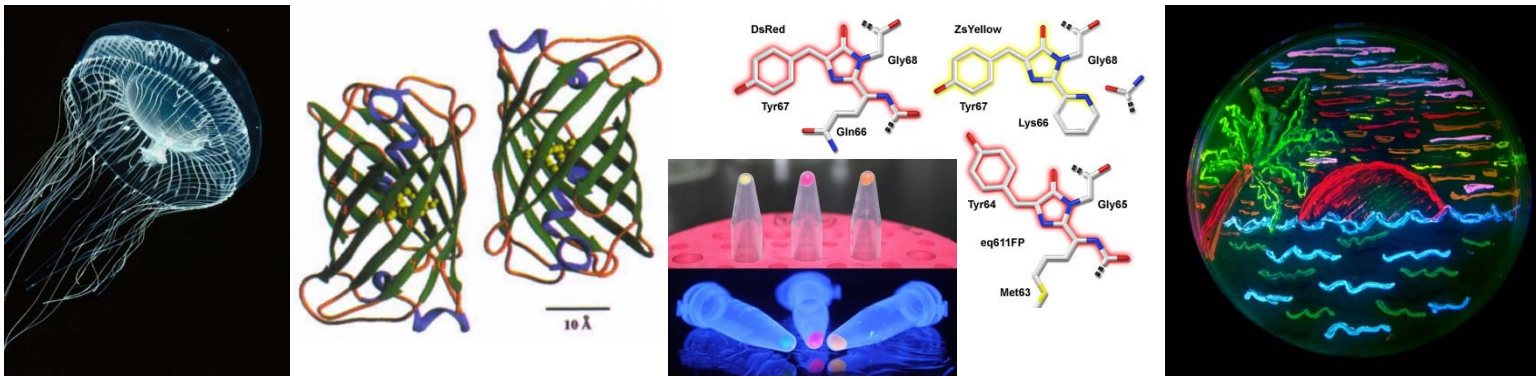
1st Martinsried Campus Bioimaging Day

Fluorophores

interface to the molecular world



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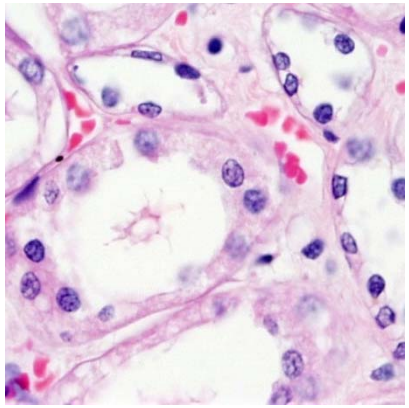
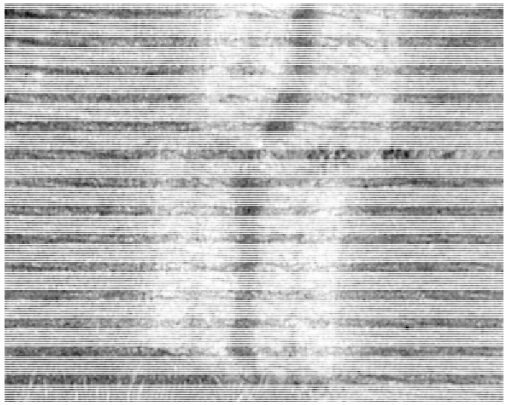
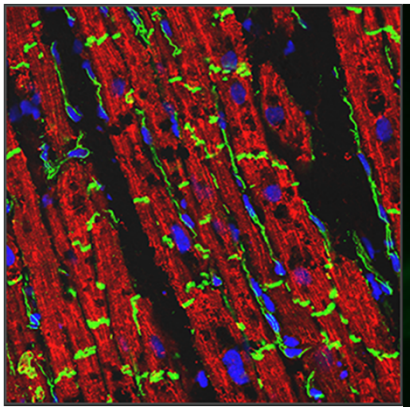
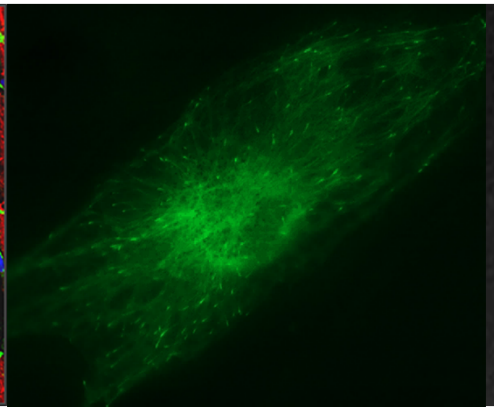
Why fluorescence? Because it lets you see more...

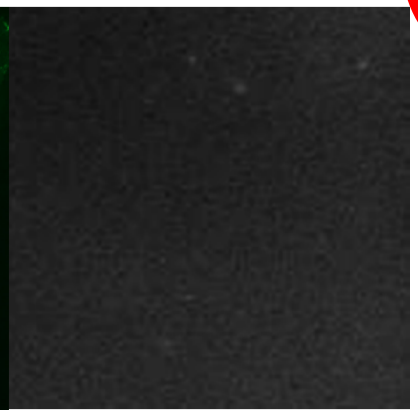




$$d = \frac{\lambda}{2\mathrm{NA}}$$







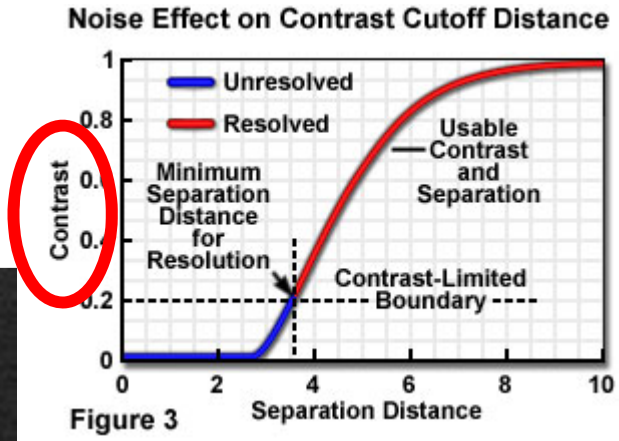


Figure 3

$$\sigma_x \geq \frac{s}{\sqrt{N}}$$

resolved volume: 28,000,000 nm^3 $\xrightarrow{1/10^8}$ visible fluorophores: 0.125 nm^3

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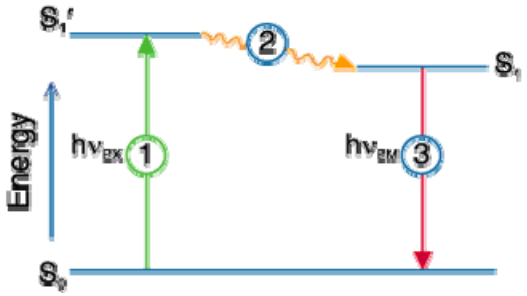
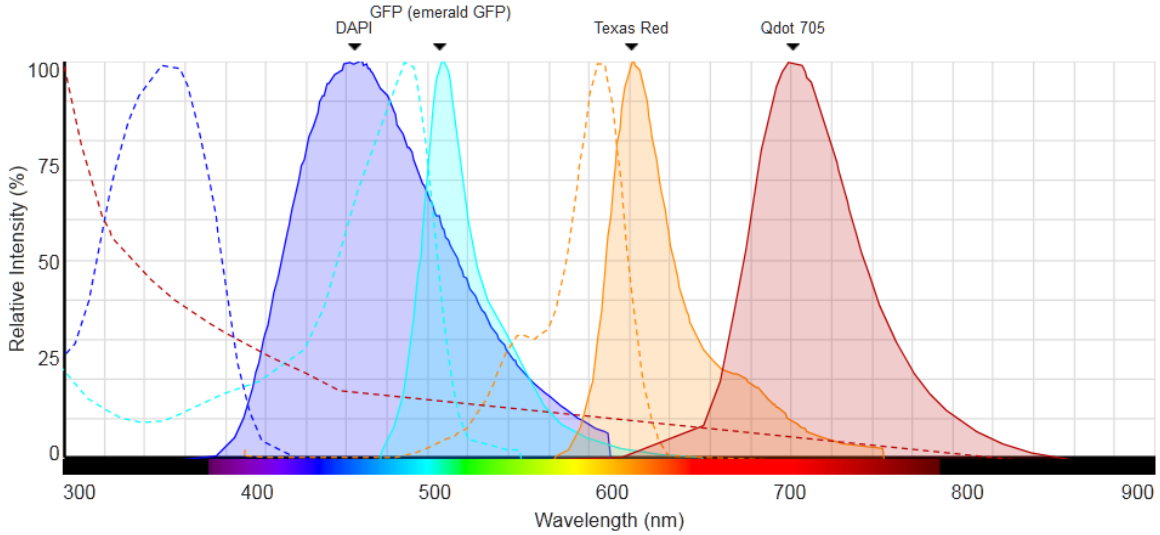
Fluorescence... how it works



Jablonski diagram:

1) excitation *(absorption of light)*
2) relaxation *(generation of heat, proportional to Stoke shift)*
3) emission *(radiation of light)*

Fluorophore	Excitation max	Emission max
DAPI	355	460
GFP	490	510
Texas Red	595	610
QDot 705	300	700



---------- *excitation spectrum* ———— *emission spectrum*

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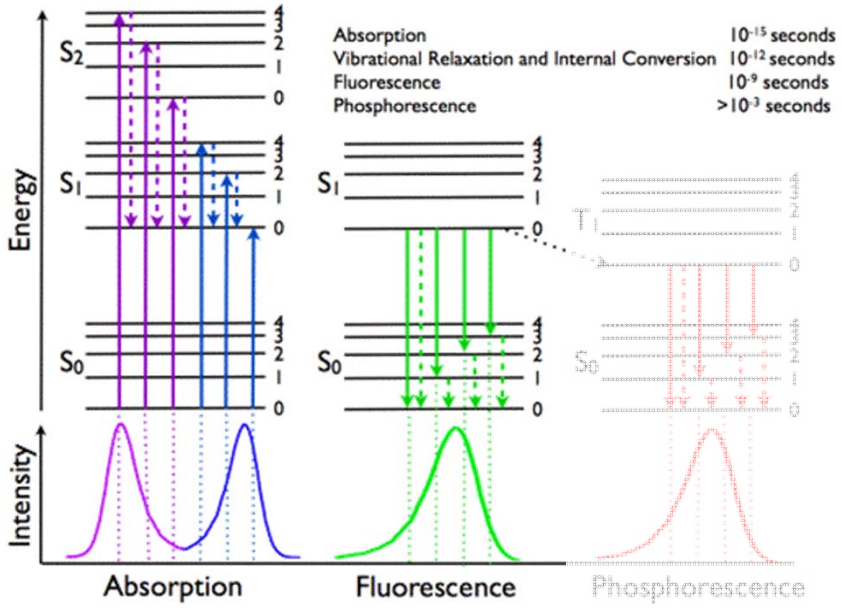
Fluorescence... how it works



λ excitation

λ emission

$\lambda = hc/\Delta E$

(h = Planck's constant)

http://www.photobiology.info/Visser-Rolinski.html

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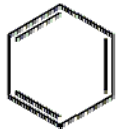
Energy levels of the helium atom

Helium

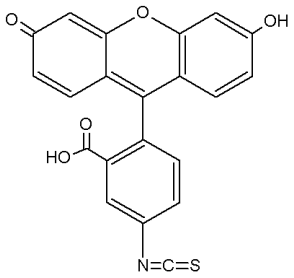
Helium Configuration			Level(cm-1)
1s2	1S	0	0.000
1s2s	3S	1	159855.9745
1s2s	1S	0	166277.4403
1s2p	3P°	2	169086.7666
		1	169086.8430
		0	169087.8309
1s2p	1P°	1	171134.8970
1s3s	3S	1	183236.7918
1s3s	1S	0	184864.8294
1s3p	3P°	2	185564.5620
		1	185564.5840
		0	185564.8547
1s3d	3D	3	186101.5463
		2	186101.5488
		1	186101.5930
1s3d	1D	2	186104.9668
1s3p	1P°	1	186209.3651
1s4p	1P°	1	191492.7120
He II (2S1/2)	Limit		198310.6691

http://physics.nist.gov/PhysRefData/Handbook/Tables/heliumtable5.htm

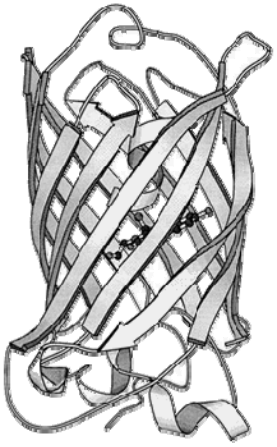
Structures of fluorophore molecules



benzene



FITC



GFP

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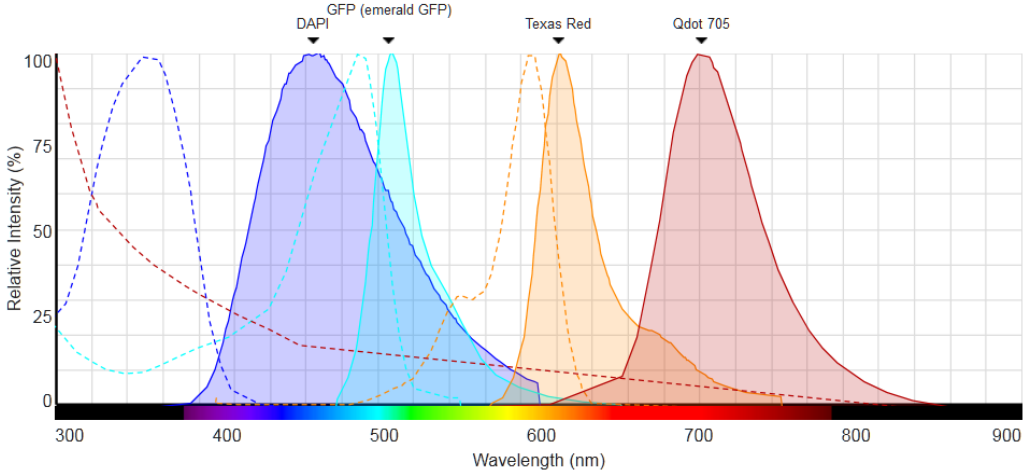
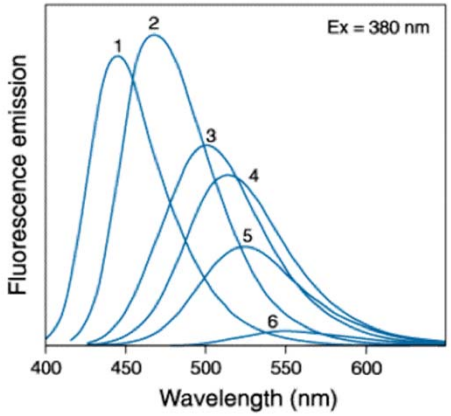
Each fluorophore has its own, specific spectral fingerprint...



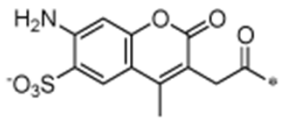
...which can change with the environment!



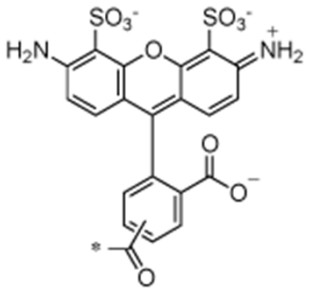
Fluorescence spectrum of Badan in
1) toluene
2) chloroform
3) acetonitrile
4) ethanol
5) methanol
6) water

Fluorescence emission spectra of the 2-mercaptoethanol adduct of badan (B6057) in: 1) toluene, 2) chloroform, 3) acetonitrile, 4) ethanol, 5) methanol and 6) water.

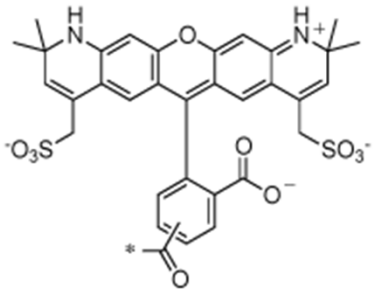
Structures and properties of fluorophores



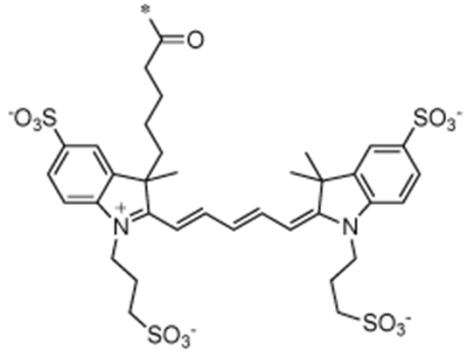
Alexa Fluor® 350



Alexa Fluor® 488



Alexa Fluor® 568



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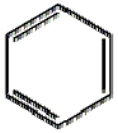
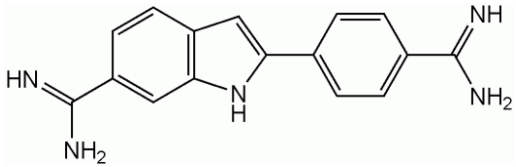
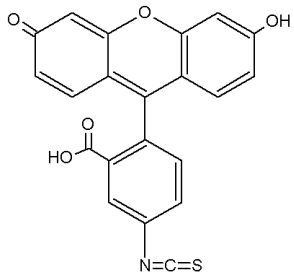
Structures and properties of fluorophores



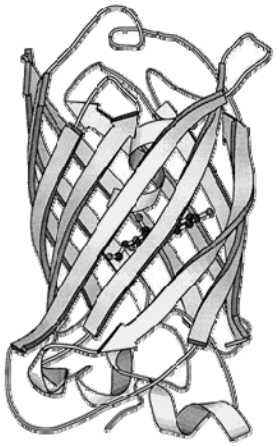
benzene



DAPI



FITC



GFP

- conjugated double-bonds, aromatic rings
- acting as “antenna” for light
- very dependent on spatial orientation
- differences between fluorophores:
  - size (also affecting wavelength → few far-red fluorescent proteins)
  - wavelength for excitation and emission
  - absorption coefficient, quantum efficiency, photostability
  - environmental sensitivity
  - hydrophobicity
  - maximal labelling density

Structures and properties of fluorophores

Fluorophores Table	Dye Category	Ex max (nm)	Em max (nm)	Extinction Coefficient (EC) [$Mol^{-1}cm^{-1}$]	Quantum Yield (QY) [emitted photons per absorbed photons]	Brightness [Ec*QY/1000]	Stokes Shift	Fluores-cence lifetime [nsec]	Molecular Weight [g/mol]
Vitamin A (retinol)	coenzymes and vitamins	324	510				186		
Hoechst 33258 (in H2O)	Nucleic acid binding	345	507	46,000	0.034	1.6	162		
Hoechst 33258 (dsDNA)	Nucleic acid binding	345	507			40,000	0.59	23.6	
Alexa Fluor 350	Organic dye	346	442	19,000			96		295.4
GFP		395/475	508	21,000	0.77	16.2	113		27,000
EGFP	Fluorescent Protein	488	507	55,000	0.6	33.0	19		27,000
Fluorescein	Organic dye	495	520	79,000	0.9	71.1	25		
Fluorescein isothiocyanate (FITC)	Organic dye	495	525	80,000	0.5	40.0	30		390
Alexa Fluor 488	Organic dye	495	519	71,000	0.94	66.7	24		
EYFP	Fluorescent Protein	514	527		0.61		13		
Alexa Fluor 532	Organic dye	532	553	81,000	0.8	64.8	21		
Atto 532	Organic dye	534	560	115,000	0.9	103.5	26	3.8	
Cy3	Organic dye	554	568	130,000	0.14	18.2	14		
DsRed	Fluorescent Protein	558	583	75,000	0.7	52.5	25		
Kaede-Green (Trachyphyllia geoffroy FP)	Fluorescent protein	572	580	60,400	0.33	19.9	8		
Alexa Fluor 568 (Alexa568)	Organic dye	578	603	91,300	0.75	68.5	25		

http://home.earthlink.net/~fluorescentdyes/

Structures and properties of fluorophores: The ideal fluorophore

- The ideal fluorophore:
  - conveniently excitable, without simultaneous excitation of the biological matrix
  - detectable with conventional instrumentation
  - bright (high molar absorption coefficient + high fluorescence quantum yield)
  - soluble in relevant buffers, cell culture media or body fluids
  - sufficiently stable under relevant conditions
  - functional groups for site-specific labeling
  - reported data about its photophysics
  - available in a reproducible quality.

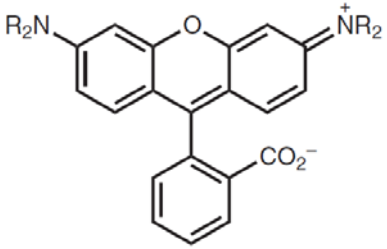
Nature Methods 5, 763 - 775 (2008)

Types of fluorophores

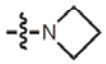
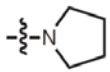
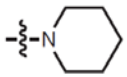
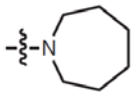
- Organic dyes
- Fluorescent proteins
- Luminescent nanocrystals (quantum dots)
- Biological structures suitable for label-free imaging

Organic dyes – structures and properties

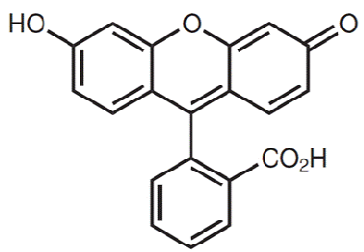
Rhodamine core



Rhodamine derivatives

Dye	NR_2	λ_{max} (nm)	ε (M^{-1} cm^{-1})	λ_{em} (nm)	ϕ	τ (ns)
1		497	76,000	520	0.88	3.26
2		548	78,000	572	0.41	2.21
3		–	–	–	–	–
4		549	101,000	571	0.88	3.84
5		553	76,000	576	0.74	3.60
6		560	80,000	586	0.10	0.59
7		560	106,000	583	0.25	1.62

fluorescein



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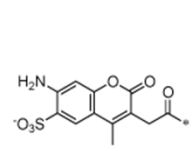
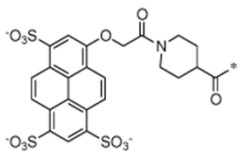
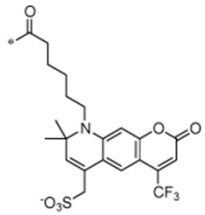
Organic dyes – structures and properties



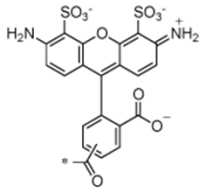
Alexa Fluor® 350



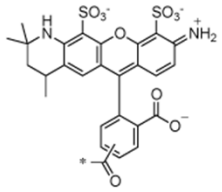
Alexa Fluor® 405



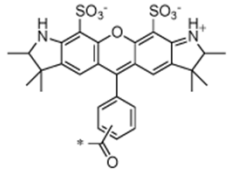
Alexa Fluor® 430



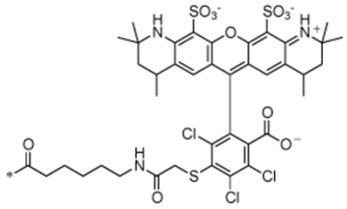
Alexa Fluor® 488



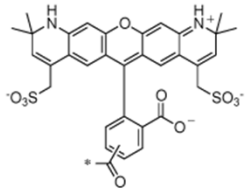
Alexa Fluor® 514



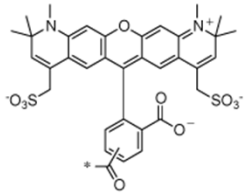
Alexa Fluor® 532



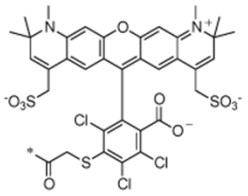
Alexa Fluor® 546



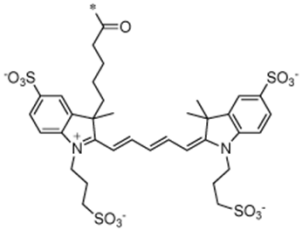
Alexa Fluor® 568



Alexa Fluor® 594



Alexa Fluor® 610



Alexa Fluor® 647

Name	λ_{max} Ex	λ_{max} Em	E at λ_{max}	τ / ns
Alexa Fluor 350	346	442	19 000	-
Alexa Fluor 405	402	421	35 000	-
Alexa Fluor 430	434	539	15 000	-
Alexa Fluor 488	495	519	73 000	4.1
Alexa Fluor 532	531	554	81 000	2.5
Alexa Fluor 546	556	573	112 000	4.1
Alexa Fluor 555	555	565	155 000	0.3
Alexa Fluor 568	578	603	88 000	3.6
Alexa Fluor 594	590	617	92 000	3.6
Alexa Fluor 633	632	647	159 000	-
Alexa Fluor 635	633	647	140 000	-
Alexa Fluor 647	650	668	270 000	1.0
Alexa Fluor 660	663	690	132 000	1.2
Alexa Fluor 680	679	702	183 000	1.2
Alexa Fluor 700	702	723	205 000	1.0
Alexa Fluor 750	749	775	290 000	0.7
Alexa Fluor 790	782	805	260 000	-

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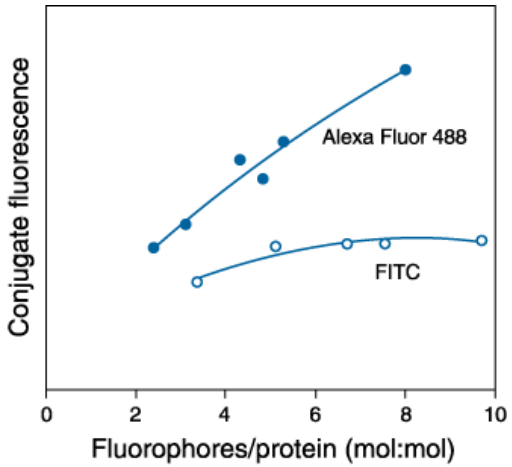
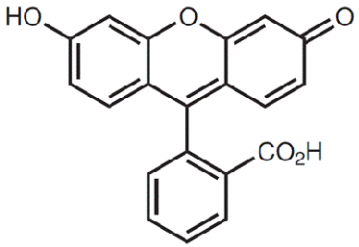
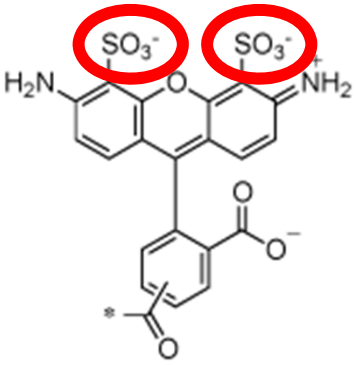
Organic dyes – labelling density

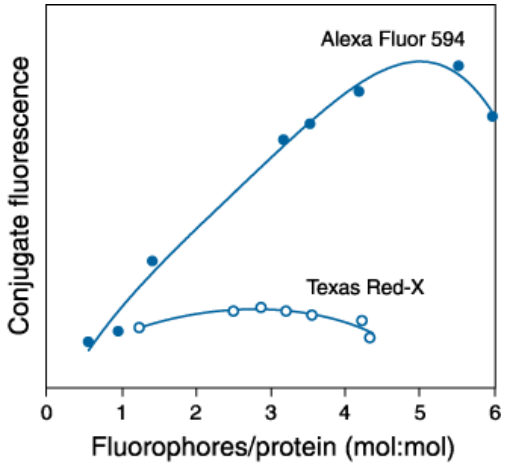


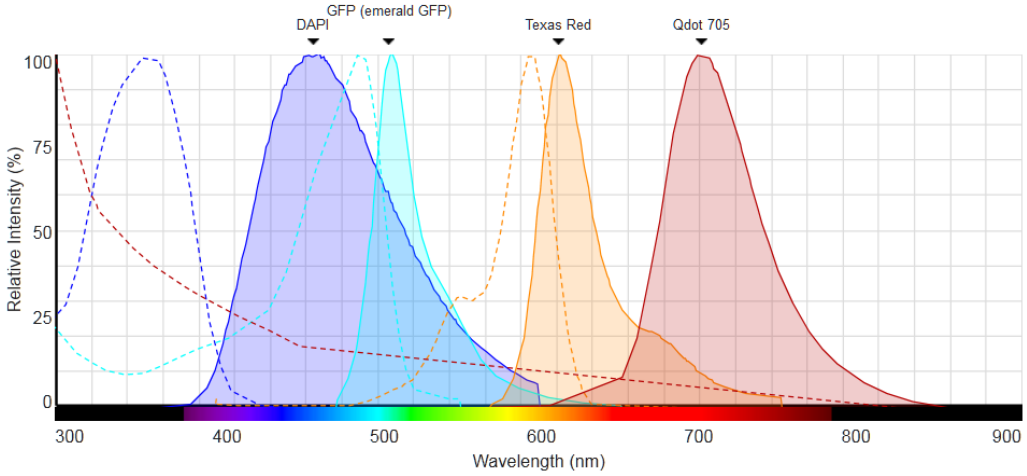
fluorescein





Alexa Fluor® 488





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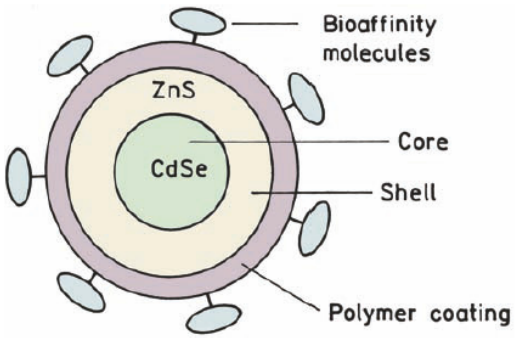
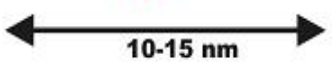
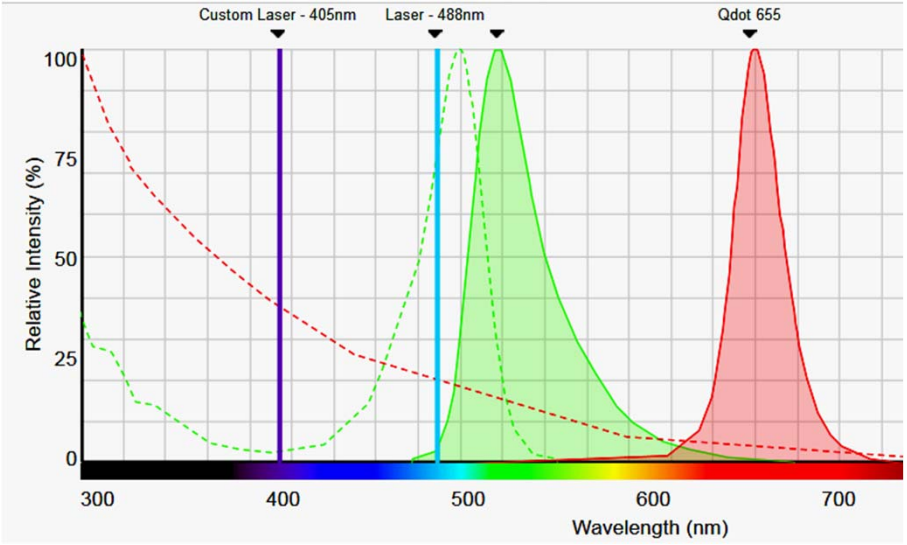
Types of fluorophores: Quantum dots





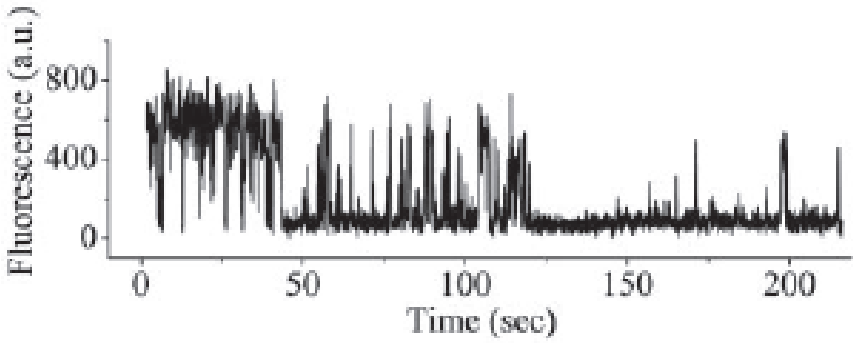
http://www.photobiology.info/Visser-Rolinski.html



- Principle of action:
  - semiconductor, photoelectric effect (photon-induced electron/hole pair, trapped in nanocrystal)
- advantages:
  - high photostability (no excited state)
  - bright (high extinction coefficient)
  - wide range of excitation
  - narrow emission peak
  - very large Stoke shift → flexible microscope setup
- disadvantages:
  - quenching (special mounting medium needed)
  - blinking
  - size (~30x larger than organic dyes)

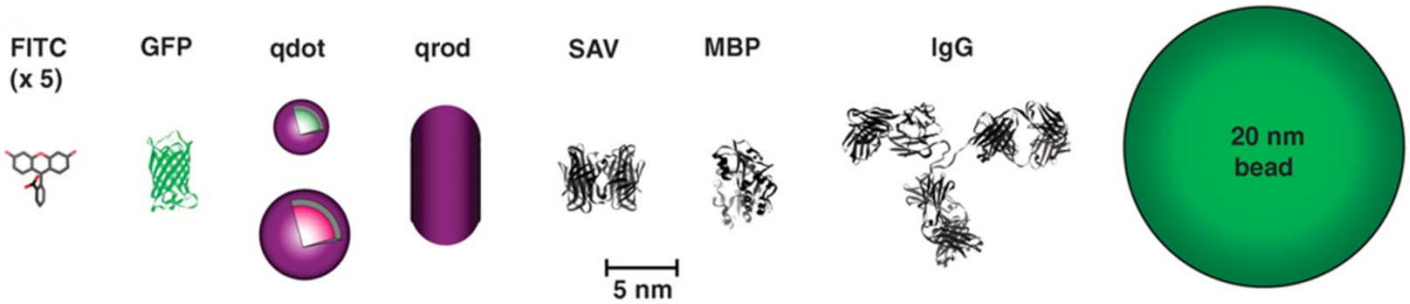
Quantum dots: properties

Quantum dots: Blinking



Yao, J., et al. , PNAS 102:14284

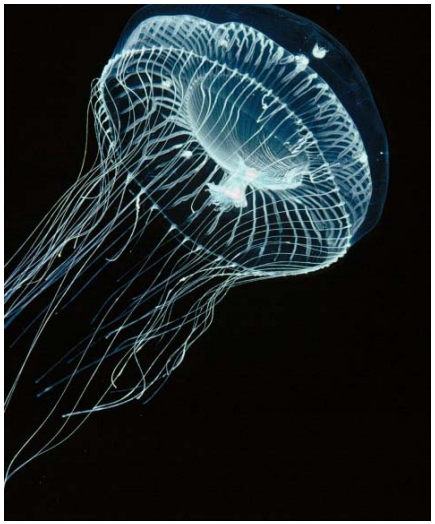
Quantum dots: size



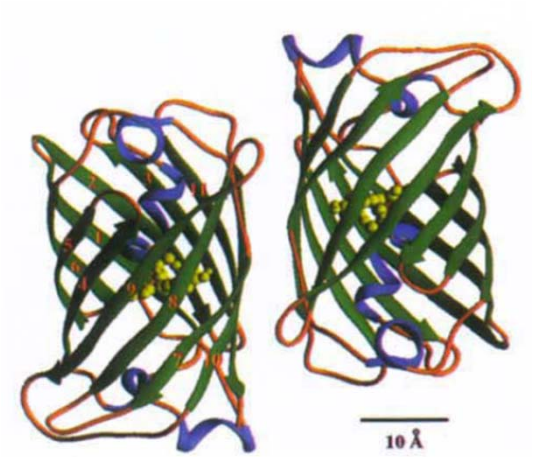
Michalet, X., et al. : Science 307(5709): 538-544., 2005

Types of fluorophores: Fluorescent proteins

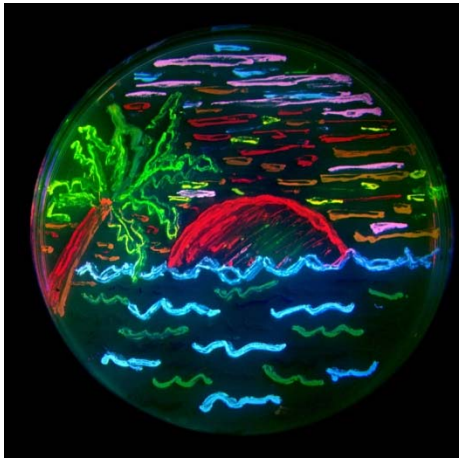
- **History:**
  - Green Fluorescent Protein first purified from Aequorea victoria by Osamu Shimomura, characterised and optimised by Martin Chalfie and Roger Tsien (discovery 1960s / 70s, joint Nobel Prize 2008)
  - fluorescent proteins found in >100 species, but biological function still unclear (light-induced electron donor?



Aequorea victoria

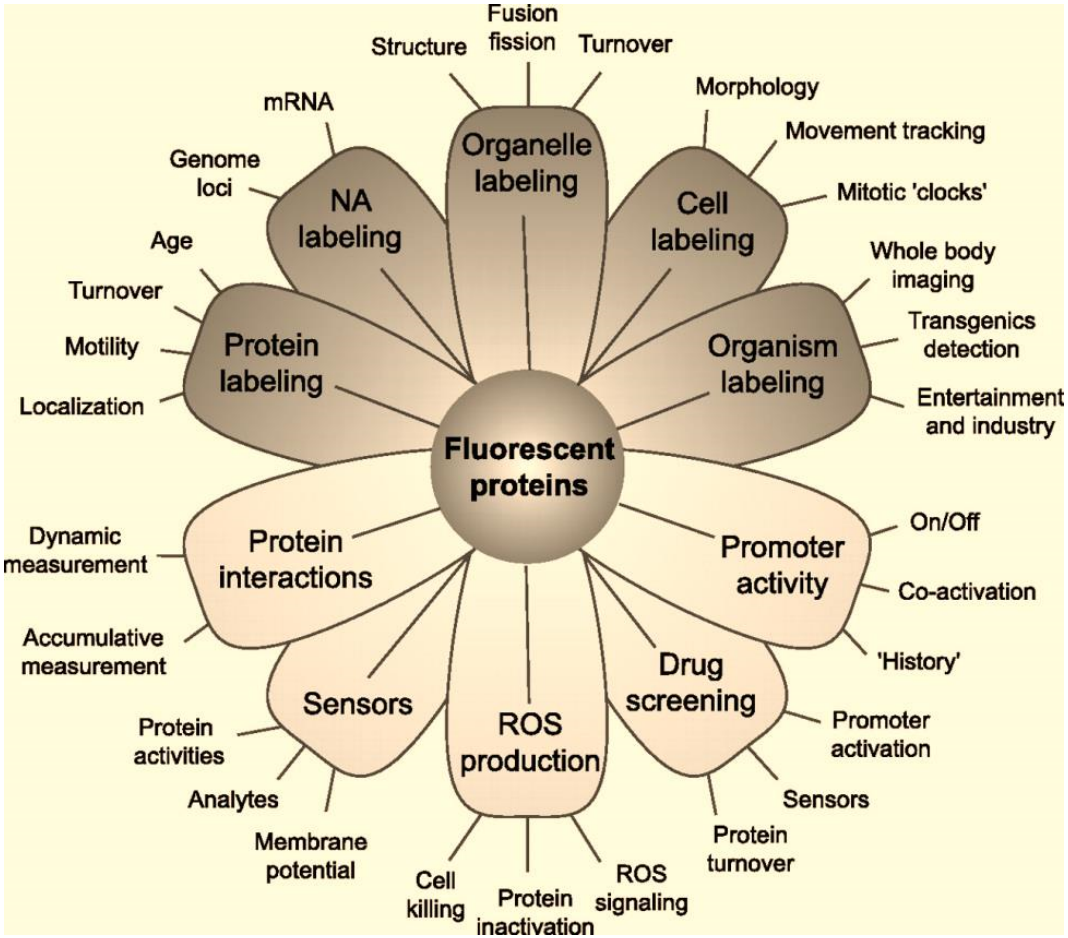


GFP fluorochrome



GFP variants

Fluorescent proteins - applications



Applications of fluorescent proteins

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Dmitriy M. Chudakov et al. Physiol Rev 2010;90:1103-1163

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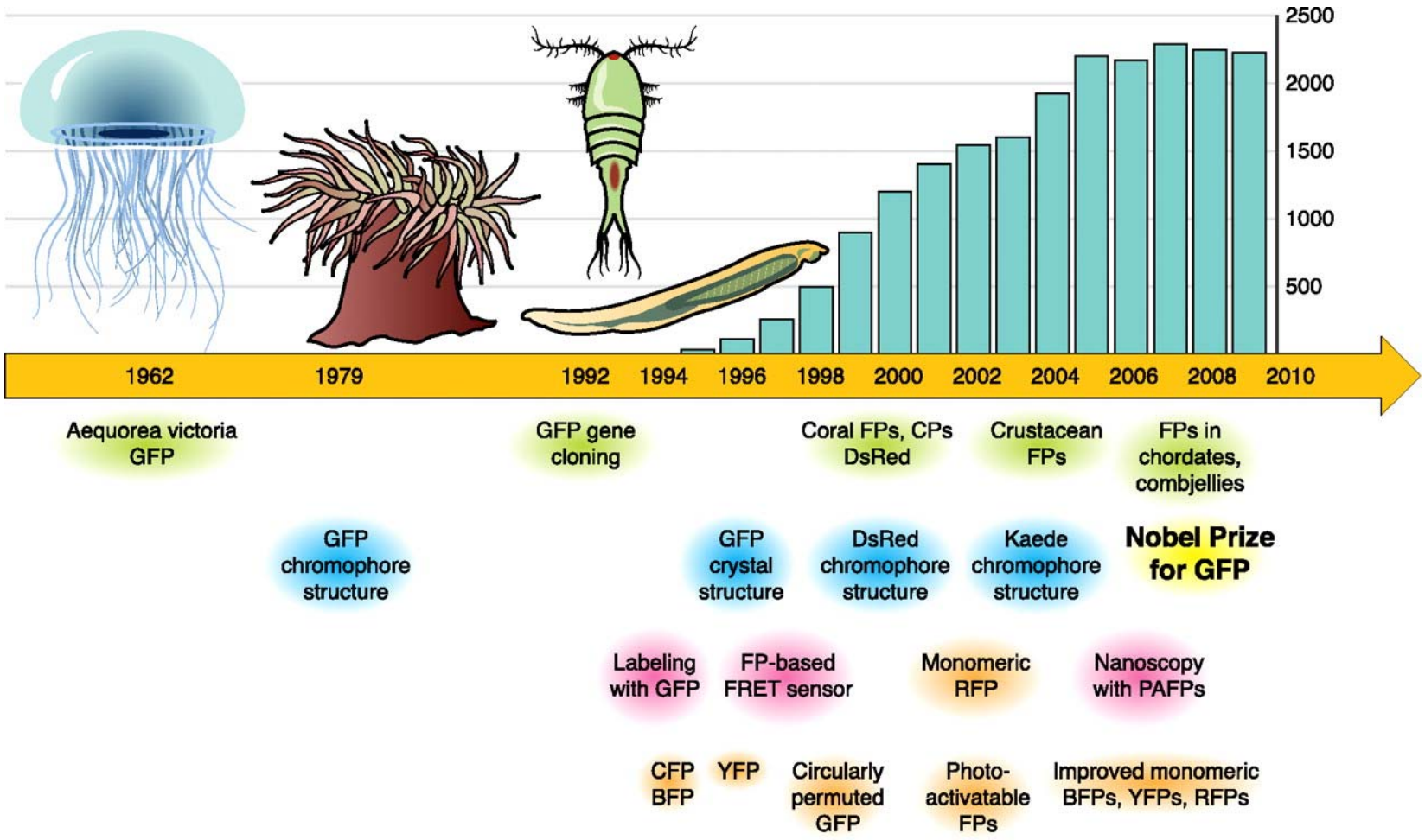
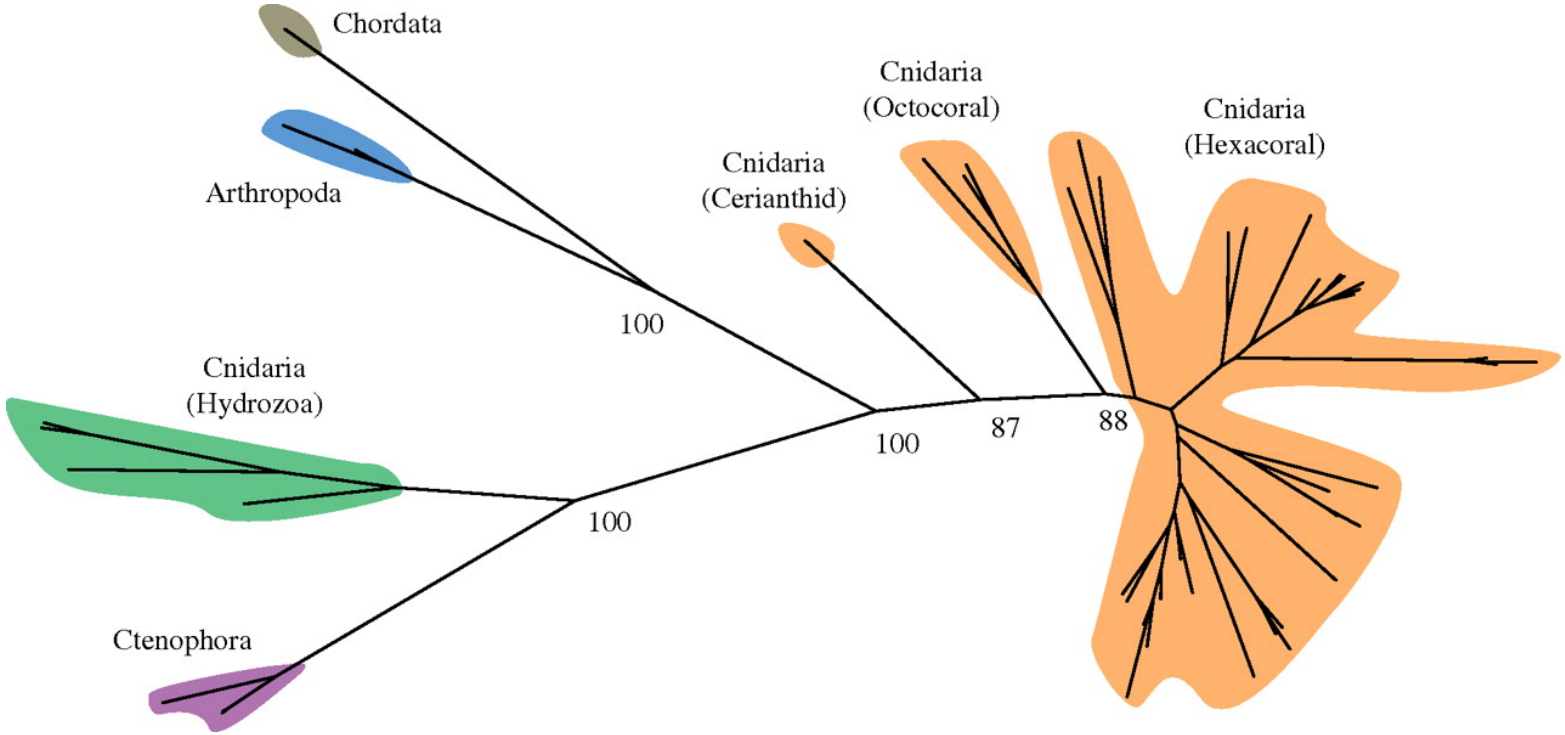
Fluorescent proteins - history



Dmitriy M. Chudakov et al. Physiol Rev 2010;90:1103-1163

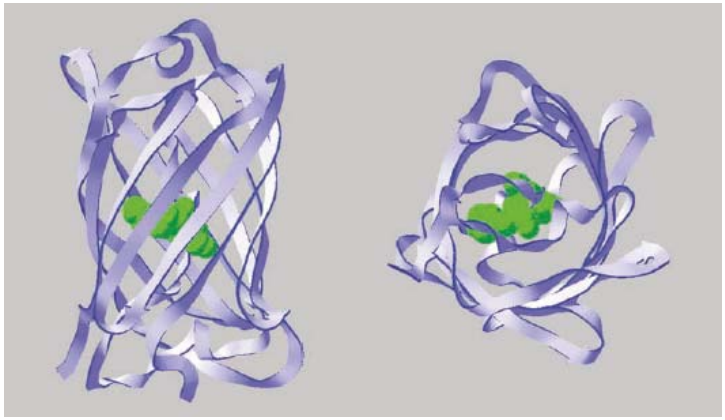
Fluorescent proteins - evolution



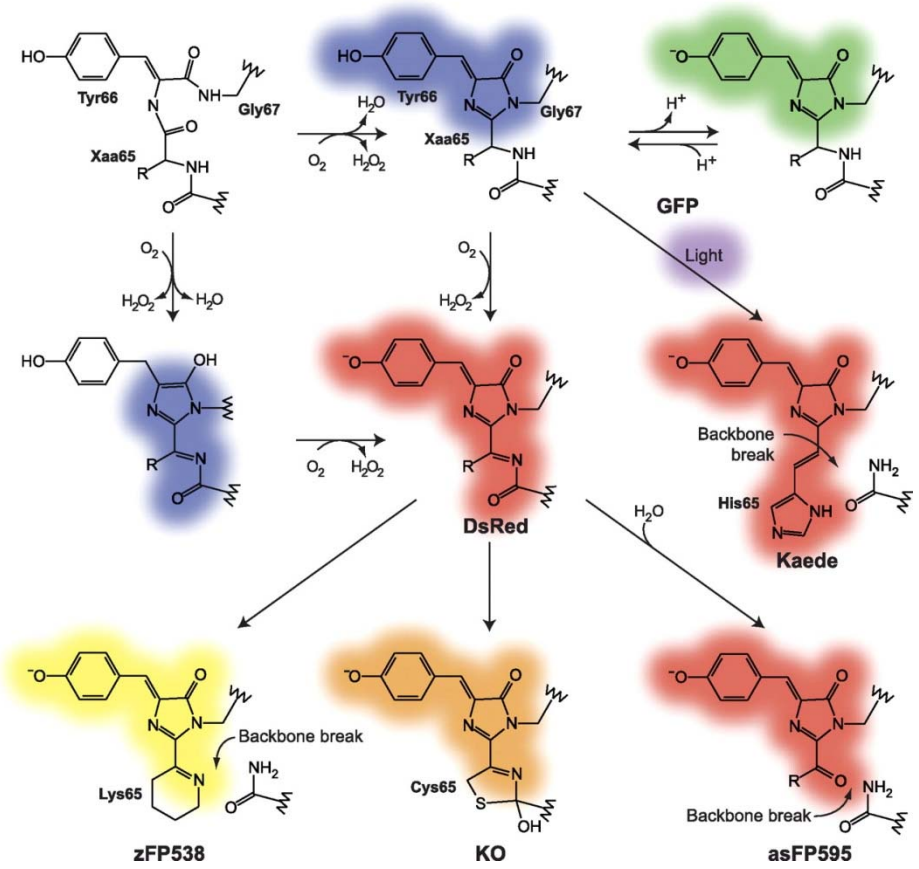
Steven H. D. Haddock et al. Proc. R. Soc. B 2010;277:1155-1160

Fluorescent proteins - structures

core structure



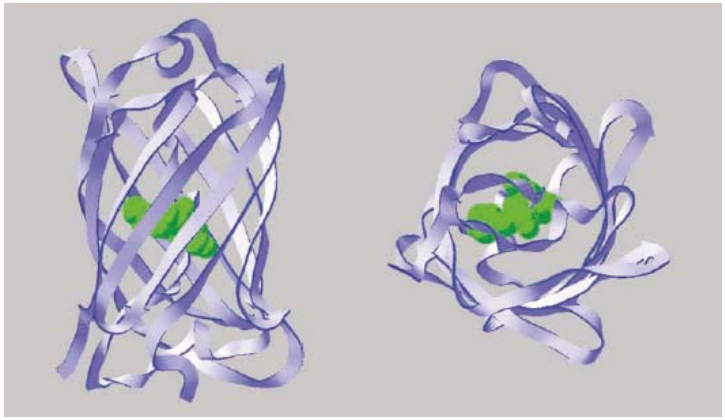
Maturation steps of naturally occurring fluorophores



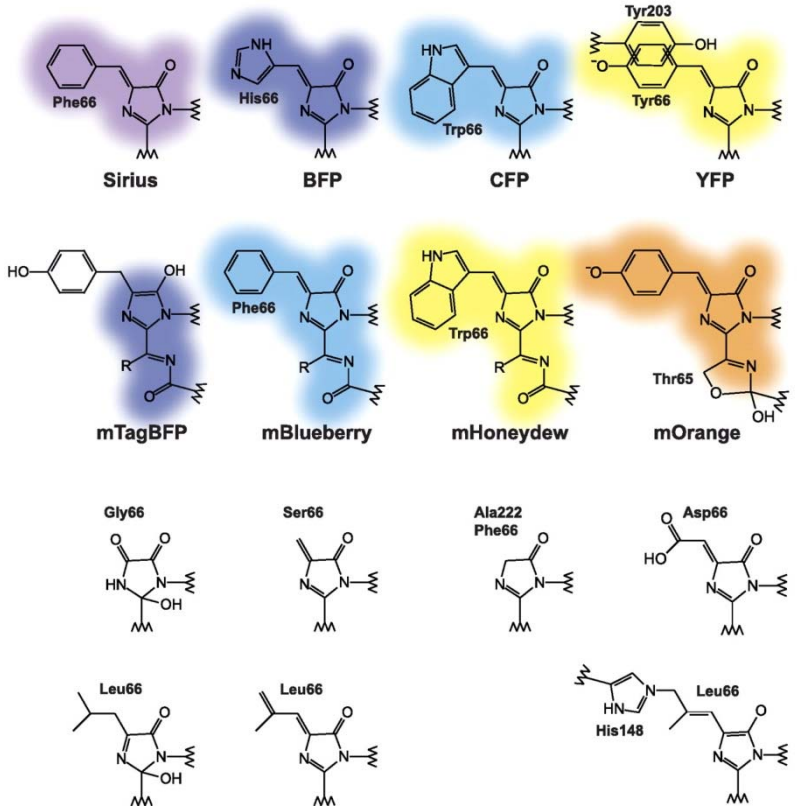
Dmitriy M. Chudakov et al. Physiol Rev 2010;90:1103-1163

Fluorescent proteins - structures

core structure



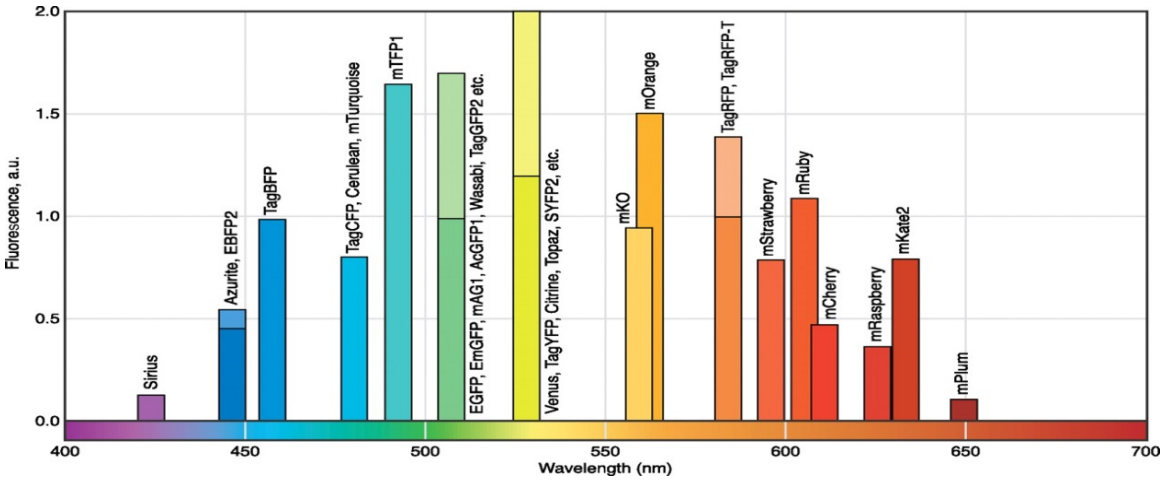
Artificial variants of the fluorescent chromophore



Dmitriy M. Chudakov et al. Physiol Rev 2010;90:1103-1163

Fluorescent proteins - structures

Representative examples of fluorescent proteins

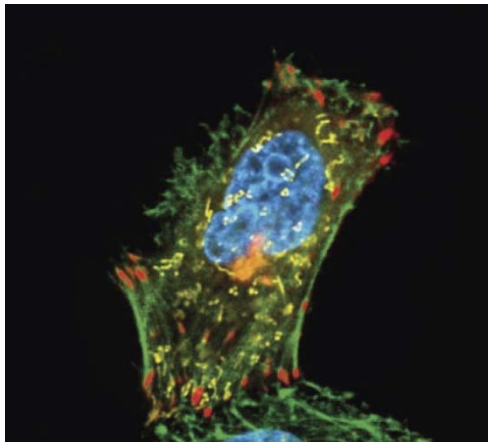


Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]	Tsien (4)	574	596	26	15	<4.5	Monomer
	J-Red[h]	Evrogen	584	610	8.8*	13	5.0	Dimer
	DsRed-monomer[h]	Clontech	556	586	3.5	16	4.5	Monomer
Orange	mOrange[g]	Tsien (4)	548	562	49	9.0	6.5	Monomer
	mKO	MBL Intl. (10)	548	559	31*	122	5.0	Monomer
Yellow-green	mCitrine[i]	Tsien (16,23)	516	529	59	49	5.7	Monomer
	Venus	Miyawaki (1)	515	528	53*	15	6.0	Weak dimer[j]
	YPet[g]	Daugherty (2)	517	530	80*	49	5.6	Weak dimer[j]
	EYFP	Invitrogen (18)	514	527	51	60	6.9	Weak dimer[j]
Green	Emerald[g]	Invitrogen (18)	487	509	39	0.69[k]	6.0	Weak dimer[j]
	EGFP	Clontech[i]	488	507	34	174	6.0	Weak dimer[j]
Cyan	CyPet	Daugherty (2)	435	477	18*	59	5.0	Weak dimer[j]
	mCFPm[m]	Tsien (23)	433	475	13	64	4.7	Monomer
	Cerulean[g]	Piston (3)	433	475	27*	36	4.7	Weak dimer[j]
UV-excitable green	T-Sapphire[g]	Griesbeck (6)	399	511	26*	25	4.9	Weak dimer[j]

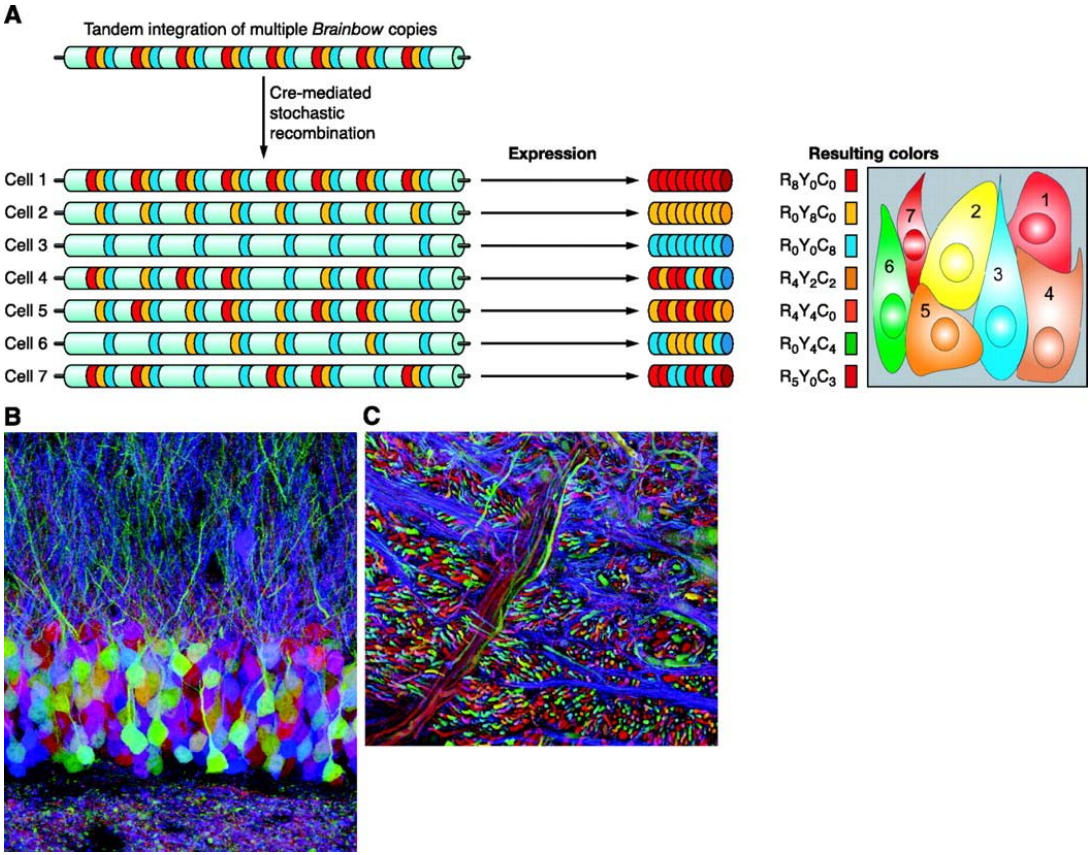
Dmitriy M. Chudakov et al. Physiol Rev 2010;90:1103-1163

Types of fluorophores – applications

multi-colour labelling



Brainbow technology

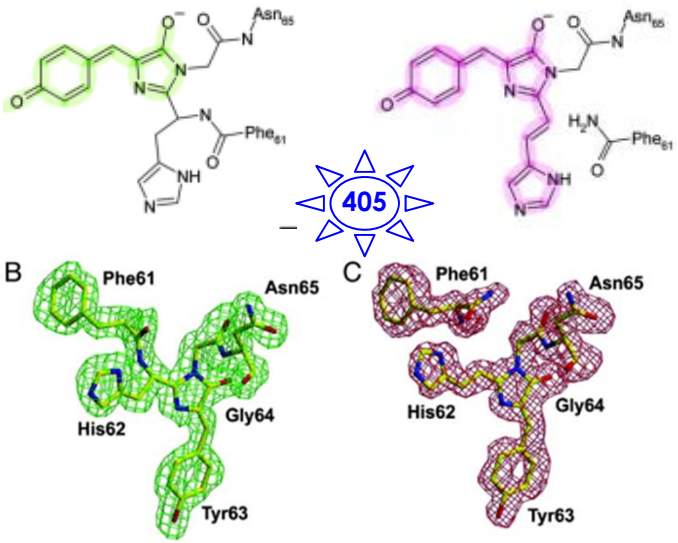


Co-transfection of various fluorescent proteins

Dmitriy M. Chudakov et al. Physiol Rev 2010;90:1103-1163

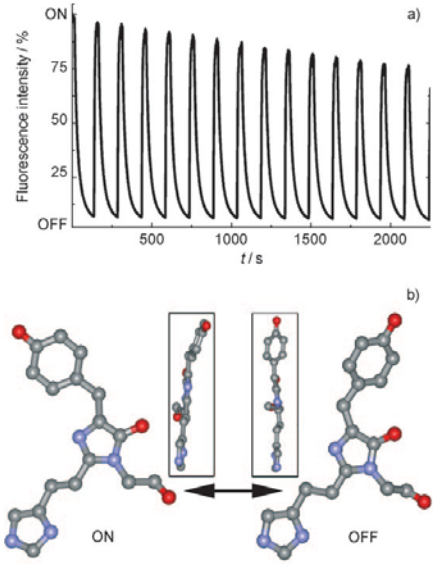
Photoswitchable fluorescent proteins ("Optical Highlighters")

Photoconversion



Example: EosFP

Photoactivation



Example: paGFP

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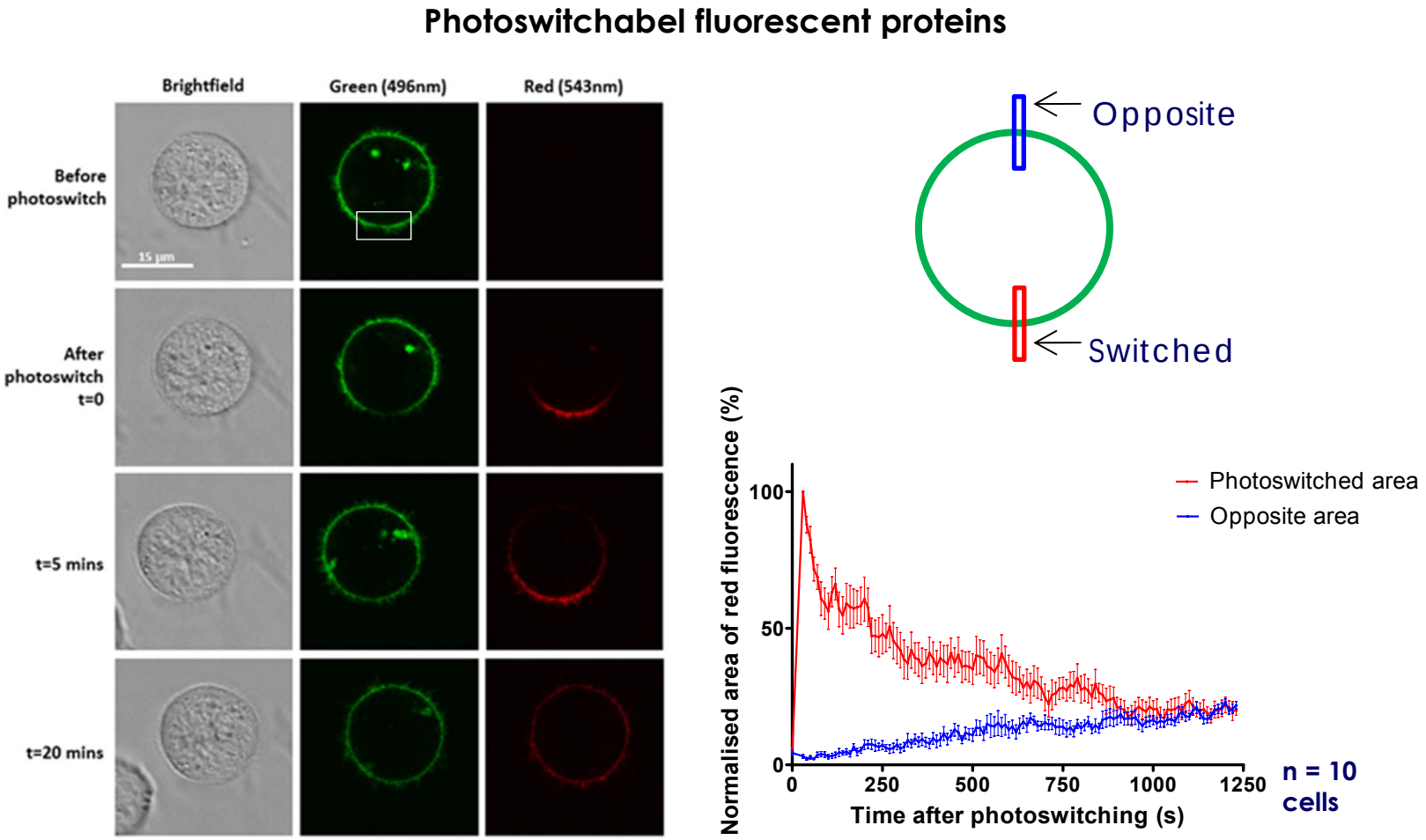
Photoswitchable fluorescent proteins ("Optical Highlighters")

Protein	λ_{ex} (nm)	λ_{em} (nm)	Aggregation	Transitions
Photoactivatible Proteins				
PA-GFP	504	517	Monomer	Off → On, 400 nm
PAmCherry1	564	595	Monomer	Off → On, 405 nm
Photoconvertible Proteins				
PS-CFP2	400	468	Monomer	
	490	511	Monomer	Cyan → Green, 405 nm
Dendra2	490	507	Monomer	
	553	573	Monomer	Green → Red, 480 nm
pcDronpa2	504	515	Tetramer	
	569	583	Tetramer	Green → Red, 405 nm
mEos2	506	519	Monomer	
	573	584	Monomer	Green → Red, 405 nm
Kaede	508	518	Tetramer	
	572	580	Tetramer	Green → Red, 380 nm
Photoswitchable Proteins				
rsEGFP2	478	503	Monomer	On → Off, 503 nm
				Off → On, 408 nm
Dronpa	503	518	Monomer	On → Off, 503 nm
				Off → On, 400 nm
Dreiklang	511	529	Monomer	On → Off, 405 nm
				Off → On, 365 nm

http://nic.ucsf.edu/FPvisualization/PSFP.html

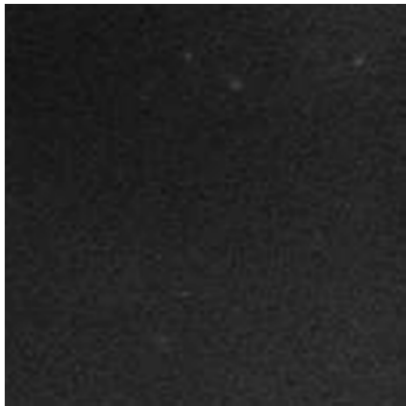
Photoswitchable fluorescent proteins (“Optical Highlighters”)



Sophie Pageon: Molecular signalling in NK cell activation measured with EOS-FP

Photoswitchable fluorescent proteins (“Optical Highlighters”)

Photoswitchable fluorescent proteins in PALM super-resolution microscopy



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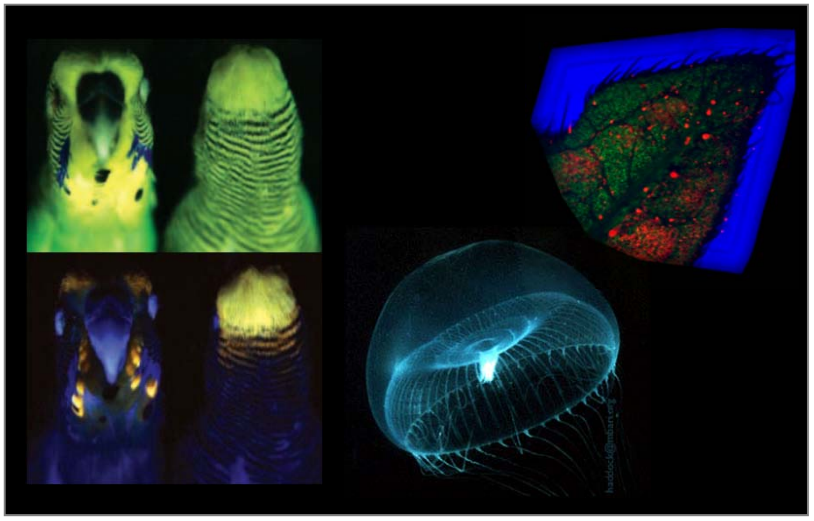
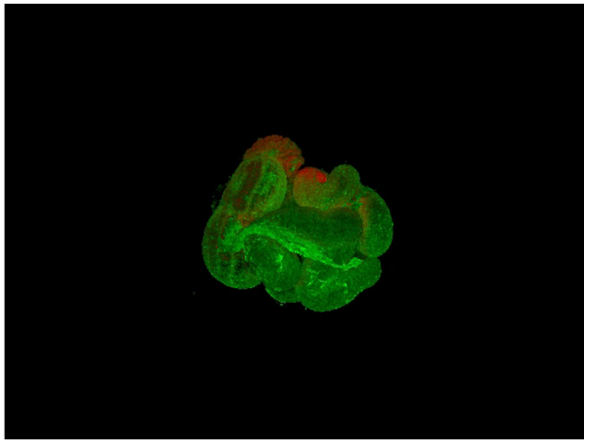
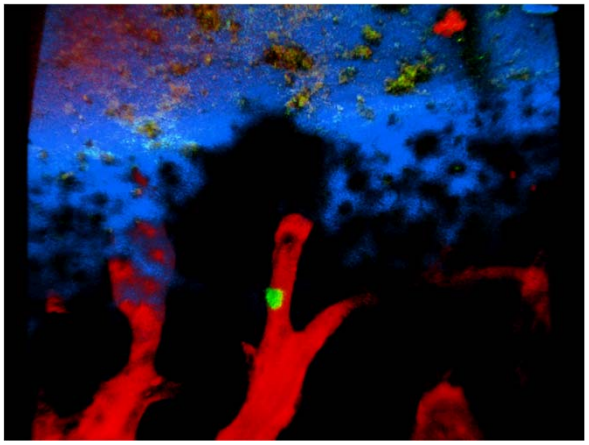
Autofluorescence and label-free imaging



Autofluorescence in nature



Angelos Skodras : **mouse oviduct**



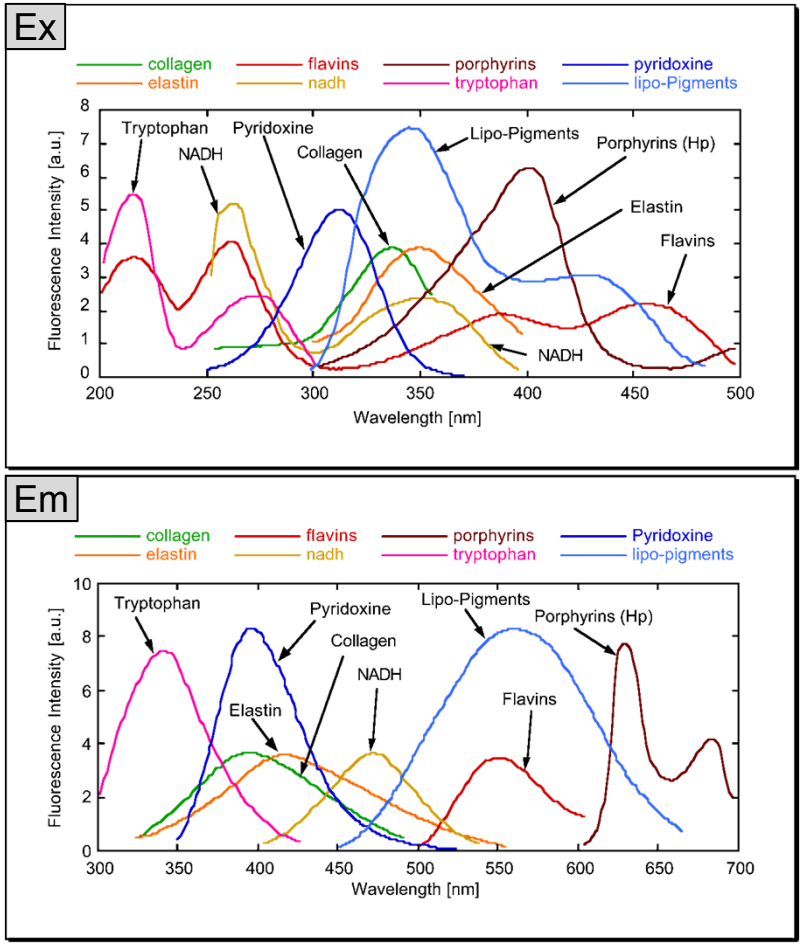
Mark Scott : **Haematopoietic stem cells in the bone marrow**

Autofluorescence and label-free imaging

Sources for autofluorescence

Autofluorescent	Excitation	Emission
Vitamin C	350	430
NAD(P)H	366	440–470
Vitamin D	390	470
Lignin	530	488
Chlorophyll	685	488
Vitamin A	340	490
Collagen and elastin	442	470–520
Flavins	380, 460	520
FMN, FAD	450	530
Lipofuscins	450–490	550
Riboflavin	450-490	500-560
Protoporphyrin IX	442	635

Fluorescence fingerprint of tissue



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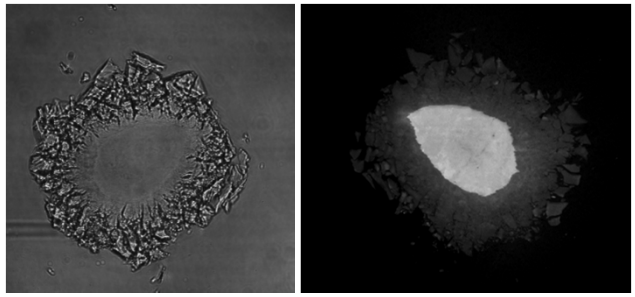
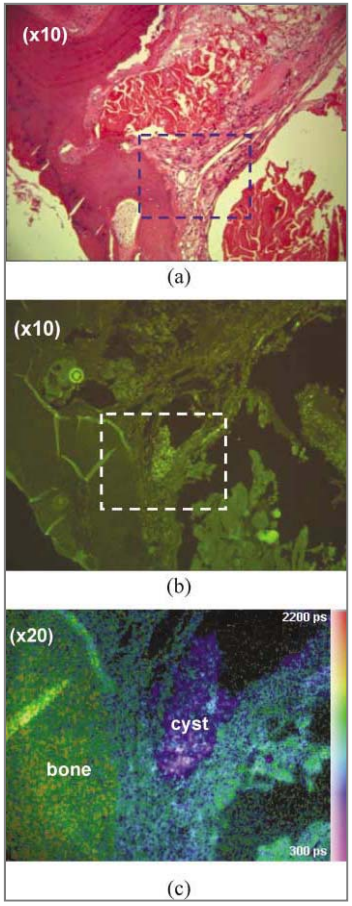
Autofluorescence and label-free imaging

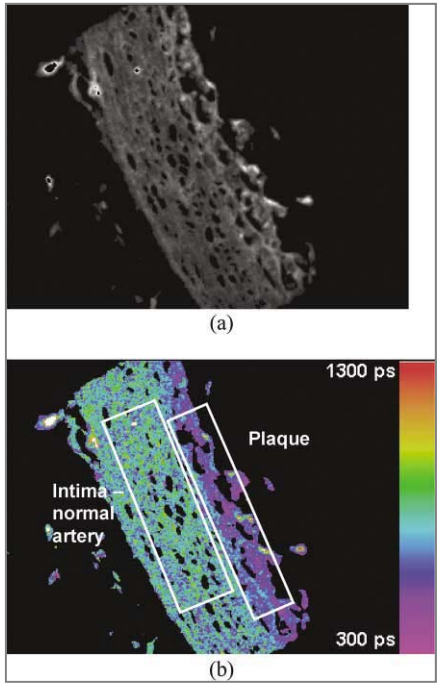
brightfield 3-photon excitation



tryptophan crystals



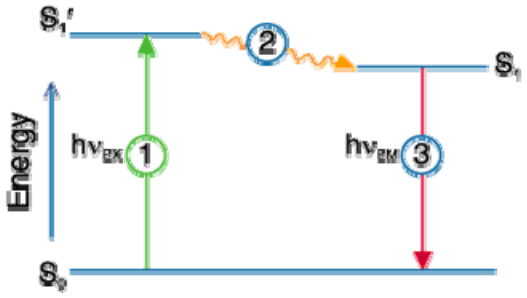
subchondral cyst in femur head



atherosclerotic plaque

Paul French: **Visualisation of disease by autofluorescence**

Fluorescence extended: 2P, SHG, Raman spectroscopy



Jablonski diagram:

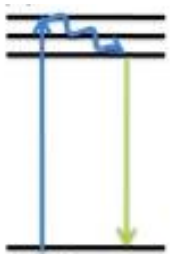
1) excitation *(absorption of light)*
2) relaxation *(generation of heat, proportional to Stoke shift)*
3) emission *(radiation of light)*

Additional interactions between light and matter used for:

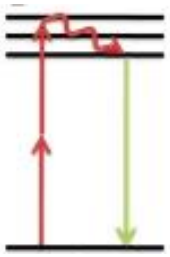
- label-free imaging
- intravital / whole-animal/ medical imaging
- super-resolution imaging

Fluorescence extended: 2P, SHG, STED, STORM Raman spectroscopy

standard fluorescence

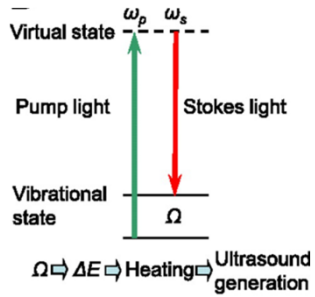


2-photon excitation

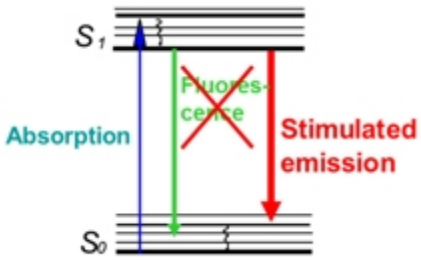


second-harmonic generation

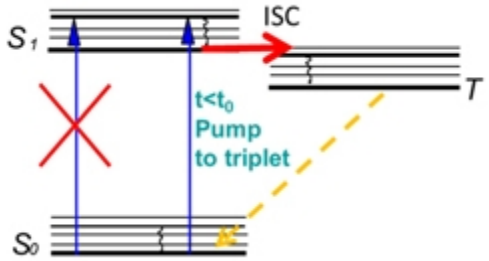
photo-acoustic effect



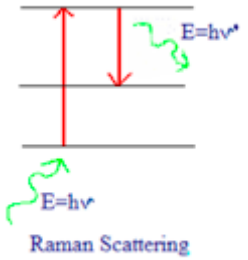
Stimulated-emission depletion (STED)



Ground-state depletion (STORM etc.)



Raman scattering



Thomas A Klar et al 2014 Phys. Scr. 2014 014049
http://www.st-andrews.ac.uk/seeinglife/science/research/Raman/Raman.html

Fluorescence extended: 2P, SHG, STED, STORM Raman spectroscopy

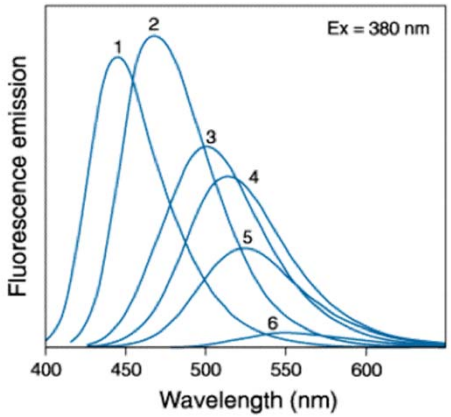
Technology	Principle	Advantage	Application
2-photon	excitation with infrared light	deep tissue penetration	intravital imaging
SHG	2 low-energy photons photons combined to 1 high-energy photon	deep tissue penetration, label-free	intravital imaging, label-free imaging
stimulated emission	wavelength-shift of emission light by depletion laser	depletion of detected emission light	STED super-resolution
ground-state depletion	majority of fluorophores pushed into invisible triplet states	only small fluorophore population visible, spacing within diffraction limit	STORM, GSD and similar super-resolution techniques
Raman spectroscopy	probing of energy levels of molecules (instead of electrons)	label-free, multi-spectral	physiological finger printing (lipids, cholesterol etc.)
photo-acoustic imaging	pressure wave produced by infrared light absorption	low scattering of ultrasound emission wave	deep-tissue and whole-animal imaging

Applications of fluorescence

- **Measuring**
  - intensity
  - single-molecule localisations
  - fluorescence lifetime
  - polarisation
- **Sensing**
  - molecular environment
  - enzymatic activities
  - molecular interactions
- **Light as a tool**
  - phototoxicity
  - light-induced localisation
  - optogenetics

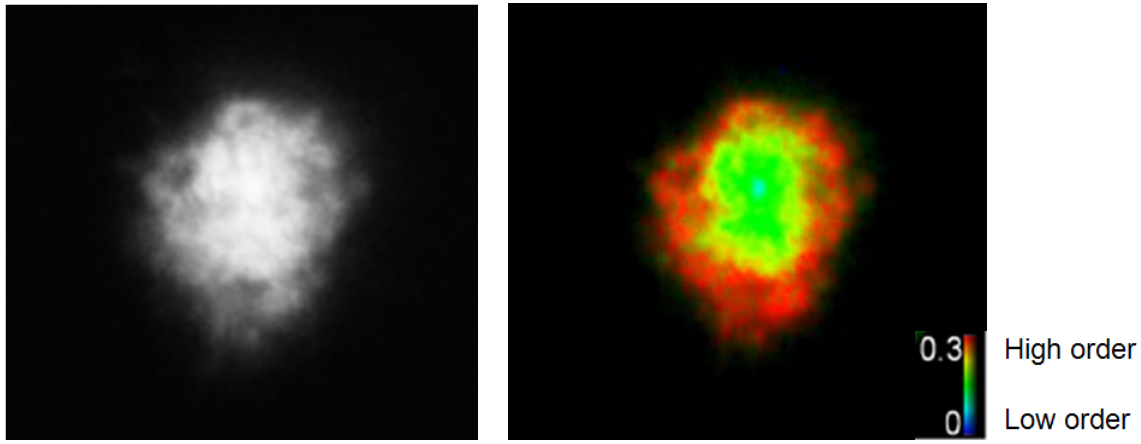
Fluorophores as sensors

Fluorescence can change with the environment!



Fluorescence spectrum of Badan in
1) toluene
2) chloroform
3) acetonitrile
4) ethanol
5) methanol
6) water

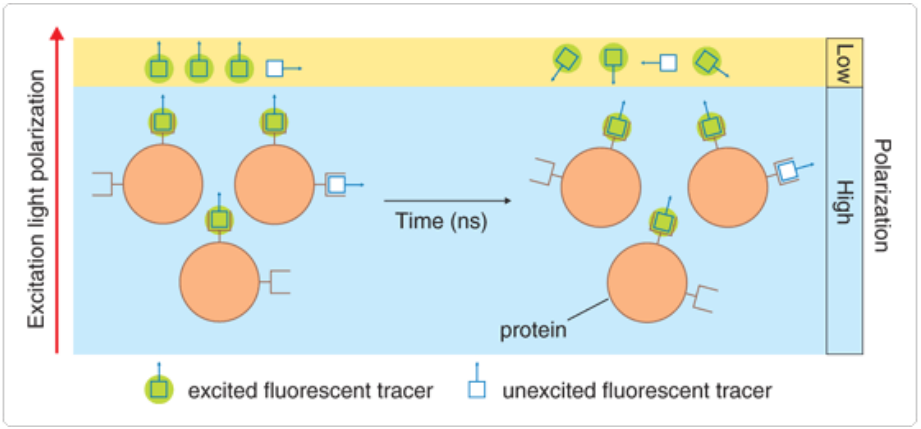
Fluorophores as sensors: membrane lipid order



Visualisation of membrane fluidity by FLIM of di-4-ANEPPDHQ

Dylan Owen, Mark Neil , Paul French, Anthony Magee,
Seminars in Cell & Developmental Biology 18 (2007) 591–598

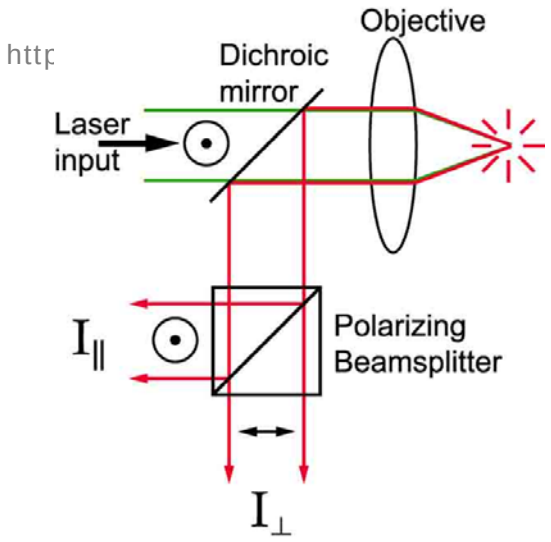
Fluorophores as sensors: viscosity (fluorescence polarisation anisotropy)



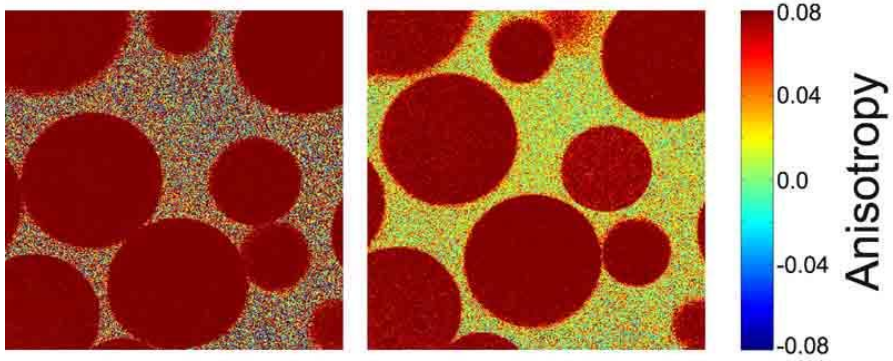
$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

$I_{\parallel}$ = *fluorescent intensity parallel to the excitation plane*

$I_{\perp}$ = *fluorescent intensity perpendicular to the excitation plane*



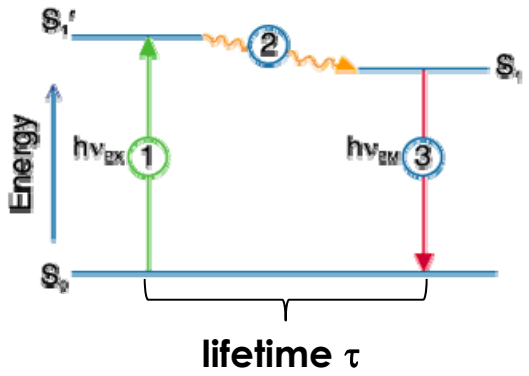
Bodipy-FL conjugated to sepharose beads

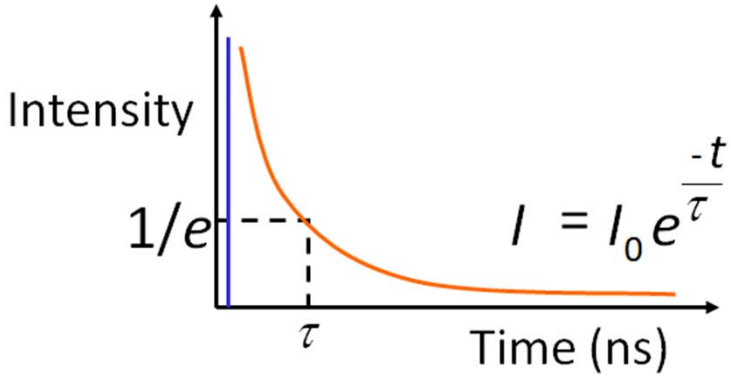


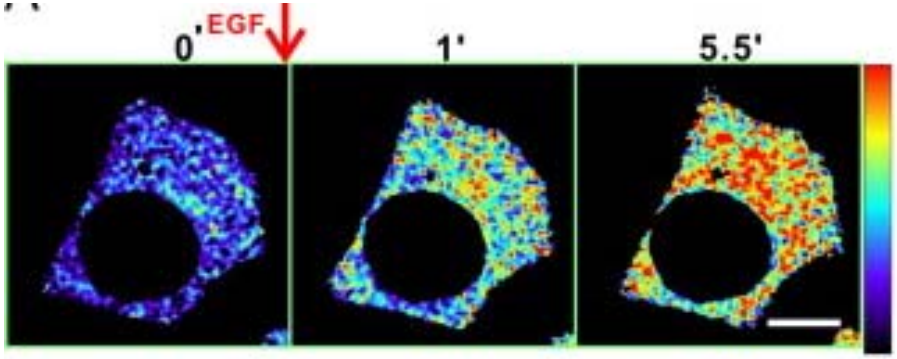
control + protease K

http://www.urmc.rochester.edu/smd/rad/foster

Fluorophores as sensors: Fluorescence Lifetime Imaging (FLIM)

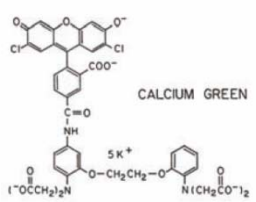


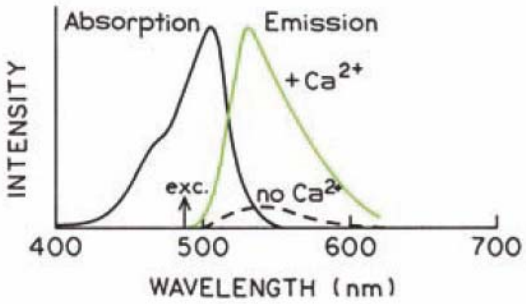




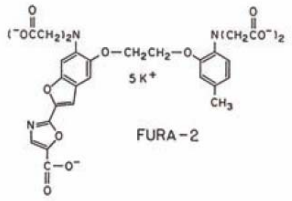
Fluorophores as sensors: environmental conditions

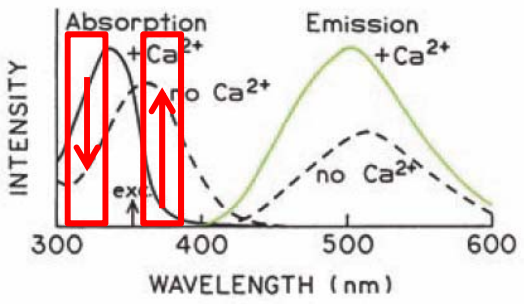
Ca^{2+}: intensity



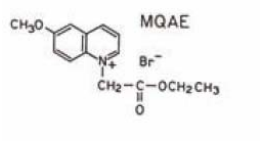


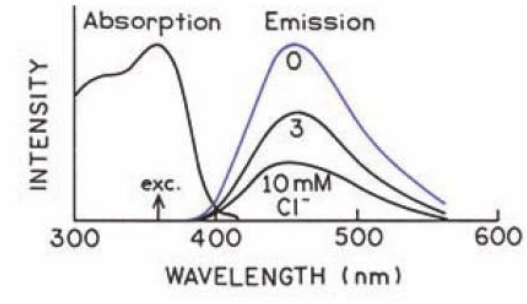
Ca^{2+}: ratiometric



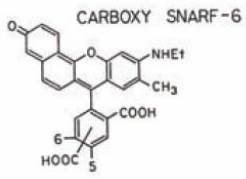


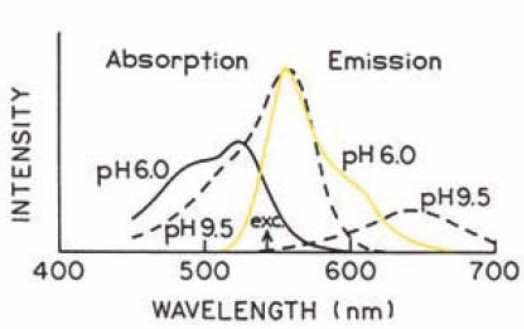
Cl^-



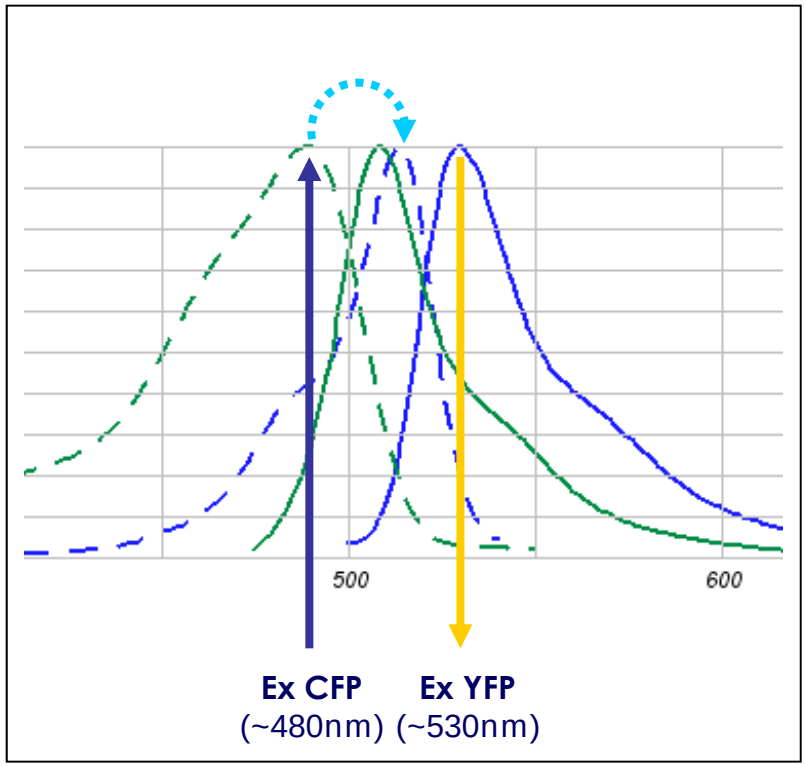


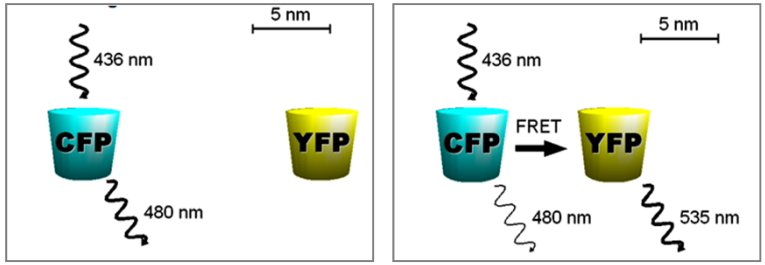
pH

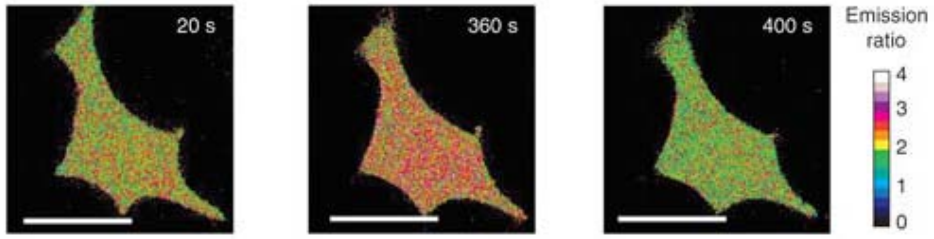




Fluorophores as sensors: Molecular interaction and Fluorescence (Förster) Resonant Energy Transfer







FRET

FRET efficiency: $E = \frac{1}{1 + (r/R_0)^6}$

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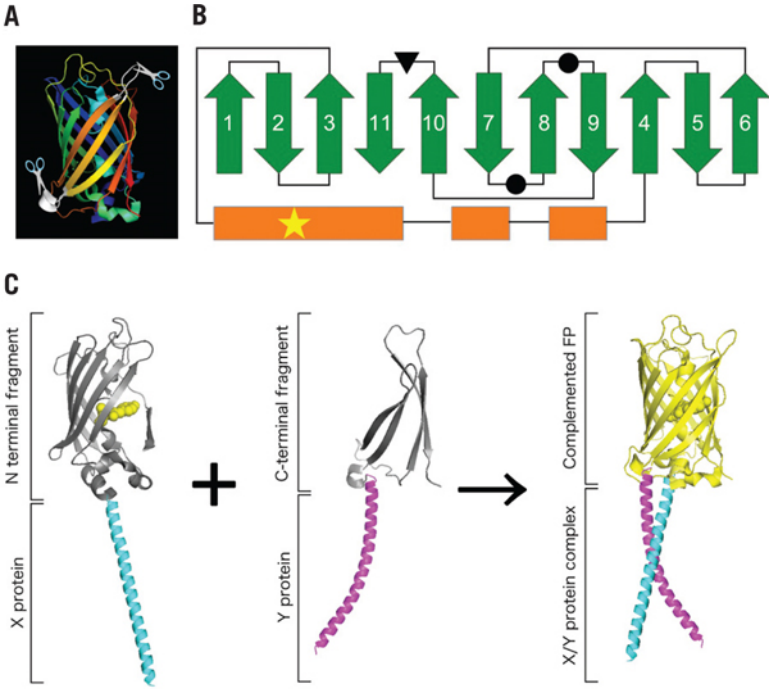
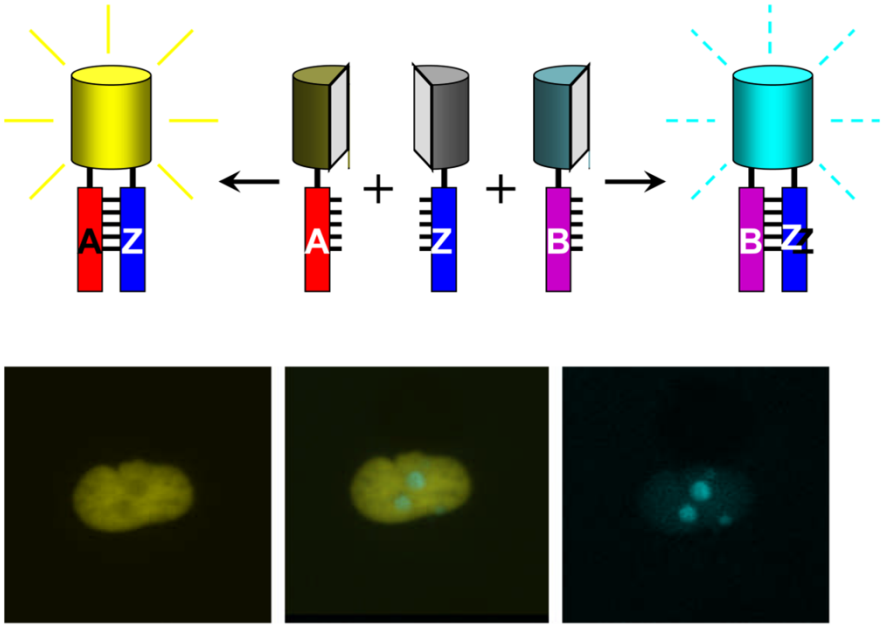
Fluorophores as sensors: molecular interaction and Bi-molecular Fluorescence Complementation (BiFC)

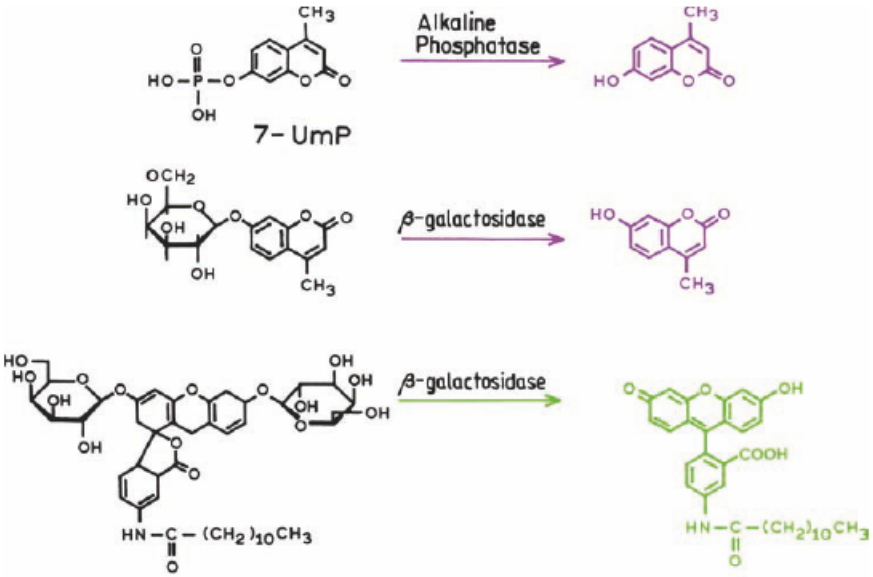


Kodama Y, Hu CD.
Biotechniques. 2012; 53(5): 285-98



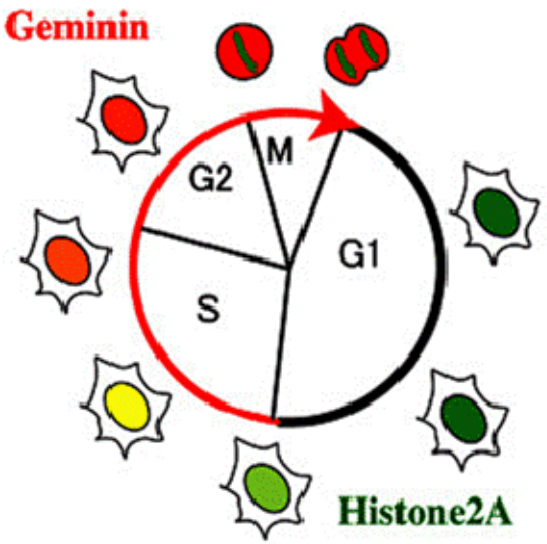
Kerppola TK.
Annu Rev Biophys. 2008; 37: 465-87

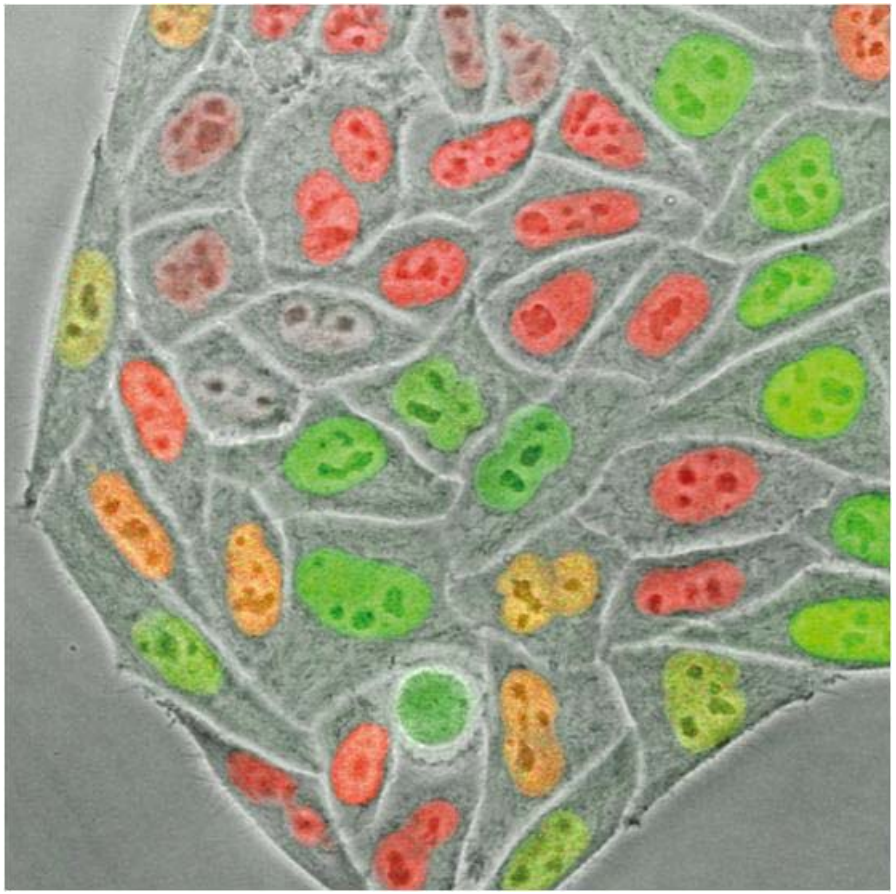
Fluorophores as sensors: enzymatic activity



Fluorophores as sensors: cell cycle

Fucci cell cycle reporter





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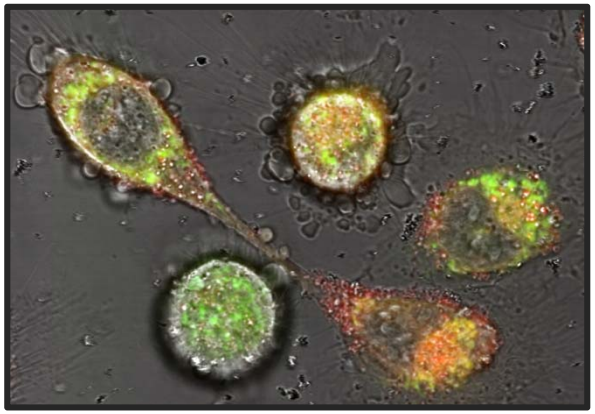
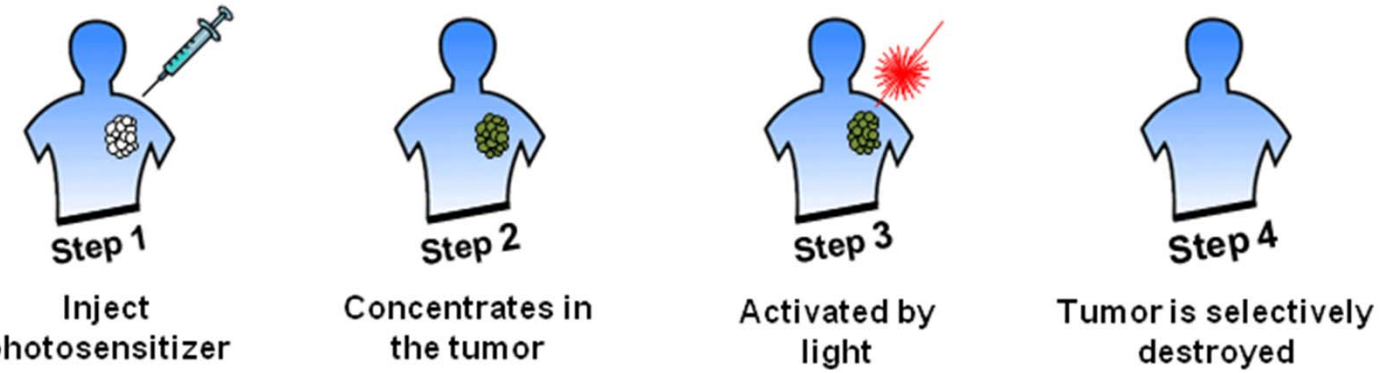
Fluorophores as tools: phototoxicity

Phototoxin-induced apoptosis

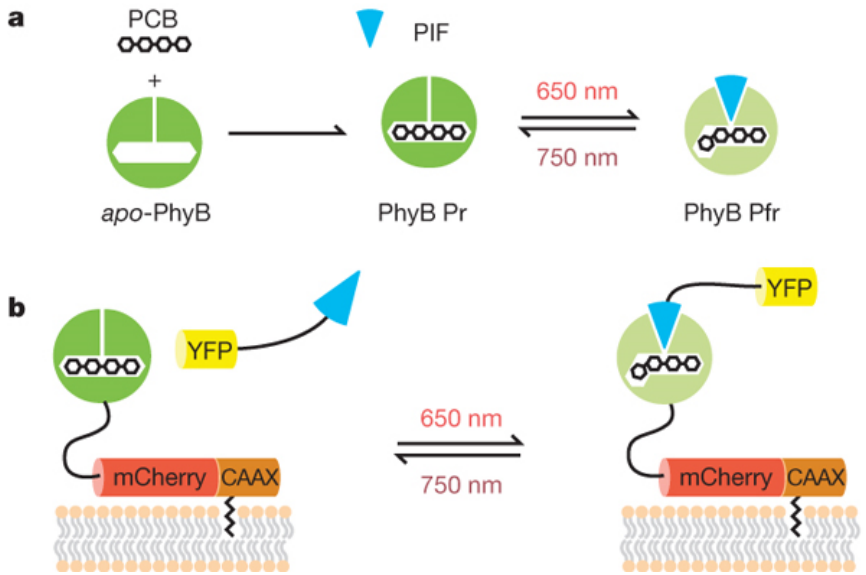


Ioanna Stamati, Imperial College London

Photo-dynamic cancer therapy



Fluorophores as tools: light-controlled interactions



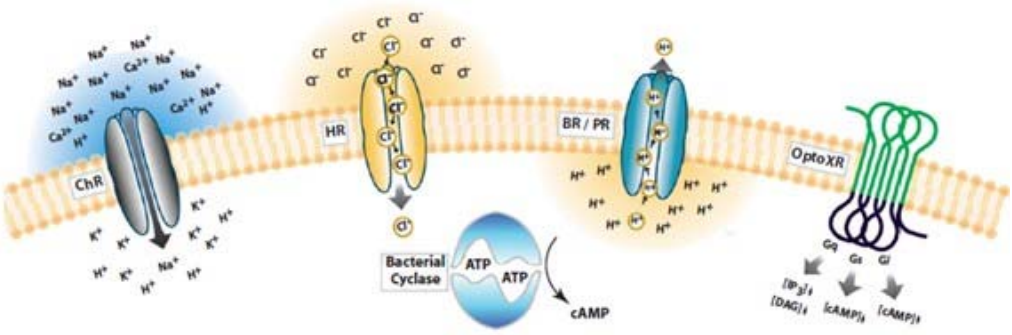
Light-controlled interaction of Phytochrome B and PIF

Levskaya1,2,3 et al, Nature 461, 997-1001 (15 October 2009

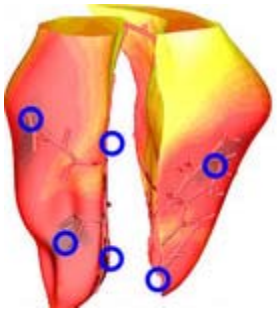
Fluorophores as tools: optogenetics

- light-controllable channels based on bacterial channelrhodopsin-2 (ChR2)
- light can induce opening or closing of channels

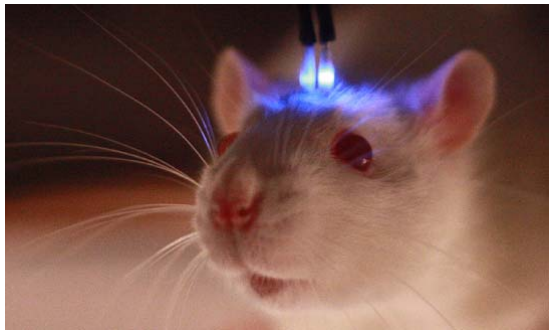
Light-controllable channels



light-induced cardiac arrythmia



light-induced neuronal stimulation



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