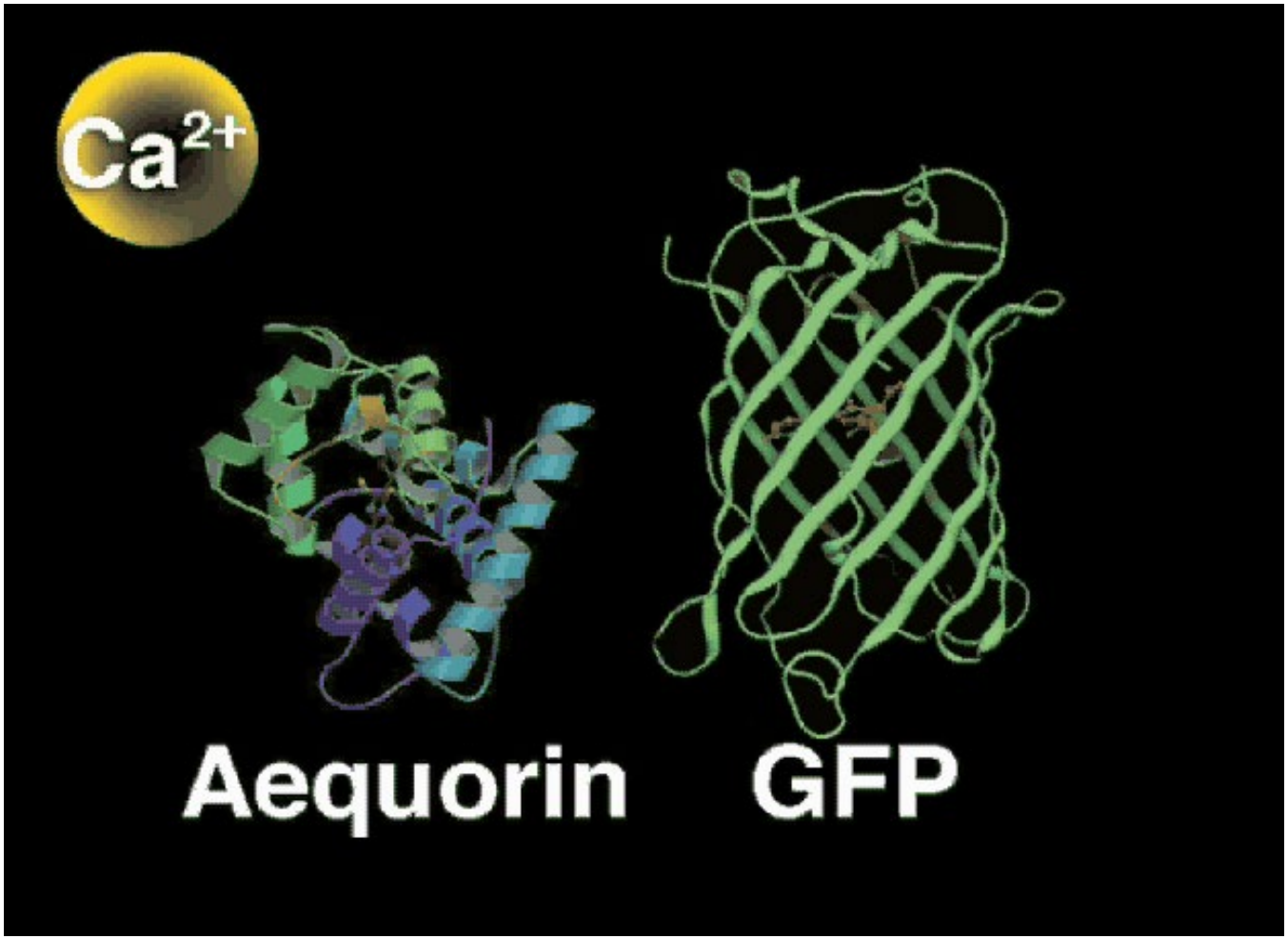
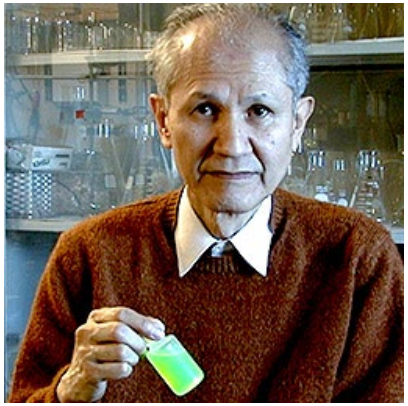


QUIZ TIME!

Please open kahoot!

Discovery and development of fluorescent proteins

- Osamu Shimomura first noticed green fluorescent protein in 1962



- Douglas Prasher cloned the GFP gene in 1992 but didn't get to test it.

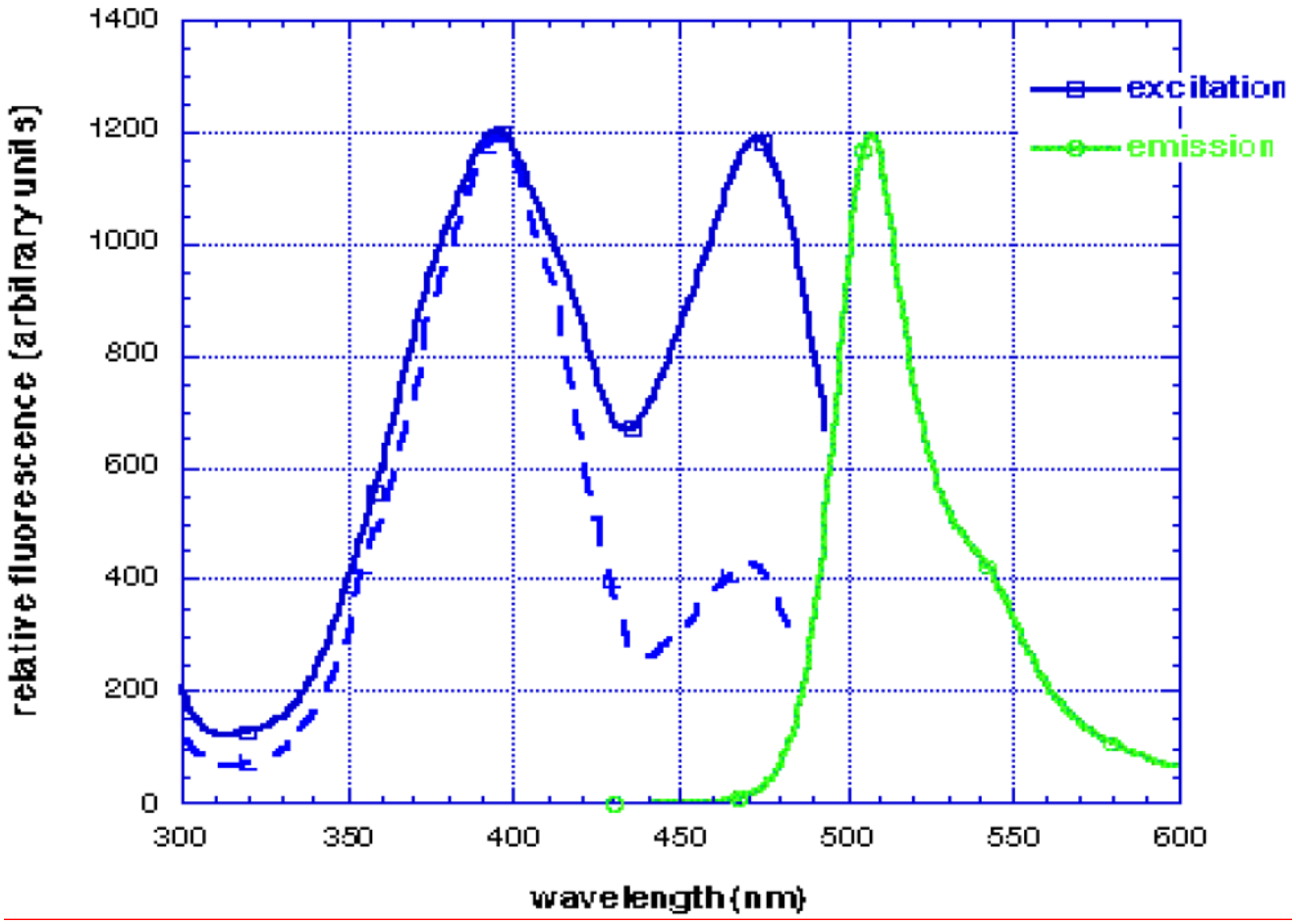
- Martin Chalfie expressed the gene in bacteria in 1994. It worked!

- Tulle Hazelrigg was among first to express GFP as a fusion protein

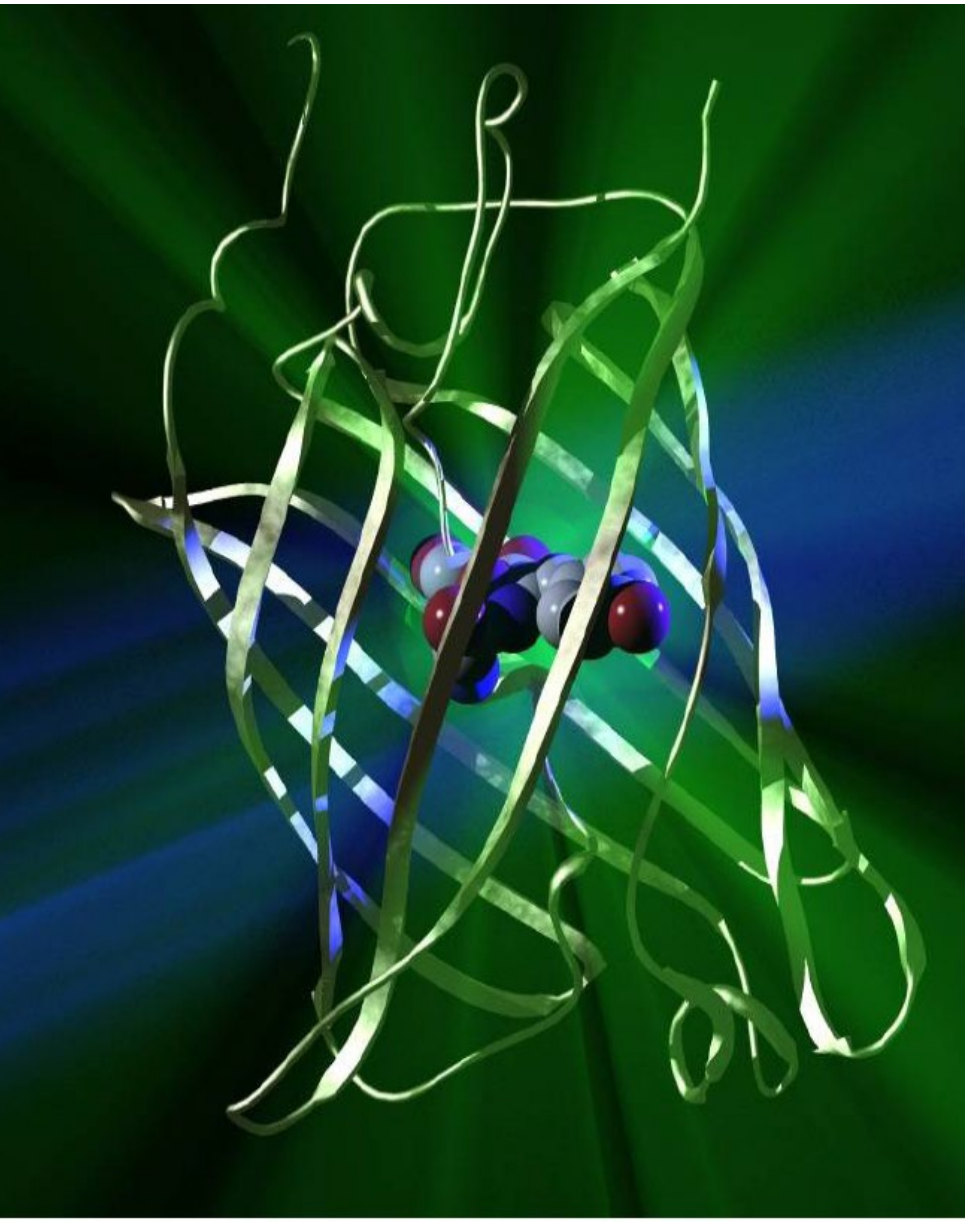
- Roger Tsien mutated GFP and obtained variants that excite at different wavelengths, emit different colors, or fold more efficiently at high temperature.

Wild type GFP wtGFP

- **wtGFP is a 238 amino acid protein that fluoresces maximally when excited at 400 nm with a lesser peak at 475 nm, and a fluorescence emission peak at 509nm.**



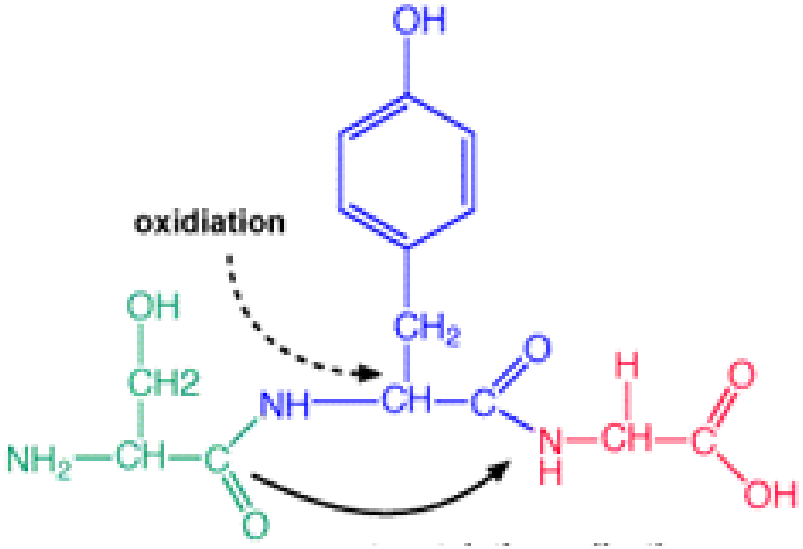
3D-Structure of GFP

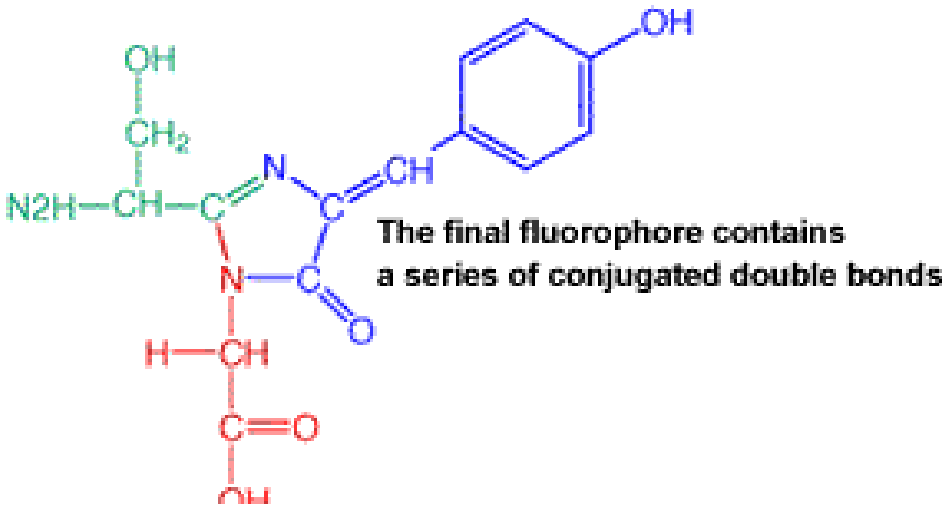


- “Paint in a can”
- Composed of 238 amino acids (27KDa).
- Each monomer composed of a central helix surrounded by an eleven stranded cylinder of anti-parallel beta-sheets (shields fluorophore from solvent)
- Cylinder has a diameter of about 30 Å and is about 40 Å long
- Fluorophore is located on the central helix
- Deprotonated phenolate of Tyr66 is the cause of fluorescence

Wild type GFP maturation

- Chromophore includes amino acids Ser65, Tyr66, and Gly67



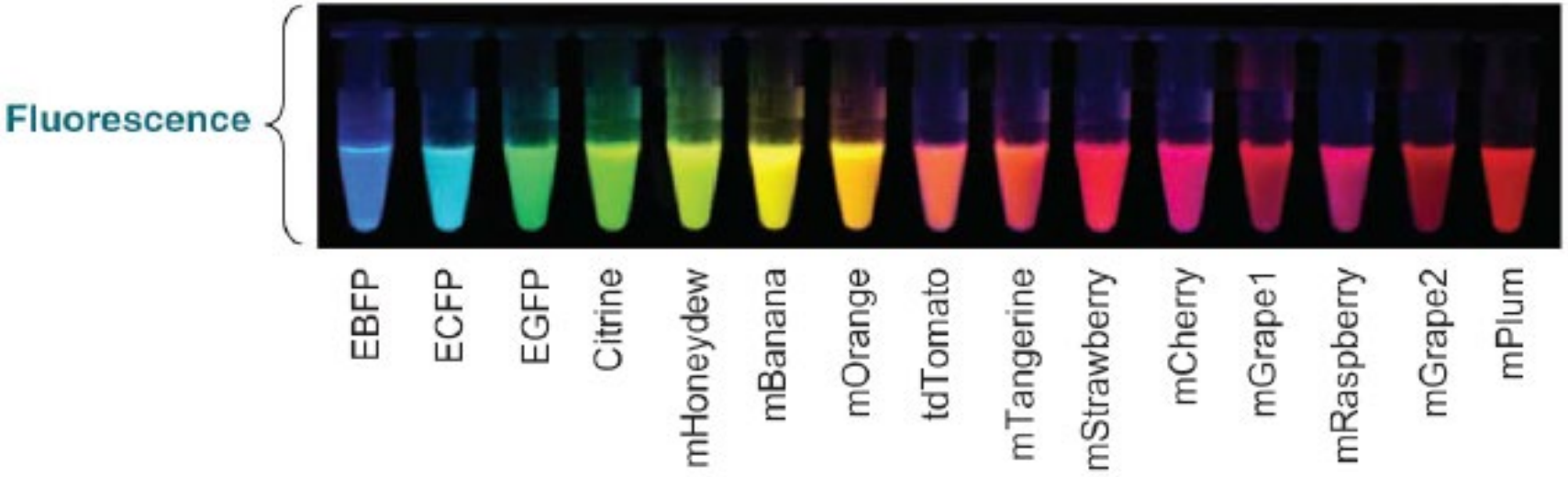


It is postulated that the protonated excited form of the fluorophore converts to the excited phenolate which is the only fluorescent species and emits light at 509 nm.

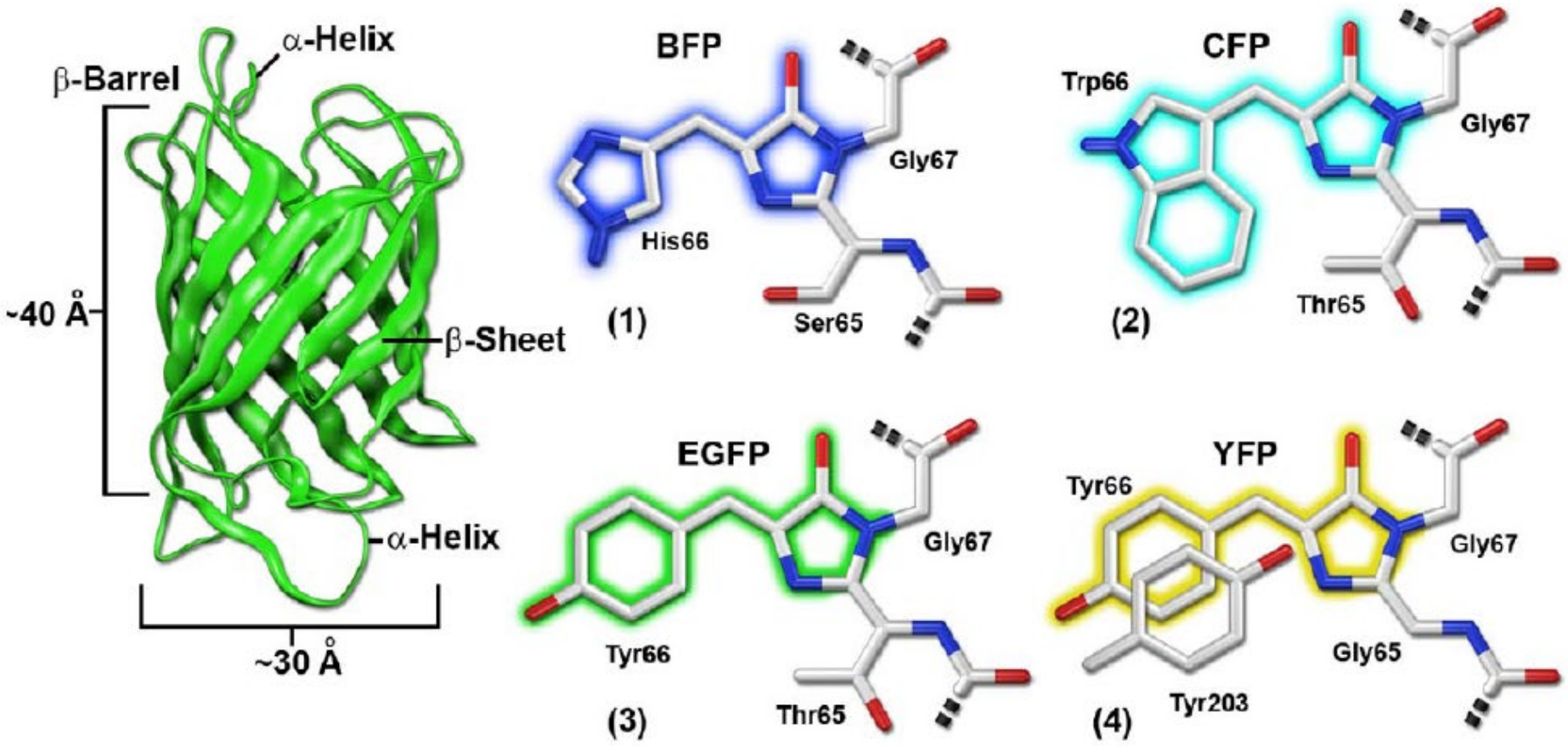
As a result, ***a cycle is formed, in which the flourophore absorbs a photon, then loses a proton, emits a photon and finally takes up a proton, returning to its original state.***

Wild type GFP

- Slow rate of fluorescence acquisition in vivo. Oxidation of fluoropore takes (2-4 hours).
- Relatively low level of fluorescence.
- Multiple excitation peaks.
- Poor expression in many types of organism.



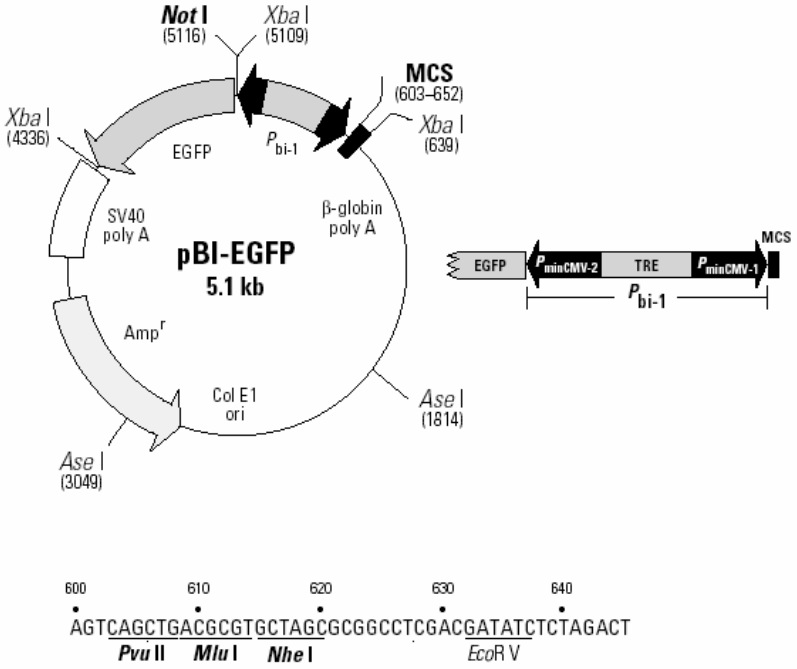
The green fluorescent protein family



Shaner et al., J. Cell Sci., 2007

Fluorescent protein variants

- Re-engineered GFP has preferred human (or plant etc.) codon usage.
- 20-fold enhancement of fluorescence because of 20-fold increase of GFP protein levels
- GFP spectral variants emit different colors and be used simultaneously to monitor independent events in cells.
- Some GFP mutants exhibit more rapid formation of the fluorophore.
- Clontech's Reef Coral Fluorescent Protein (RCFP) family

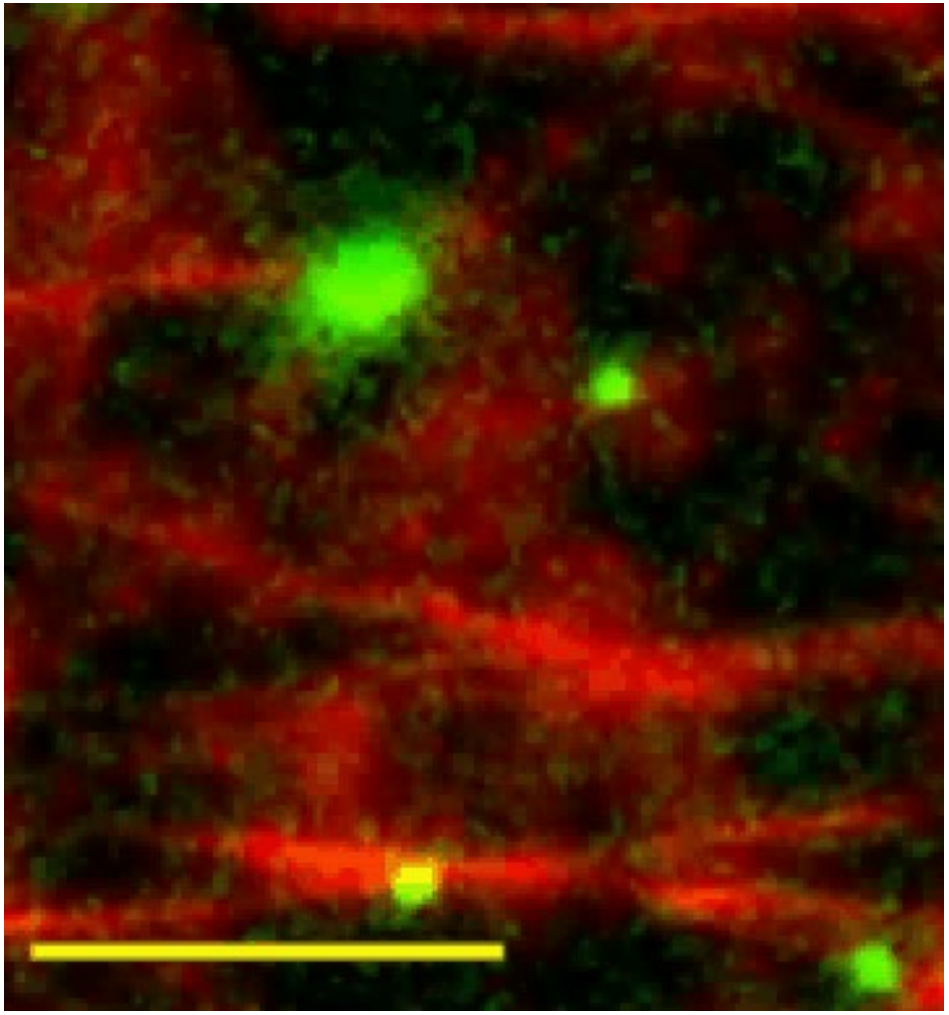


Vector	Size	Cat. No.
pAmCyan[a,b]	20 µg	632440
pAmCyan1-N1[c]	20 µg	632442
pAmCyan1-C1[c]	20 µg	632441
pZsGreen[a,b]	20 µg	632446
pZsGreen1-N1[c]	20 µg	632448
pZsGreen1-C1[c]	20 µg	632447
pZsYellow[a,b]	20 µg	632443
pZsYellow1-N1[c]	20 µg	632445
pZsYellow1-C1[c]	20 µg	632444
pAsRed2[a]	20 µg	632451
pAsRed2-N1[c]	20 µg	632449
pAsRed2-C1[c]	20 µg	632450

QUIZ TIME!

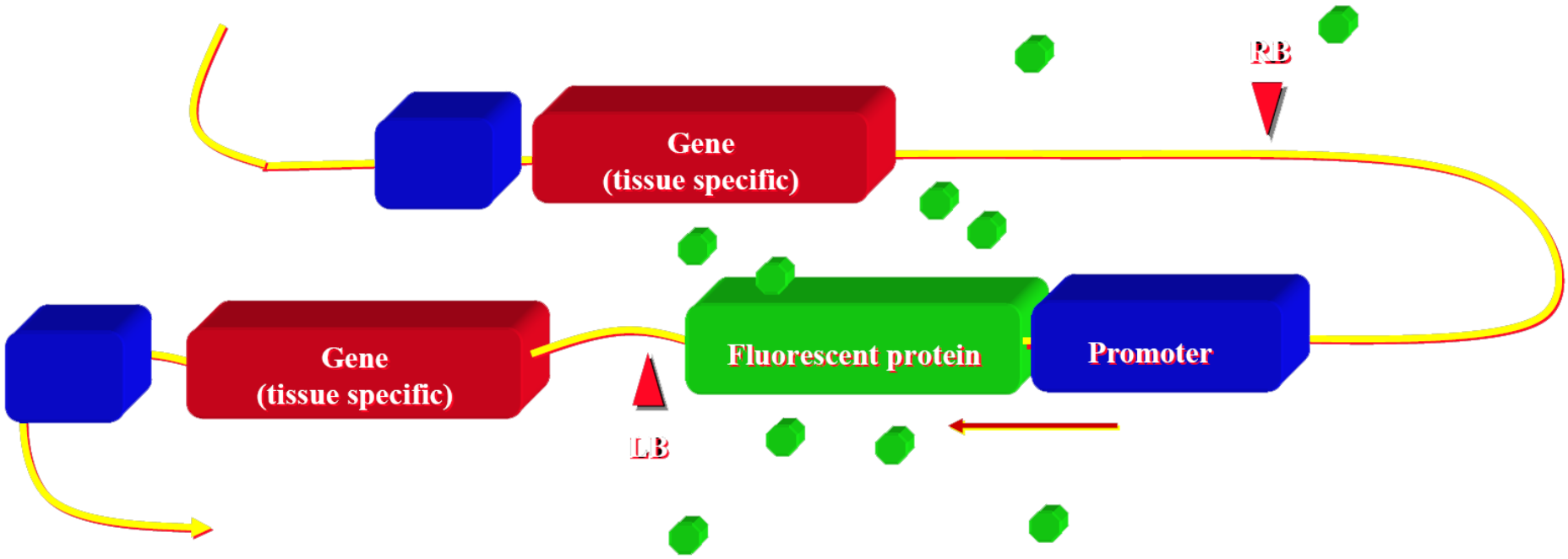
Please open kahoot!

How do we use fluorescent proteins?



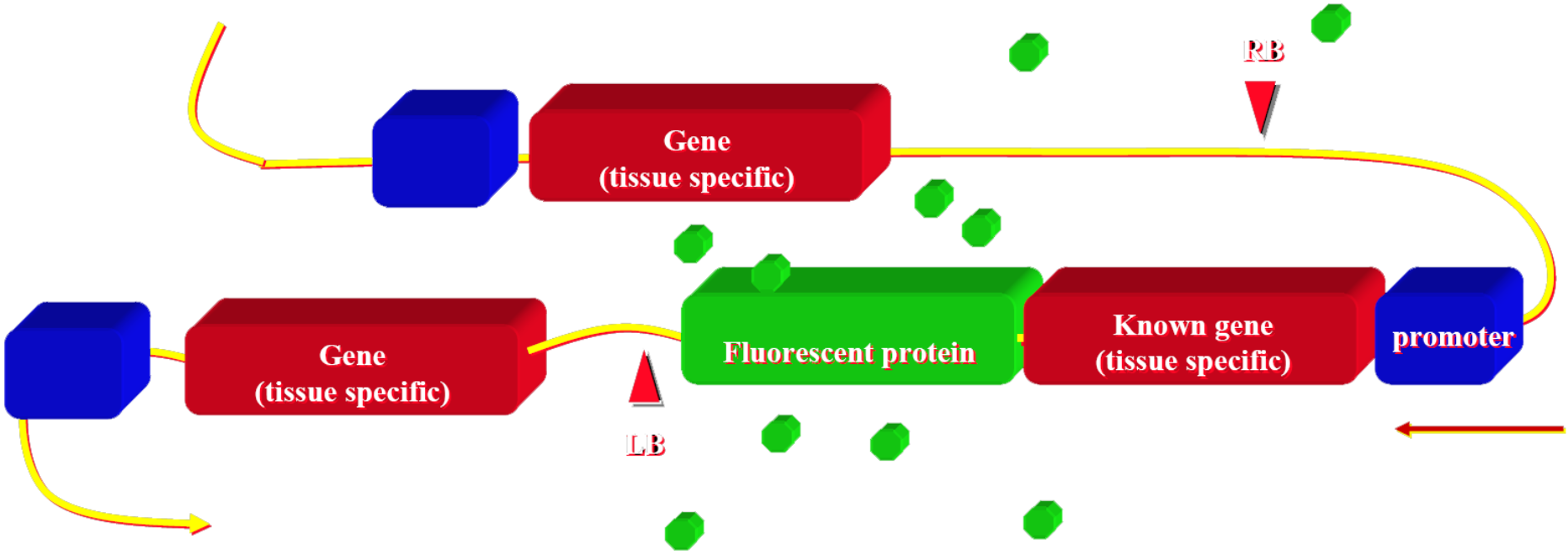
How do we use fluorescent proteins?

- Structural studies Genetic modification of an organism for fluorescent protein expression. Promotor can be constitutive or tissue-specific.



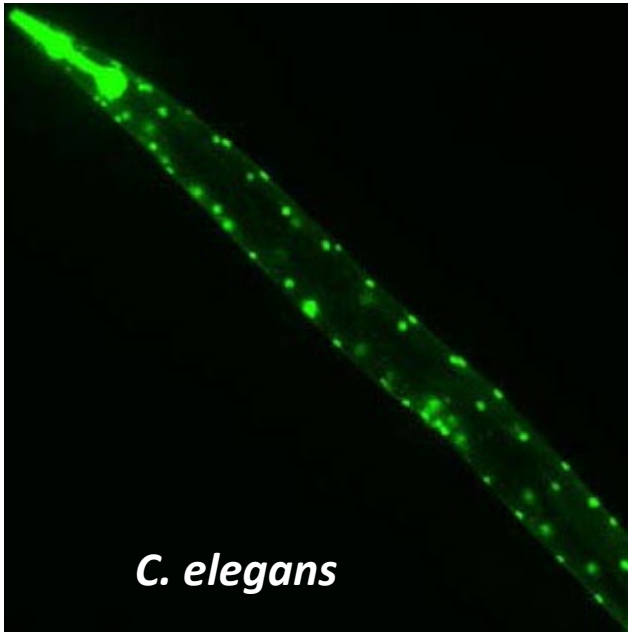
How do we use fluorescent proteins?

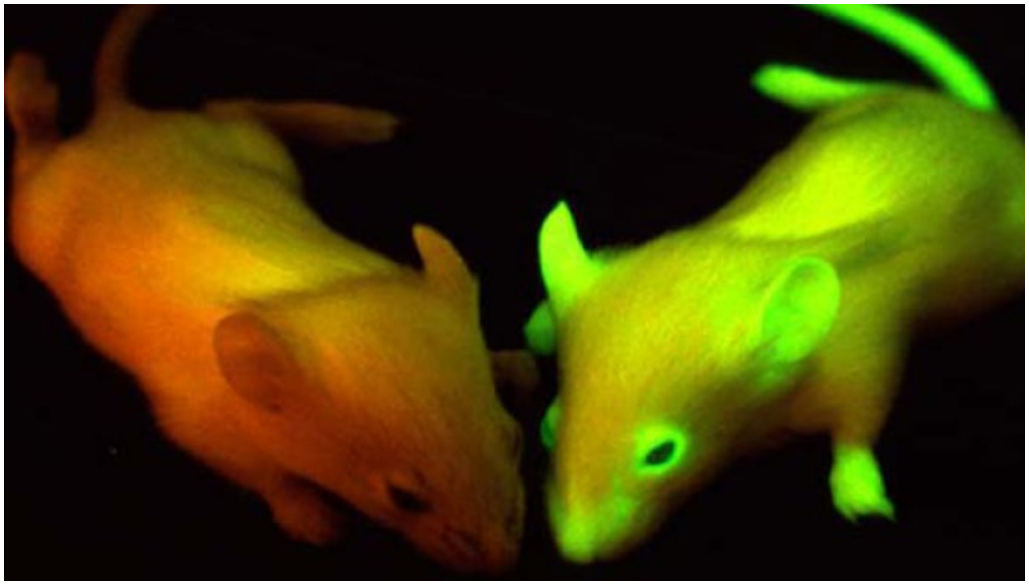
- Fluorescent protein gene can be fused to a known gene for sub-cellular targeting.

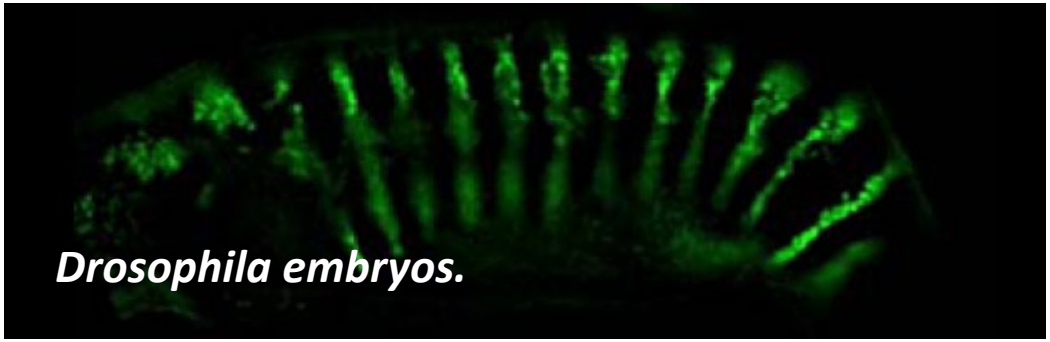


What organisms have been transformed?

bacteria, fungi, Dictyostelium, C. elegans, plants, Drosophila, mammals



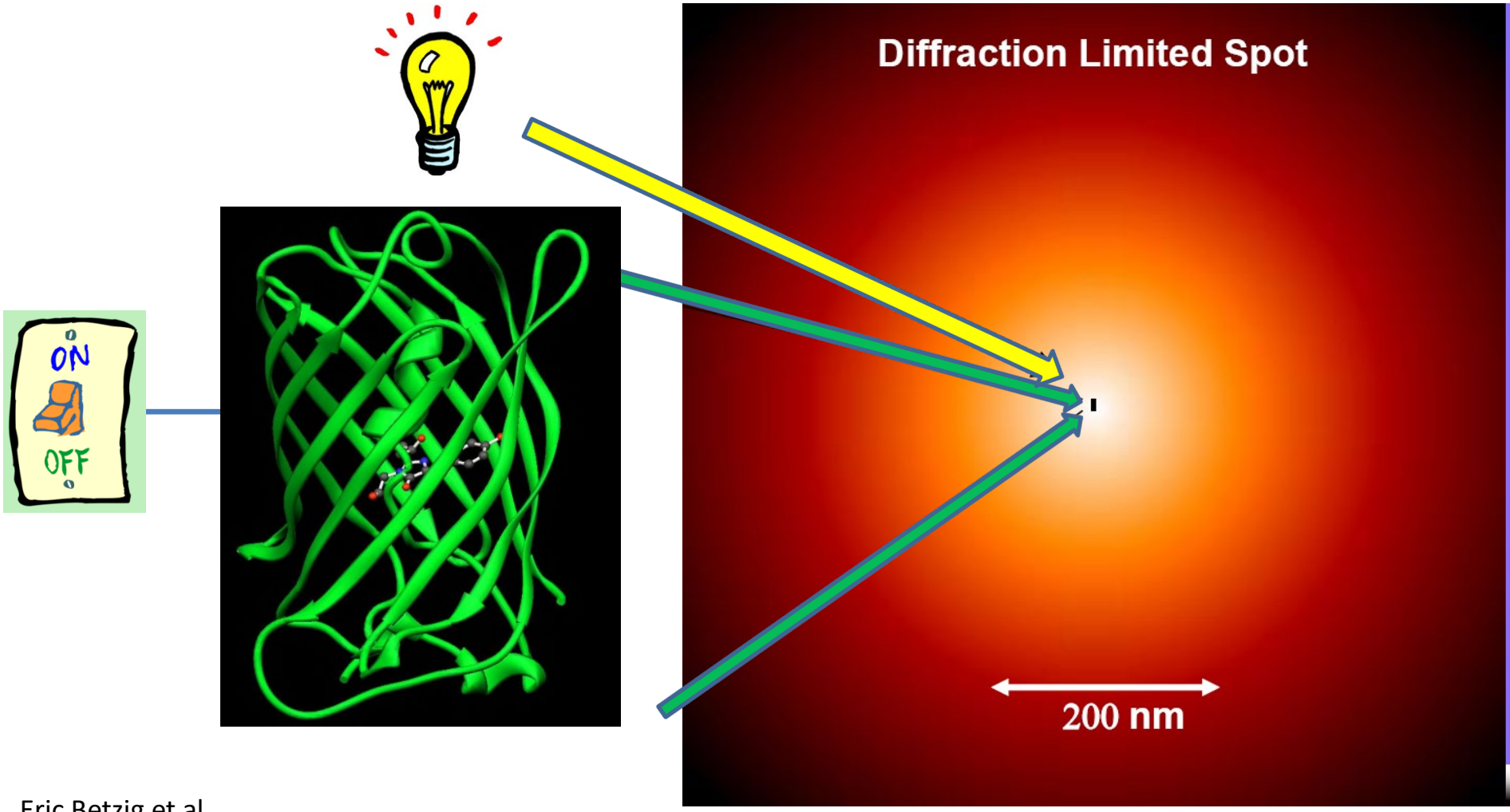




GloFish, the first pet sold with these proteins artificially present.

Photo-Activatable Green Fluorescent Proteins

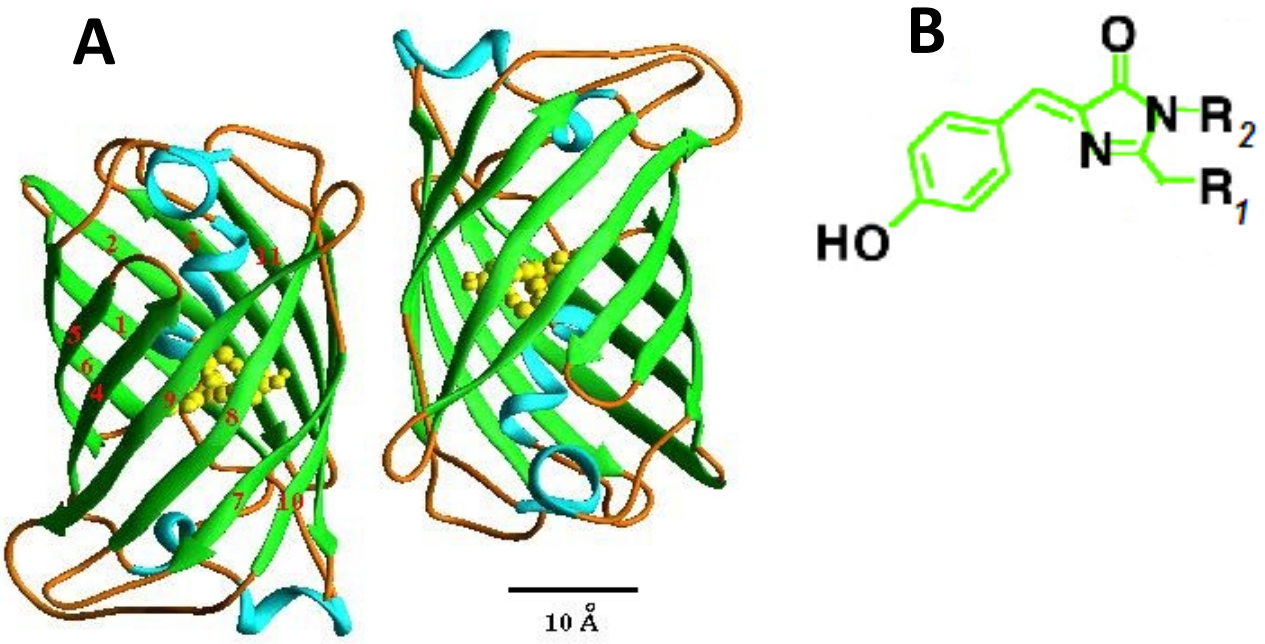
fluorescent "optical highlighters "



Eric Betzig et al

Photo-Activatable Green Fluorescent Protein (PA-GFP)

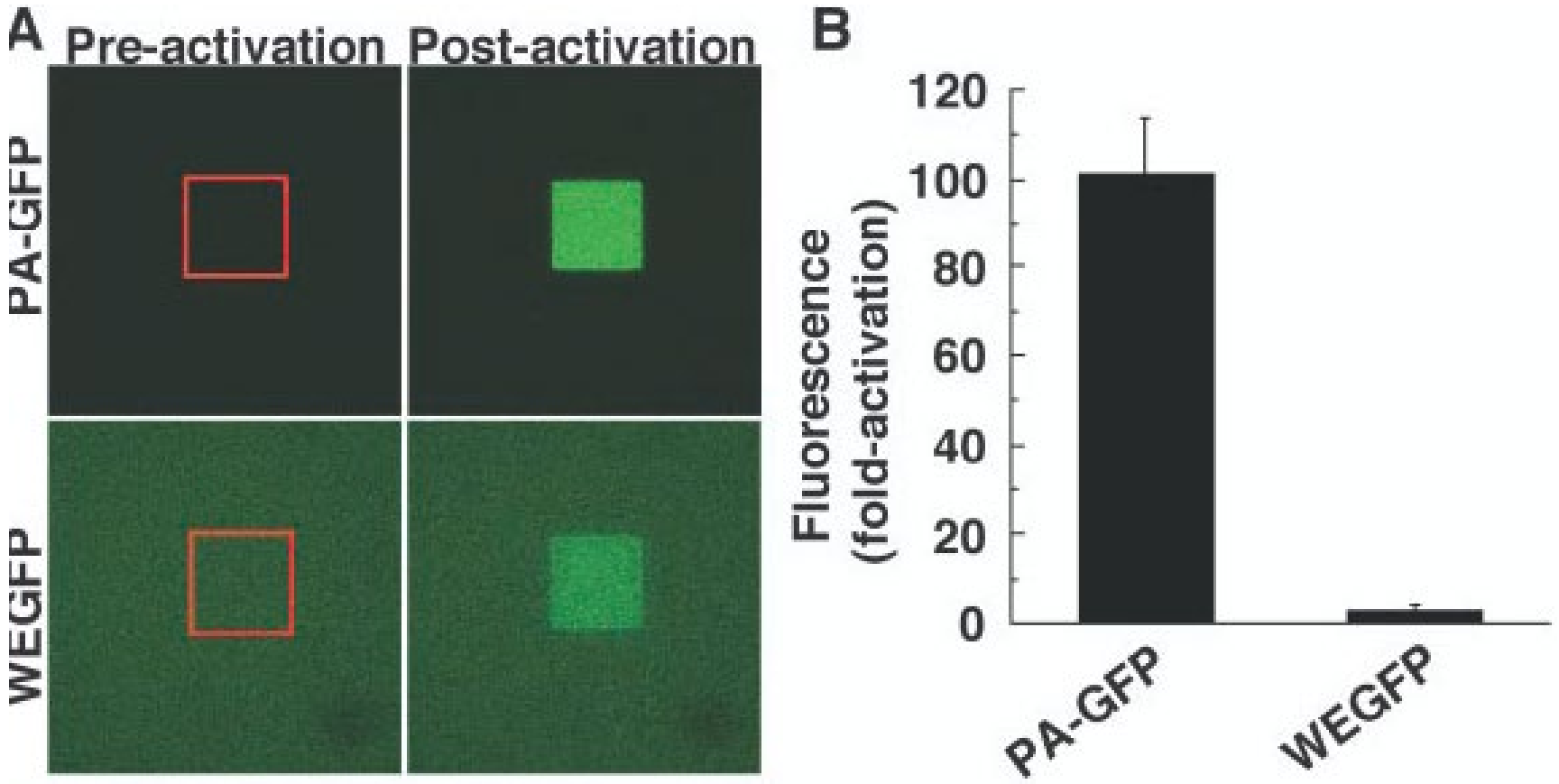
- The wild type of this protein is extracted from a Pacific Ocean jelly fish "*Aequorea Victoria*" The protein has 238 amino acids and the mutation at the amino acid site **203 Threonine with a Histidine (T203H)** is known as PA-GFP.



A) β barrel structure of GFP. Has a diameter of ~30Å and a length of ~40 Å. (B) chemical structure of the chromophore also shown at the center in fig 1. A (yellow structure).

G. H. Patterson et al., Science 297, 1873 (2002)

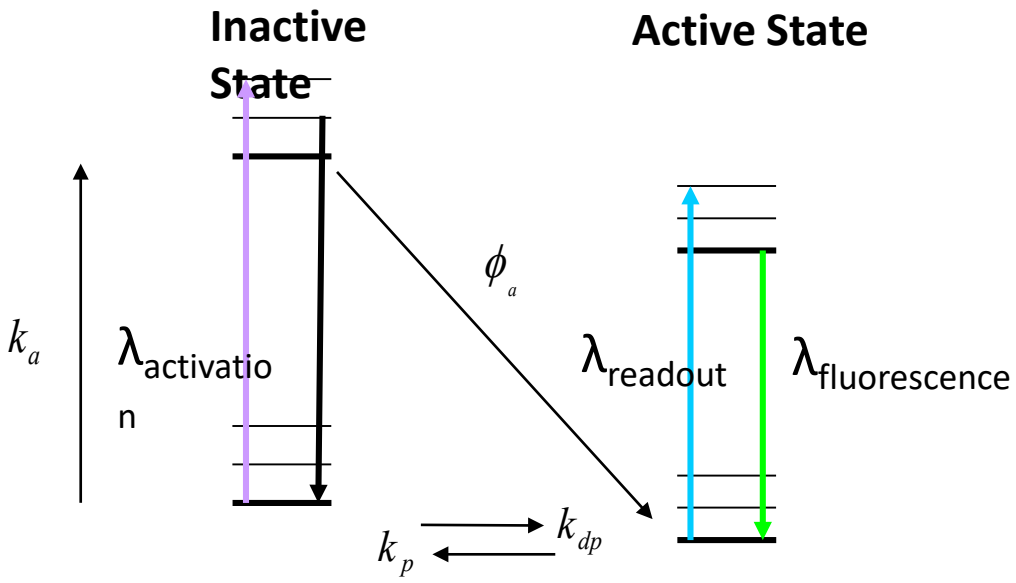
PA-GFP



G. H. Patterson et al., Science 297, 1873 (2002)

Photoconversion Scheme

- Common Photoconversion Scheme for PAGFP and wEosFP k_{dp} *and* k_p are the rate constants for ground state inter-conversions.



Can show that the functional dependence of the active type molecules upon activation time is given by,

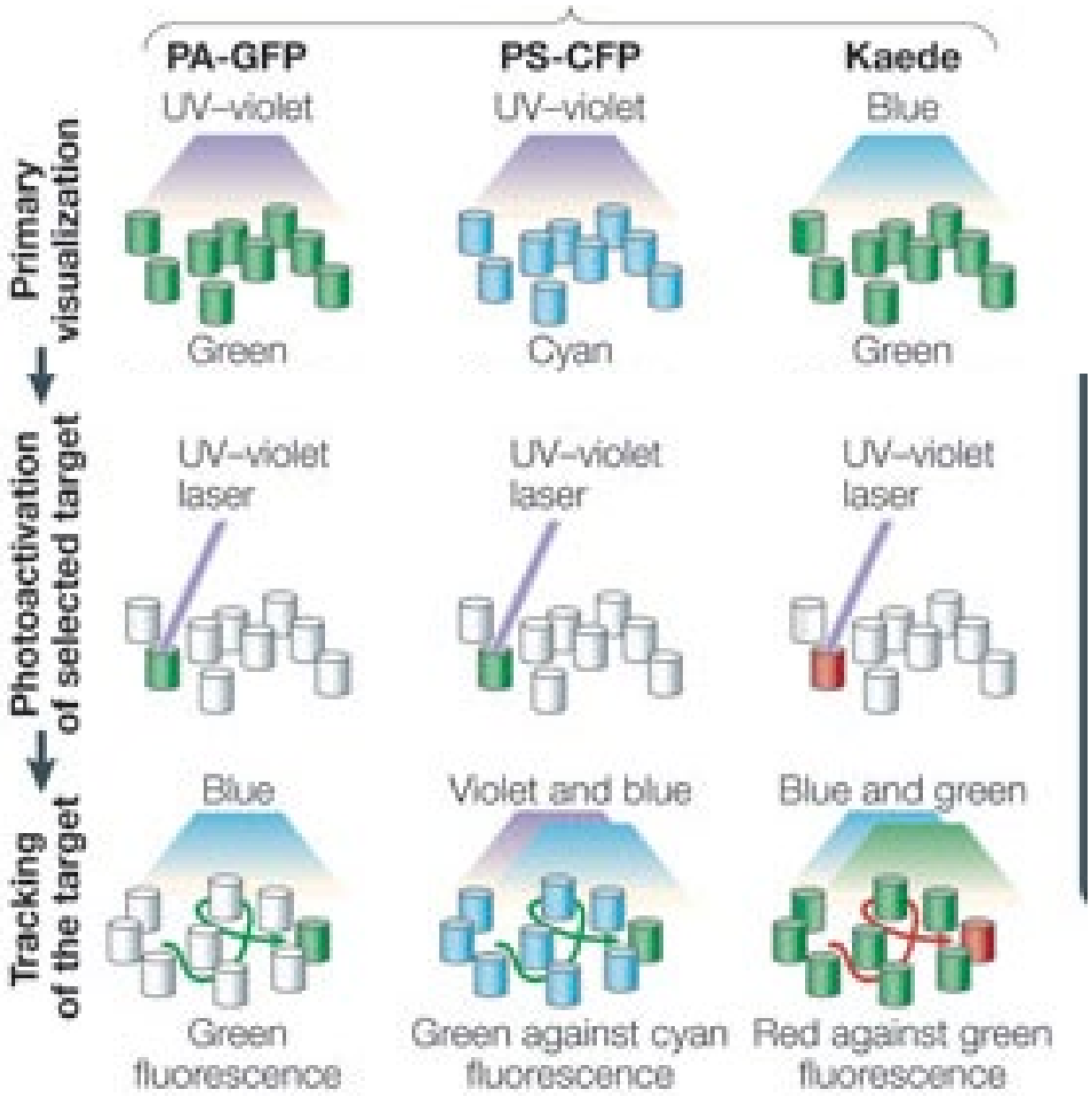
$$n_a(t) = n_{0a}e^{-k_b\phi_b t} + \frac{k_a\phi_a}{k_b\phi_b - k_a\phi_a} n_{0n}\left(e^{-k_a\phi_a t} - e^{-k_b\phi_b t}\right)$$

Where, k_a / k_b – Activation / Bleaching excitation rates per molecule.

Φ_a / Φ_b - Activation / Bleaching Yields.

n_a / n_n - Number of Active / Inactive type molecules, the 0 subscript denotes the initial numbers.

Irreversible Photoactivation / Photoswitching



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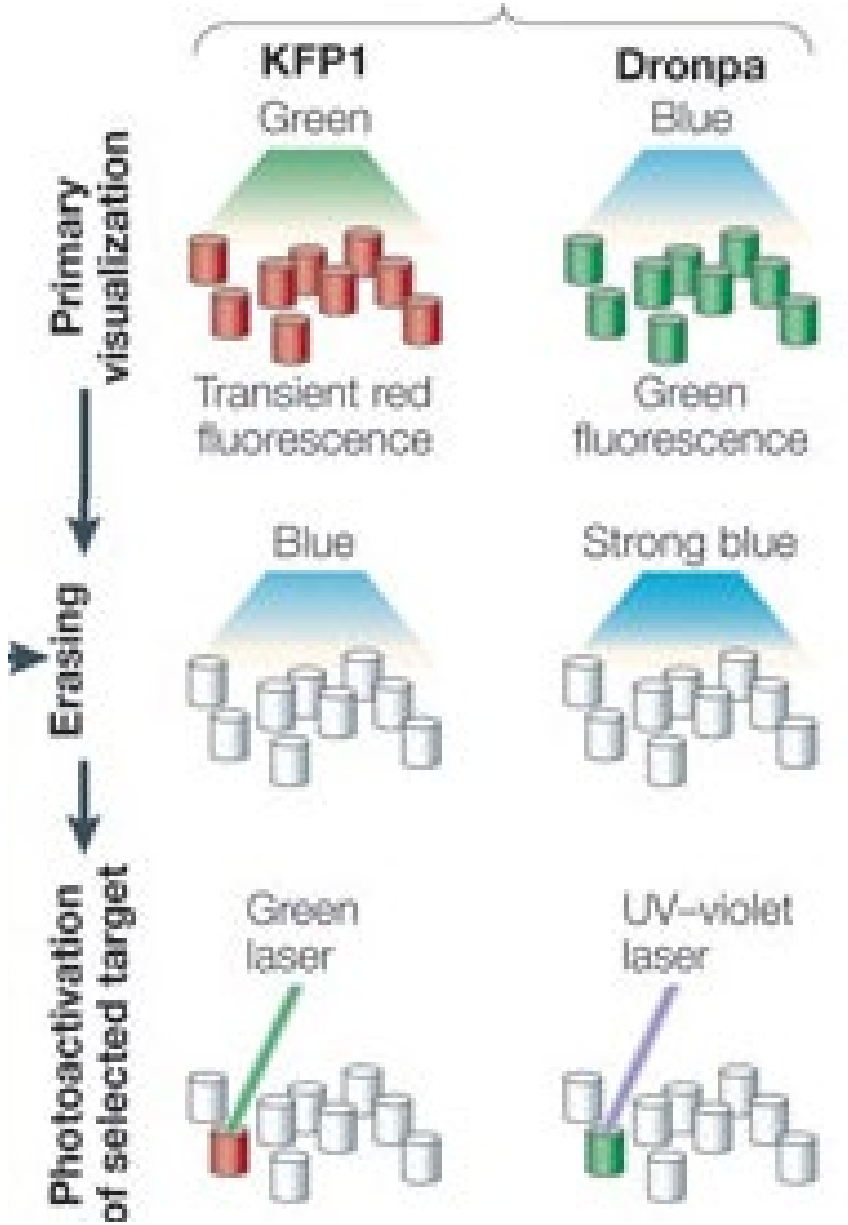
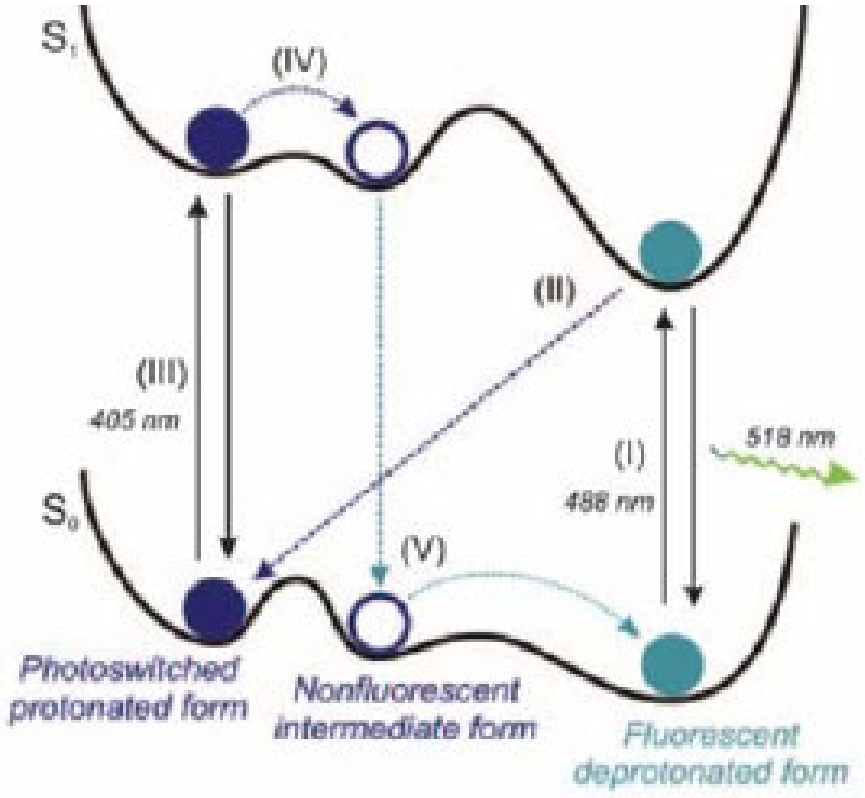
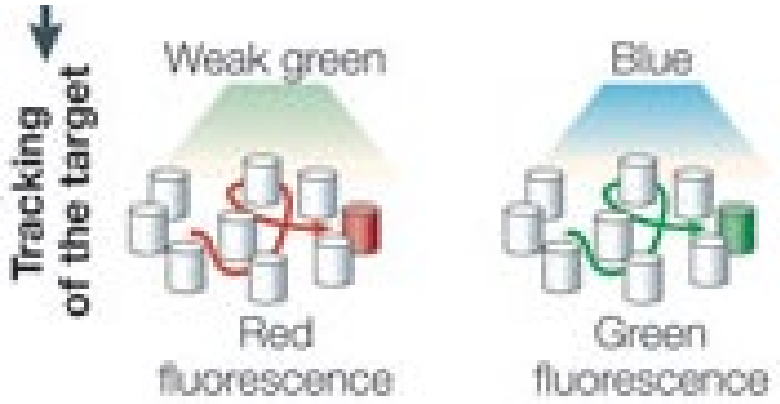
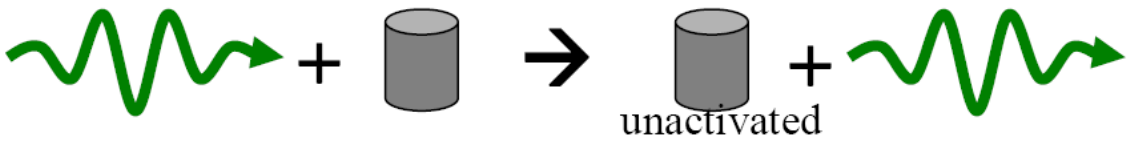
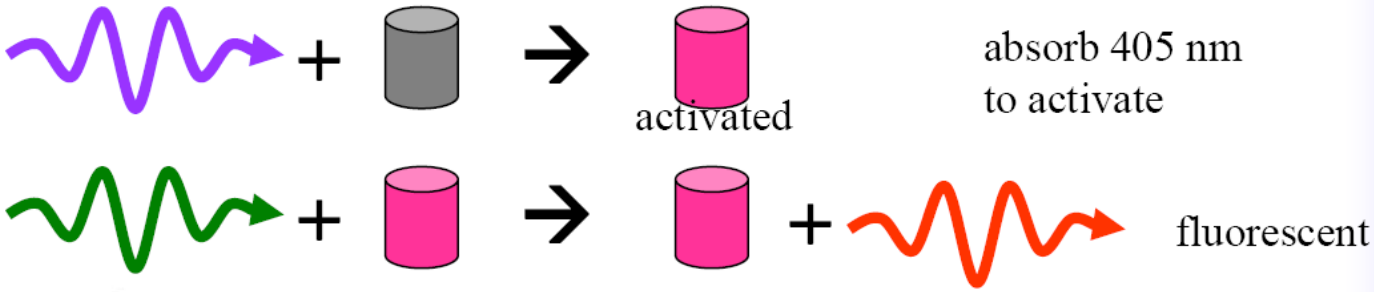
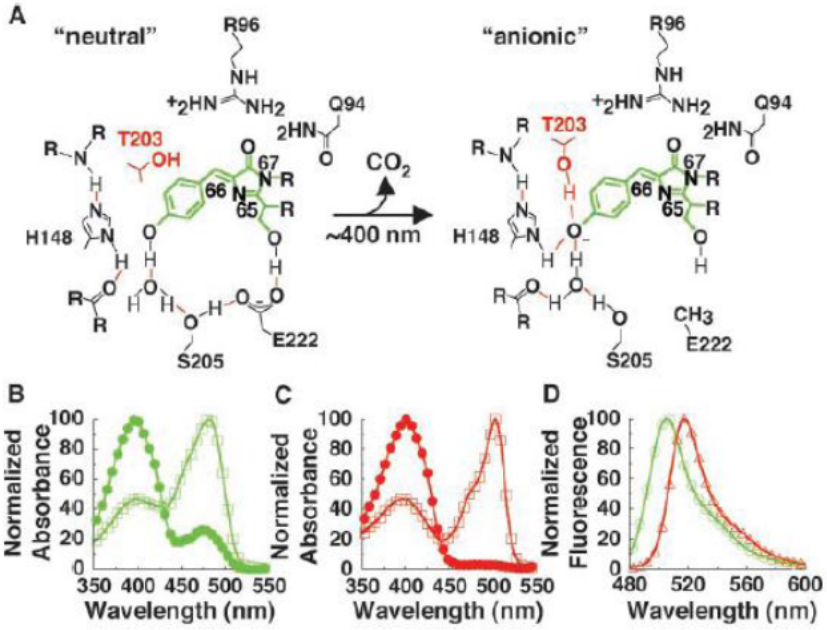







non-absorbing
unactivated


absorb 405 nm
to activate
activated
fluorescent


A
"neutral"
"anionic"
R96
Q94
T203
H148
S205
E222
CO2
~400 nm
B
C
D
Normalized Absorbance
Normalized Fluorescence
Wavelength (nm)

Comparison of the spectroscopic properties of selected photoactivatable fluorescent proteins (PFAPs)

PAFP properties	PA-GFP	PS-CFP	PS-CFP2	PAmRFP1-1	Kaede	mEosFP	KikGR	KFP1*	Dronpa
Oligomeric state	Monomer‡	Monomer‡	Monomer‡	Monomer‡	Tetramer§	Monomer‡	Tetramer§	Tetramer§	Monomer‡
Activating light	UV–violet§	UV–violet§	UV–violet§	UV–violet§	UV–violet§	UV–violet§	UV–violet§	Green‡	UV–violet§
Quenching light	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Blue, max at ~450 nm	Blue, max at ~490 nm
Change of absorbance spectrum (nm)	400 to 504	402 to 490	400 to 490	Increase at 578	508 to 572	505 to 569	507 to 583	Increase at 590	Increase at 503
Change of emission spectrum (nm)	Increase at 517	468 to 511	470 to 511	Increase at 605	518 to 580	516 to 581	517 to 593	Increase at 600	Increase at 518
Reversibility of photoactivation	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	Reversible and irreversible‡	Reversible‡
Increase in fluorescence intensity (fold)	100	300‡	>400‡	70	800‡	ND	ND	70 or 35	ND
Fluorescence contrast (fold)	~200	1,500‡	>2,000‡	N/A	2,000‡	ND	>2,000‡	N/A	N/A
Before activation:QY	0.13	0.16	0.2	ND	0.88	0.64	0.7	<0.001	ND
Before activation: EC	20,700 at 400 nm	34,000 at 402 nm	43,000 at 400 nm	ND	98,800 at 508 nm	67,200 at 505 nm	28,200 at 507 nm	123,000 at 590 nm	ND
Before activation: pK_a	4.5‡	4.0‡	4.3‡	ND	5.6	ND	7.8	ND	ND
Before activation: brightness‖	0.08§	0.17§	0.26	ND	2.64‡	1.3‡	0.60‡	<0.004	ND
After activation: QY	0.79	0.19	0.23	0.08	0.33	0.62	0.65	0.07	0.85
After activation: EC	17,400 at 504 nm	27,000 at 490 nm	47,000 at 490 nm	10,000 at 578 nm	60,400 at 572 nm	37,000 at 569 nm	32,600 at 583 nm	59,000 at 590 nm	95,000 at 503 nm
After activation: pK_a	ND	6.0	6.1	4.4‡	5.6‡	ND	5.5‡	ND	5.0‡
After activation: brightness‖	0.42	0.16§	0.33	0.03§	0.60‡	0.70‡	0.64‡	0.13§	2.45‡
Source organism (class)	*Aequorea victoria* (hydrozoa)	*Aequorea coerulescens* (hydrozoa)	*Aequorea coerulescens* (hydrozoa)	*Discosoma* spp. (anthozoa)	*Trachyphyllia geoffroyi* (anthozoa)	*Lobophyllia hemprichii* (anthozoa)	*Favia favus* (anthozoa)	*Anemonia sulcata* (anthozoa)	*Pectinlidae* spp. (anthozoa)
Reference	8	9	–	10	22	12	23	25	11
Commercially available	No	No	Yes, Evrogen	No	Yes, MBL Intl	No	Yes, MBL Intl	Yes, Evrogen	Yes, MBL Intl

Few recent Photo-Activatable Proteins

Photoactivatable mCherry for high-resolution two-color fluorescence microscopy

Fedor V Subach[1,3], George H Patterson[2,3], Suliana Manley[2], Jennifer M Gillette[2], Jennifer Lippincott-Schwartz[2] & Vladislav V Verkhusha[1]

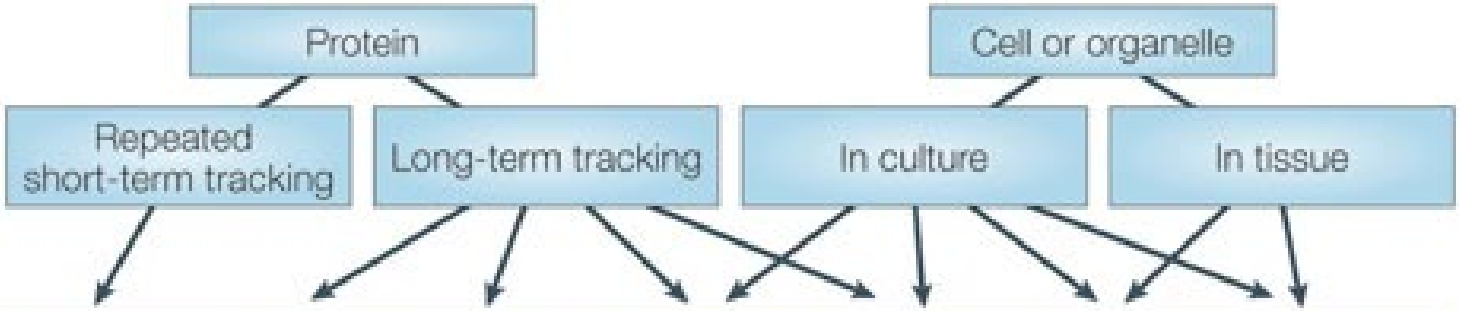
Photoswitchable fluorescent proteins enable monochromatic multilabel imaging and dual color fluorescence nanoscopy

Martin Andresen[1], Andre C Stiel[1], Jonas Fölling[1], Dirk Wenzel[2], Andreas Schönle[1], Alexander Egner[1], Christian Eggeling[1], Stefan W Hell[1] & Stefan Jakobs[1]

Super-resolution imaging in live *Caulobacter crescentus* cells using photoswitchable EYFP

Julie S Biteen[1], Michael A Thompson[1], Nicole K Tselentis[1], Grant R Bowman[2], Lucy Shapiro[2] & W E Moerner[1]

Photo-Activatable Proteins



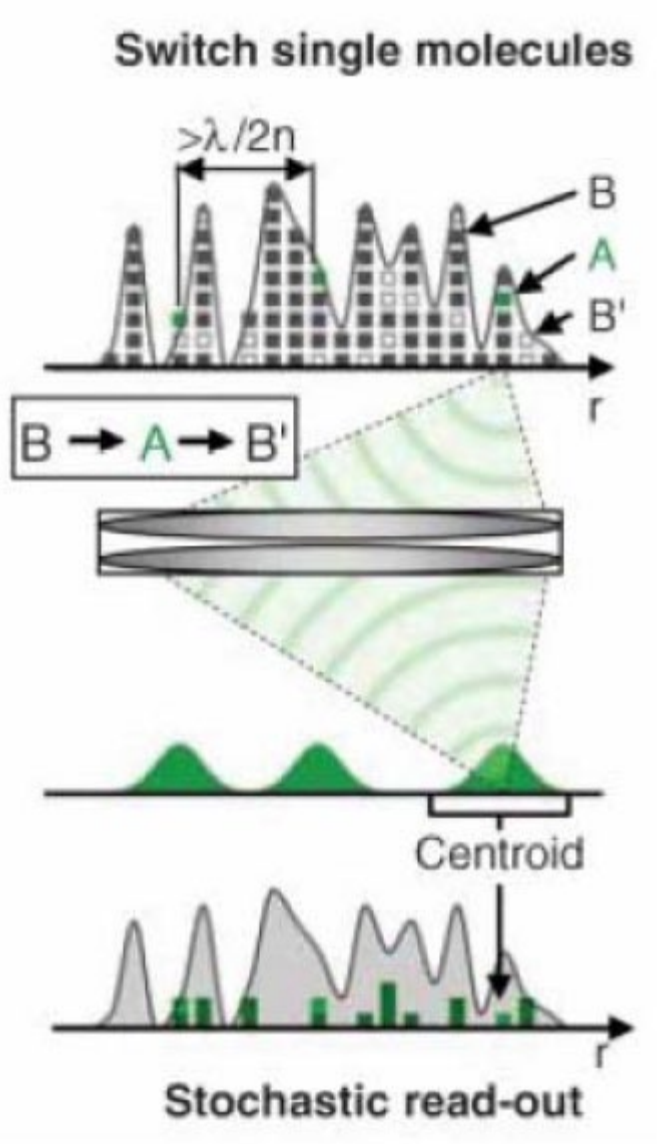
	Dronpa	PAmRFP1	mEosFP	PA-GFP	PS-CFP2	Kaede	KFP1
	Monomer	Monomer	Monomer	Monomer	Monomer	Tetramer	Tetramer
	Reversible	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	(Ir)reversible
Fluorescence changes during photoactivation							
High brightness	✓			✓		✓	
High contrast			✓		✓	✓	
Dual labelling with red and green fluorescent proteins	✓	✓		✓	✓		✓
Low phototoxicity of the activation light							✓

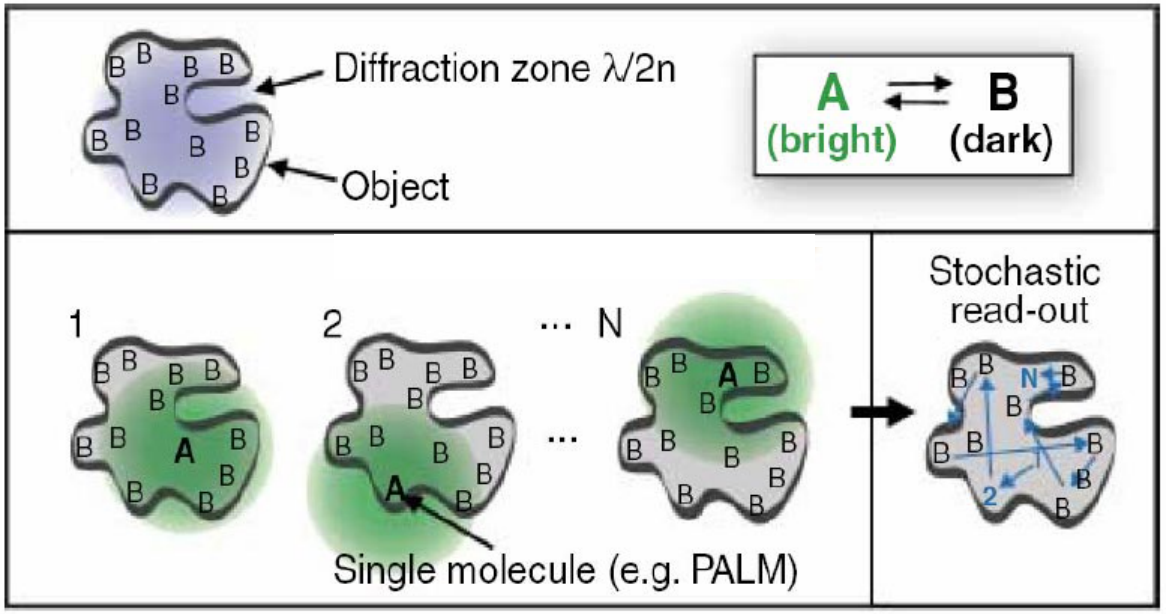
Nature Reviews | Molecular Cell Biology

QUIZ TIME!

Please open kahoot!

PhotoActivated Localization Microscopy (PALM) principle

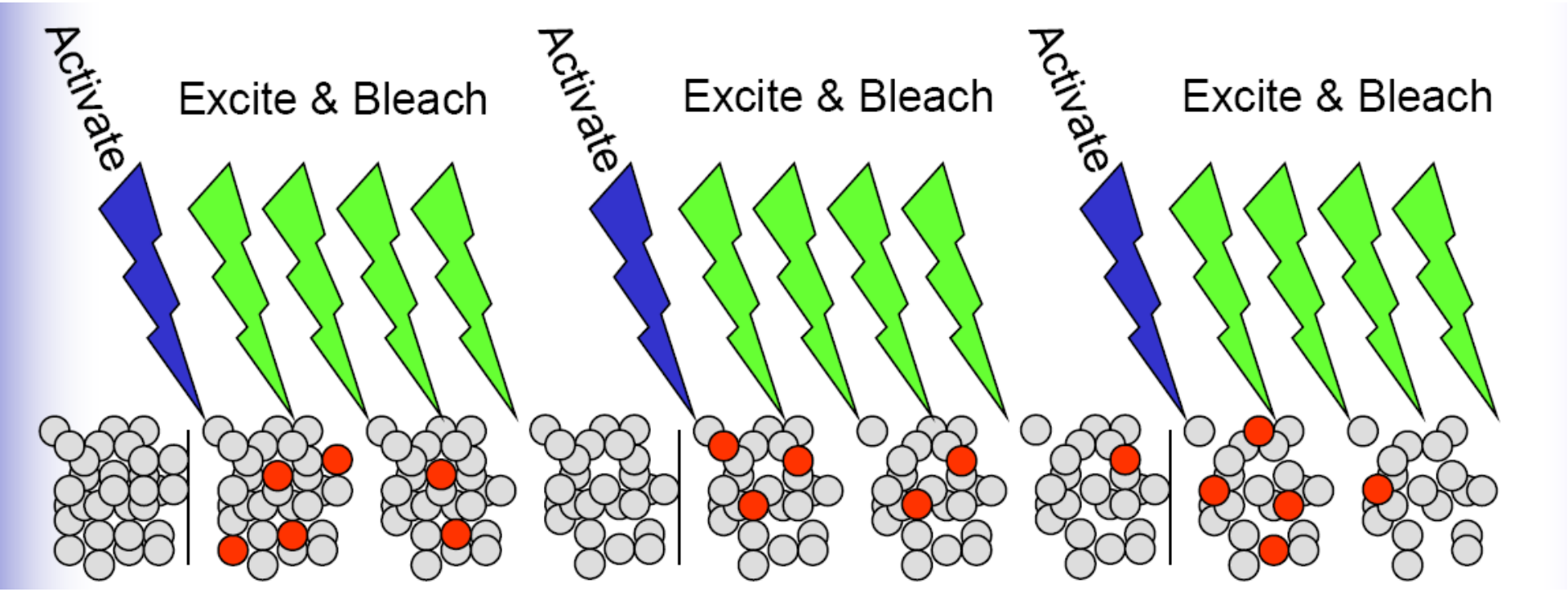




Eric Betzig et al

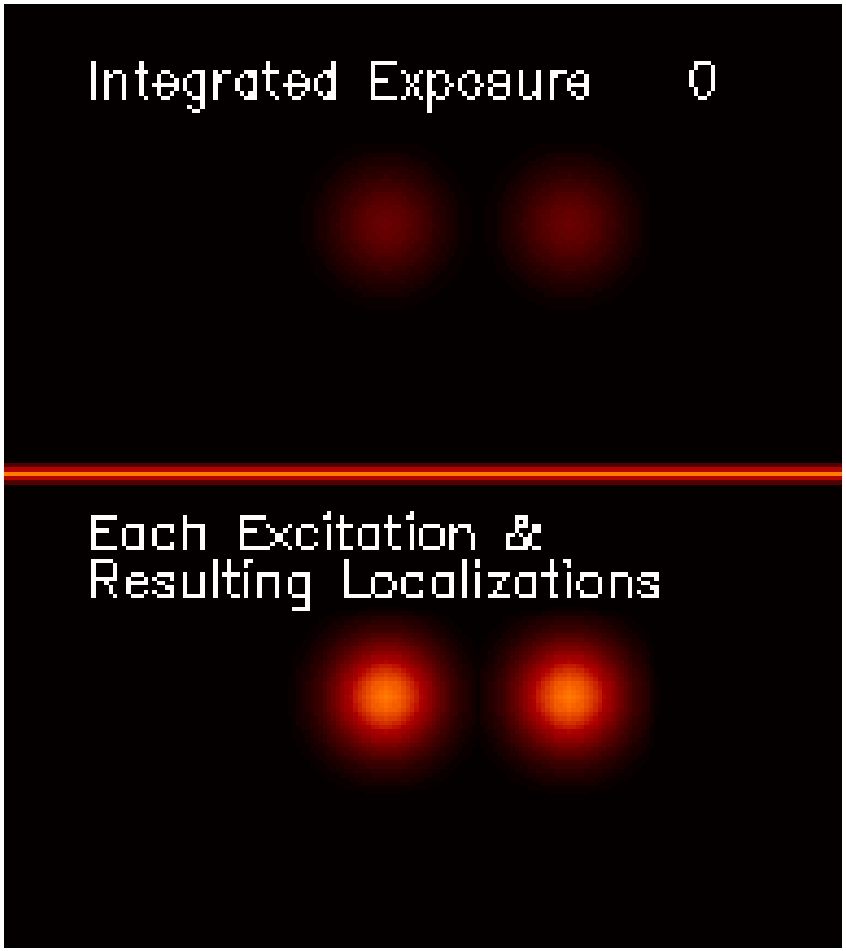
PALM principle

- 1. Establish Sparse Subset with Fractional Activation



- 2. Localize:
  - Fit Point Spread Function *s ~ 200 nm*
  - Center Location w Error σ~ *seN ~ 10 nm*

PhotoActivated Localization Microscopy (PALM) principle



A small fraction of a sample's fluorescent proteins are put into an "on" state, where they glow red when illuminated with yellow light. At the highest optical magnification each molecule looks like a fuzzy ball about 250 nm in diameter. However, the center where the fluorescent label is located can be determined to a fraction of that size.

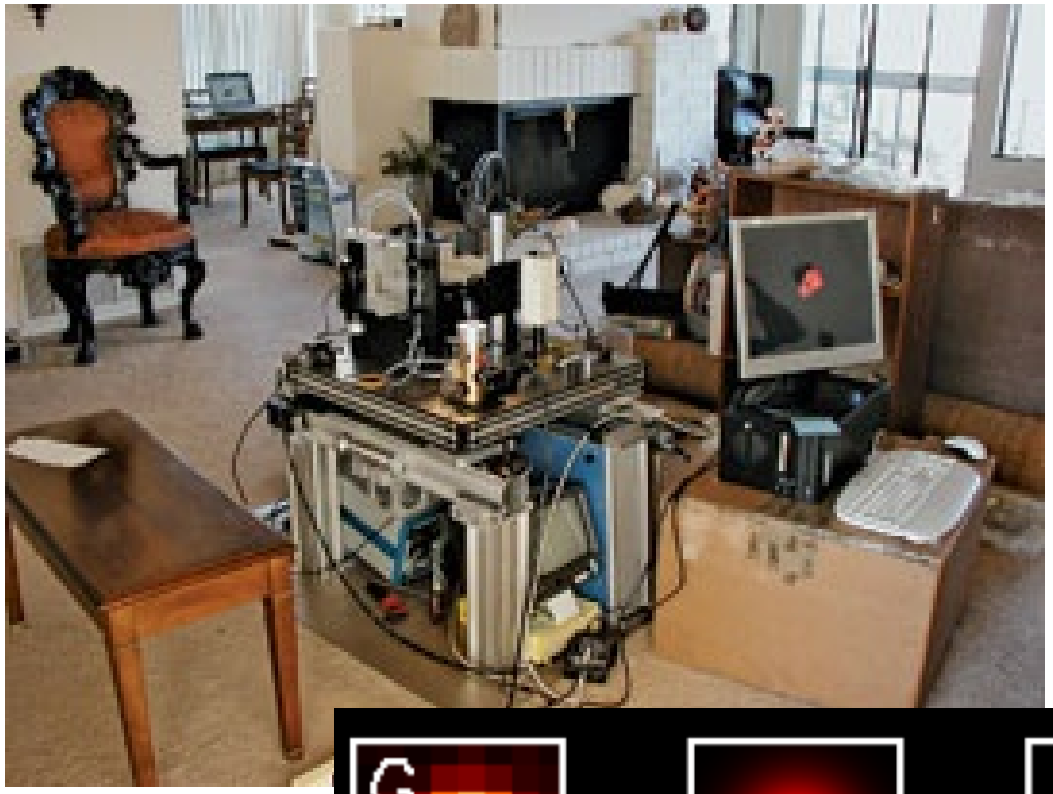
Next, a new sparse set of fluorescent proteins is turned on and the process iterates. In the top frame the accumulation of all the fuzzy balls forms the diffraction-limited image seen in a far-field microscope. The bottom frame shows the accumulation of the center spots, which builds a higher resolution PALM image

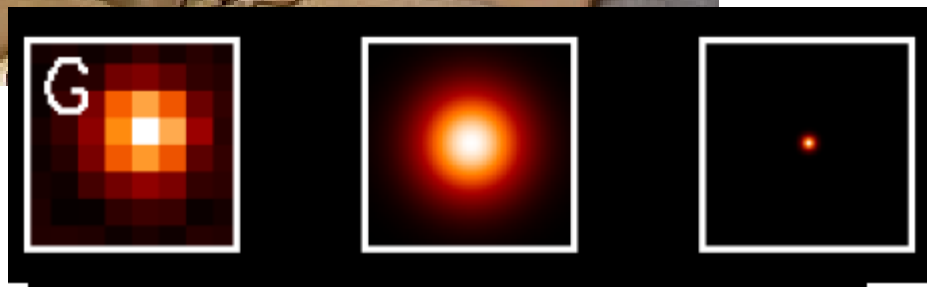
Harald Hess

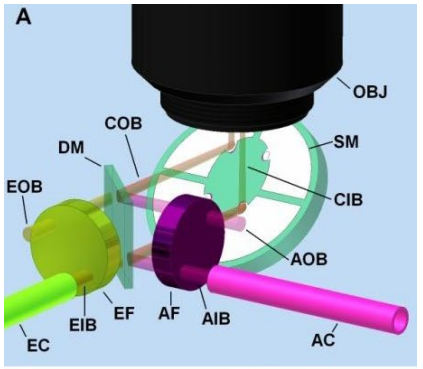
http://www.hhmi.org/research/fellows/figs/hess_fig.html

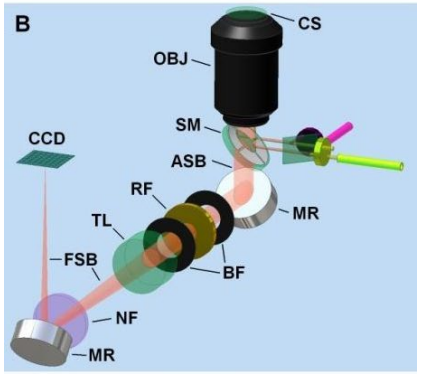
PALM: Photo-Activatable Localization Microscopy

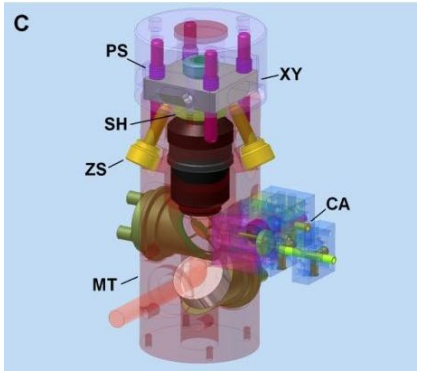
- The World's highest resolution (1-10 nm !!!) optical microscope was developed and built in a living room in Michigan. PALM: Photo-Activatable Localization Microscopy







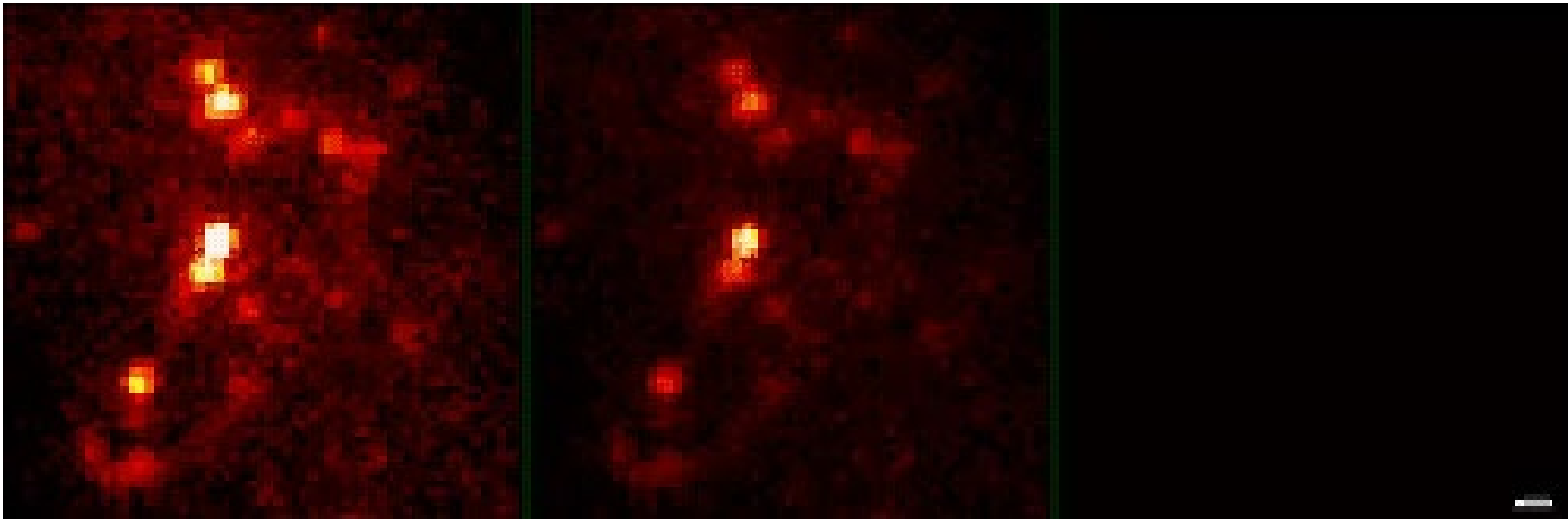




September 2006!

Betzig, E. *et al*. 313, 1642–1645 (2006)

Gradual assembly of a PALM image

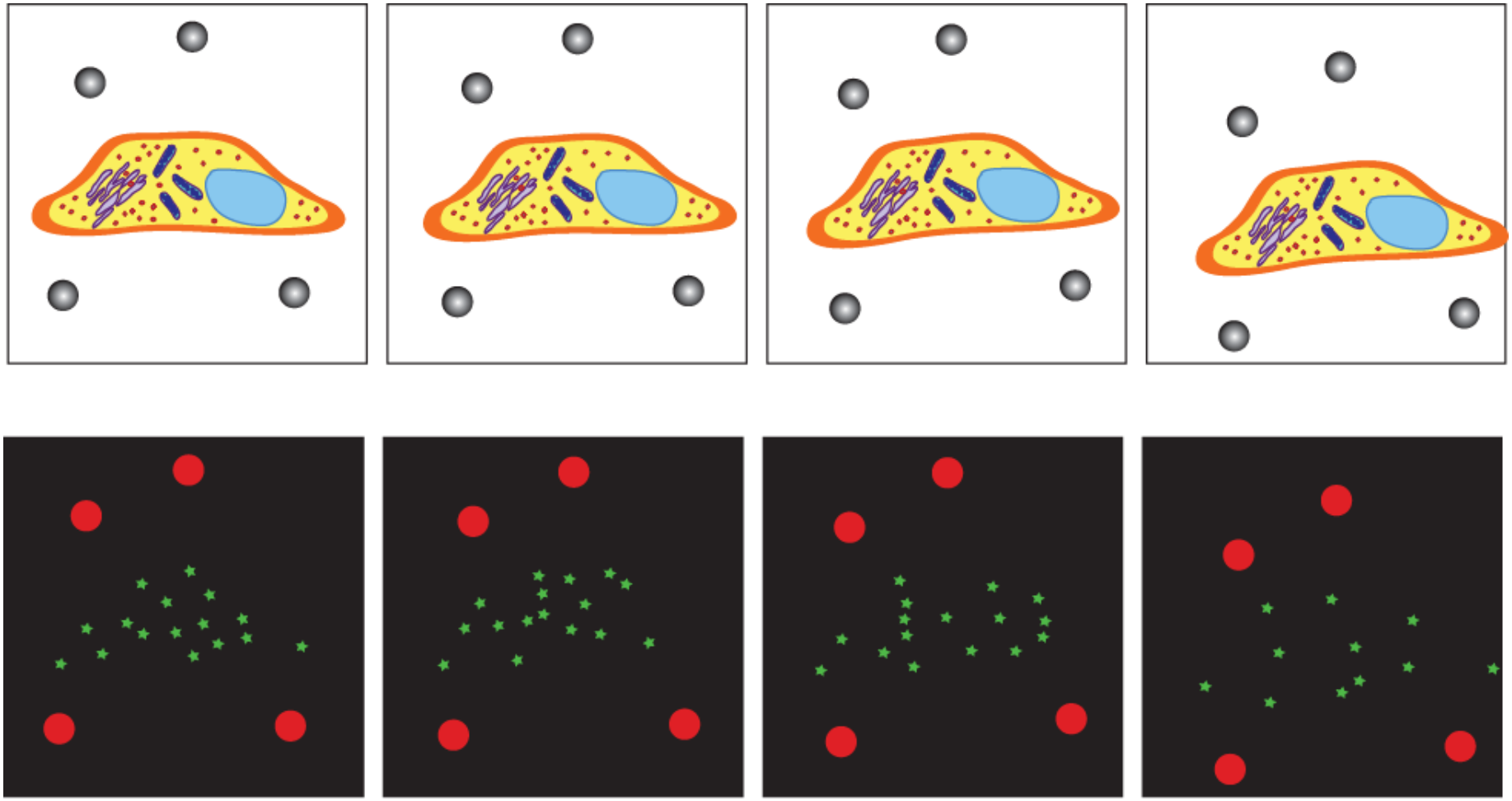


Gradual assembly of a photoactivated localization microscope (PALM) image (right) from the center coordinates of the images of many single molecules (flashing spots at left), activated and localized one-at-a-time. If the images are summed without localization, the conventional optical image is eventually recovered (center).

The process takes anywhere **from 2–12 hours** per sample and generates 10^4–10^5 images.

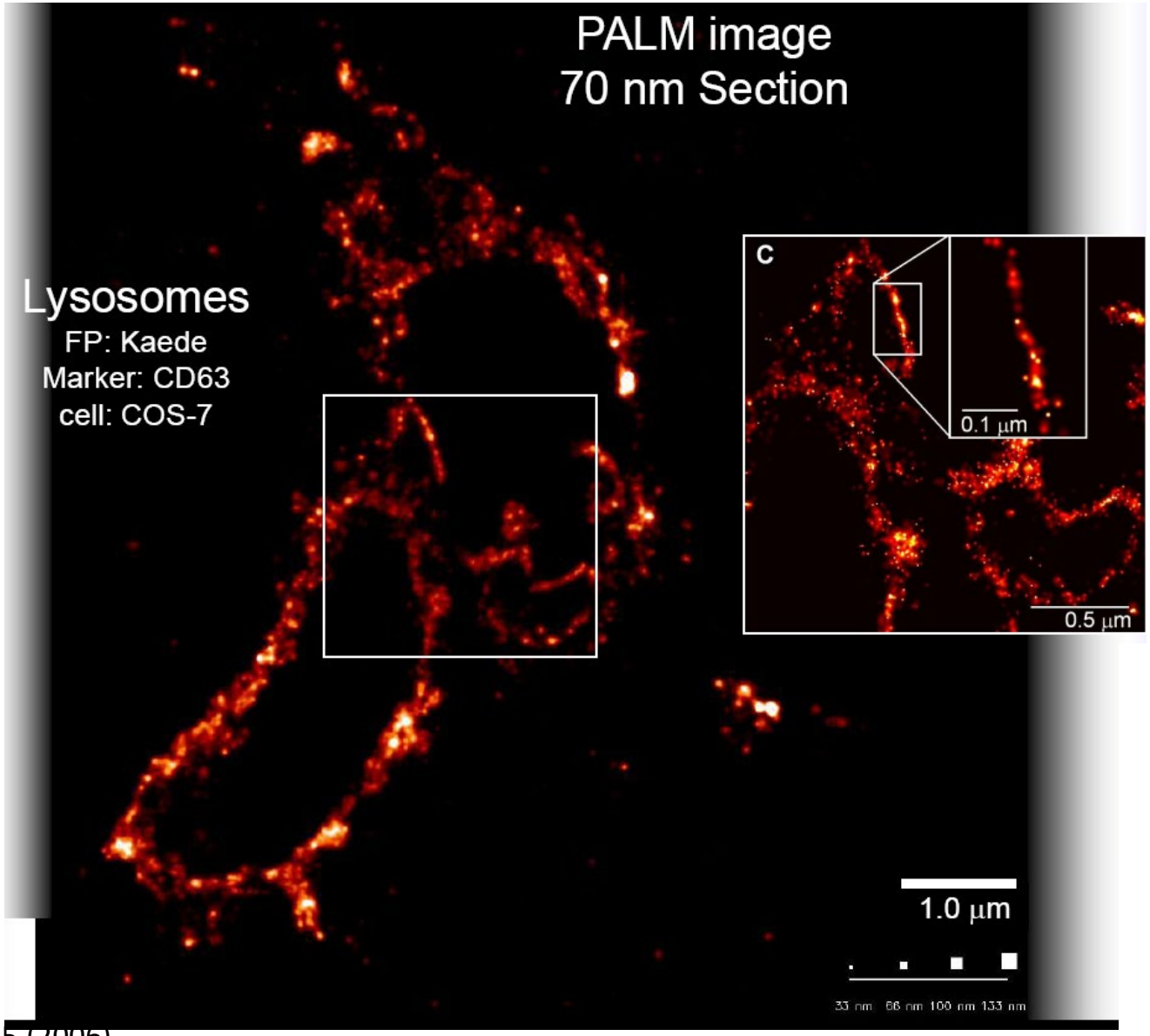
Obtaining Fiducial Data

- PALM /STORM fiducials are identified as stable light sources during imaging this light sources are always on and it could be tracked to



Betzig, E. *et al*. 313, 1642–1645 (2006)

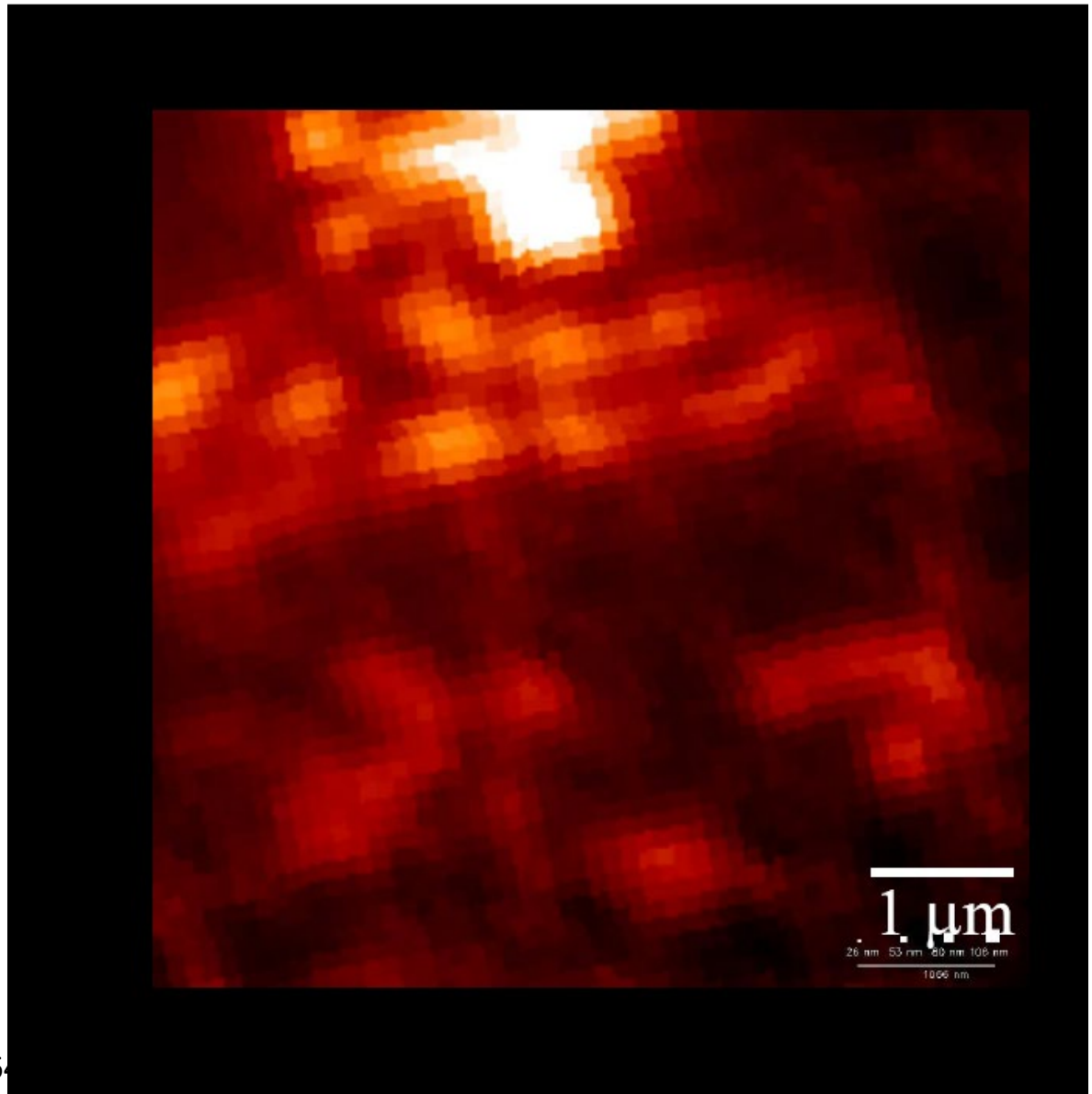
First PALM images fixed cells



Betzig, E. *et al*. 313, 1642–1645 (2006)

First PALM images fixed cells

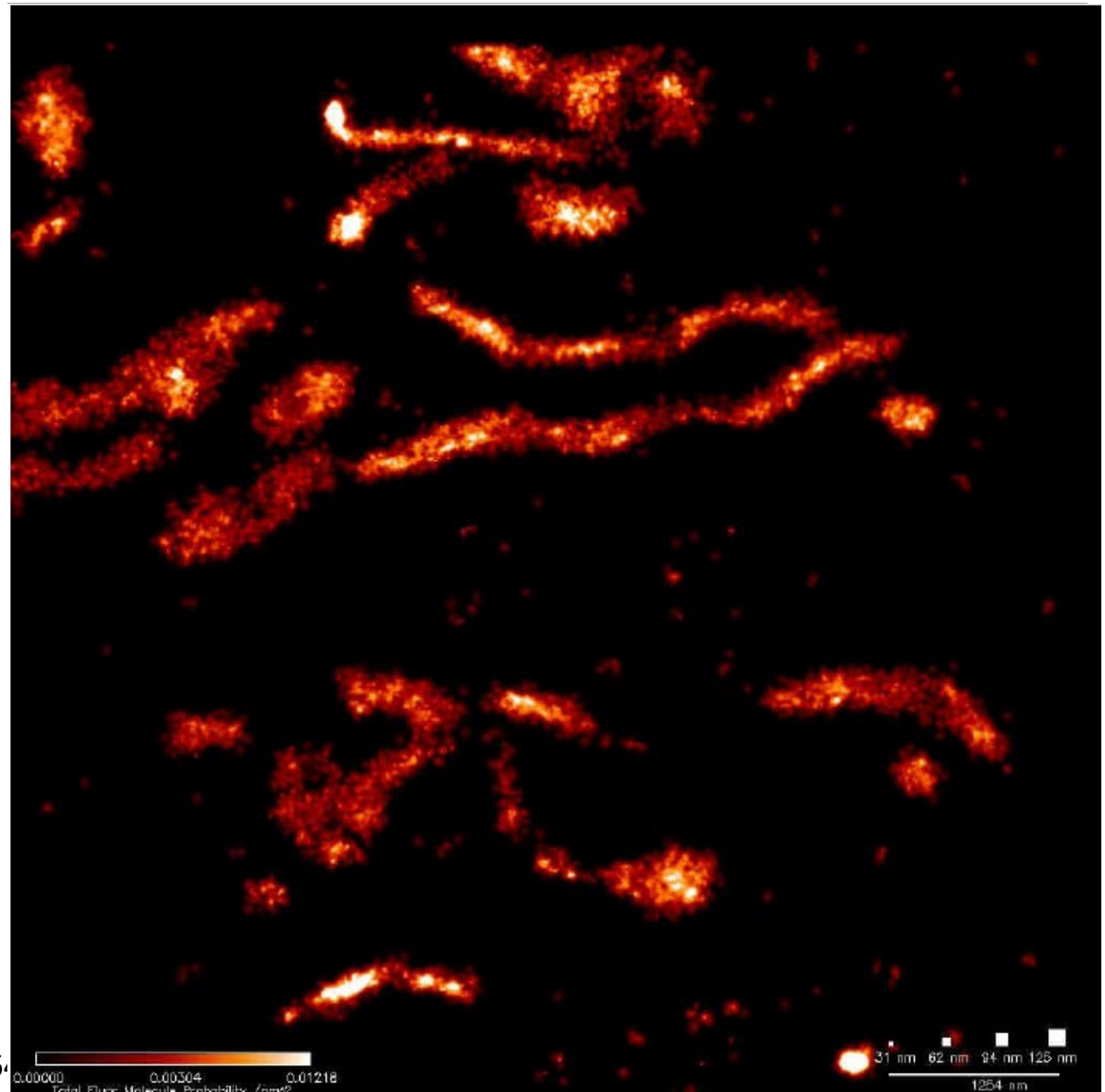
- Mitochondria Section Eos in lumin
-



Betzig, E. *et al*. 313, 164

First PALM images fixed cells

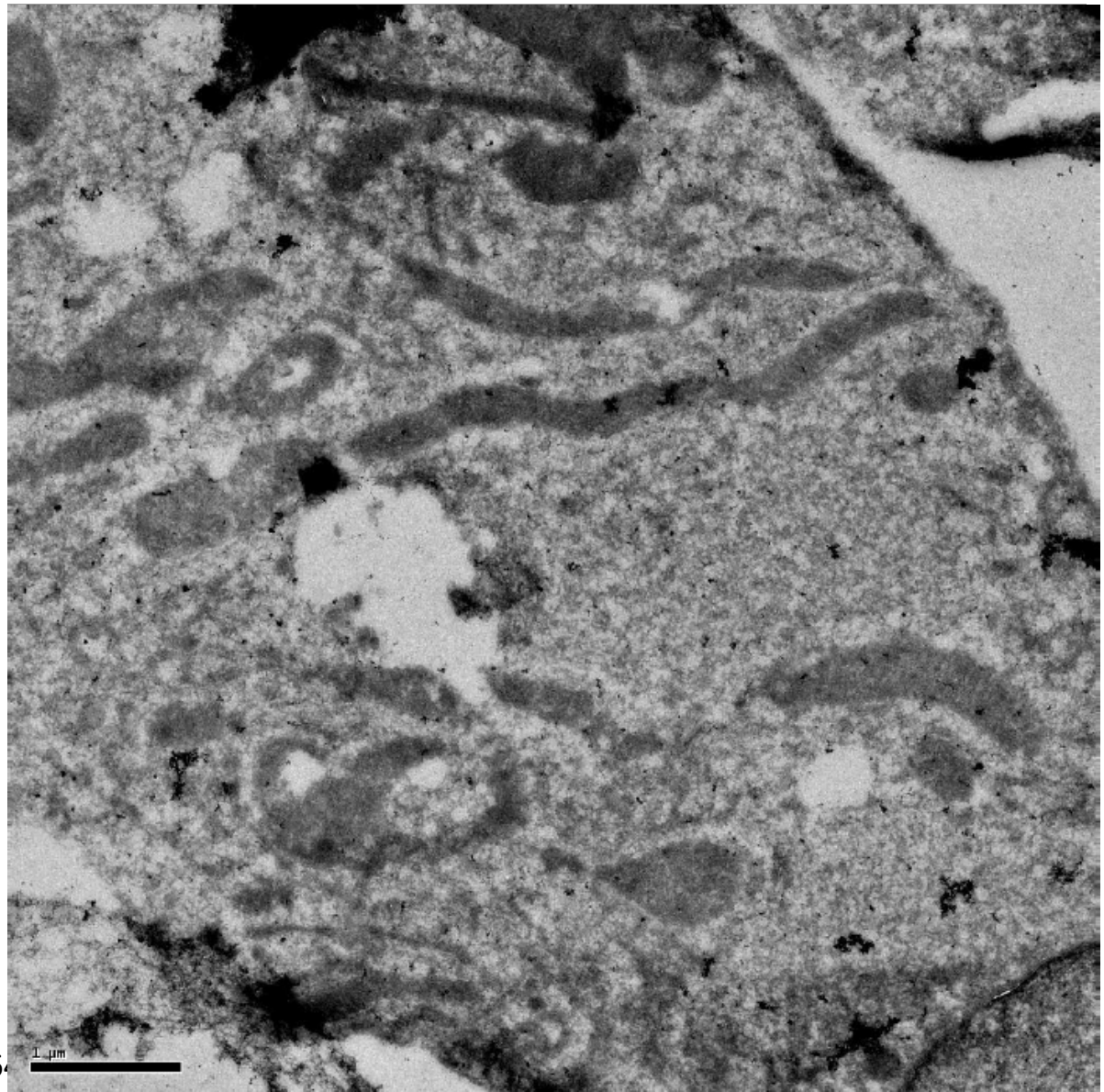
- Mitochondria Section Eos in lumin,



Betzig, E. *et al*. 313, 16

First PALM images fixed cells

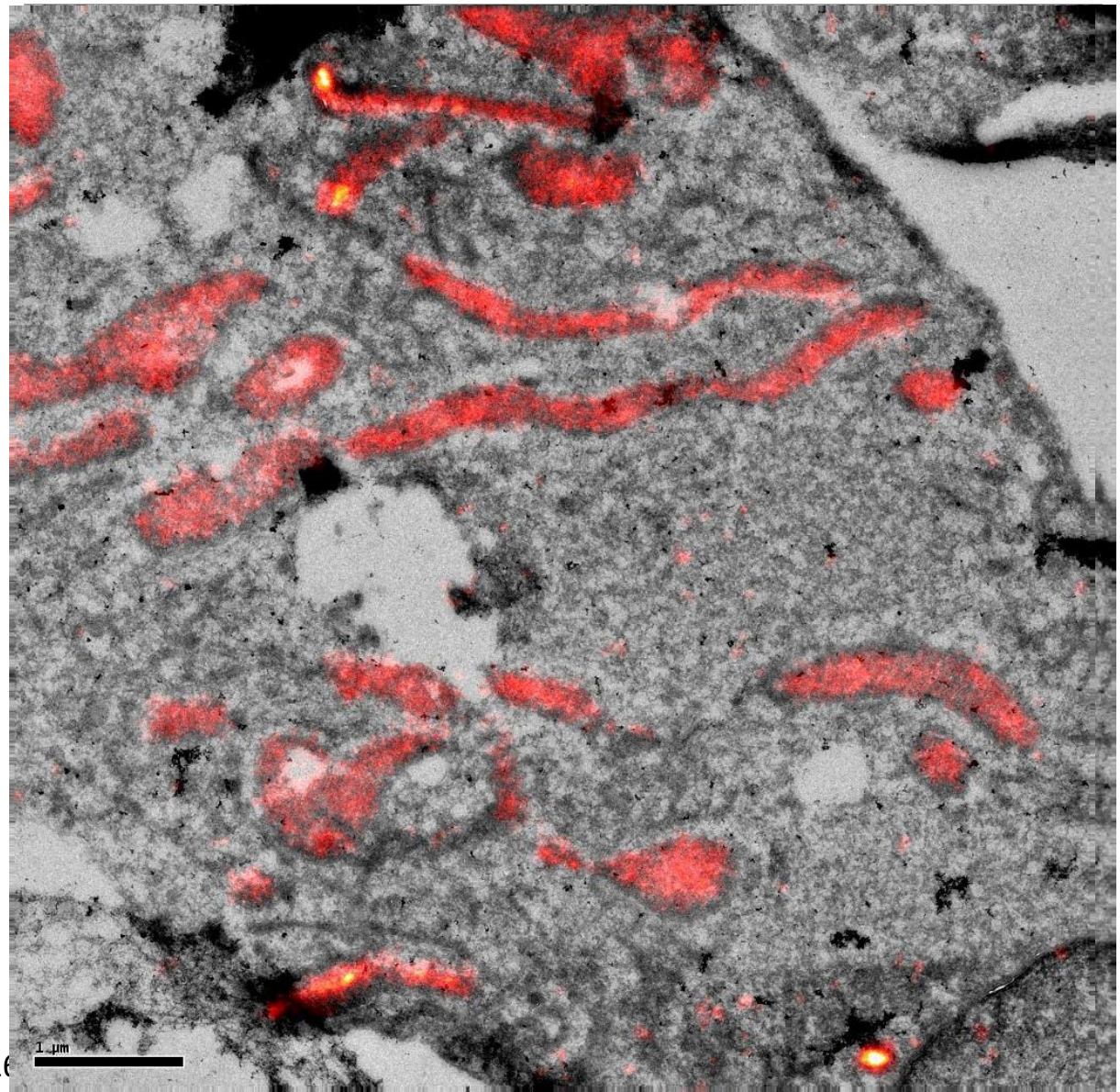
- Mitochondria Section Eos in lumin, TEM image,



Betzig, E. *et al*. 313, 16

First PALM images fixed cells

- PALM TEM Composite Image



Betzig, E. *et al*. 313, 16