Studying dynamic processes and interactions in living cell by fluorescence microscopy

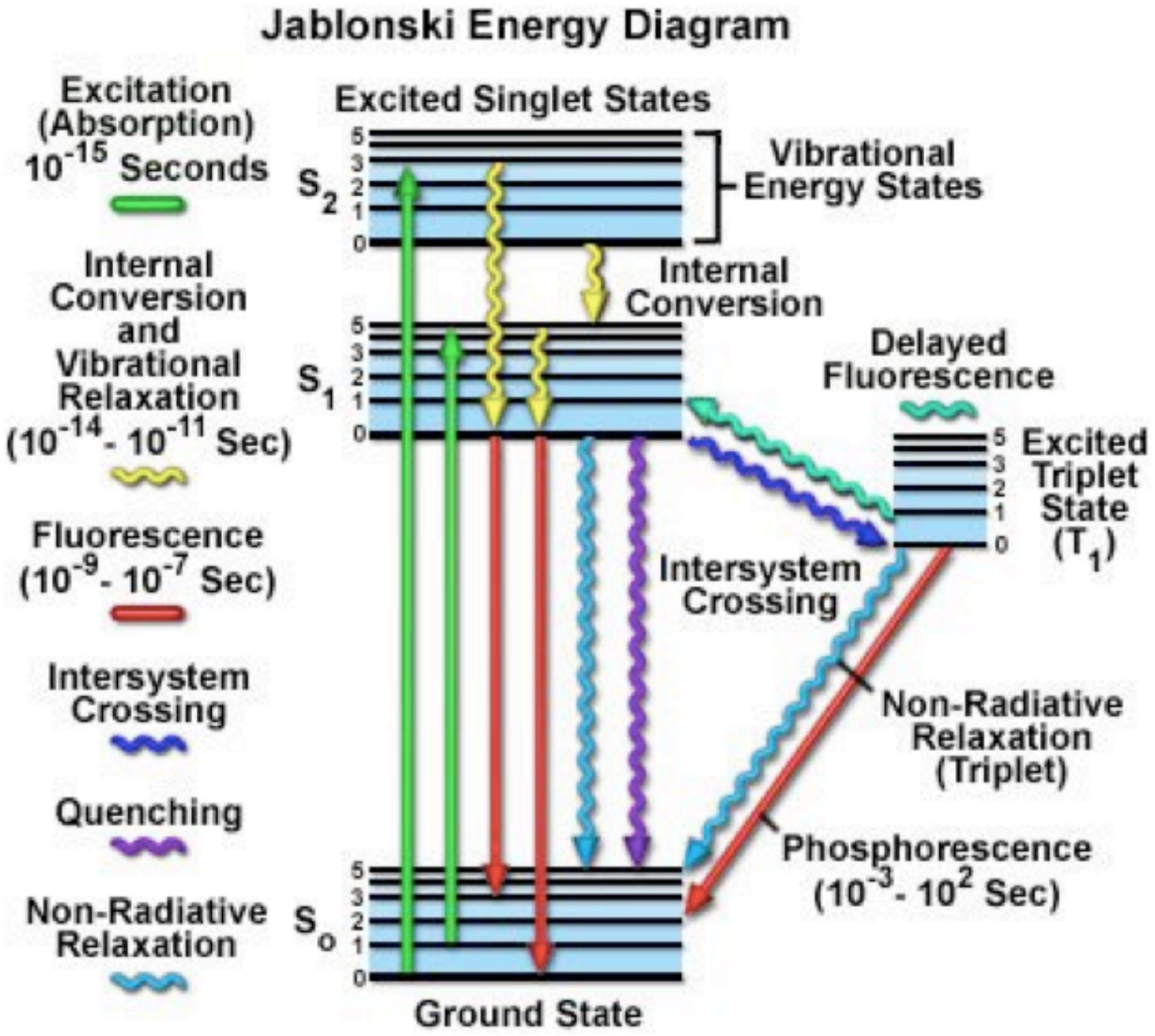
Gin Tonic & Fluorescence: Quinine

UV

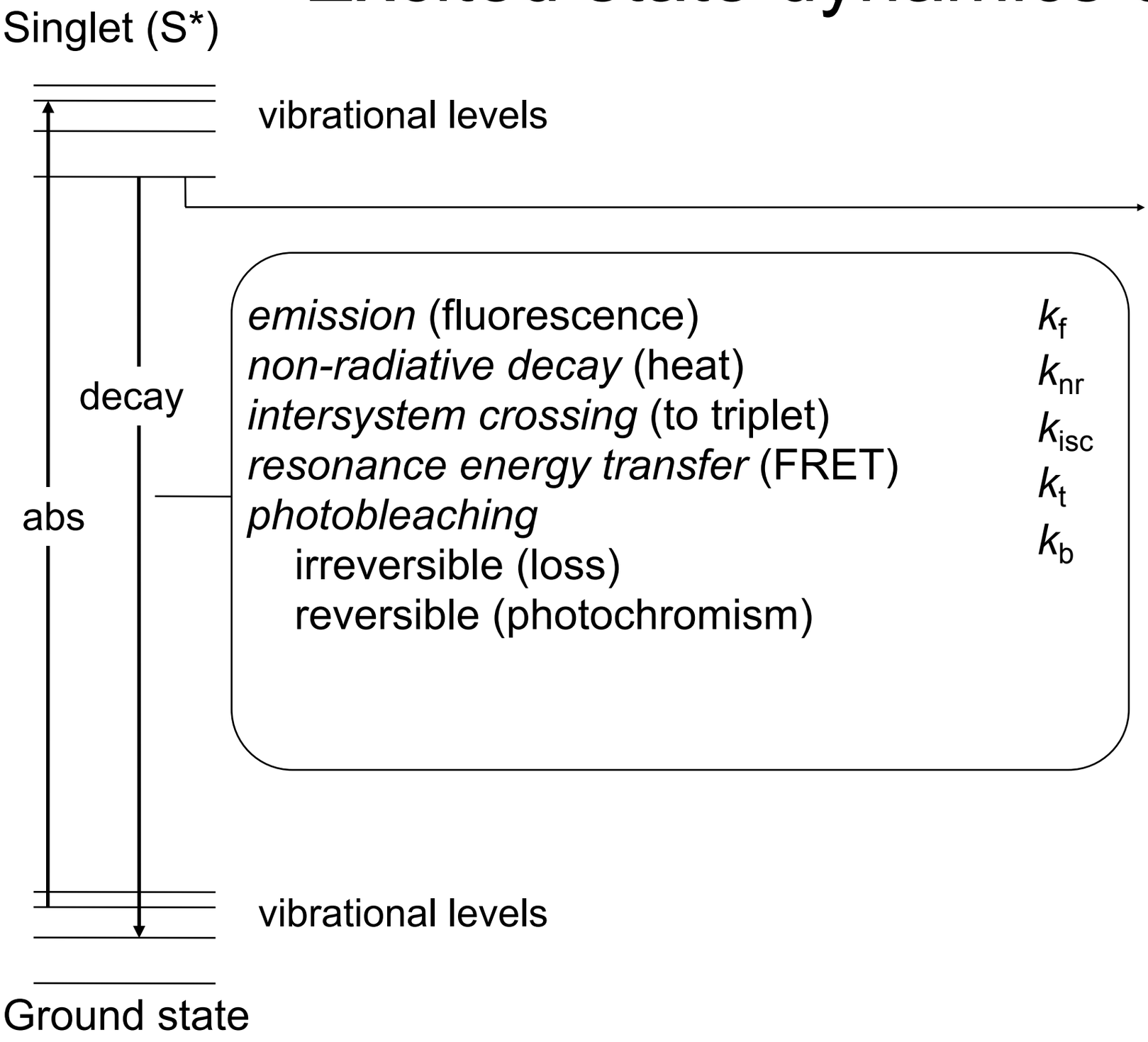
blue

What is fluorescence?

A dye is excited with a short wavelength and emits at a longer wavelength (in an isotropic fashion)



Excited state dynamics of a fluorophore



After absorption of a photon, the fluorophore spends some time in the electronically excited state S*, characterized by an exponential decay law (lifetime t):

$I(t) = I_o \exp(-t/\tau)$

$\tau^{-1} = \sum k = (k_f + k_{nr} + k_{isc} + k_t + k_b)$

Q = quantum yield =
# emitted photons/
# absorbed photons
$= k_f \tau$

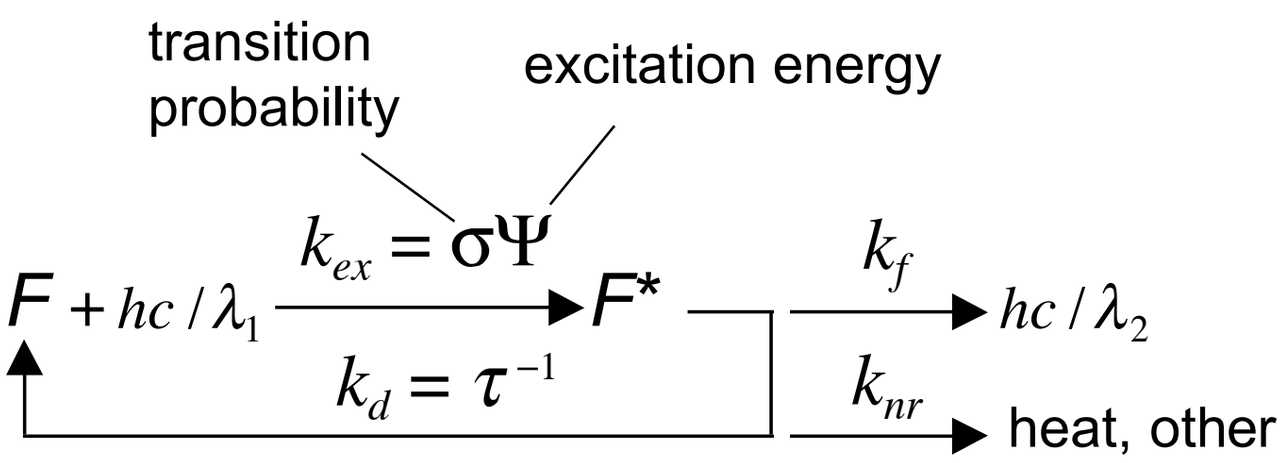
A fluorophore is a photophysical “enzyme”

(Tom Jovin, MPI Göttingen)

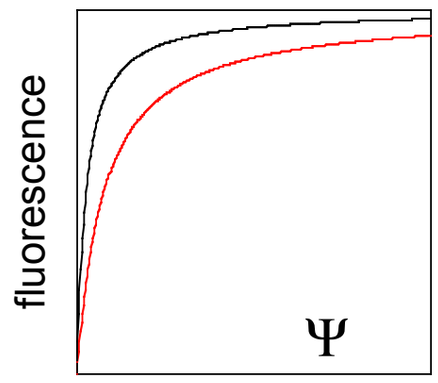
$$K_m = (k_2+k_3)/k_1$$

$$\text{enzyme} + S \underset{k_2}{\overset{k_1}{\leftrightarrow}} E{\cdot}S \xrightarrow{k_3} E + P$$

$$\frac{v}{v_{max}} = \frac{S}{S + K_m}$$



Saturation curve



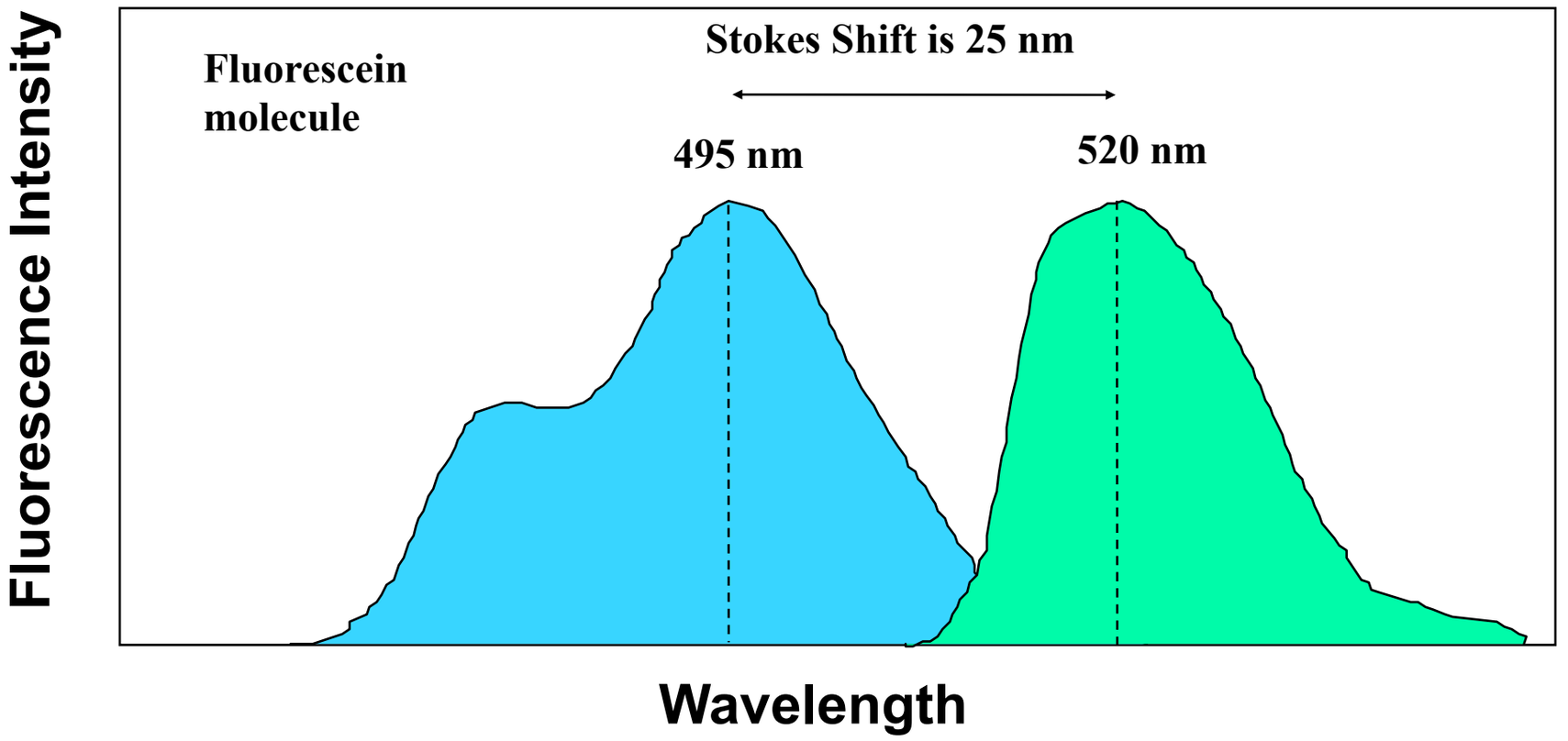
$$\text{fluorescence}(hc/\lambda_2) \propto_1 \left(\frac{k_f \Psi}{\Psi + [\sigma\tau]^{-1}}\right); \quad \text{low}\,\Psi \rightarrow \sigma Q \Psi; \quad \text{high}\,\Psi \rightarrow k_f$$

quantum yield

Fluorescence

Stokes Shift

is the energy difference between the lowest energy peak of absorbance and the highest energy of emission



Parameters that characterize fluorophore

- Extinction and emission wavelength
- Stability toward bleaching
- Extinction Coefficient ε
  - ε refers to a single wavelength (usually the absorption maximum)
- Quantum Yield ϕ

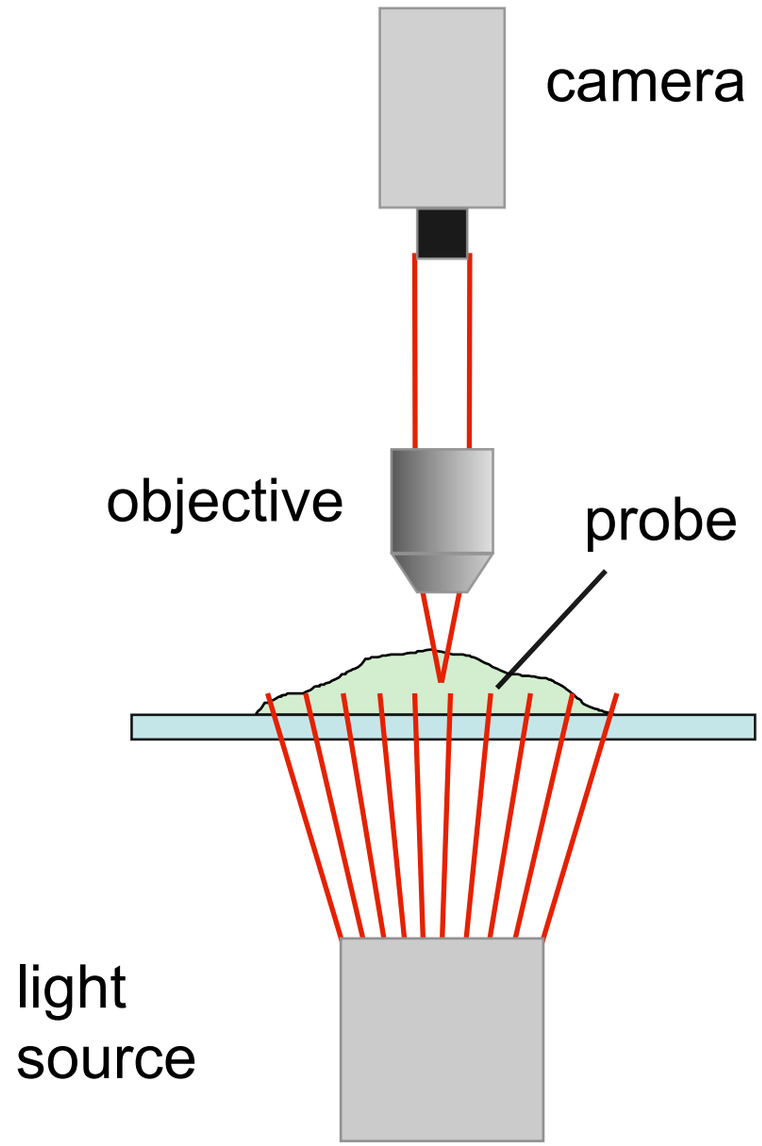
  ϕ is a measure of the integrated photon emission over the fluorophore spectral band

$$\phi = \frac{Number\ of\ emitted\ photons}{Number\ of\ absorbed\ photons}$$

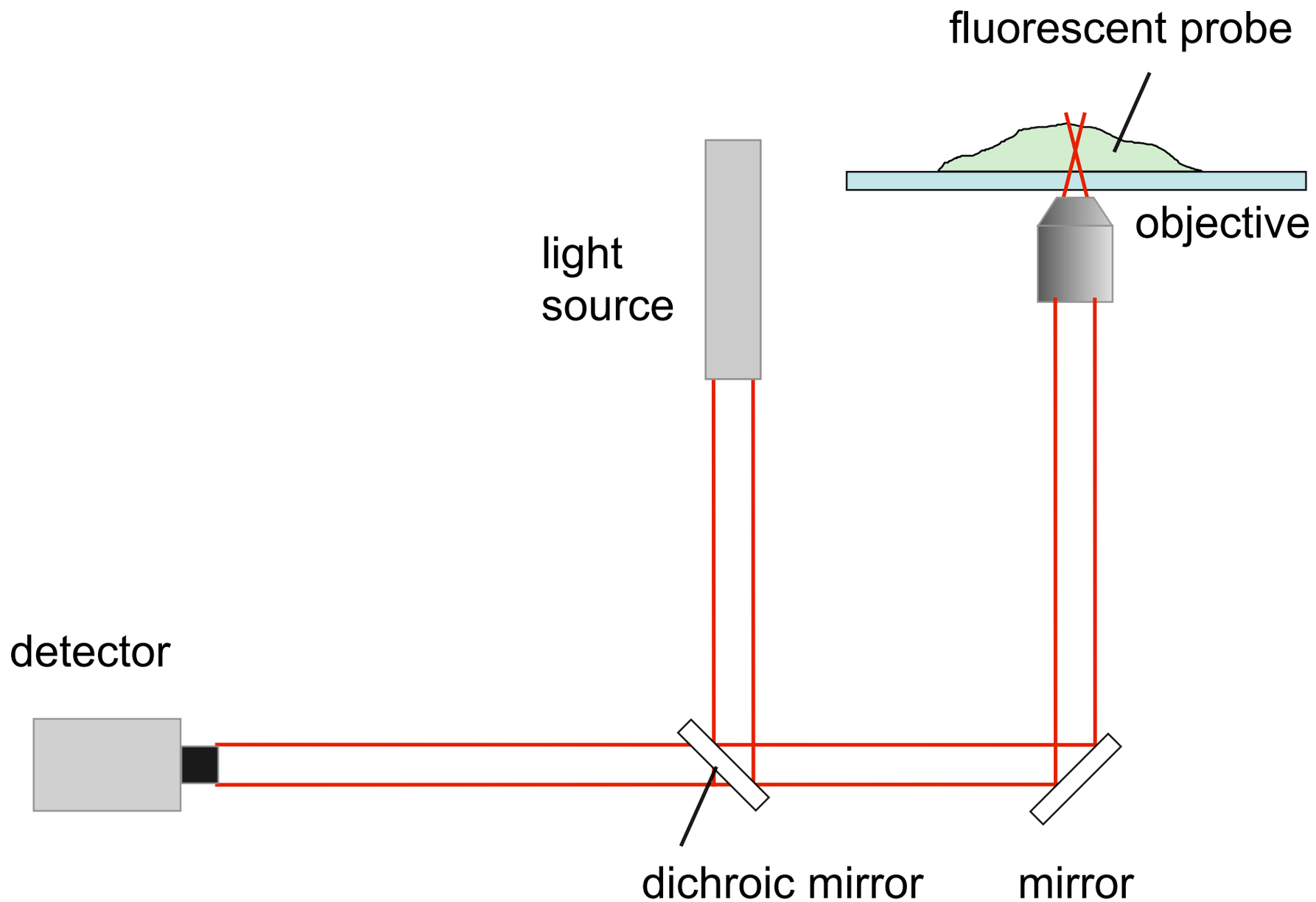
- At sub-saturation excitation rates, fluorescence intensity or brightness is proportional to the product of ε and ϕ

$$I = const \cdot \varepsilon \cdot \phi$$

Wide field microscope setup



Fluorescence microscope setup

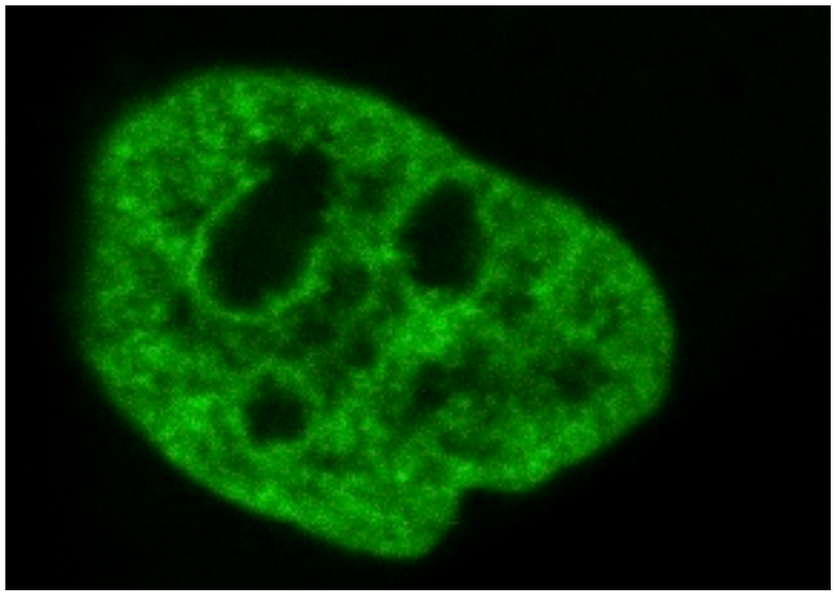


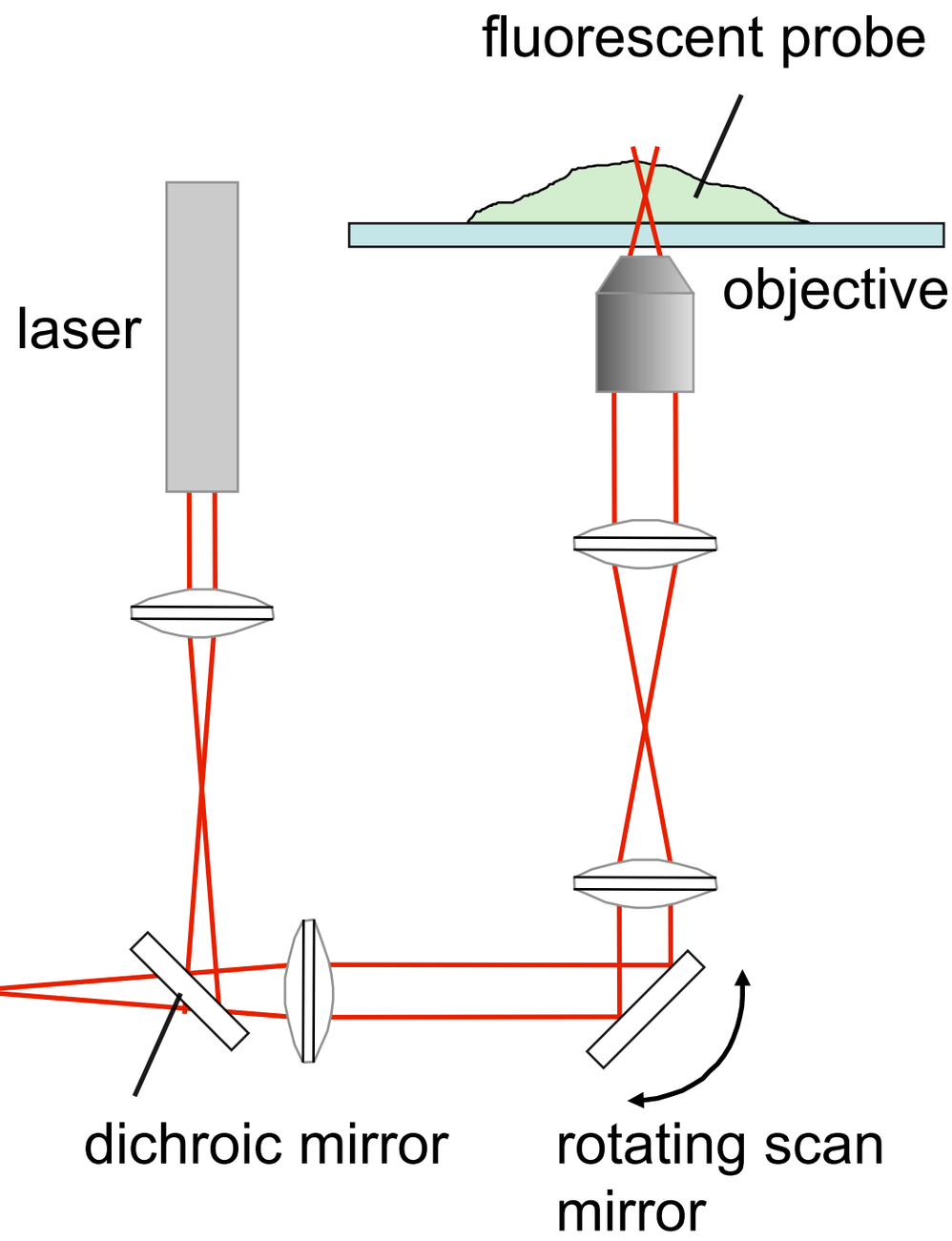
Confocal microscope setup

synchronized beam scanning
and signal acquisition

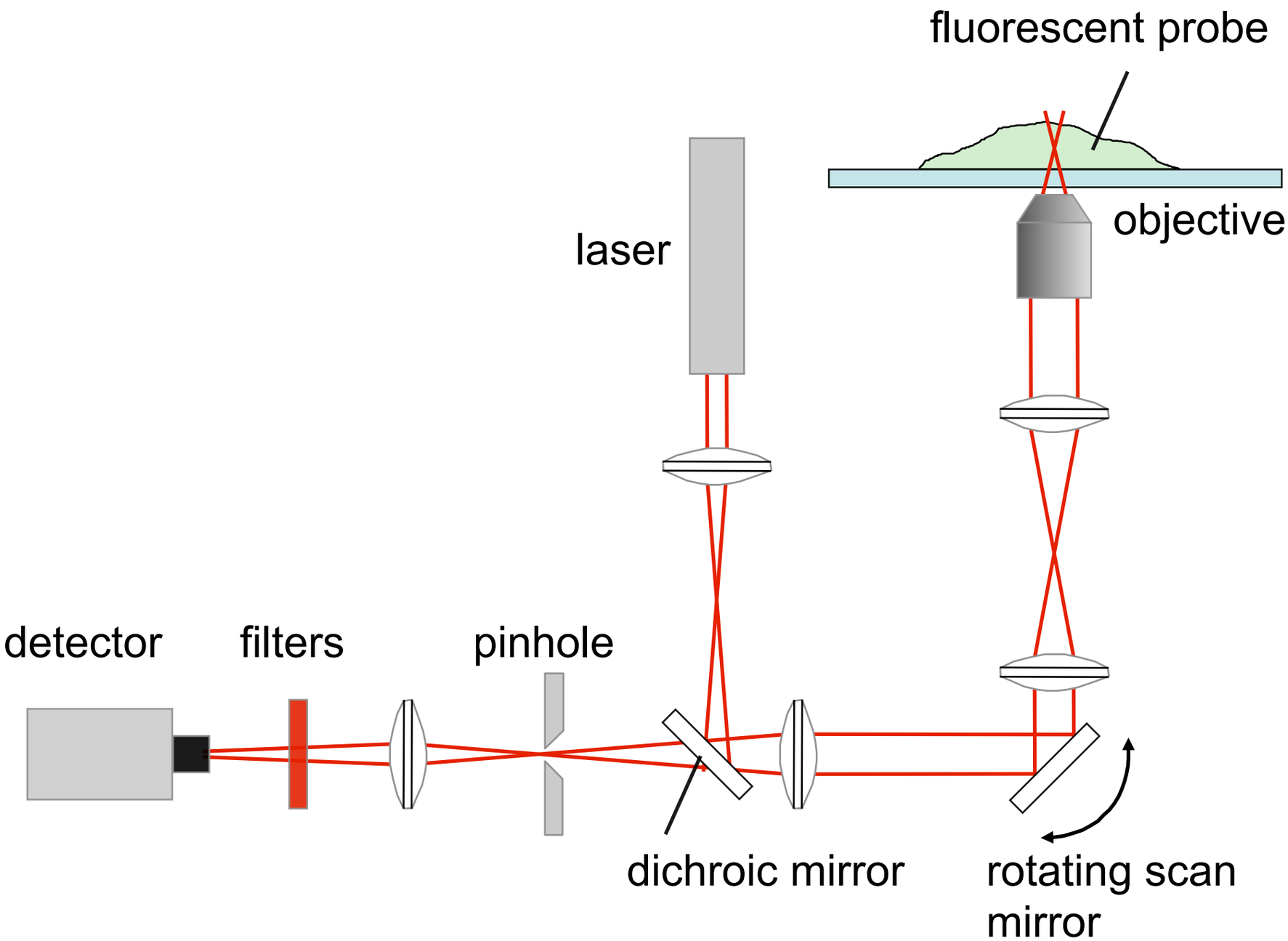
↓

reconstruction of the spatial
fluorescence distribution

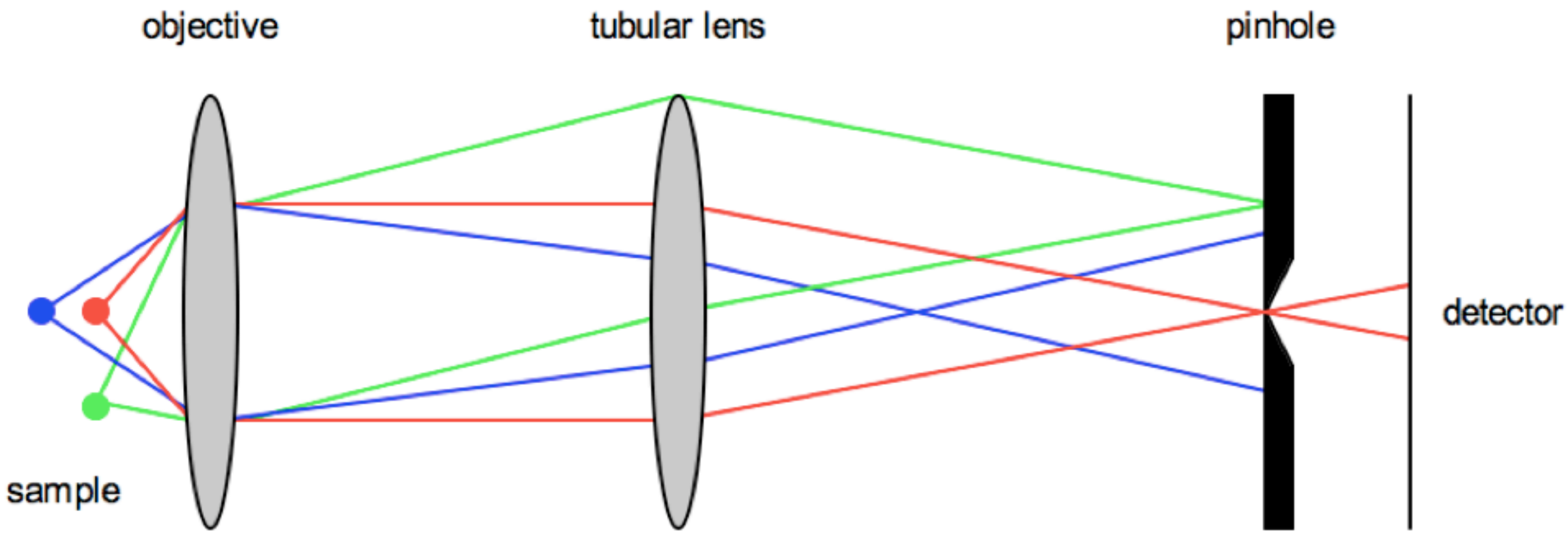




Confocal microscope setup

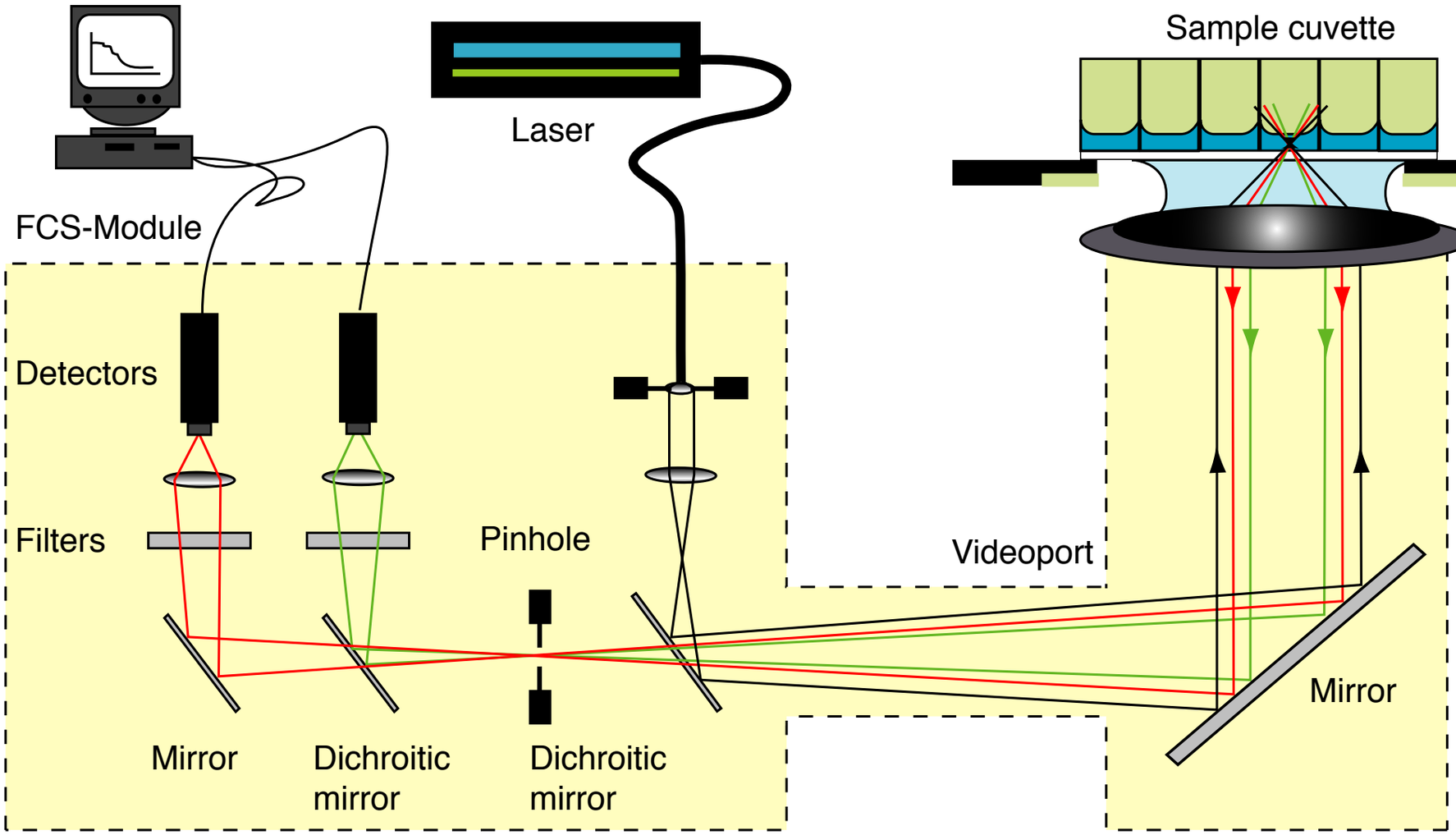


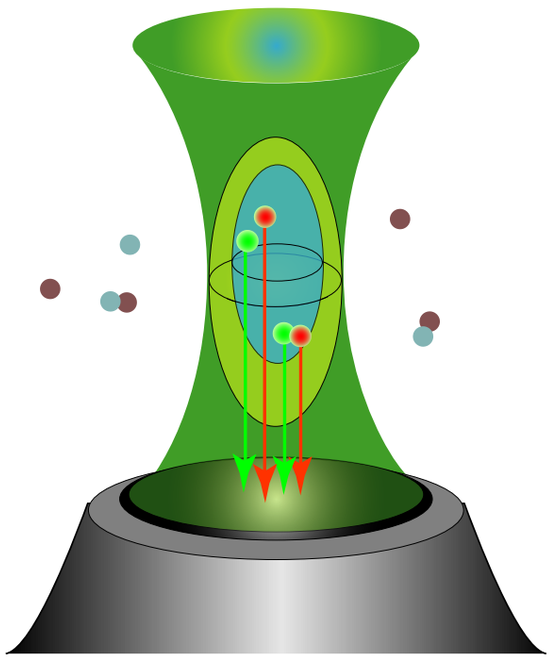
Optical sectioning with the pinhole



Resolution in all three dimensions, fluorescence from „wrong“ axial positions is blocked

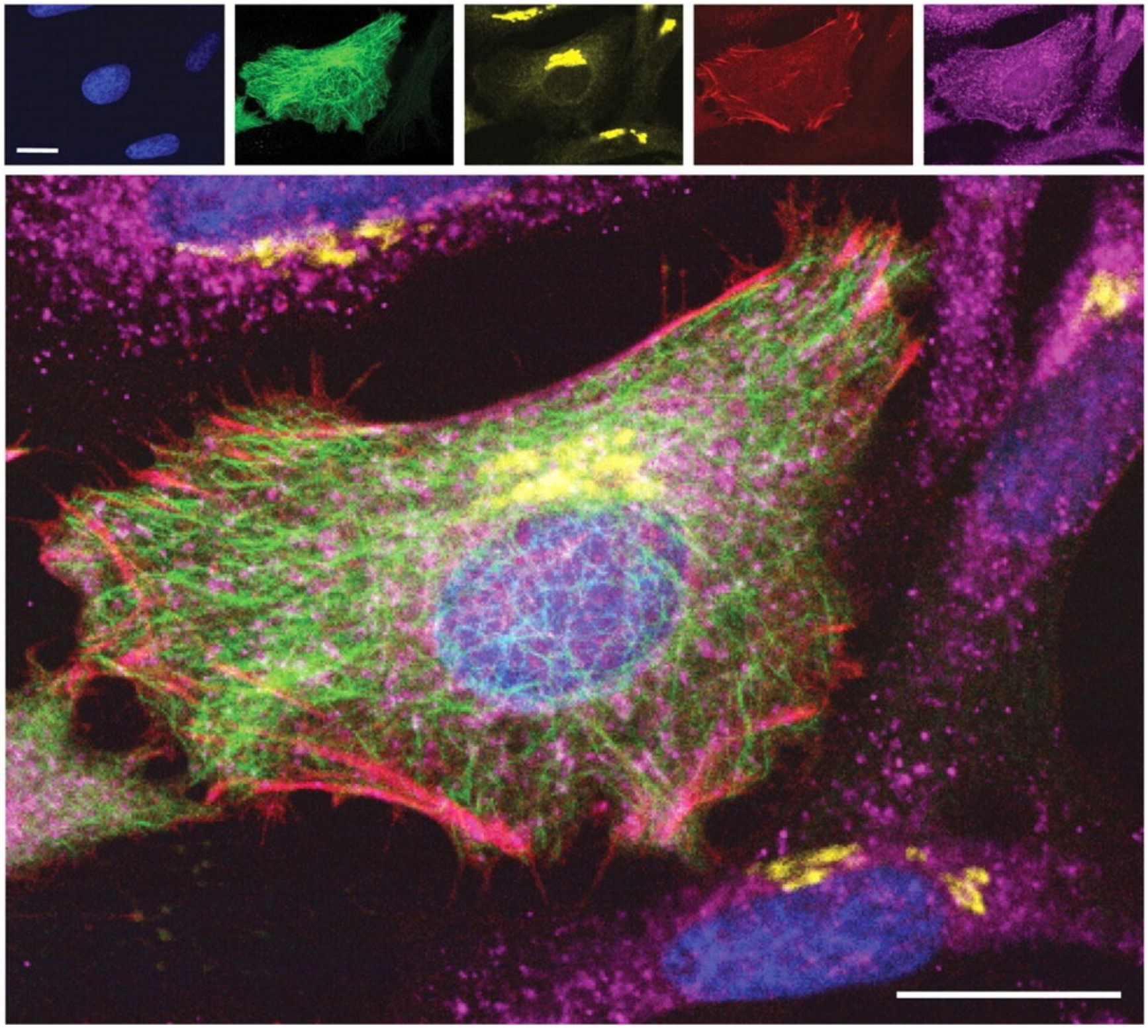
Instrument setup for confocal fluorescence microscopy



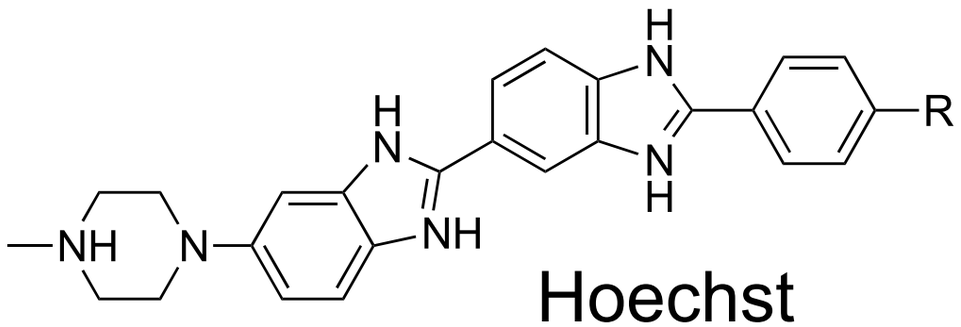


excitation volume
200-300 nm (x,y)
600-700 nm (z)

Excitation (nm):	800 (2 photon)	488	432	568	637
Emission (nm):	410-490	500-530	555-565	580-620	>660
Fluorophore:	Hoechst	GFP	QD565	ReAsH	Cy5
Targeting:	direct affinity	genetic	immuno	genetic	immuno
Target:	DNA	α-tubulin	giantin	β-actin	Cytochrome c
Structure:	nuclei	microtubules	golgi	stress fibers	mitochondria



DNA staining with Hoechst



Hoechst

DAPI

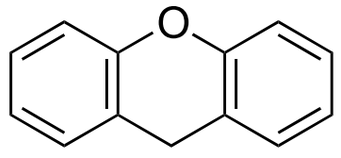
Hoechst 33258: most common, minor groove binder, ex 350 nm, em: 461 nm

Hoechst 33342: very similar but 10 x higher cell permeability, live cell staining

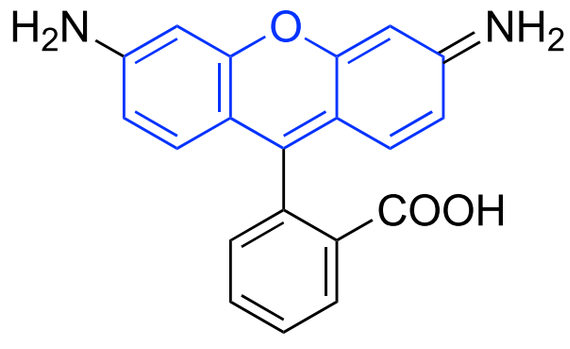
DAPI: = half of Hoechst, very similar spectra, works well with fixed cells

Some popular small molecule dyes derived from xanthene

- Xanthene

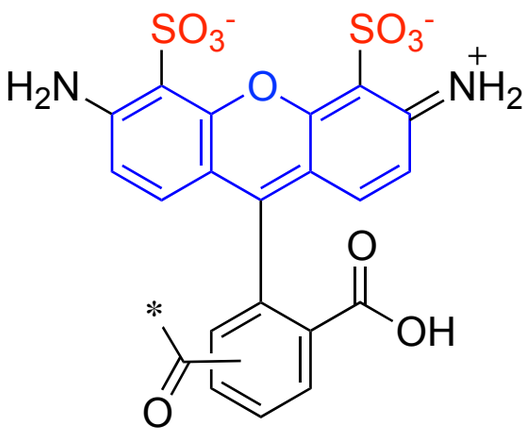


Xanthen

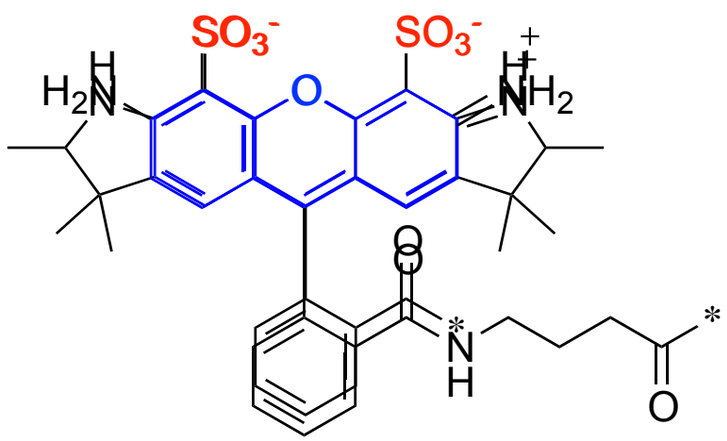


Rhodamine 110

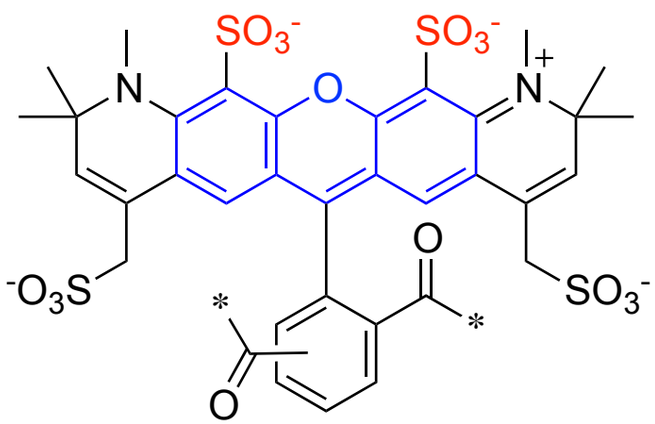
  - Fluorescein
  - Rhodamine
    - **Rhodamine B, 110, 123, 6G**
    - **ROX, TAMRA, Texas Red**
    - **Alexa 488, 514, 532, 546, 568, 594, 610**
    - **ATTO 488, 514, 520, 532, 565, 590**



Alexa Fluor 488

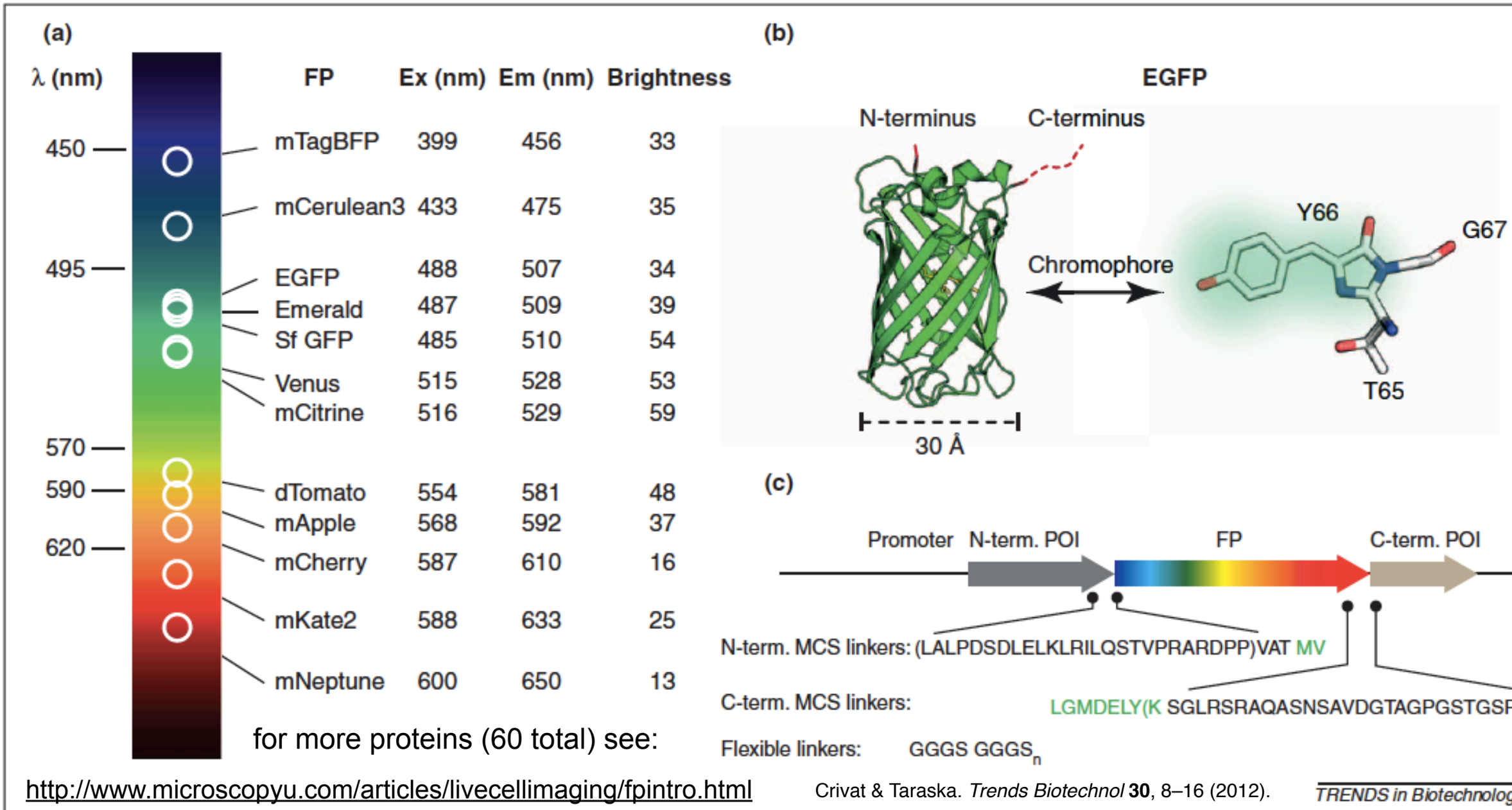


Alexa Fluor 532



Alexa Fluor 610

Broad range of fluorescent proteins for many applications



Desired features of fluorescent tag: right color, bright, stable, monomeric, pH insensitive, fast formation of fluorescent state (GFP 30 min maturation time)

Checks for artefacts: N- vs. C-terminal tag, intracellular localization in comparison to immunostaining, complex composition (GFP pull down), in vitro activity test, stable cell lines

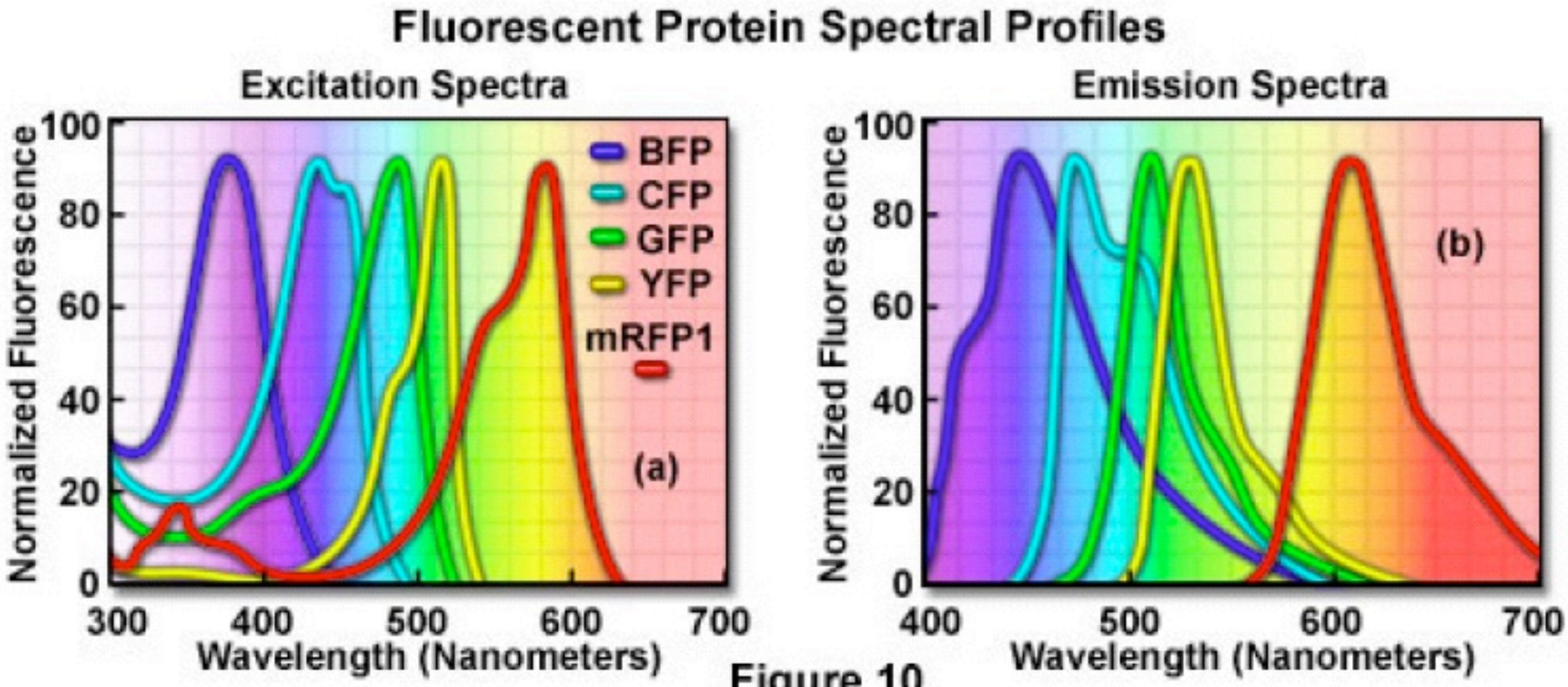

Fluorescent Protein Spectral Profiles
Excitation Spectra
Emission Spectra
Normalized Fluorescence
100
80
60
40
20
0
300
400
500
600
700
Wavelength (Nanometers)
BFP
CFP
GFP
YFP
mRFP1
(a)
(b)

Figure 10

Autofluorescent proteins

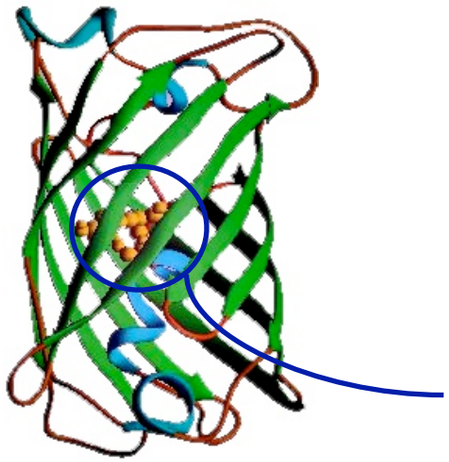
Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Structure	Relative Brightness (% of EGFP)
GFP (wt)	395/475	509	21,000	0.77	Monomer*	48
Green Fluorescent Proteins						
EGFP	484	507	56,000	0.60	Monomer*	100
Emerald	487	509	57,500	0.68	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
mTurquoise	434	474	30,000	0.84	Monomer*	75
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

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

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
YPet	517	530	104,000	0.77	Monomer*	238
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

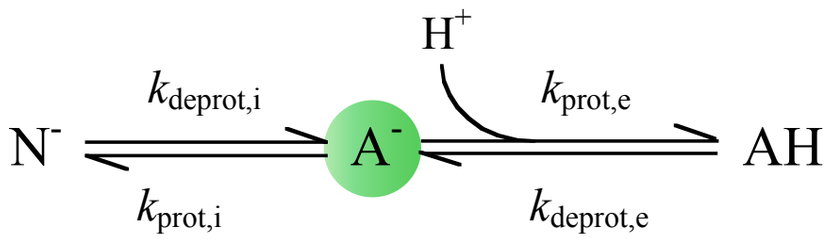
Intramolecular conformation changes of GFP

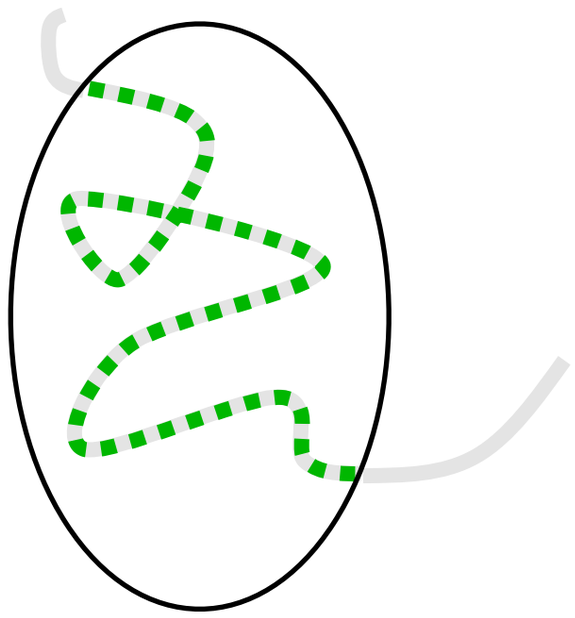
EGFP has 2 nonfluorescent states due to an internal and a pH-dependent external protonation of the chromophore:

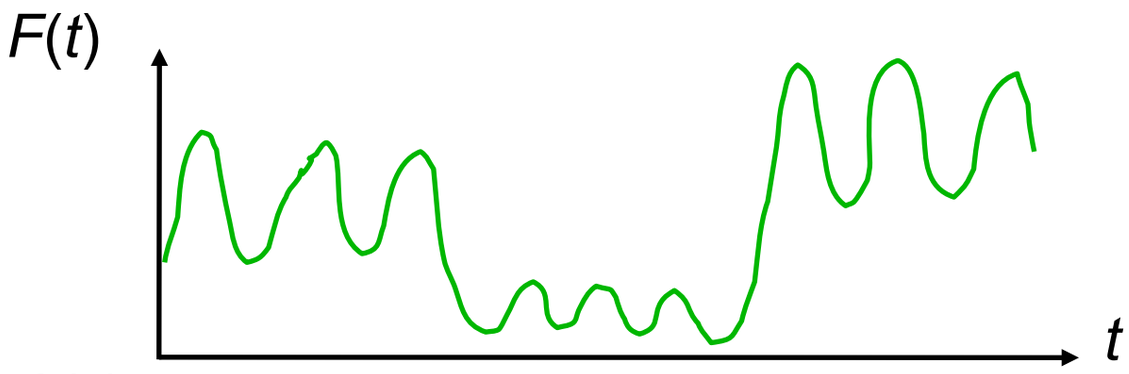


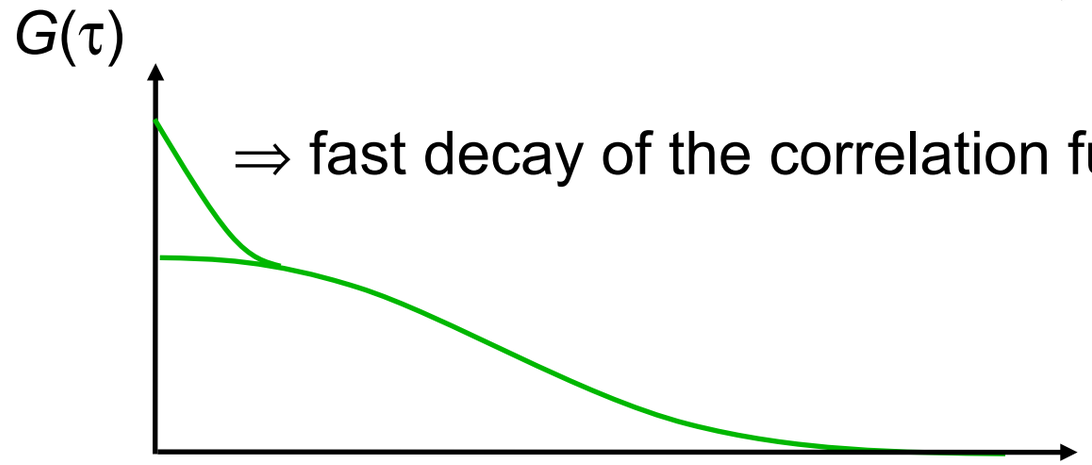
time constant for blinking 300 µs (pH 7) and 45 µs (pH 5)

⇒ EGFP can be used as an intracellular pH indicator









⇒ fast decay of the correlation function due to blinking

Expression systems based on protein fusions

	added kDa	target
Visible fluorescent proteins from jelly fish and corals (VFPs)	27	—
spectral variants (Tsien, Miyawaki, etc.)		
“cameleon” dual-VFP constructs for sensing ions, pH, covalent modification,…		
photoactivatable, photoconvertible, photochromic VFPs		
bimolecular complementation (half VFP molecules)	2x 27/2	
CCxxCC motifs [bisarsenicals FlAsH, ReAsH; biotin, photochromic derivativesl]	0.7-1.3	cys_4
N-terminal cysteine (from protein cleavage) [thioester-coupled probesl]	–	cys
acyl and peptidyl carrier protein (ACP, PCP) [CoA-linked probes]	9	ser
DNA alkyltransferase (SNAP-tag, CLIP-tag) [para-substituted benzylguanines]	20	cys
mutant dehalogenase (HaloTag) [probe-substituted haloalkanes]	33	asp
biotin ligase acceptor protein (AP)	0.9	lys
oligohistidine and cys-his-Zn finger [NTA-linked biotin or probes]	0.7	his_{4-6}
biotin-mimetic peptide (Nano-tags) [avidin, streptavidin, anti-biotin linked probes]	1-1.7	–