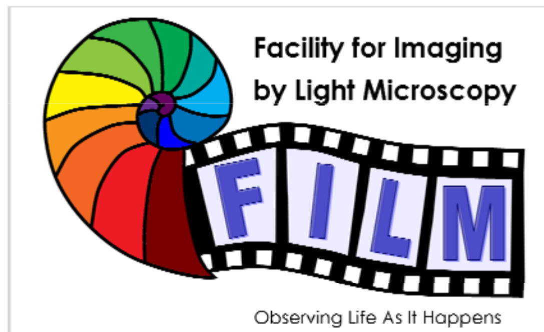
Imperial College London

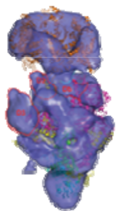
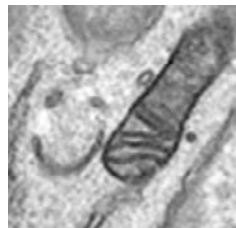
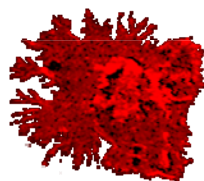
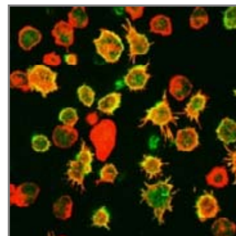
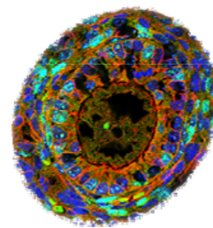
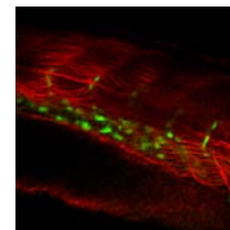


MICROSCOPY DAY 2011:

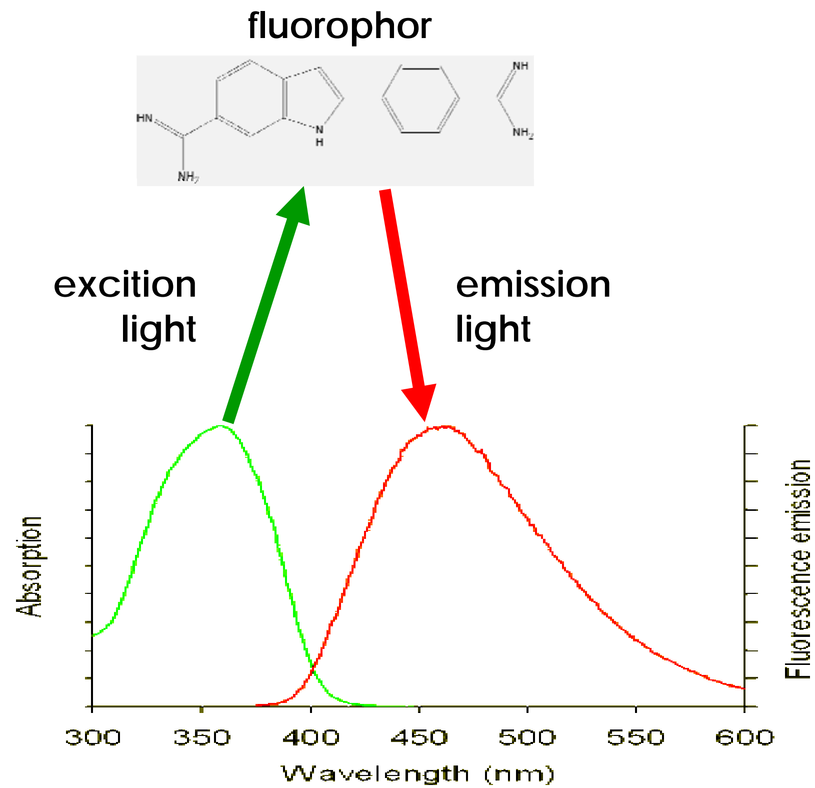
Principles of fluorescence

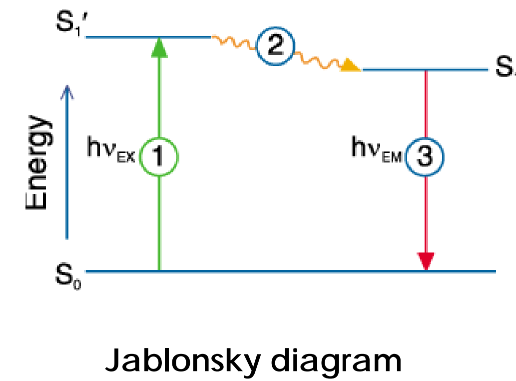
Martin Spitaler

- the fluorescence process
- technical considerations for epifluorescence microscopy
- fluorophores:
  - chemical fluorophores:
    - labels
    - environmental sensors
  - quantum dots
  - antibody labelling
- fluorescent proteins:
  - variants
  - switchable FPs
- nanobodies
- autofluorescence

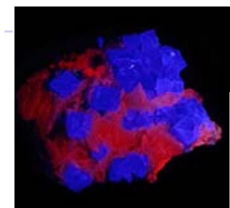
The fluorescence process



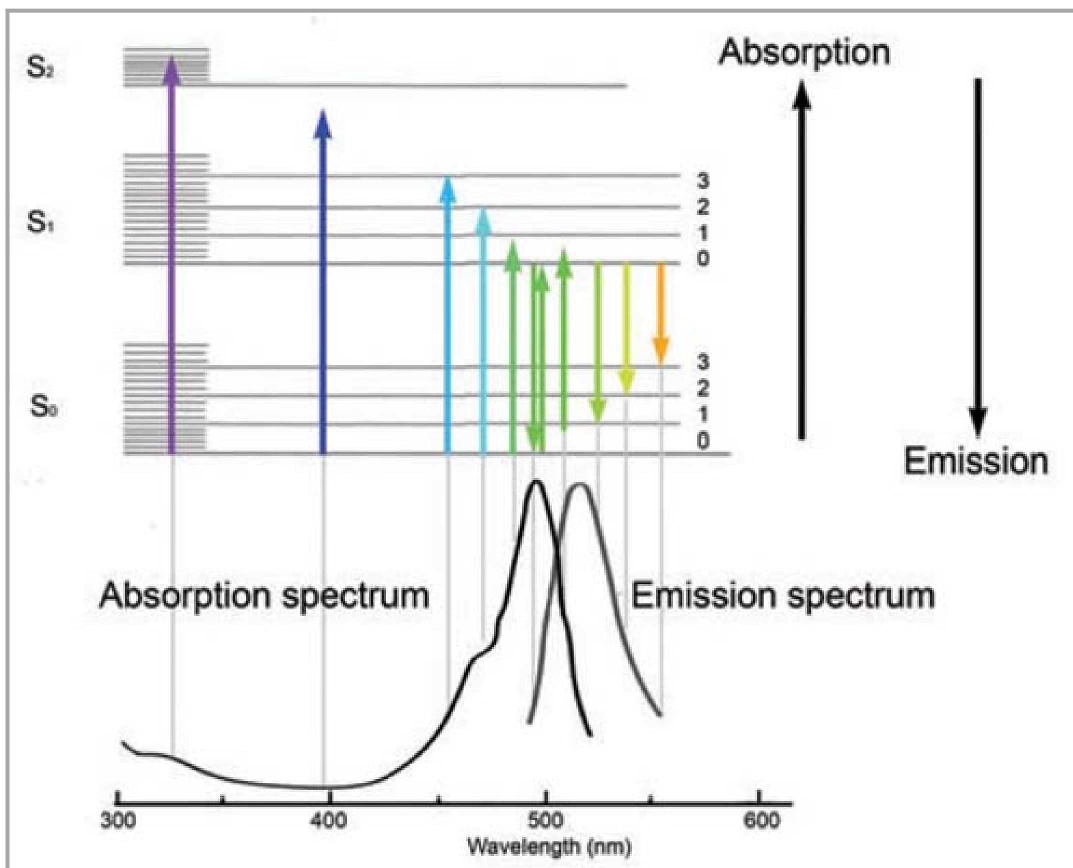


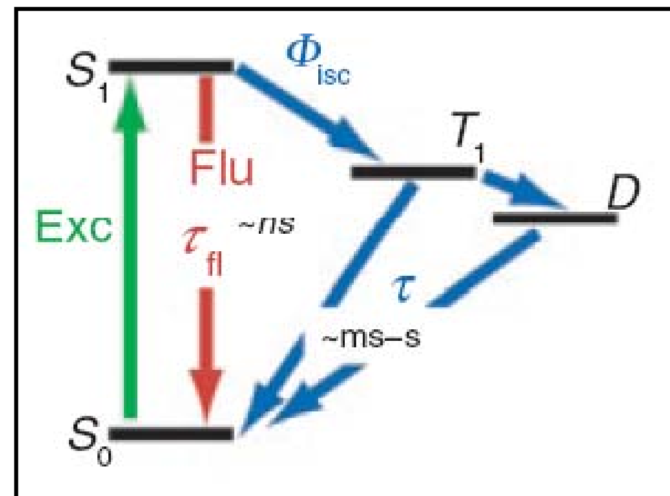
Jablonsky diagram

"fluorescence": named by George Gabriel Stokes (1852) after the mineral fluorite which lights up when illuminated with UV



The fluorescent process

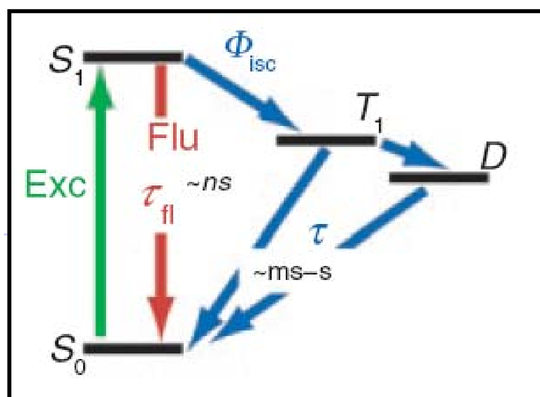




source: Nature_Meth._5_943-945

Factors affecting intensity of fluorescence images:

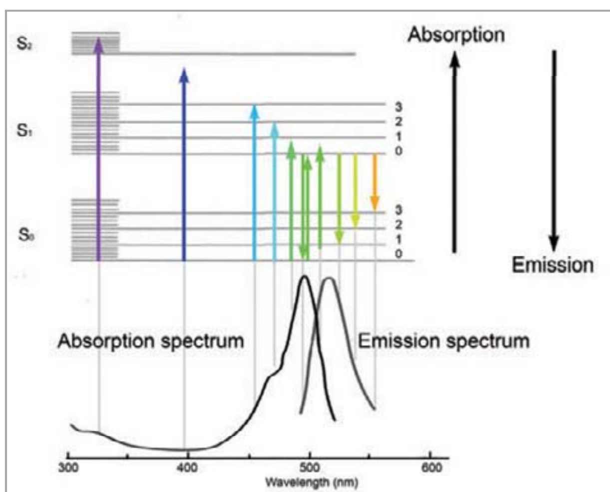
- wavelengths of excitation and detection
- intensity of excitation light
- quantum efficiency of fluorophore
- saturation of fluorophore (transition to dark states) - photon output in linear range
- resonant energy transfer partners (fluorescent = Fret, absorbing = quenching)
- oxidating / reducing agents in medium

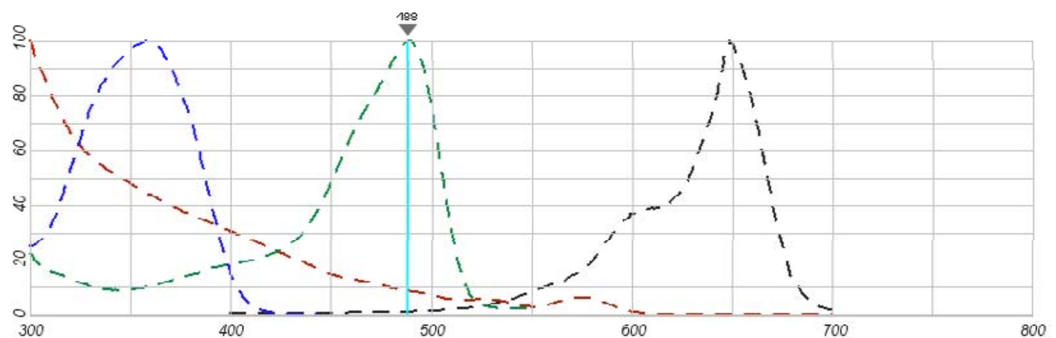


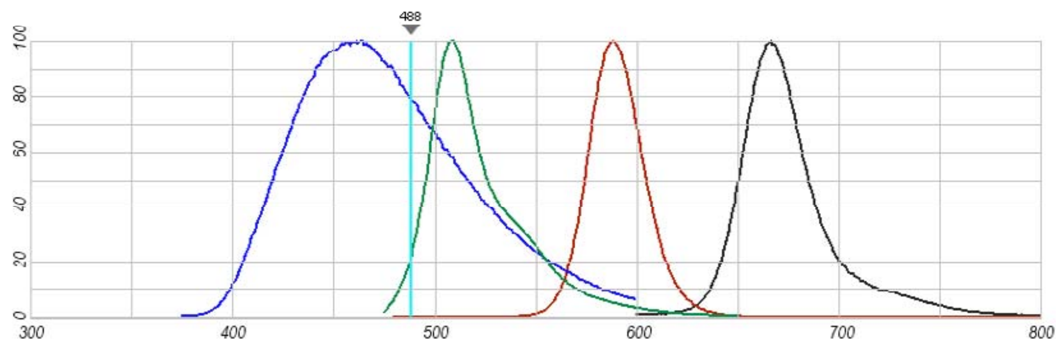
Quantum efficiency

$$\Phi = \frac{\#\ \text{photons emitted}}{\#\ \text{photons absorbed}}$$

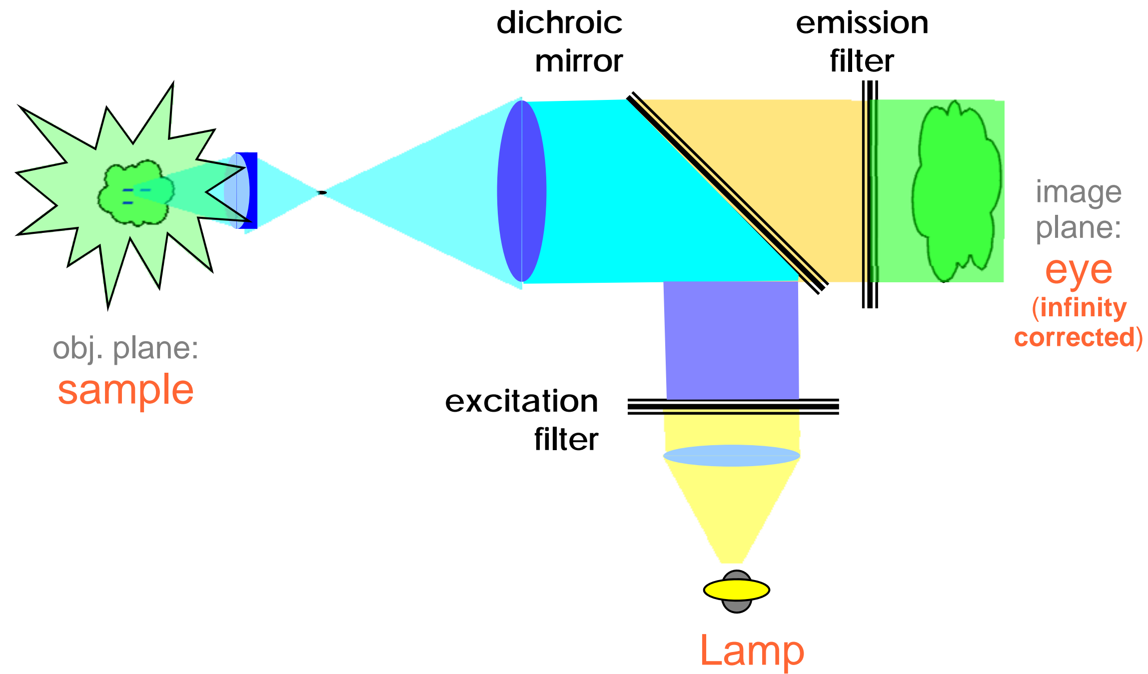
The fluorescent process







Epifluorescent instrumentation



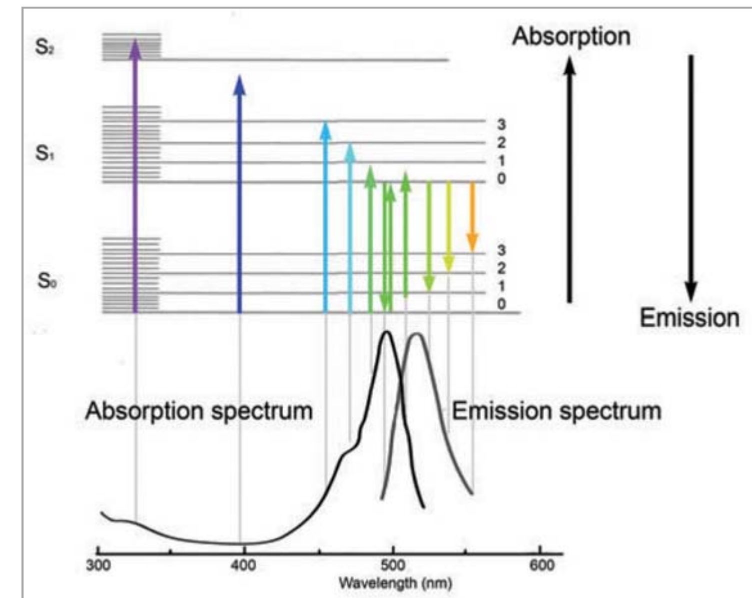
Invented 1965 by Johan Ploem

Advantages of fluorescence:

- excellent signal-to-noise ratio (black background)
- linear correlation fluorophore molecules / signal output
- large number of fluorophores
- additional parameters (FLIM, FRET)

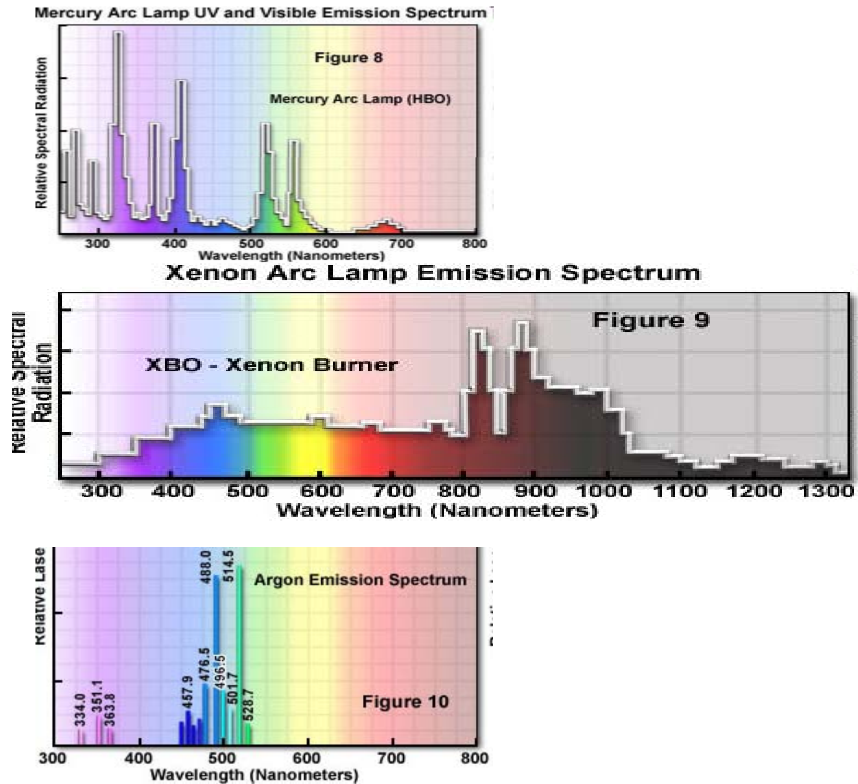
Limitations of fluorescence:

- limit of linear intensity
- no intensity correlation between fluorophores (different quantum efficiency, bleaching, wavelength, ...)
- bleaching and dark states
- high-energy light (phototoxicity / mutagenicity)
- autofluorescence

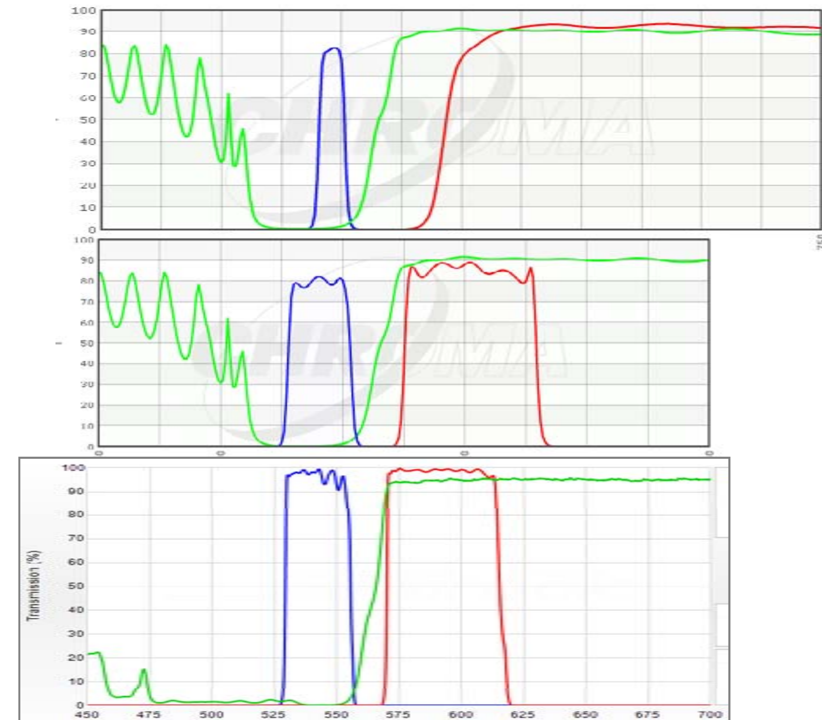


Technical considerations for epifluorescence microscopy

Excitation light spectra



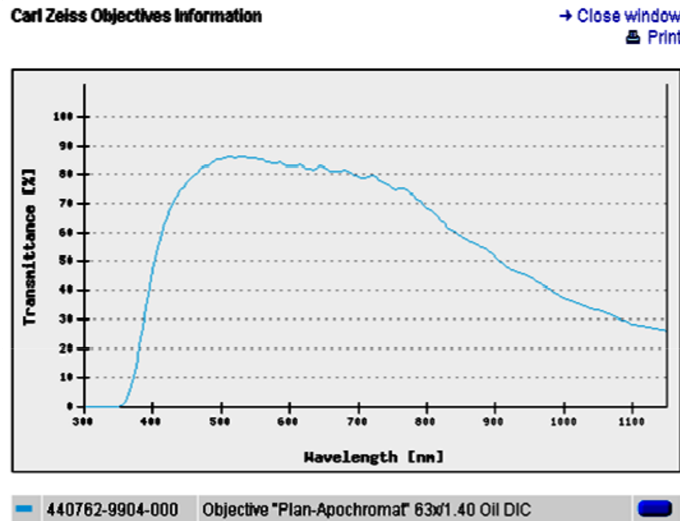
Filter set spectra



http://micro.magnet.fsu.edu/primer/anatomy/sources.html

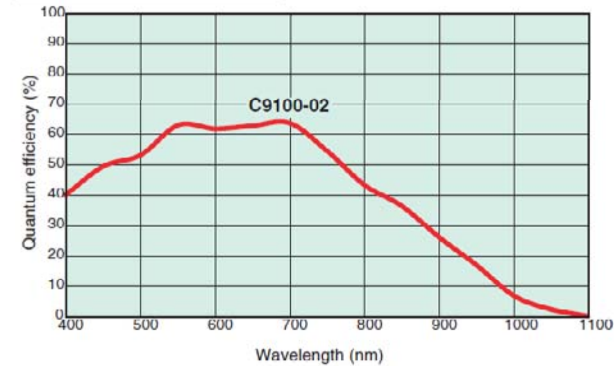
Technical considerations for epifluorescence microscopy

Transmission spectrum of typical objective

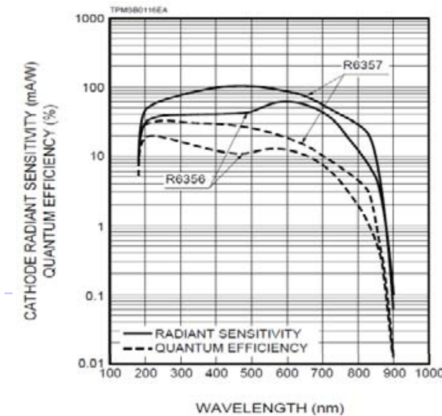


Spectral sensitivity of detectors

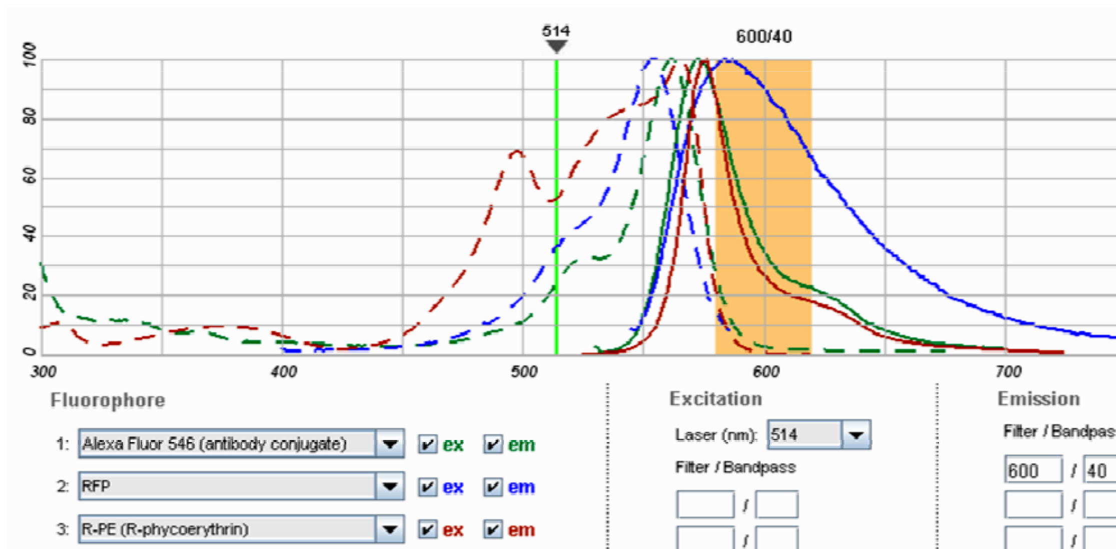
CCD camera (widefield)

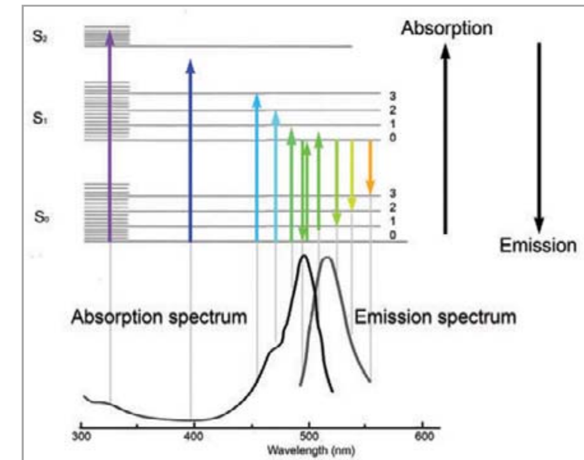


PMT (confocal)

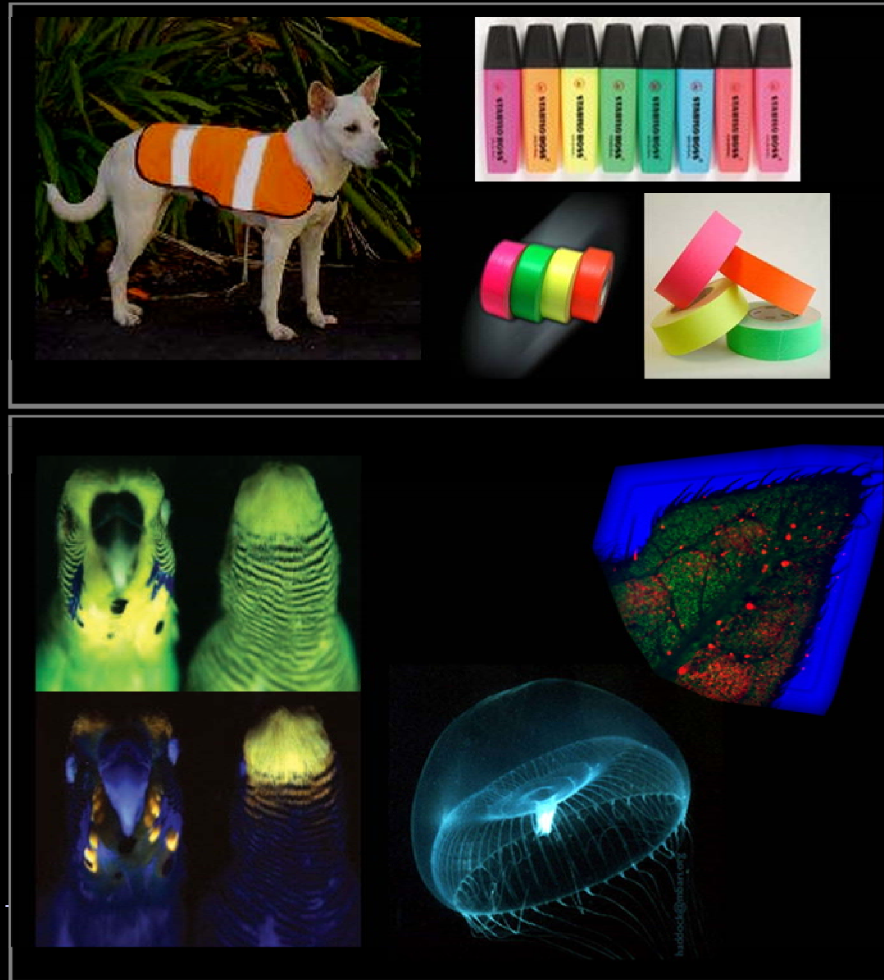


Technical considerations for epifluorescence microscopy





Fluorophores

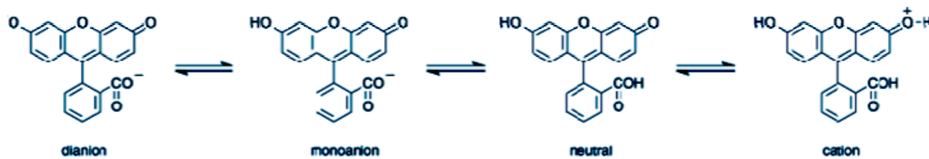


Structural basis of fluorophores:

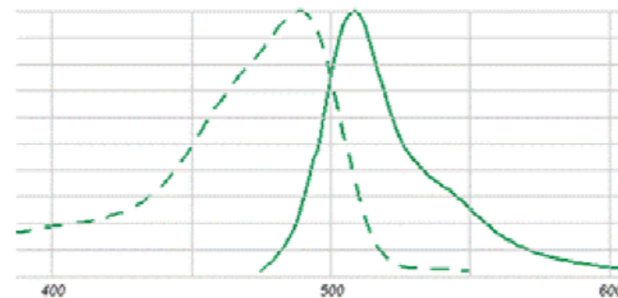
- conjugated double bonds acting as 'antenna'

Important consideration for microscopy:

- quantum efficiency
- photostability (essential for microscopy, irrelevant for FACS)
- chemical stability
- (no) binding to cellular structures, binding specificity
- autoquenching
- variability of fluorescence (e.g. with pH, …)



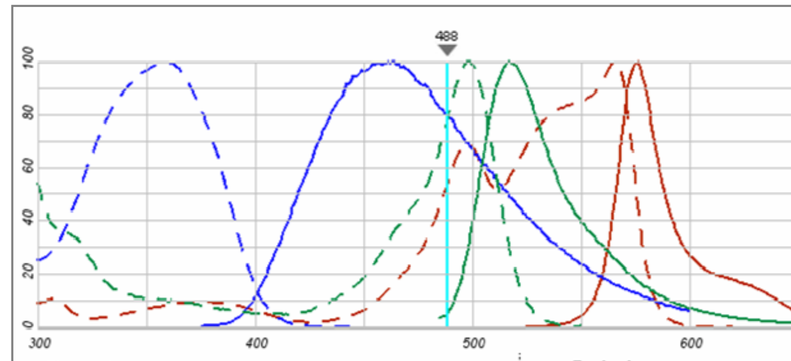
fluorescein



Fluorophores: Chemical fluorophores

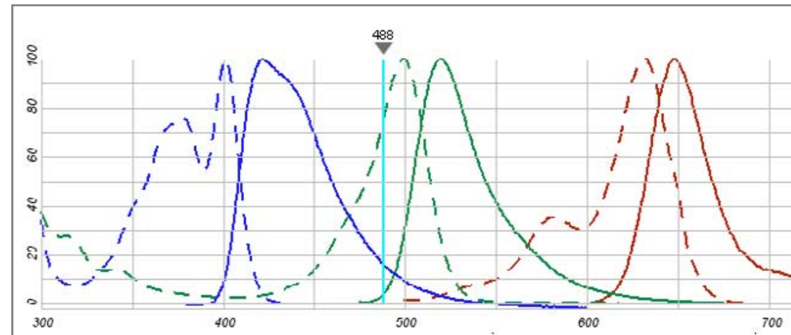
traditional fluorophores:

Dapi, fluorescein, PE, …



'designer' fluorophores:

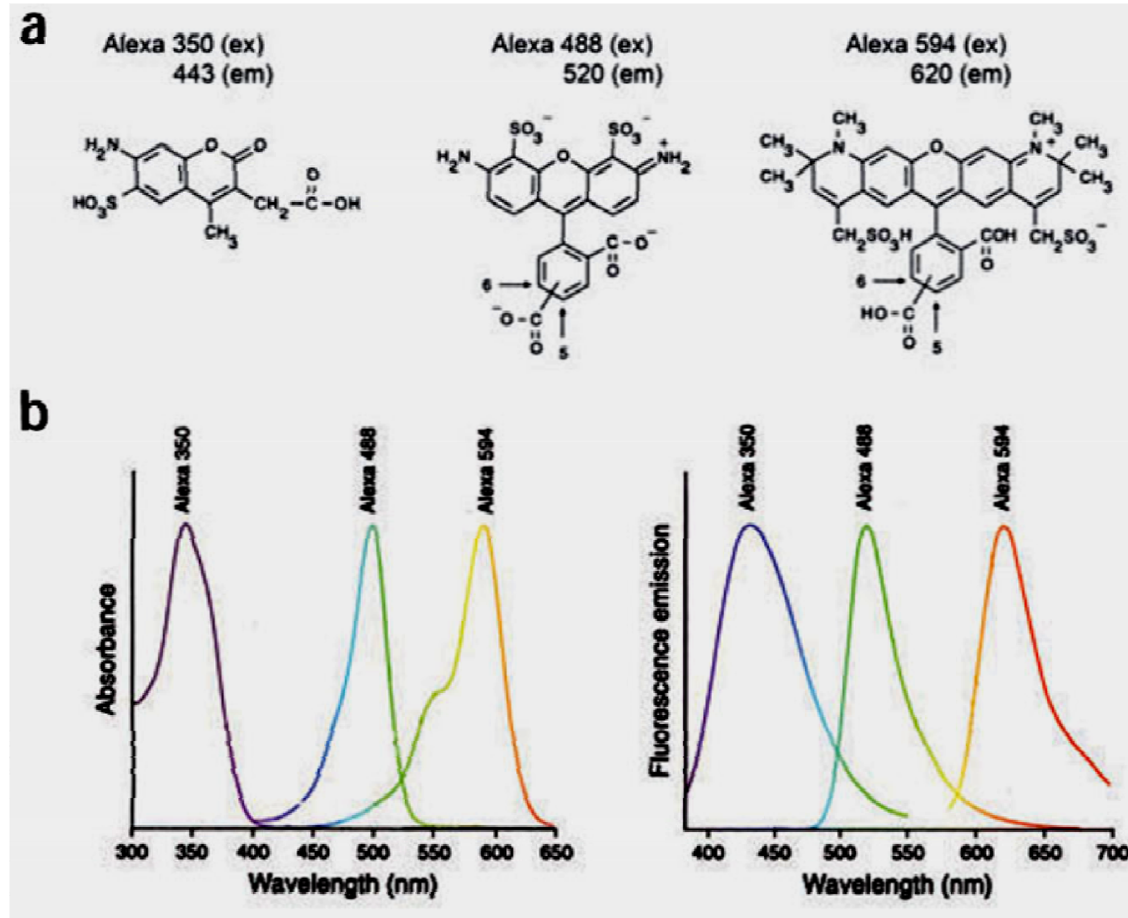
Cy2, Cy3, Cy5, Alexa dyes, Draq5, Atto, …



- chemical stability
- photostability
- bright (high QE)
- narrow excitation and emission peaks
- no unspecific binding
- measurable lifetime (~1-10 nsec)
- suitable for super-resolution (STORM, STED)

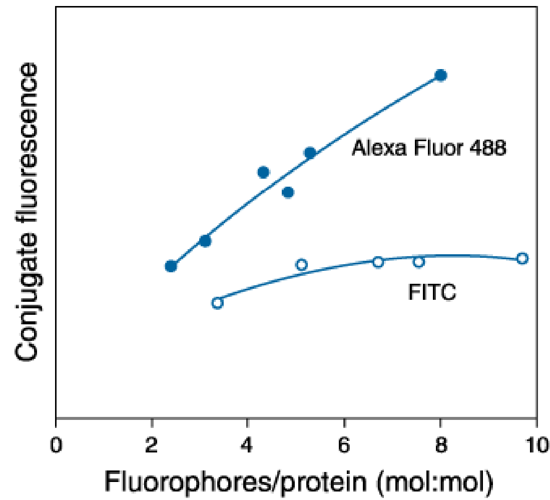
- for live imaging:
- cell permeability
- non-toxic

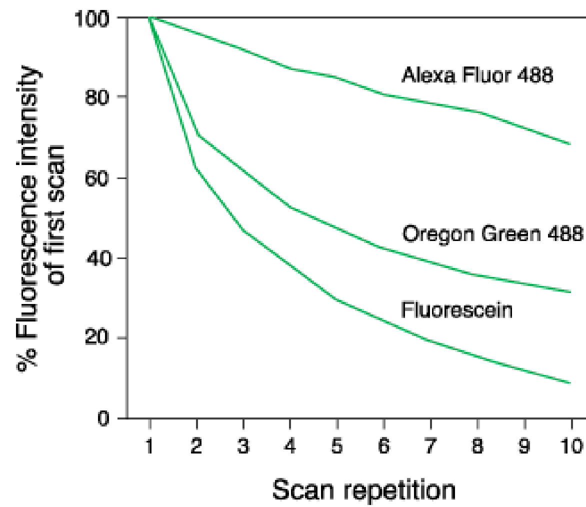
Fluorophores: Chemical fluorophores

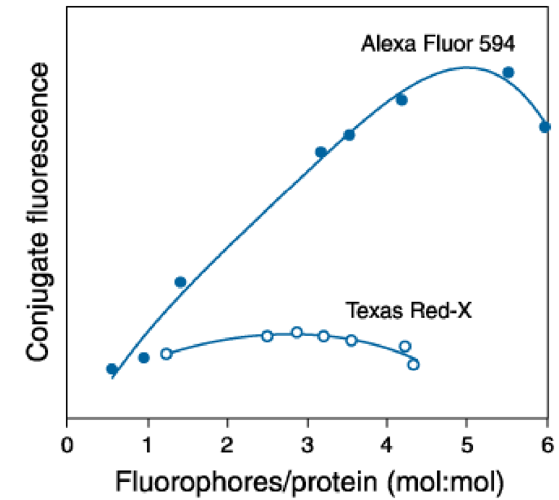


Nature Methods 2, 910 - 919 (2005)

Fluorophores: photochemical properties







quantum efficiency **bleaching** **auto-quenching**

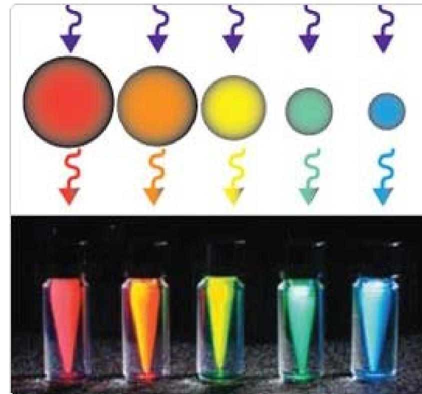
Nature Methods 2, 910 - 919 (2005)

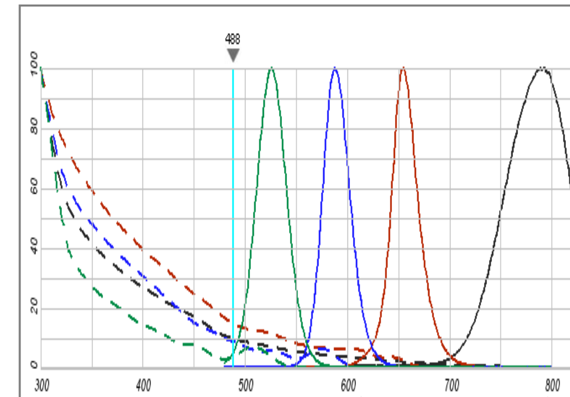
Advantages:

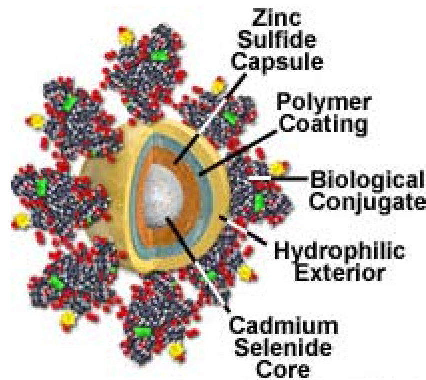
- Tunable to any wavelength
- Large Stokes shift
- Extreme photostability
- Visible in EM (correlative microscopy)

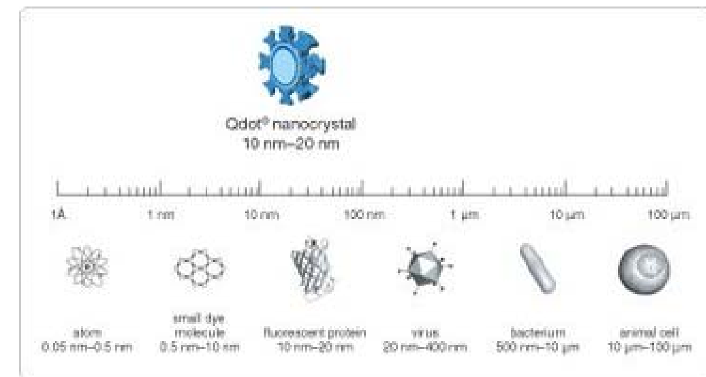
Disadvantages:

- Blinking
- Quenched by DAPI, antifade mounting media, …
- Large size

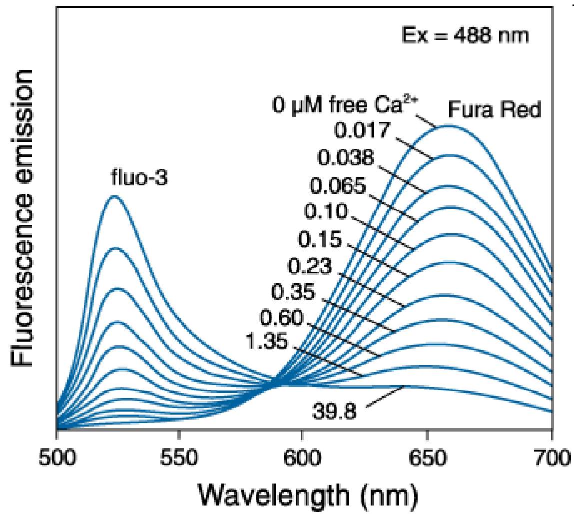




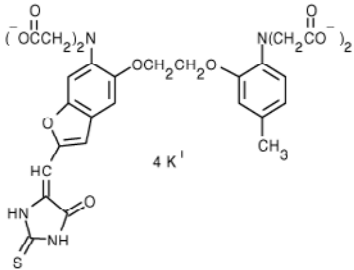


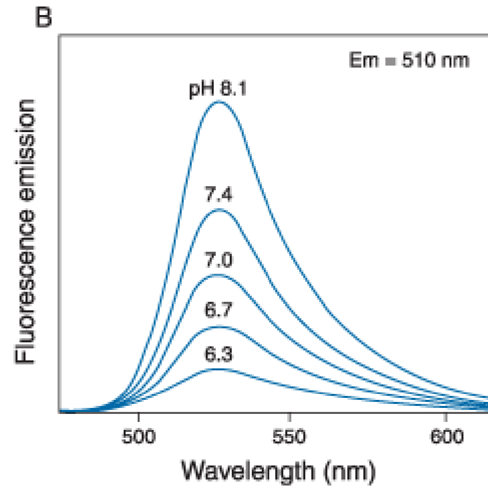


Fluorophores: Environmental sensors

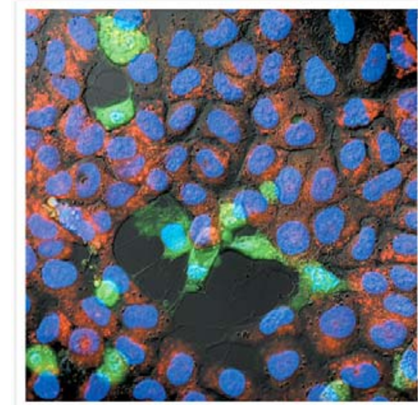


Ca^{2+} indicator Fura-Red





pH indicator BCECF



[image: Molecular Probes]

apoptosis indicator rhodamine 110, bis -L-aspartic acid amide

Fluorophores: Environmental sensors

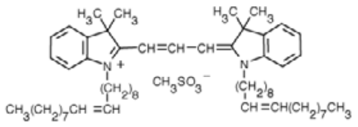
Physiological events that can be measured with fluorecent indicators:

- ion concentrations (calcium, zinc, copper, iron, mercury, …)
- pH
- reactive oxigen, nitric oxide
- membrane potential
- cell viability, apoptosis
- membrane microdomains (‘lipid rafts’)
- uptake (phagocytosis, endocytosis, receptor internalisation, transferrin, glucose, …)
- …

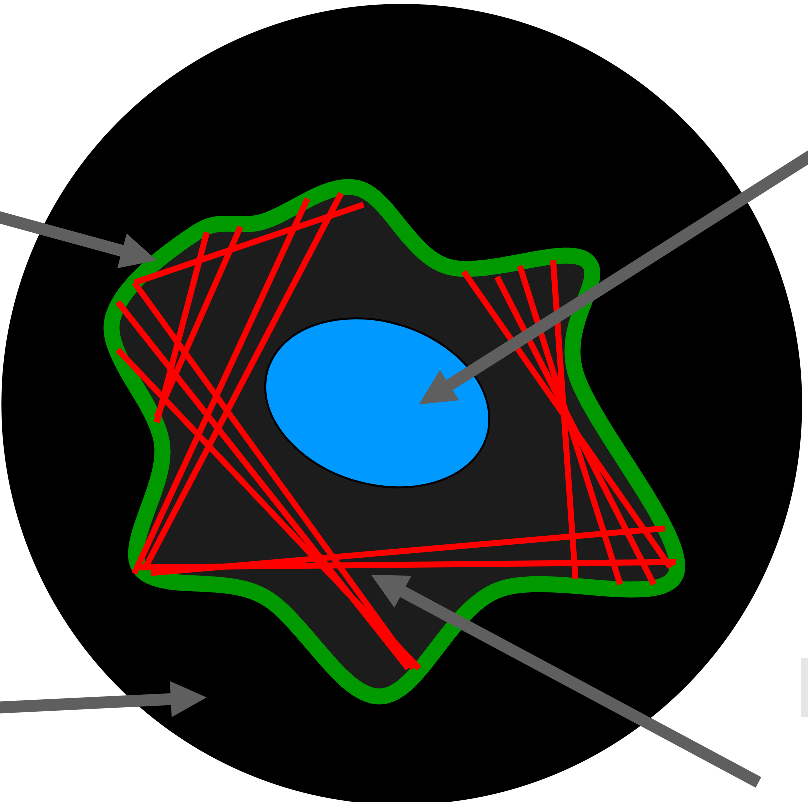
Fluorescent staining: Fluorophores with binding specificity

Lipophilic dyes

lipids (membrane)

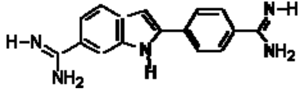


DiI



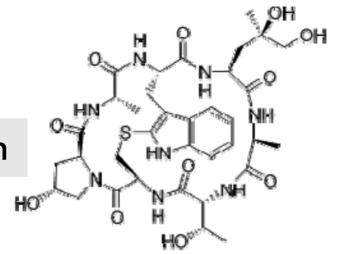
DAPI, DRAQ5

DNA (nucleus)



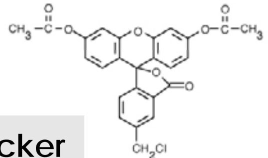
DAPI

phalloidin



Cytosolic dyes

membrane-permeable, intracellularly modified (e.g. by GST) and trapped

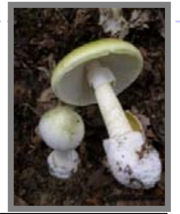


Celltracker

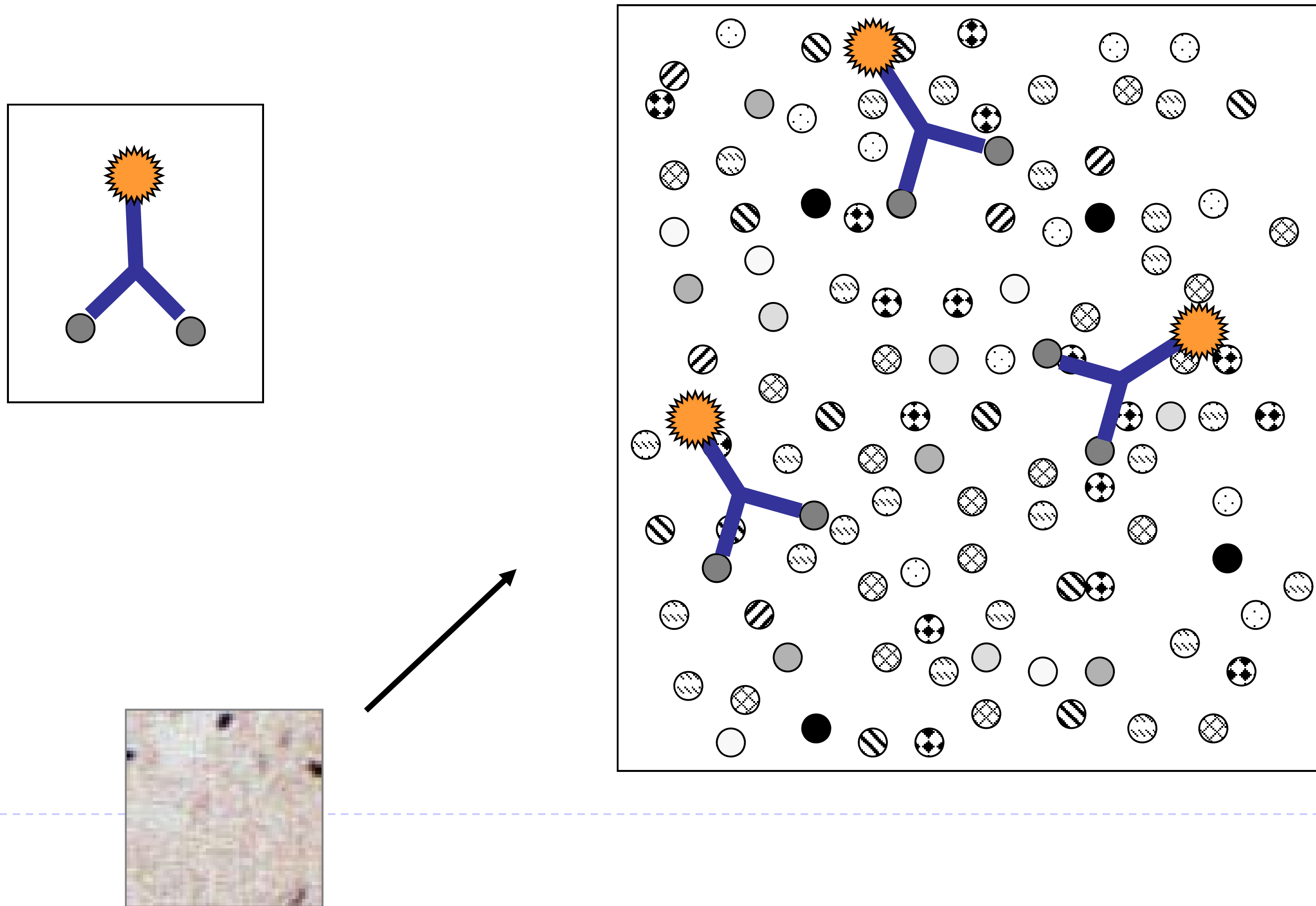
Rhodamine phalloidine

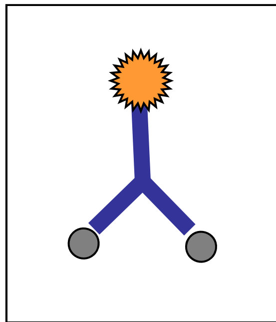
polymerised actin (cytoskeleton)

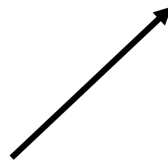
Death cap \ (Amanita phalloides)

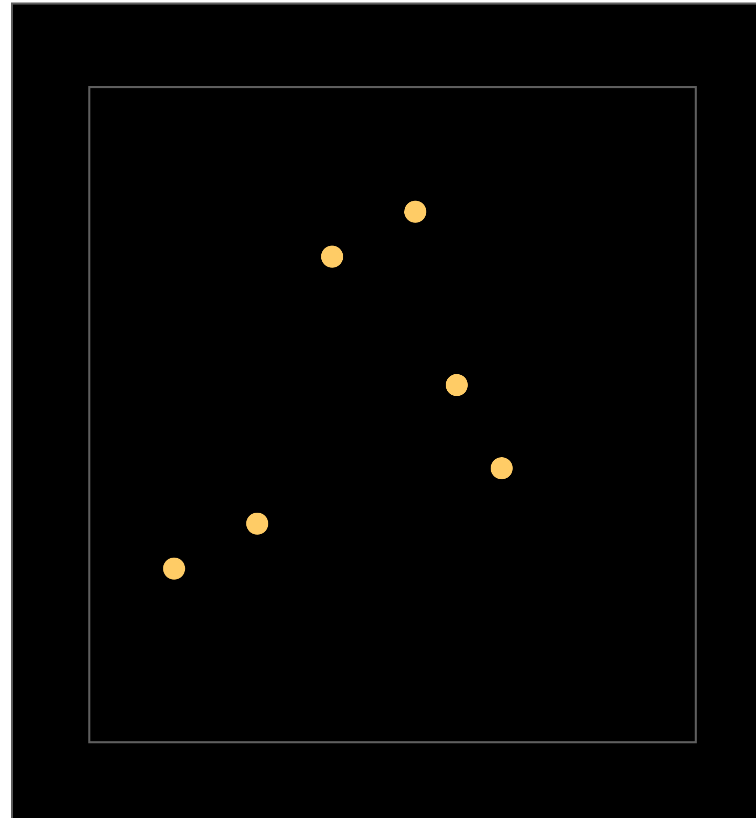


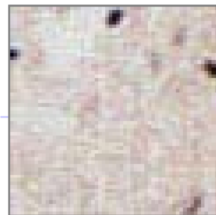
Fluorescent staining: Antibodies











Antibody labelling – important considerations:

- Antibodies are large proteins → unspecific binding of 1st and 2nd antibody (good blocking!)
- Must detect native protein (not denatured, as for western blots)
- Must only detect one single protein (no size control as in western blot)
- For quantifications: good quality, reliable ratio fluorophores per antibody
- labelling density:
  - too dense → autoquenching
  - important for single-molecule analysis, STORM

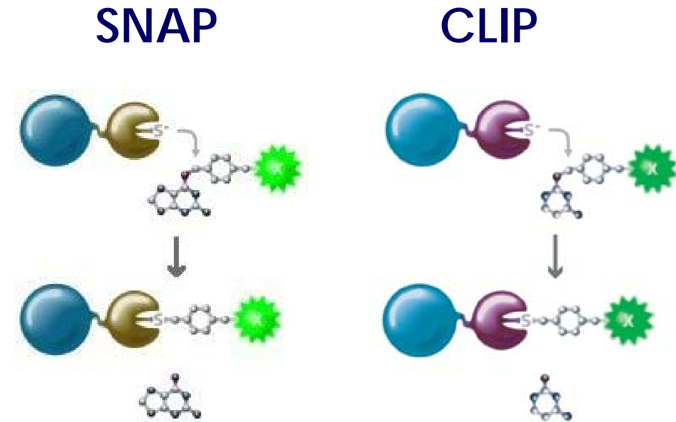
Controls

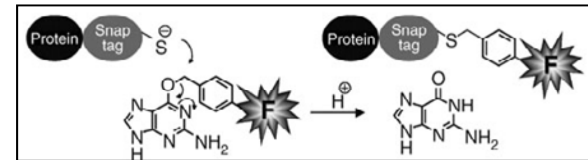
- No primary antibody (unspecific background from secondary)
- Positive **control**: high expression of protein in question (full staining)
- Negative **control**: no expression of protein in question (full staining)
- If possible: GFP fusion of protein in question – GFP and antibody must give same staining (ideal: induced translocation)

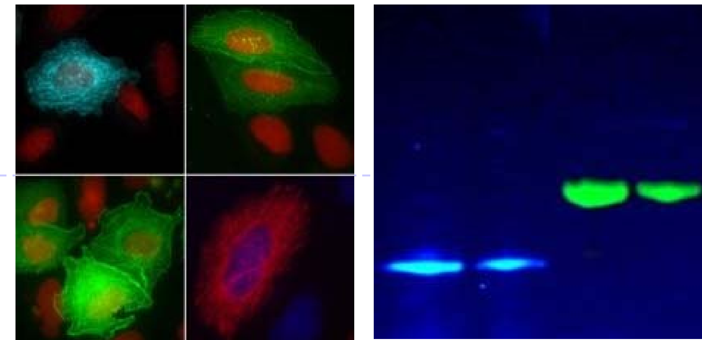
Fluorescent staining: Direct labelling

Click chemistry

- Small tag (20 kD)
- based on intracellular enzymes (e.g. DNA repair enzyme O6 –alkylguanine-DNA-alkyltransferase)
- monomeric
- requires incubation of only 10 min
- specifically reacts with its benzylguanine (BG) substrates
- covalent, thus highly stable thioether bond
- substrates membrane-permeable
- different chemistry / specificity of SNAP and CLIP, so can be labelled with different colours
- excitation wavelegths 360-750nm

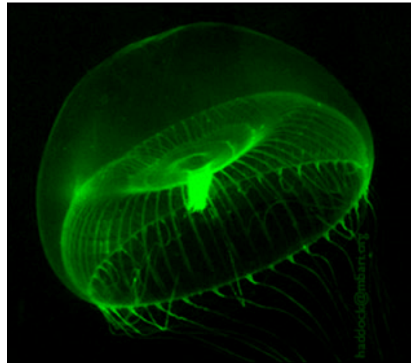




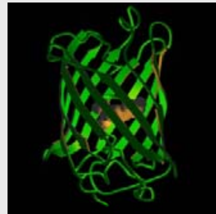


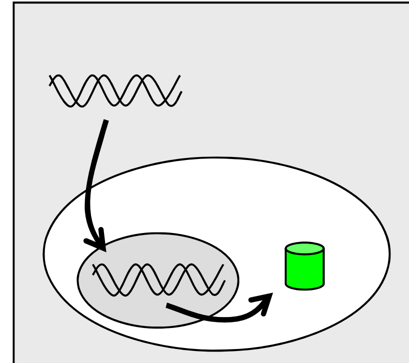
Fluorescent proteins

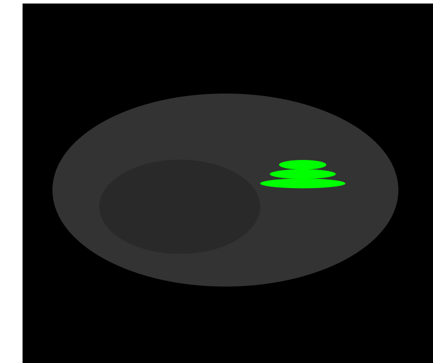
Aequorea victoria

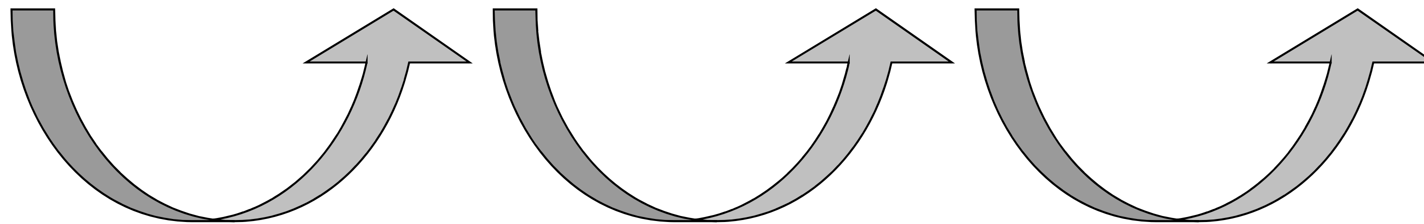


Green Fluorescent Protein (GFP)









Nobel Prize in Chemistry 2008 for the development of GFP

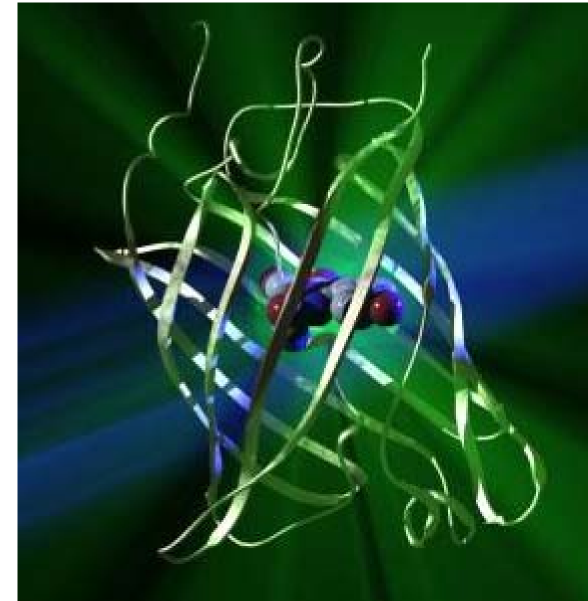
Osamu Shimomura first isolated GFP from the jellyfish *Aequorea victoria*, and discovered that it glowed bright green under ultraviolet light.

Martin Chalfie demonstrated the value of GFP genetic tag for biological phenomena

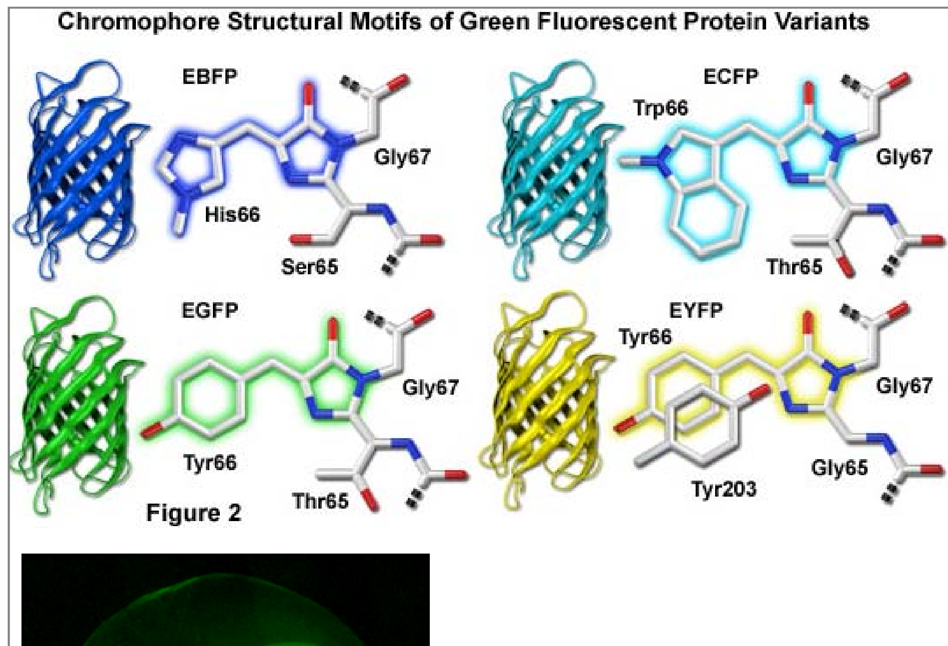
Roger Y. Tsien contributed to our general understanding of how GFP fluoresces and extended the colour palette

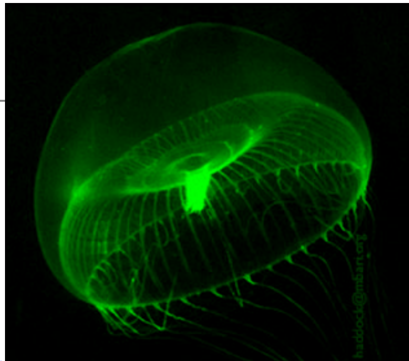
Structural features of GFP
('paint in a tin')

- Main motif: β-can
- 11 antiparallel beta strands (green) form a very compact cylinder
- Inside this beta-structure there is an alpha-helix (dark blue), in the middle of which is the chromophore (red)
- The rigid barrel protects the fluorophore against photochemical damage and the passage of unwanted, diffusible ligands

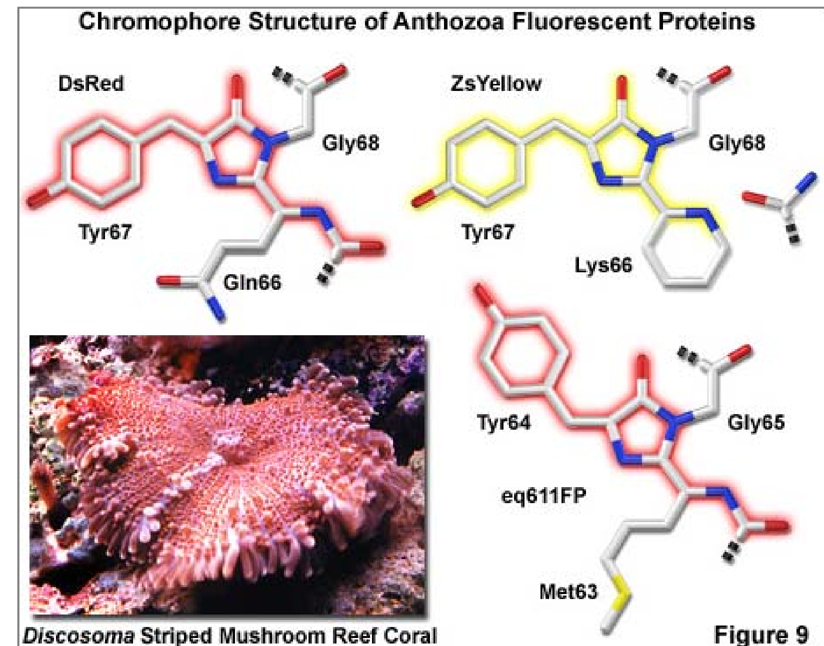


Fluorescent proteins: Structure





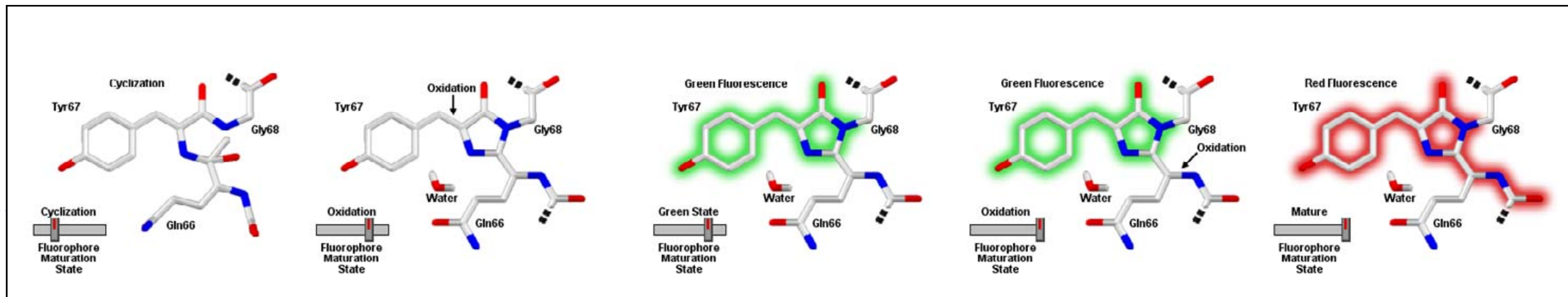
Aequorea victoria



http://www.olympusfluoview.com/applications/fpcolorpalette.html

Fluorescent proteins: Features

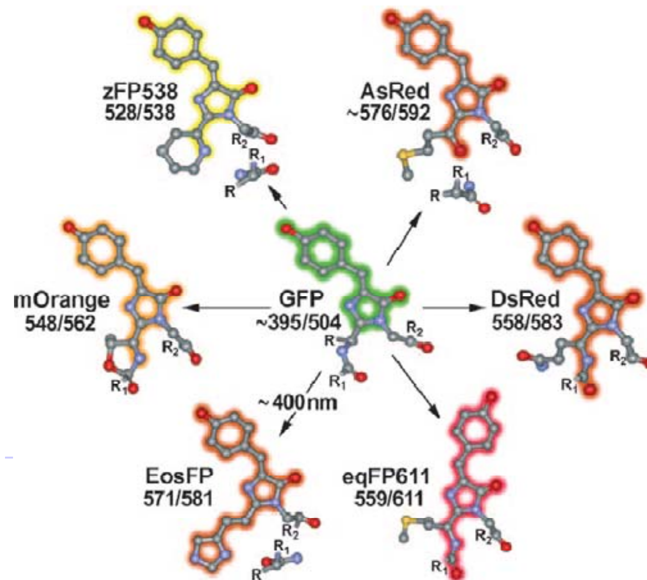
- IMPORTANT:
  - Excitation / emission wavelength
  - Quantum efficiency
  - Stokes shift ($\lambda_{ex} \rightarrow \lambda_{em}$)
  - Protein stability
  - Physiological temperature (from sea animals, some only mature at 30 C)
  - Aggregation tendency (dimerisation / tetramerisation)
  - Maturation time
  - Intermediate fluorescence states during maturation
  - No signalling or localisation function in the cell
  - **EXPRESSION LEVEL!!!**



Mutations of GFP:

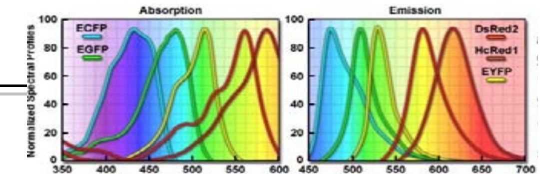
- Tyr-66-Phe (in the fluorophore): shifts in excitation bands loss of intensity
- Ser-65-Thr: increase in fluorescence intensity
- Tyr-66-His: wavelength shift, Ex383 Em448 nm
- Tyr-66-Trp: blue-shifted, weaker fluorescence
- Ser-65-[Thr, Ala, Cys or Leu]: loss of the 395 nm excitation peak
- Ile-167-Thr: reversed ratio of 395 to 475 nm sensitivity
- Val-163-Arg: increased temperature tolerance for functional GFP expression
- Mutations of His148: affects the pH dependence of the excitation bands

- …and many more…



Uli Nienhaus et al., ChemPhysChem

Fluorescent proteins: Variants

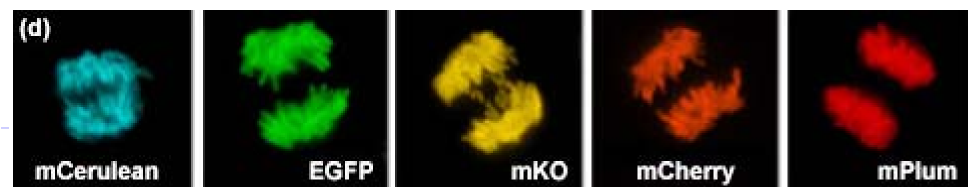


Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Structure	Relative Brightness (% of EGFP)
GFP (wt)	395/475	509	21,000	0.77	Monomer*	48
Green Fluorescent Proteins						
EGFP	484	507	56,000	0.60	Monomer*	100
Emerald	487	509	57,500	0.68	Monomer*	116
Superfolder GFP	485	510	83,300	0.65	Monomer*	160
Azami Green	492	505	55,000	0.74	Monomer	121
mWasabi	493	509	70,000	0.80	Monomer	167
TagGFP	482	505	58,200	0.59	Monomer*	110
TurboGFP	482	502	70,000	0.53	Dimer	102
AcGFP	480	505	50,000	0.55	Monomer*	82
ZsGreen	493	505	43,000	0.91	Tetramer	117
T-Sapphire	399	511	44,000	0.60	Monomer*	79
Blue Fluorescent Proteins						
EBFP	383	445	29,000	0.31	Monomer*	27
EBFP2	383	448	32,000	0.56	Monomer*	53
Azurite	384	450	26,200	0.55	Monomer*	43
mTagBFP	399	456	52,000	0.63	Monomer	98
Cyan Fluorescent Proteins						
ECFP	439	476	32,500	0.40	Monomer*	39
mECFP	433	475	32,500	0.40	Monomer	39
Cerulean	433	475	43,000	0.62	Monomer*	79
CyPet	435	477	35,000	0.51	Monomer*	53
AmCyan1	458	489	44,000	0.24	Tetramer	31
Midori-Ishi Cyan	472	495	27,300	0.90	Dimer	73
TagCFP	458	480	37,000	0.57	Monomer	63
mTFP1 (Teal)	462	492	64,000	0.85	Monomer	162
Yellow Fluorescent Proteins						
EYFP	514	527	83,400	0.61	Monomer*	151
Topaz	514	527	94,500	0.60	Monomer*	169
Venus	515	528	92,200	0.57	Monomer*	156
mCitrine	516	529	77,000	0.76	Monomer	174
TagYFP	508	524	64,000	0.60	Monomer	118
PhiYFP	525	537	124,000	0.39	Monomer*	144
ZsYellow1	529	539	20,200	0.42	Tetramer	25
mBanana	540	553	6,000	0.7	Monomer	13
Orange Fluorescent Proteins						
Kusabira Orange	548	559	51,600	0.60	Monomer	92
Kusabira Orange2	551	565	63,800	0.62	Monomer	118
mOrange	548	562	71,000	0.69	Monomer	146
mOrange2	549	565	58,000	0.60	Monomer	104
dTomato	554	581	69,000	0.69	Dimer	142
dTomato-Tandem	554	581	138,000	0.69	Monomer	283
TagRFP	555	584	100,000	0.48	Monomer	142
TagRFP-T	555	584	81,000	0.41	Monomer	99
DsRed	558	583	75,000	0.79	Tetramer	176
DsRed2	563	582	43,800	0.55	Tetramer	72
DsRed-Express (T1)	555	584	38,000	0.51	Tetramer	58
DsRed-Monomer	556	586	35,000	0.10	Monomer	10
mTangerine	568	585	38,000	0.30	Monomer	34
Red Fluorescent Proteins						
mRuby	558	605	112,000	0.35	Monomer	117
mApple	568	592	75,000	0.49	Monomer	109
mStrawberry	574	596	90,000	0.29	Monomer	78
AsRed2	576	592	56,200	0.05	Tetramer	8
mRFP1	584	607	50,000	0.25	Monomer	37
JRed	584	610	44,000	0.20	Dimer	26
mCherry	587	610	72,000	0.22	Monomer	47
HcRed1	588	618	20,000	0.015	Dimer	1
mRaspberry	598	625	86,000	0.15	Monomer	38
dKeima-Tandem	440	620	28,800	0.24	Monomer	21
HcRed-Tandem	590	637	160,000	0.04	Monomer	19
mPlum	590	649	41,000	0.10	Monomer	12
AQ143	595	655	90,000	0.04	Tetramer	11

http://www.microscopyu.com/articles/livecellimaging/fpintro.html

Fluorescent proteins: Variants

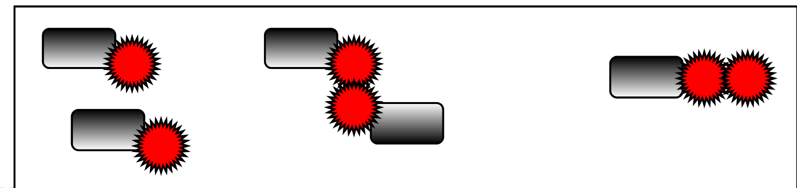
Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]	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[l]	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]



Shaner NC, Steinbach PA, Tsien RY.: *A guide to choosing fluorescent proteins.* Nat Methods. 2005 Dec;2(12):905-9. Review.

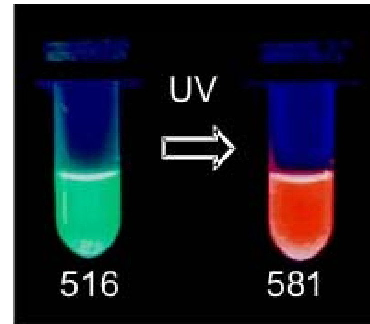
Far-red fluorescent protein variants

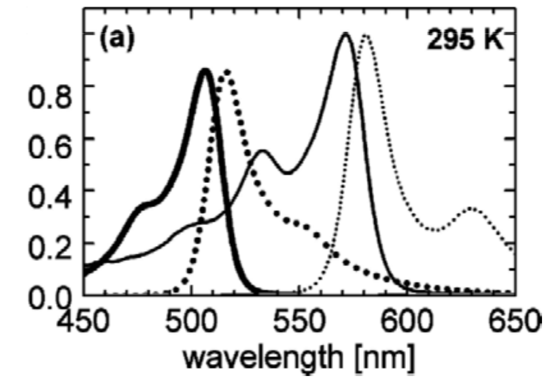
FP Variant	Ex (nm)	Em (nm)	Quantum Yield	Relative Brightness	Maturation at 37°C ($t_{0.5}$, hr)	Oligomerisation
eqFP611	559	611	0.45	2.07	n.a.	Dimer
td-RFP611	558	609	0.47	2.13	3.75	Tandem dimer
RFP639	588	639	0.18	0.63	1.5	Dimer
td-RFP639	589	631	0.16	0.55	<8	Tandem dimer
hcRed	594	645	0.05	0.11		Dimer
mCherry	587	615	0.22	0.5	0.25	Monomer

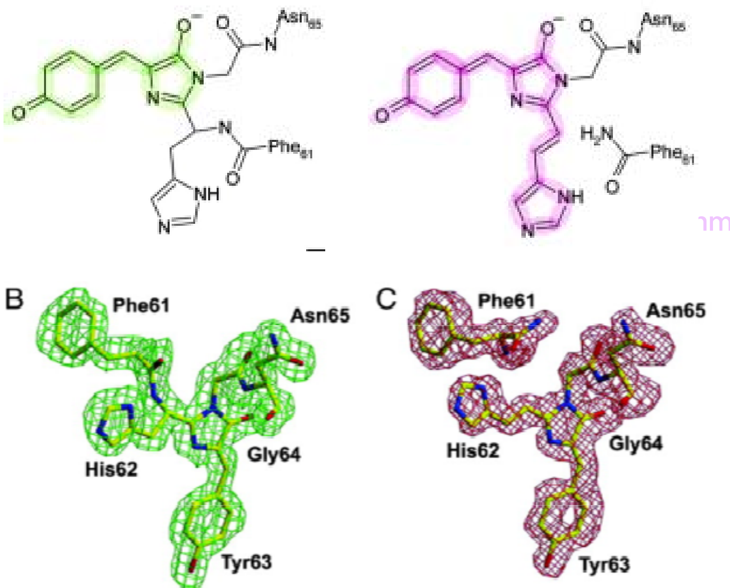


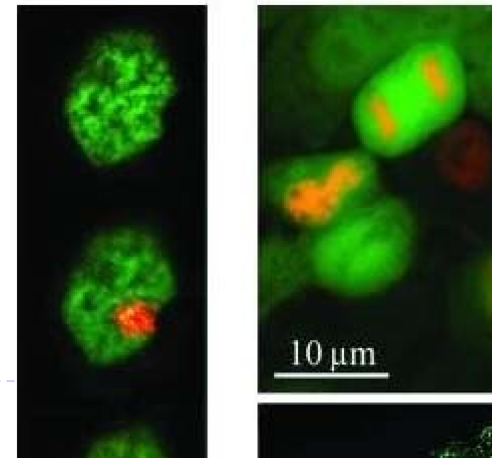
Uli Nienhaus, University of Ulm, Germany

Photoconvertible fluorescent proteins
example: EosFP
- monomeric
- conversion wavelength separate from excitation wavelength (no bleaching during conversion!)
- stable photoconversion (irreversible: cleavage)









Fluorescent proteins: Variants

Photoconvertible fluorescent proteins

- EosFP
- pDendra2
- PA-GFP
- PS-CFP
- KFP-Red

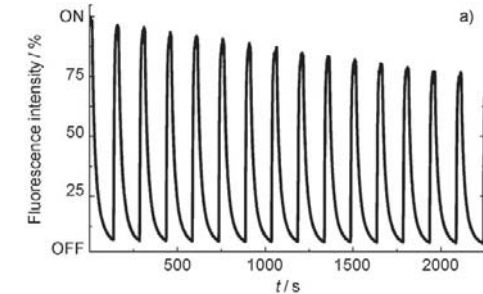
Applications

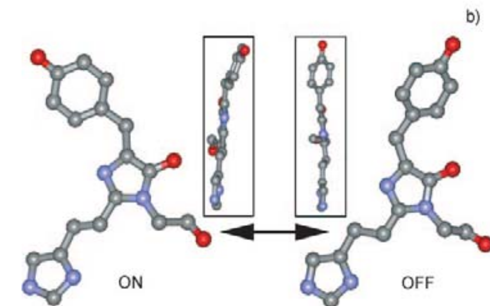
- Photoactivation (e.g. FRET)
- Motility
- High-resolution microscopy (PALM)

Fluorescent proteins: Variants

Table 1 | **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)	*Pectiniidae* 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

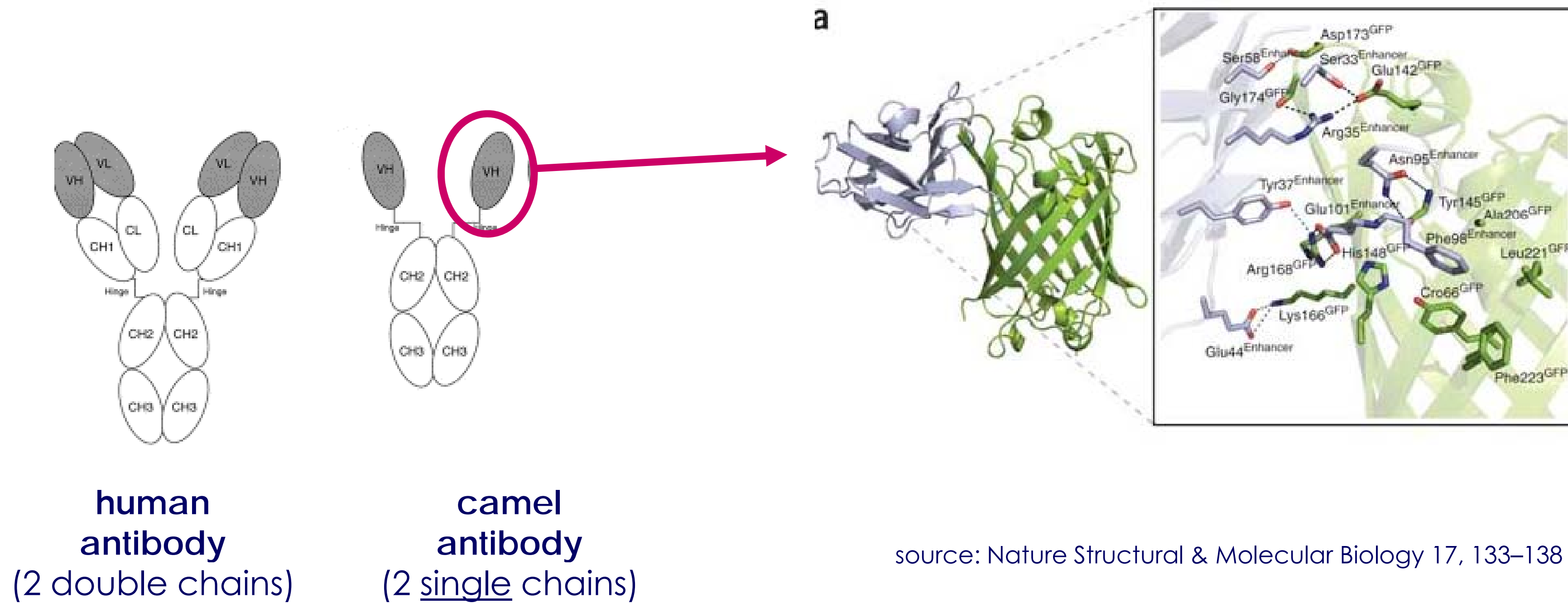




Konstantin A. Lukyanov, Dmitry M. Chudakov, Sergey Lukyanov & Vladislav V. Verkhusha, Nature Reviews Molecular Cell Biology 6, 885-890 (November 2005)

Uli Nienhaus et al., ChemPhysChem

Antibody + fluorescent protein = nanobody

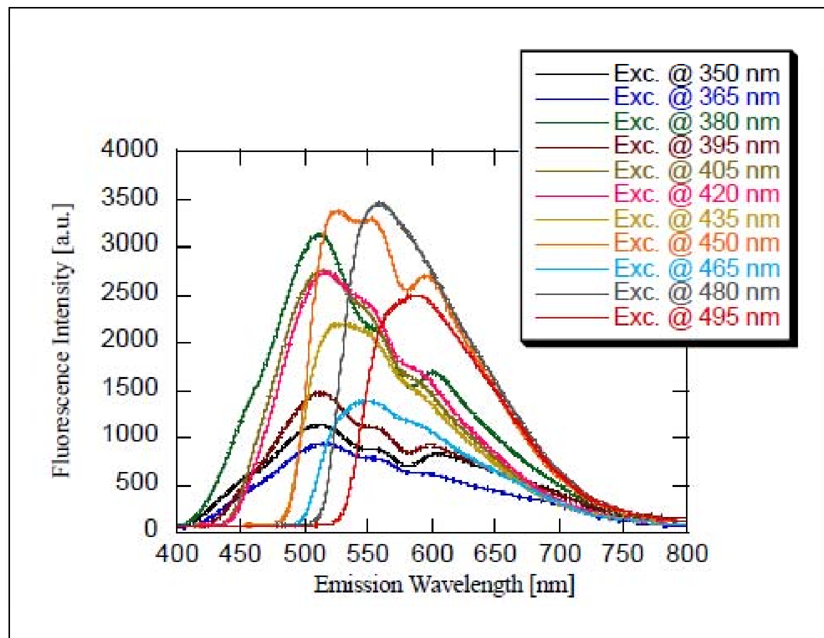


Applications

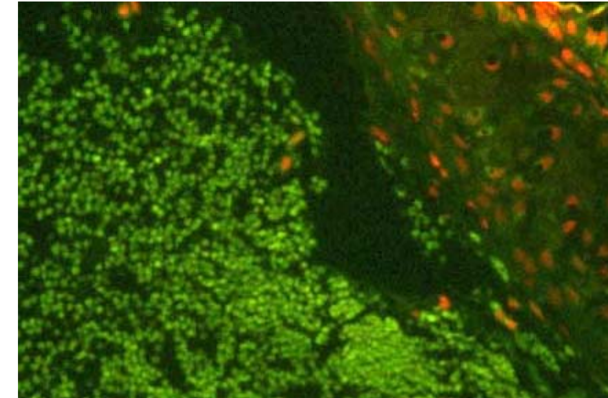
- small (17kD)
- can be targetted against any domain (not just C and N terminal)
- potentially inducible expression

Uli Nienhaus et al., ChemPhysChem

Autofluorescence



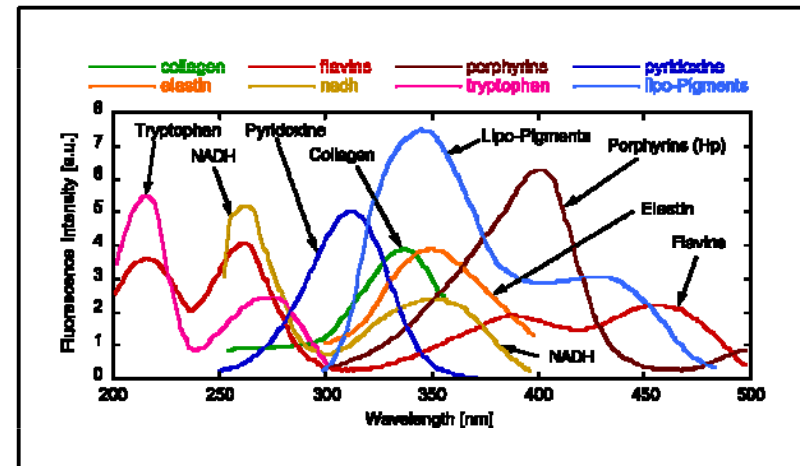
Emission spectra of normal bronchial tissue in vivo

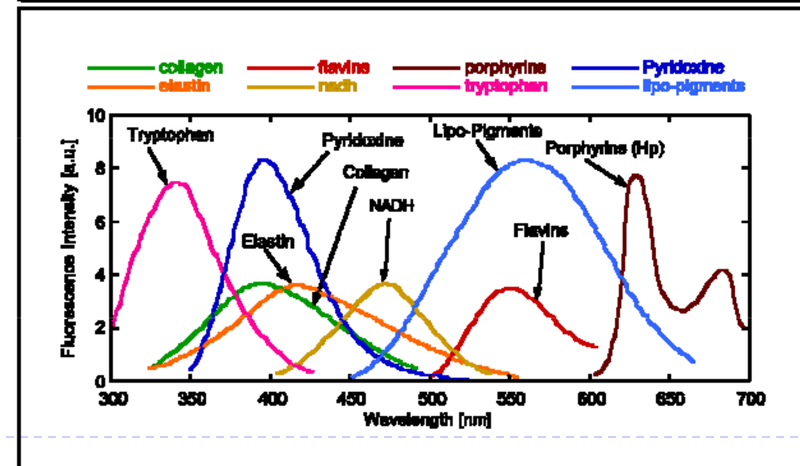


Matthieu Zellweger, PhD thesis, ETH Lausanne

Autofluorescence

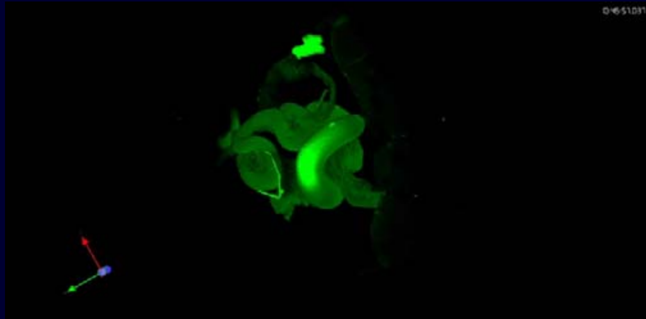
Autofluorescent	Excitation	Emission
Vitamin C	350	430
NAD(P)H	366	440–470
Vitamin D	390	470
Lignin	530	488
Chlorophyll	685	488
Vitamin A	340	490
Collagen and elastin	442	470–520
Flavins	380, 460	520
FMN, FAD	450	530
Lipofuscins	450–490	550
Riboflavin	450-490	500-560
Protoporphyrin IX	442	635



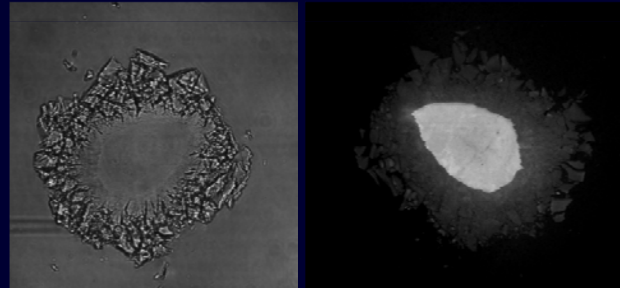


Matthieu Zellweger, PhD thesis, ETH Lausanne

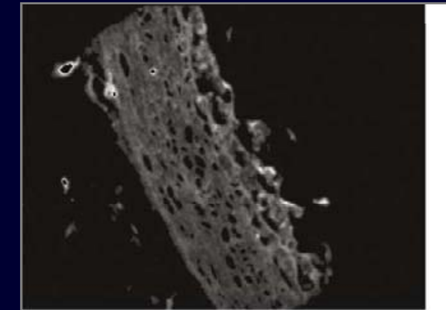
Autofluorescence

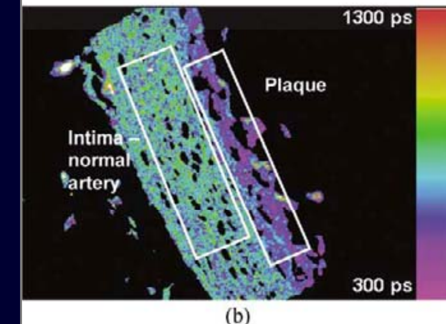


Angelos Skodras:
mouse oviduct



Tryptophan crystals,
(3-photon excitation)





Paul French:
Atheroslerotic plaque
(autofluorescence / Flim)

Pitfalls in fluorescence imaging: Background fluorescence

Source:

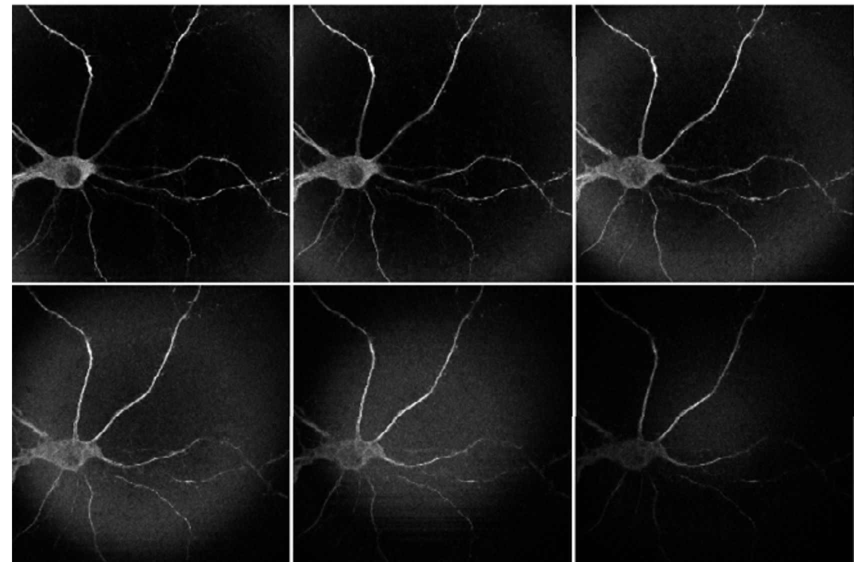
- autofluorescence of metabolic compounds (NADH, FADH, collagen, …)
- unspecific staining
- fixatives and mounting media

Effect:

- wrong signal intensity

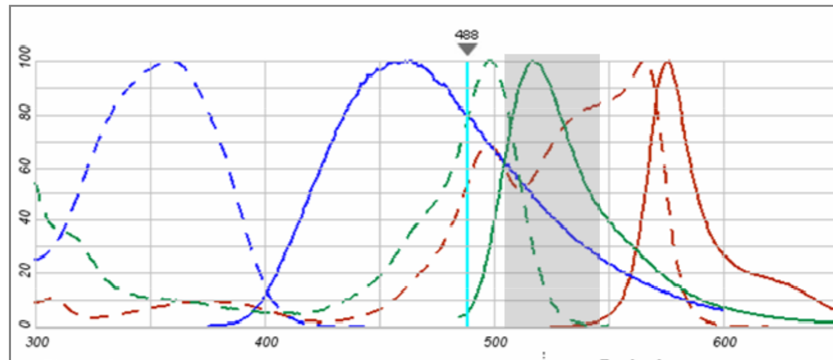
Implications:

- quantification error, false-positive results



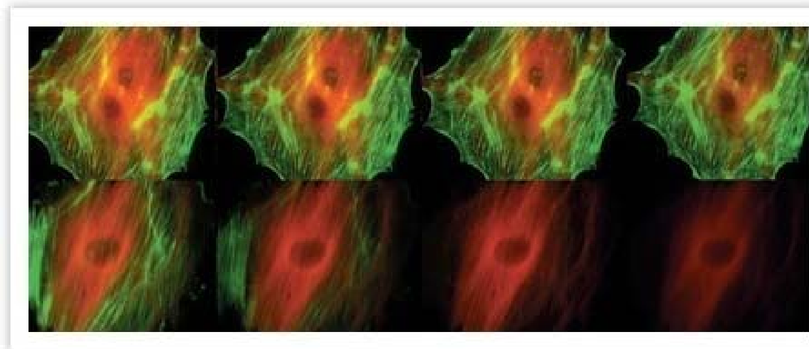
Pitfalls in fluorescence imaging: Fluorophore crosstalk

- **Source:**
  - spectral overlap of excitation and / or emission spectra of fluorophores
  - wrong settings or instrument limitations
- **Effect:**
  - wrong signal intensity and structural information
- **Implications:**
  - quantification error, false-positive results



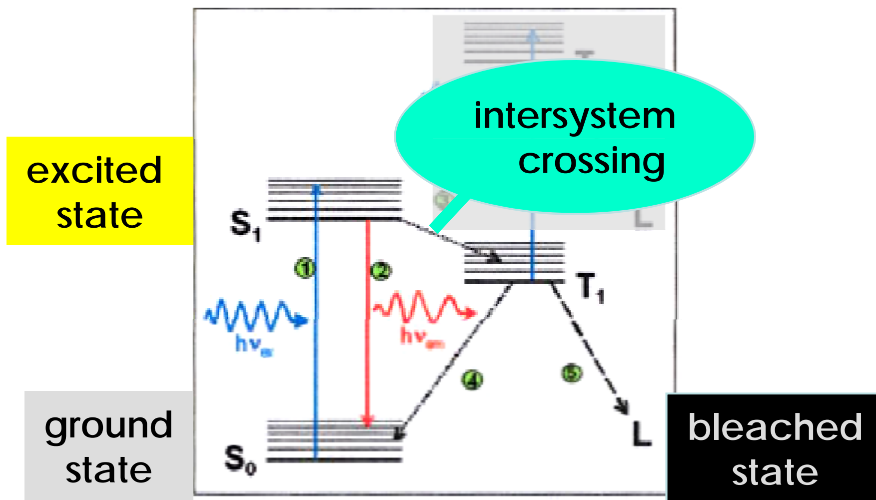
Pitfalls in fluorescence imaging: Bleaching and phototoxicity

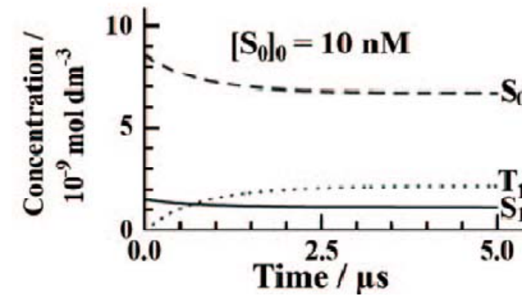
- **Source:**
  - Excitation light and oxigen radicals produced during fluorescence process
  - DNA damage by UV light
- **Effect:**
  - Reversible (triplet state) or irreversible (oxidation) inactivation of fluorophor
  - oxidation (destruction) of biological compounds
  - Mutagenesis (in long live experiments)
- **Implications:**
  - cytotoxicity
  - loss of visible fluorophore, loss of linear fluorophore-signal relationship
  - quantification error, false-negative results



http://probes.invitrogen.com

Bleaching of fluorophores





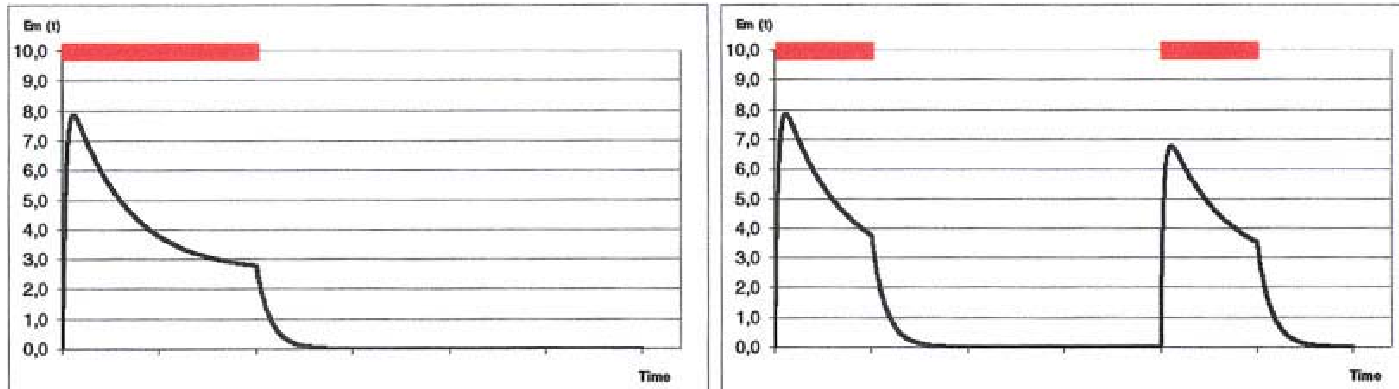
ontimes (pixel dwell times) for confocal microscopes:
from some 50 ns up to ca. 100 ms (typically ~1-2µs)

Applications:

- FRAP, FLIP
- single-molecule analysis
- superresolution (STORM)

Pitfalls: Bleaching and phototoxicity

Bleaching of fluorophores



Consequences for (confocal) imaging:

•At high light intensities, linearity between fluorophore concentration and detection intensity is lost;

•fast scanning → fewer photons per pixel →lower signal-to-noise

BUT

fast scanning → better emission vs. triplet → higher photo efficiency

ROLF T. BORLINGHAUS: MRT Letter: High Speed Scanning Has the Potential to Increase Fluorescence Yield and to Reduce Photobleaching. MICROSCOPY RESEARCH AND TECHNIQUE 69:000–000 (2006)