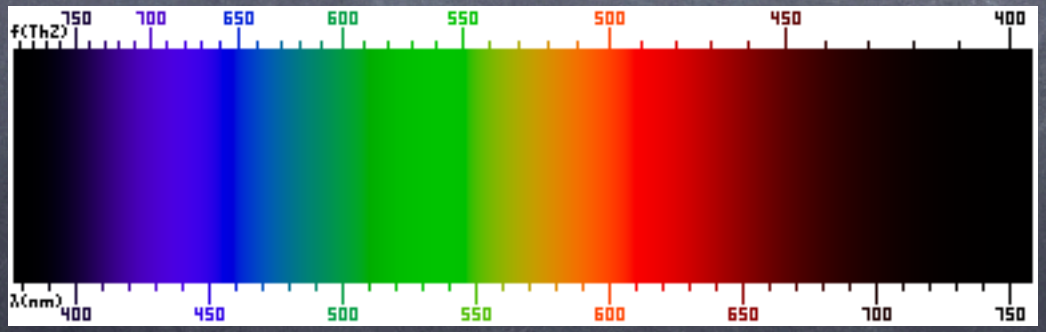
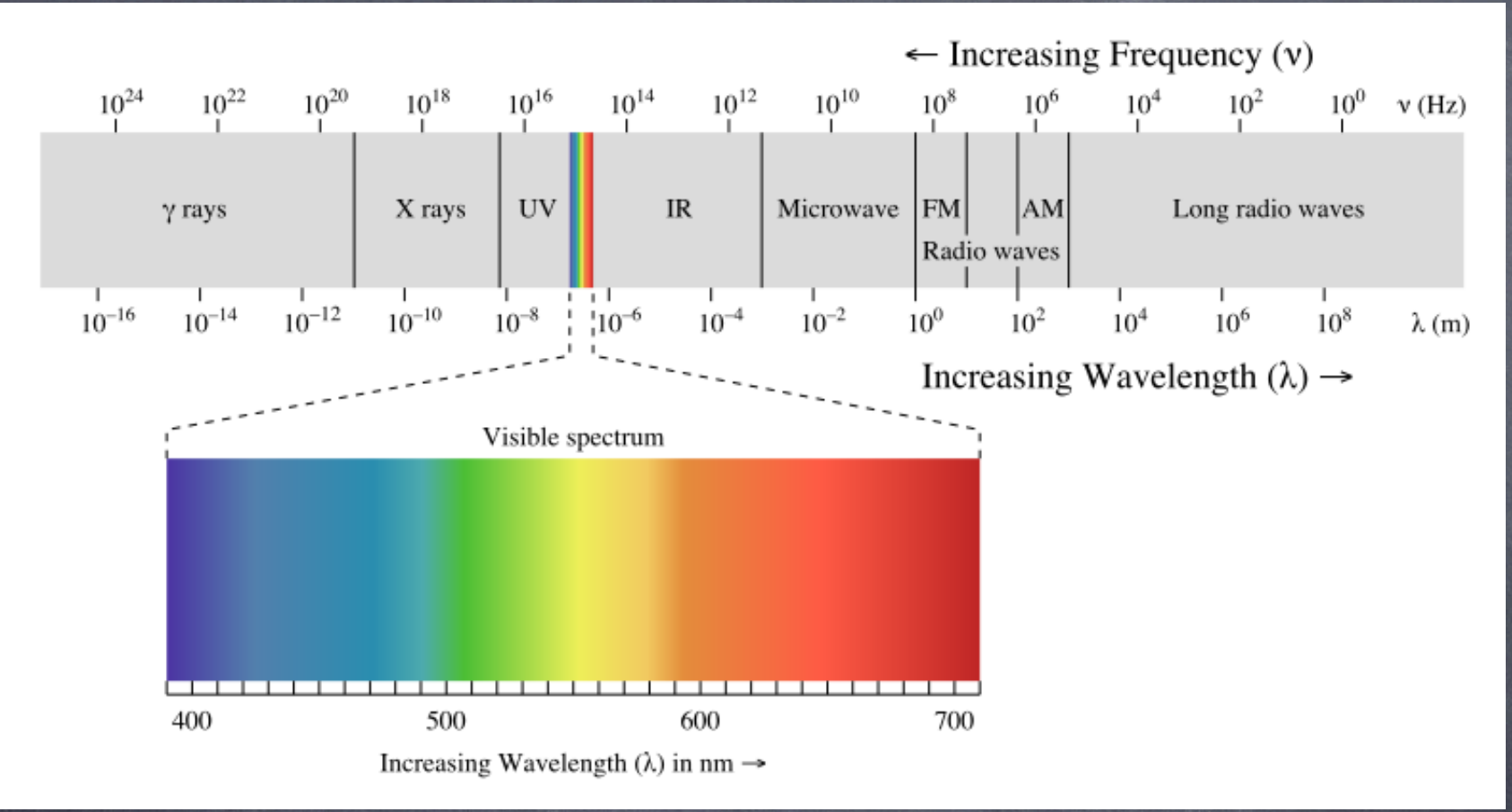
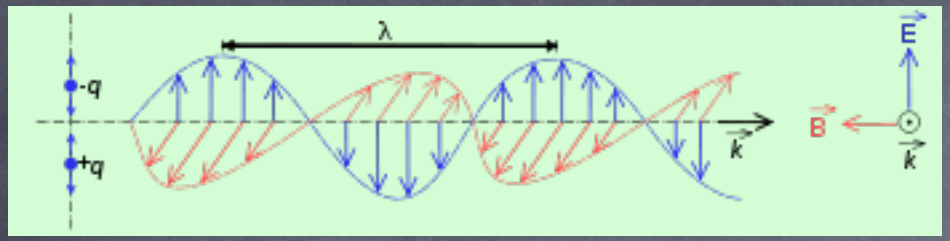
The properties of light





The speed of light in vacuum (c) = 299,792,458 m/s $E=mc^2$



Radiation
vs
corpuscolar properties

$$c = f\lambda$$

photon energy

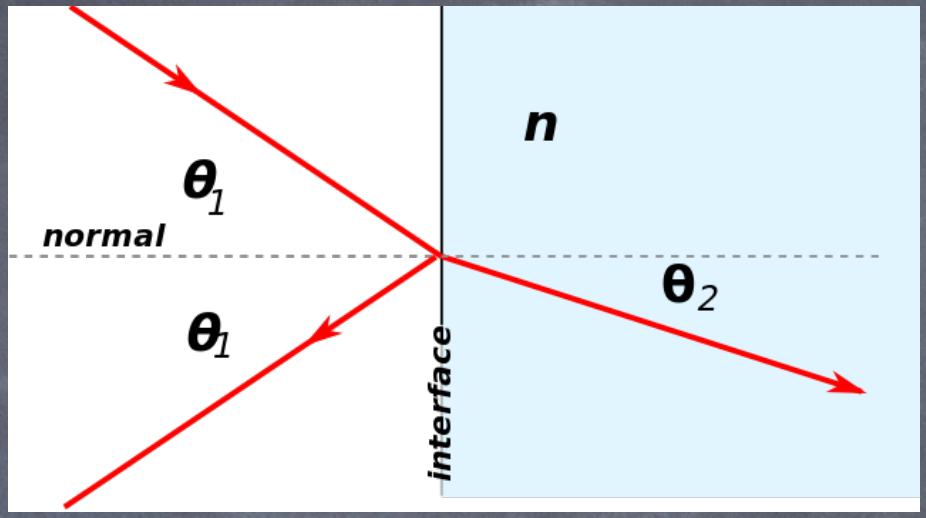
$$E = hf = \frac{hc}{\lambda}$$ Planck - Einstein

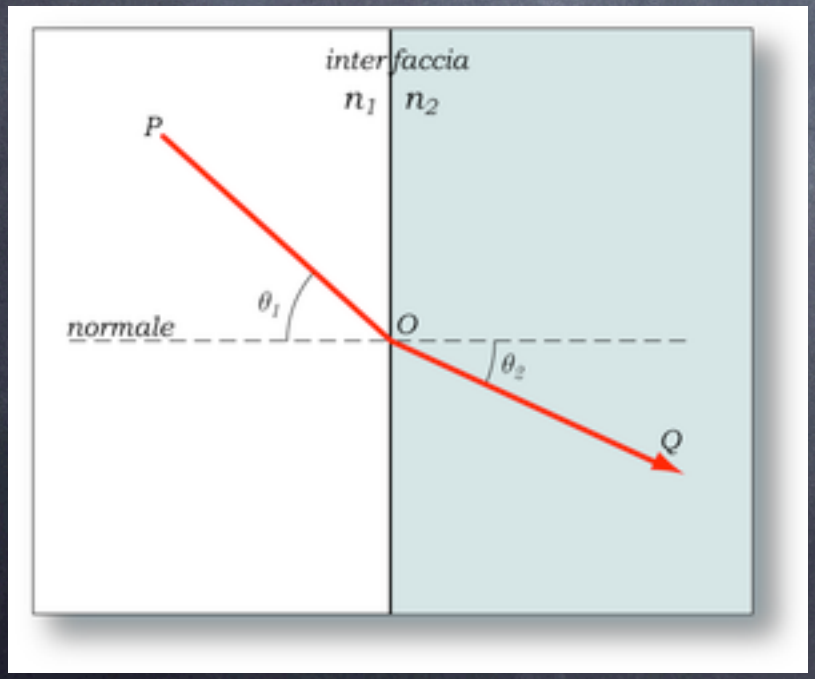
h = Planck constant

f = frequency

CLASS	FREQUENCY	WAVELENGTH	ENERGY
	300 EHz	1 pm	1.24 MeV
γ	30 EHz	10 pm	124 keV
HX	3 EHz	100 pm	12.4 keV
SX	300 PHz	1 nm	1.24 keV
	30 PHz	10 nm	124 eV
EUV	3 PHz	100 nm	12.4 eV
NUV	300 THz	1 μm	1.24 eV
NIR	30 THz	10 μm	124 meV
MIR	3 THz	100 μm	12.4 meV
FIR	300 GHz	1 mm	1.24 meV
EHF	30 GHz	1 cm	124 μeV
SHF	3 GHz	1 dm	12.4 μeV
UHF	300 MHz	1 m	1.24 μeV
VHF	30 MHz	10 m	124 neV
HF	3 MHz	100 m	12.4 neV
MF	300 kHz	1 km	1.24 neV
LF	30 kHz	10 km	124 peV
VLF	3 kHz	100 km	12.4 peV
VF/ULF	300 Hz	1 Mm	1.24 peV
SLF	30 Hz	10 Mm	124 feV
ELF	3 Hz	100 Mm	12.4 feV

reflection
refraction

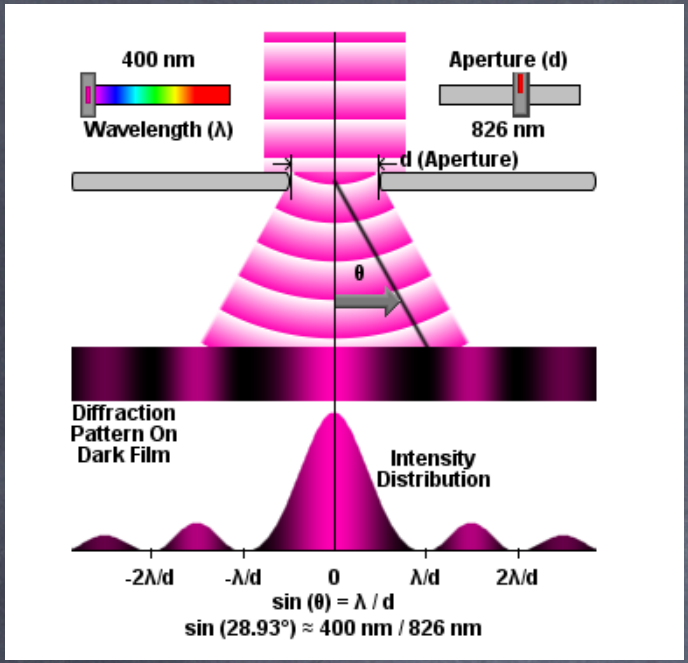




Snell law

$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

$$n = \frac{c}{v}$$ refractive index



Interference (diffraction and scattering)

diffraction limit (Abbe)

$$d = 1.22\lambda \frac{f}{a}$$

f = focal distance

a = light beam diameter

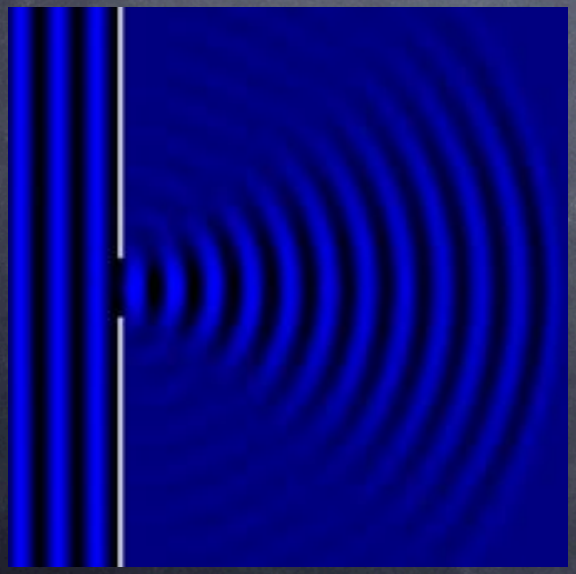
angolar resolution is

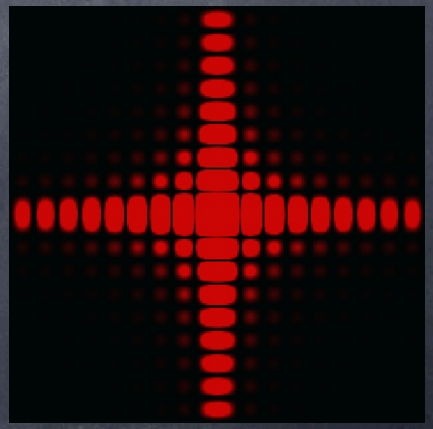
$$\sin\Theta = 1.22 \frac{\lambda}{D}$$

Abbe can be written as :

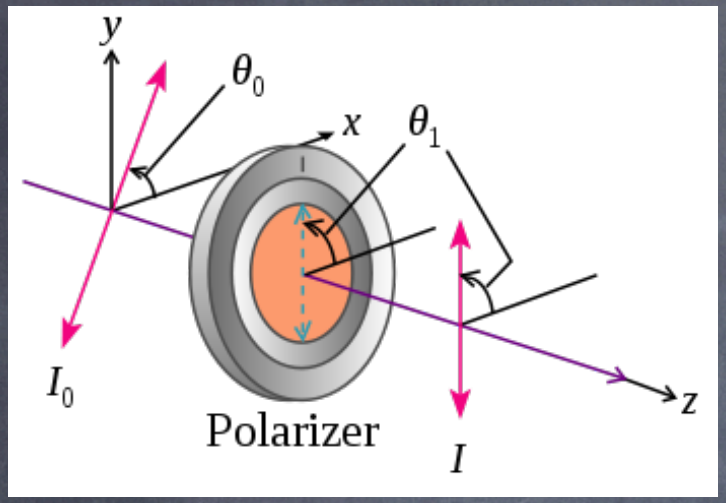
$$d = \frac{\lambda}{2(n \sin\Theta)}$$

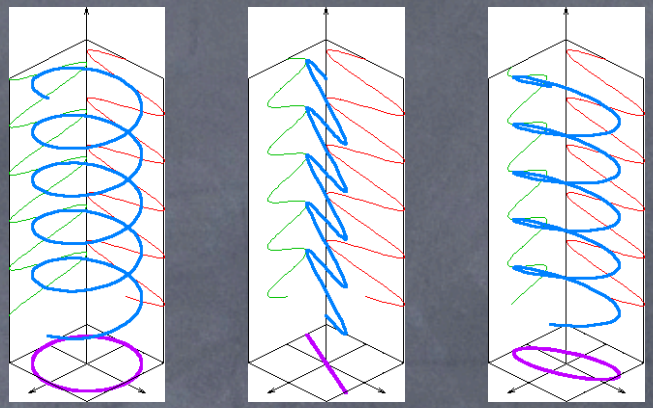
$n \sin\Theta = NA$ (numerical aperture)



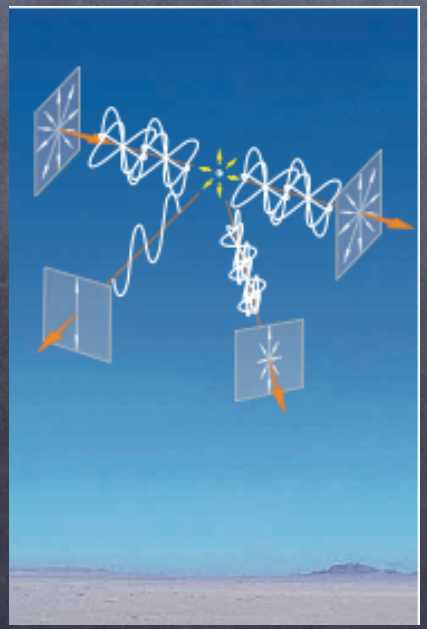


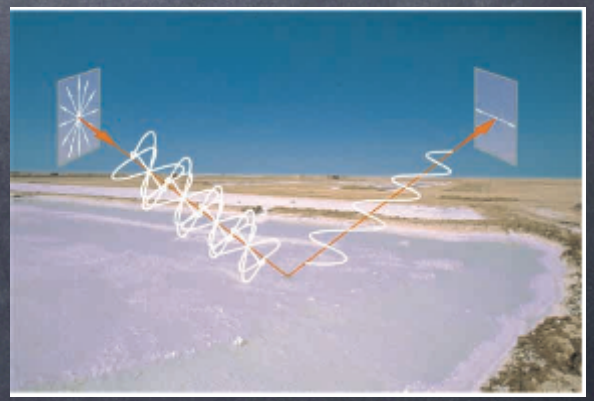
Polarization & scattering (dispersion)





$$I = I_0 \cos^2 \Theta_i$$





LAMBERT BEER law

LAMBERT - BEER

T = transmittance

$$T = \frac{I}{I_0} = 10^{-\varepsilon lc}$$

ε = molar extinction coefficient

c = concentration

l = path length

A = absorbance

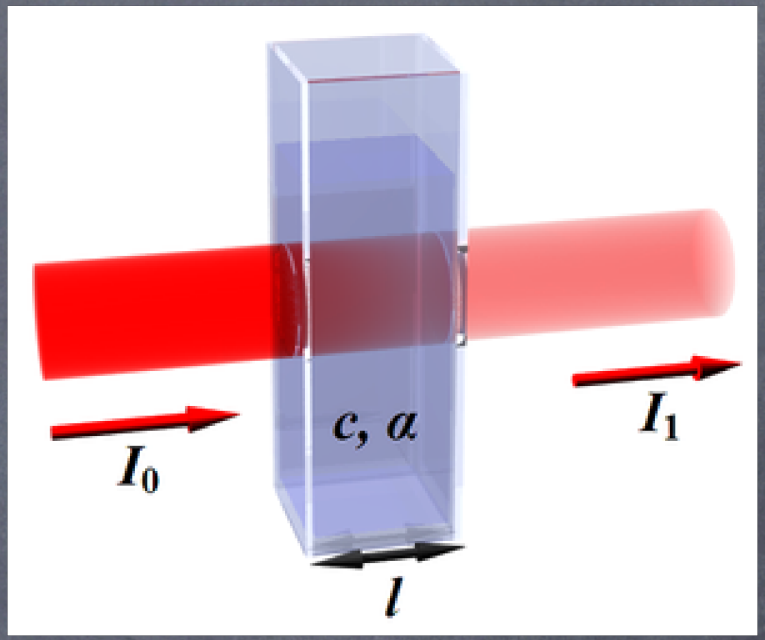
$$A = -\log_{10}\left(\frac{I}{I_0}\right)$$

$$A = \varepsilon lc = \alpha l$$

$$\alpha = \frac{4\pi n}{\lambda_0}$$

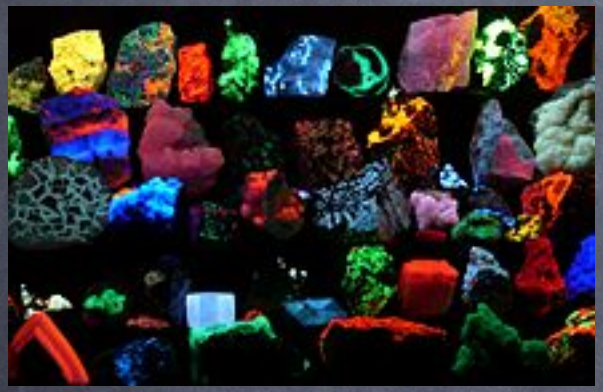
$$n = \text{refractive index} = \frac{c \text{ (light velocity in vacuum)}}{v_p \text{ (light velocity in the medium)}}$$

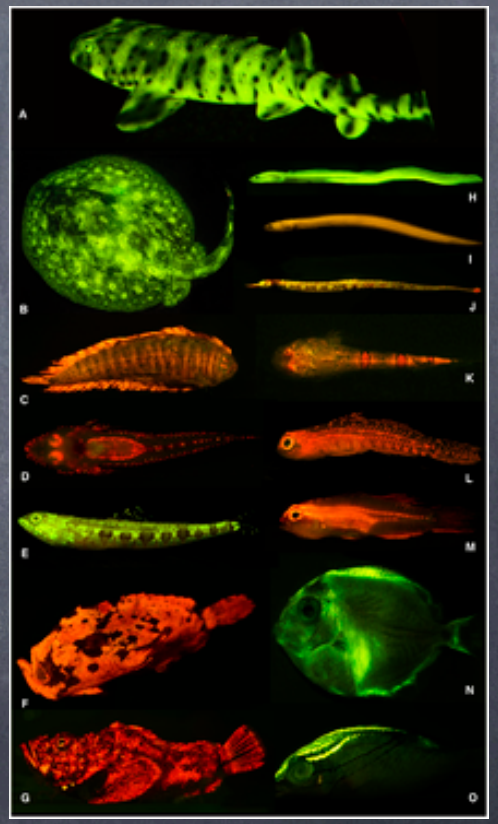
$\lambda_0 = \lambda$ of light in vacuum



FLUORESCENCE is the emission of light by a substance that has absorbed light or other electromagnetic radiation.

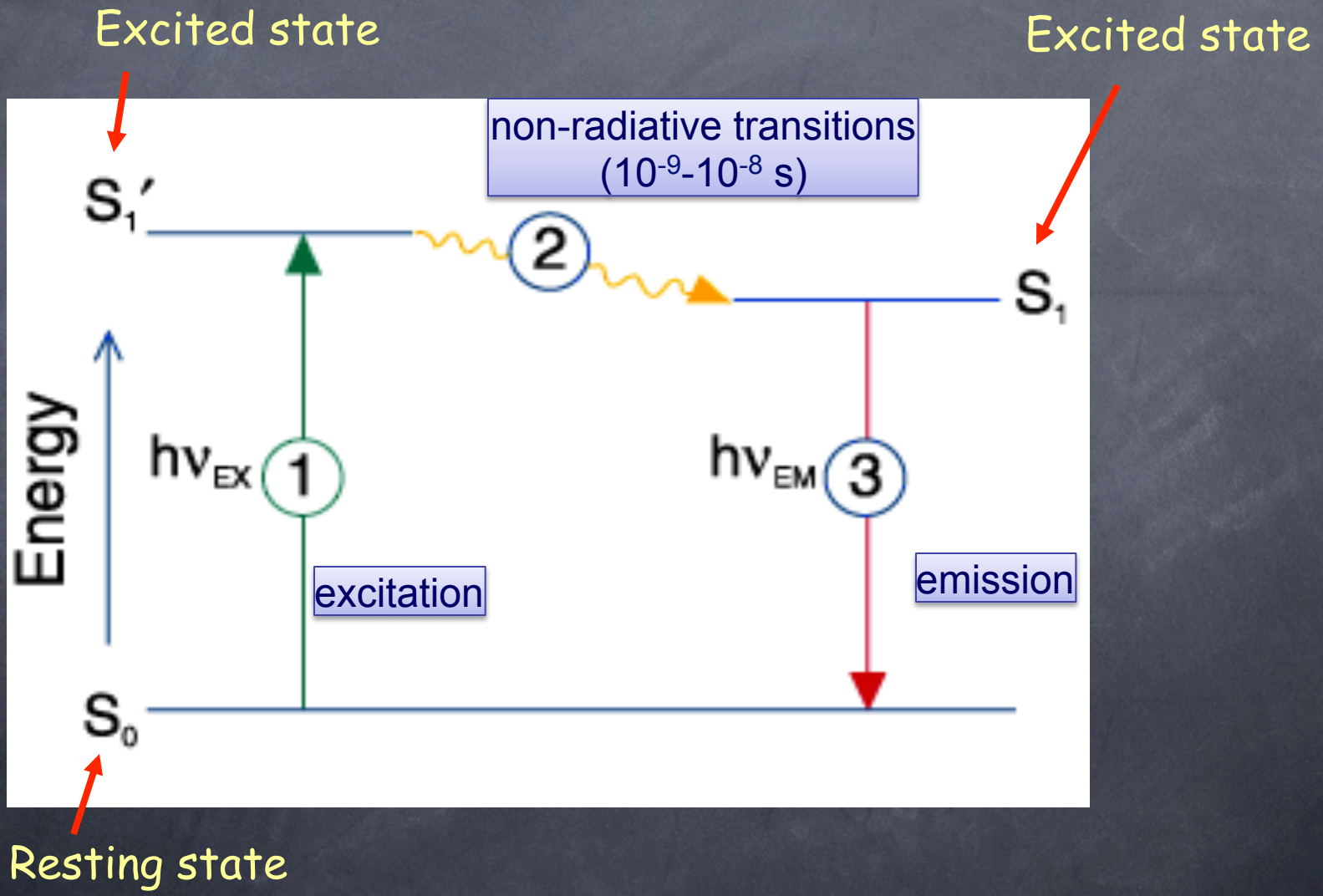
FLUORESCENCE in nature…





and in lab…

Jablonski diagram



$$S_0 + hf_{ex} \rightarrow S_1$$

h = Planck constant

f = light frequency

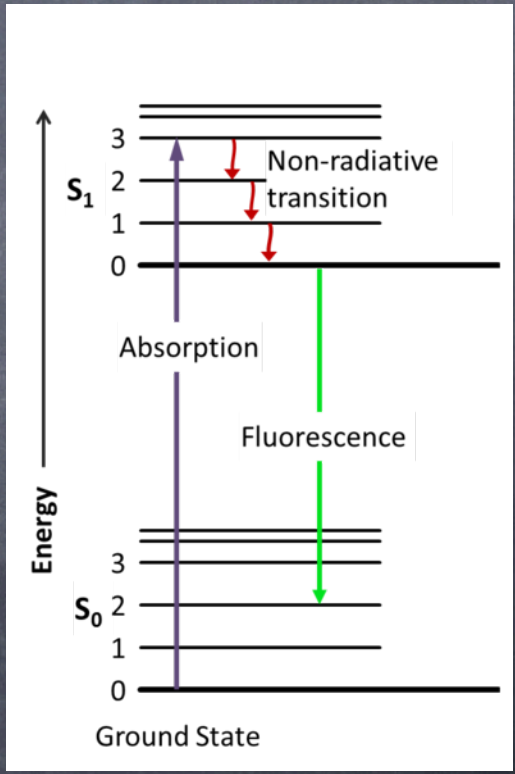
$$S_1 \rightarrow S_0 + hf_{em} + heat$$

$$\Phi = \text{quantum yield} = \frac{\text{number of emitted photons}}{\text{number of absorbed photons}}$$

$$\Phi = \frac{k_f}{\sum_i k_i}$$

k_f = spontaneous emission of radiation

$\sum_i k_i$ = sum of all rates of excited state decay



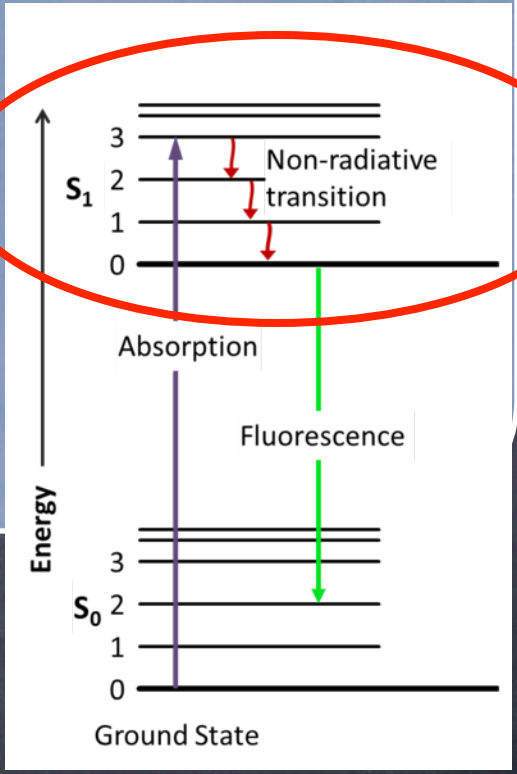
Fluorescence lifetime

Average time at which a molecule stays in excited state before photon emission

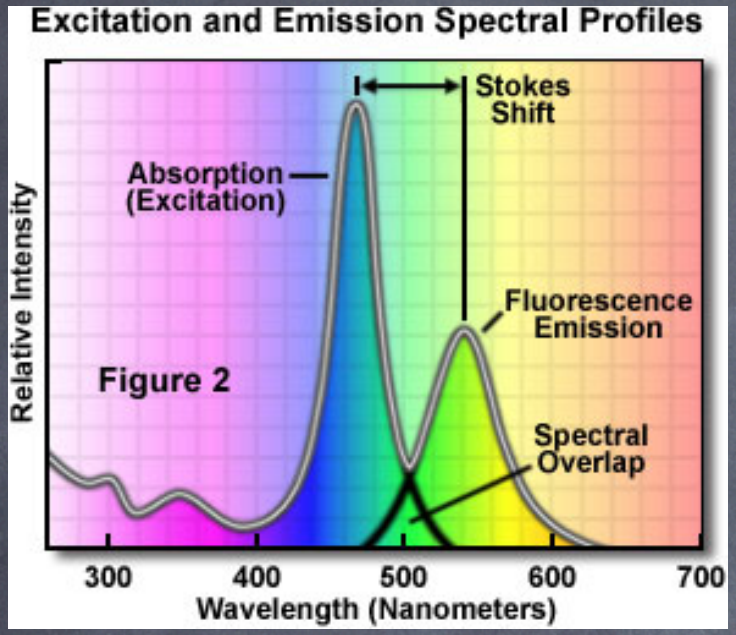
$$[S1] = [S1]_0^{-\Gamma t}$$

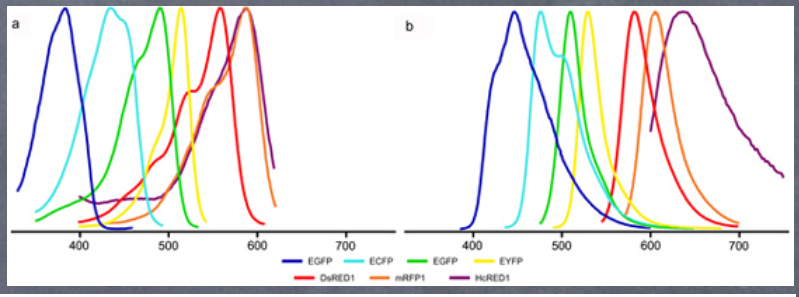
$$\Gamma = \frac{1}{\text{fluorescence lifetime}} = \text{decay rate}$$

$$\Gamma_{tot} = \Gamma_{rad} + \Gamma_{nonrad}$$



Fluorescence spectrum



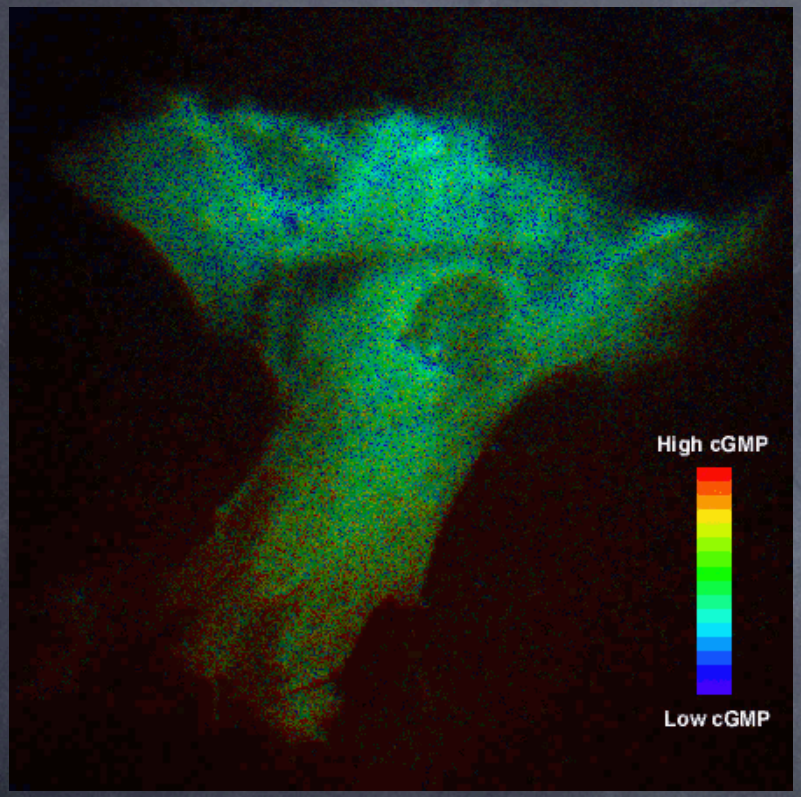


	Color†	Absorb (nm)	Emit (nm)	MM (g/mol)	ε ($cm^{-1}M^{-1}$)	Quantum Yield [1]
Alexa Fluor 350	blue	346	442	410	19,000	-
— 405	violet	401	421	1028	34,000	-
— 430	green	434	541	702	16,000	-
— 488	cyan-green	495	519	643	71,000	0.92
— 500	green	502	525	700	71,000	-
— 514	green	517	542	714	80,000	-
— 532	green	532	554	721	81,000	0.61
— 546	yellow	556	573	1079	104,000	0.79
— 555	yellow-green	555	565	~1250	150,000	0.1
— 568	orange	578	603	792	91,300	0.69
— 594	orange-red	590	617	820	90,000	0.66
— 610	red	612	628	1172	138,000	-
— 633	red	632	647	~1200	100,000	-
— 647	red	650	665	~1300	239,000	0.33
— 660	red	663	690	~1100	132,000	0.37
— 680	red	679	702	~1150	184,000	0.36
— 700	red	702	723	~1400	192,000	0.25
— 750	red	749	775	~1300	240,000	0.12

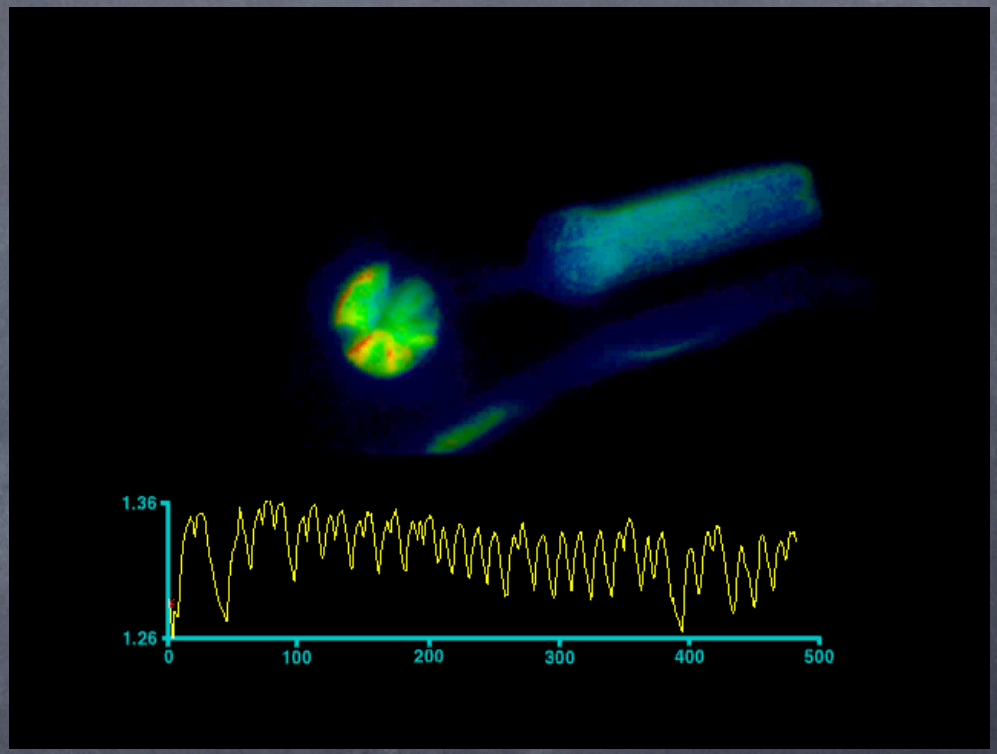
† = approximate color of the emission spectrum

ε = extinction coefficient

http://flowcyt.salk.edu/fluo.html



cGMP wave in RFL Cells



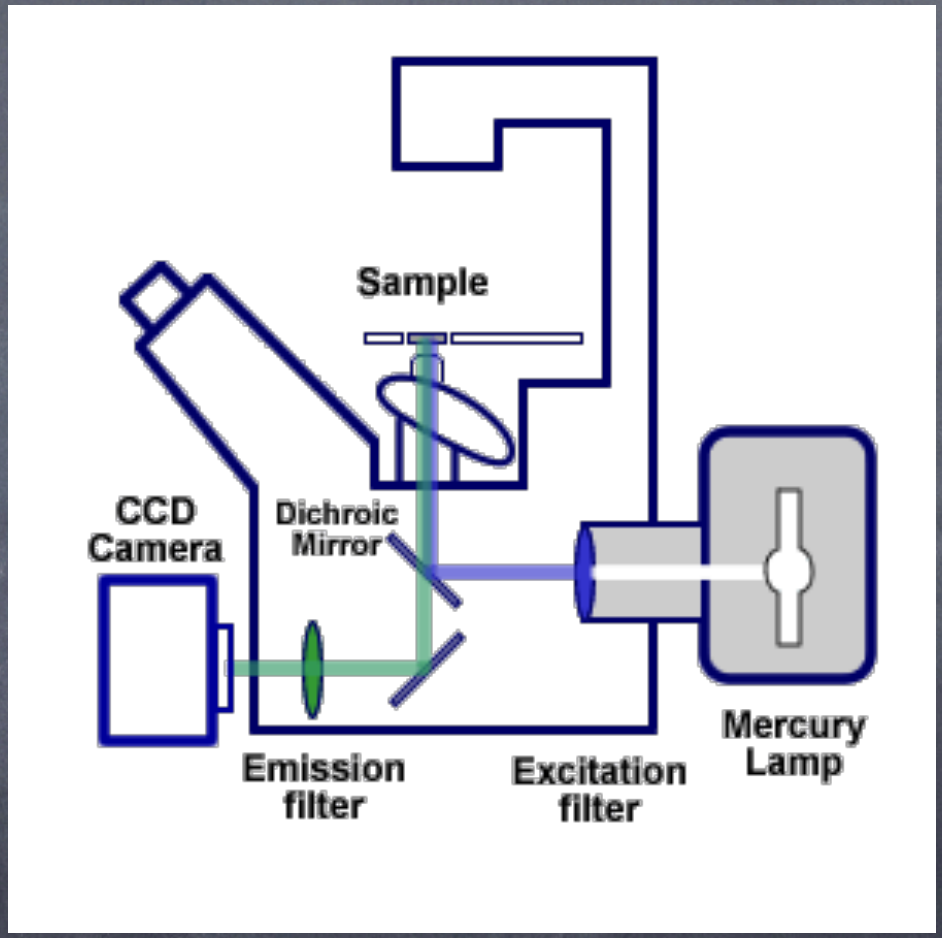
Pseudocolor movie of calcium transients in the pharyngeal muscle of an intact Caenorhabditis elegans. The calcium indicator is cameleon (Miaywaki et al., Nature 388:882), a ratiometric fluorescent calcium sensor containing CFP and YFP.

Overview of the Alexa Fluor Dyes

The Alexa Fluor dyes produce exceptionally bright and photostable conjugates (Alexa Fluor active esters and kits for labeling proteins and nucleic acids—Table 1.4, The Alexa Fluor Dye Series—Note 1.1). The Alexa Fluor dyes share several significant attributes, including:

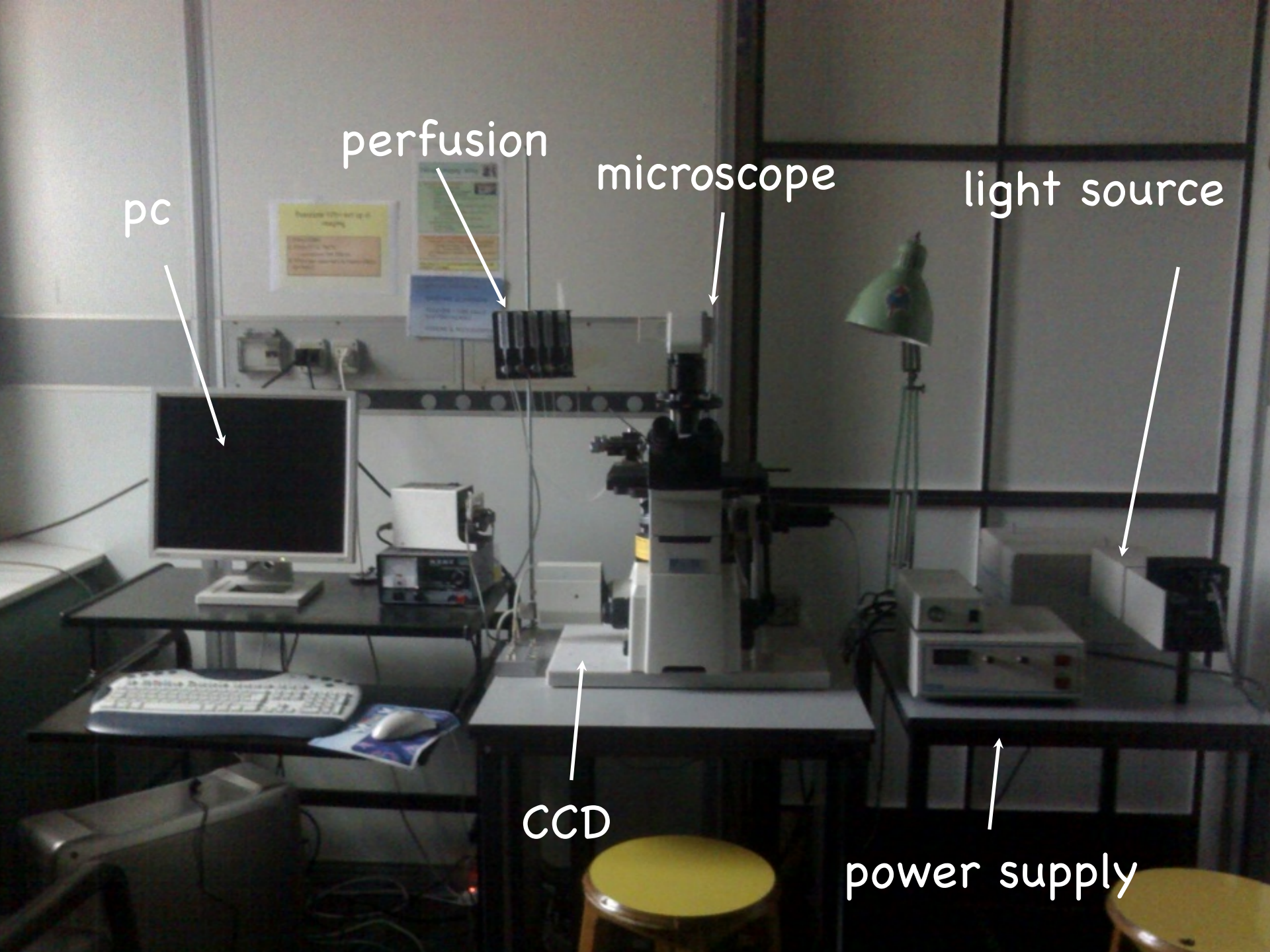
- Strong absorption at wavelengths of maximal output of common excitation sources
- Bright and unusually photostable fluorescence of their bioconjugates
- Good water solubility, which makes the reactive dyes easy to conjugate and the conjugates resistant to precipitation and aggregation
- Insensitivity of their absorpion and emission spectra to pH over a broad range
- Well-differentiated spectra, providing many options for multicolor detection and fluorescence resonance energy transfer (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2)
- High quantum yields and long fluorescence lifetimes (Fluorescence quantum yields (QY) and lifetimes (T) for Alexa Fluor dyes—Table 1.5)
- Extremely high FRET efficiency, with calculated R_0 values of up to 84 Å between pairs of Alexa Fluor dyes (R<0> values for some Alexa Fluor dyes—Table 1.6) and up to 77 Å between Alexa Fluor dyes and some nonfluorescent quenchers (R<0> values for QSY and dabcyl quenchers—Table 1.11)

Fluorescence microscopy: what do you need?



Look at this movie:

https://www.youtube.com/watch?v=AhzhOzgYoqw

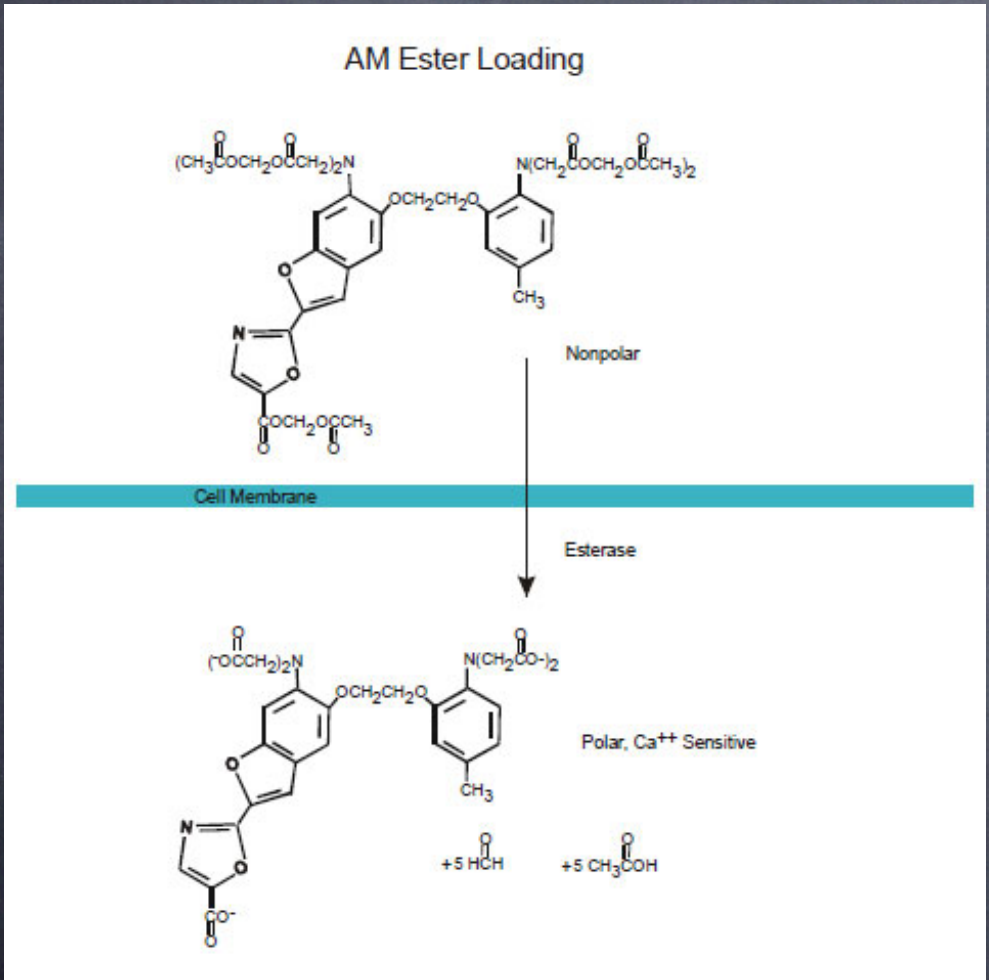

perfusion
microscope
pc
light source
CCD
power supply


Nikon

How to choose the right probe?

- Calcium concentration (dissociation constant K_d: from $0.1K_d$ to $10K_d$)
- Qualitative or quantitative measurement
- Emission brightness
- Multiparametric measurements

How to put the probe inside the cell (if it is not a protein)?



drawbacks:

Compartmentalization

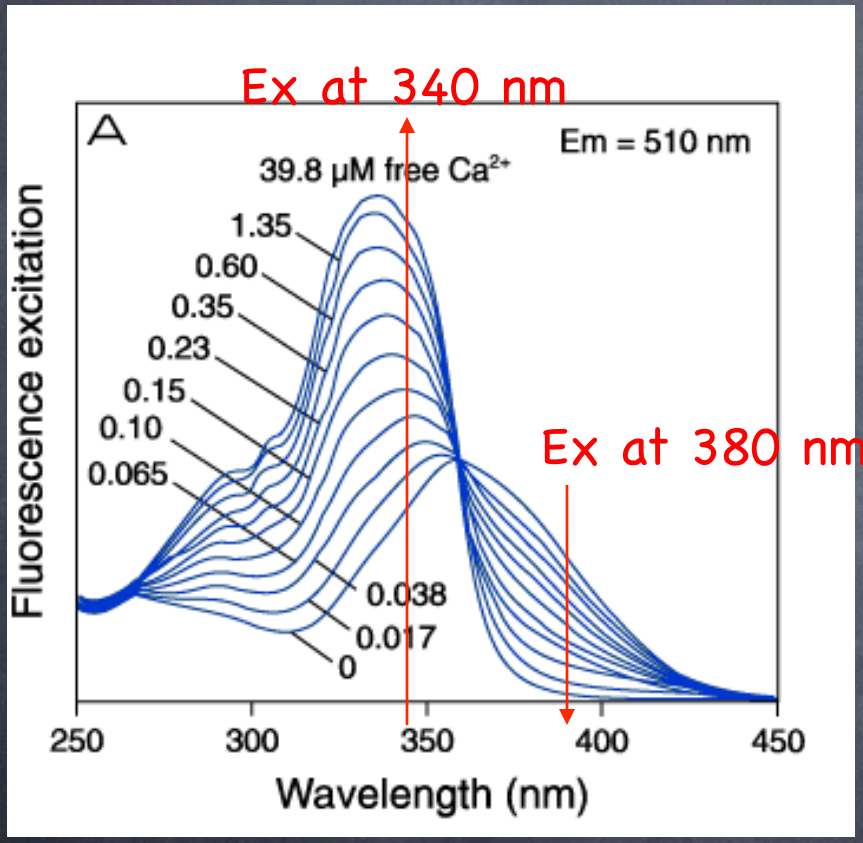
Quenching

UV-excited fluorescent probes

- Excitation or emission shift
- Measurement at two wavelenghts to avoid all the limitations imposed by Lambert-Beer law
- Ratiometric data

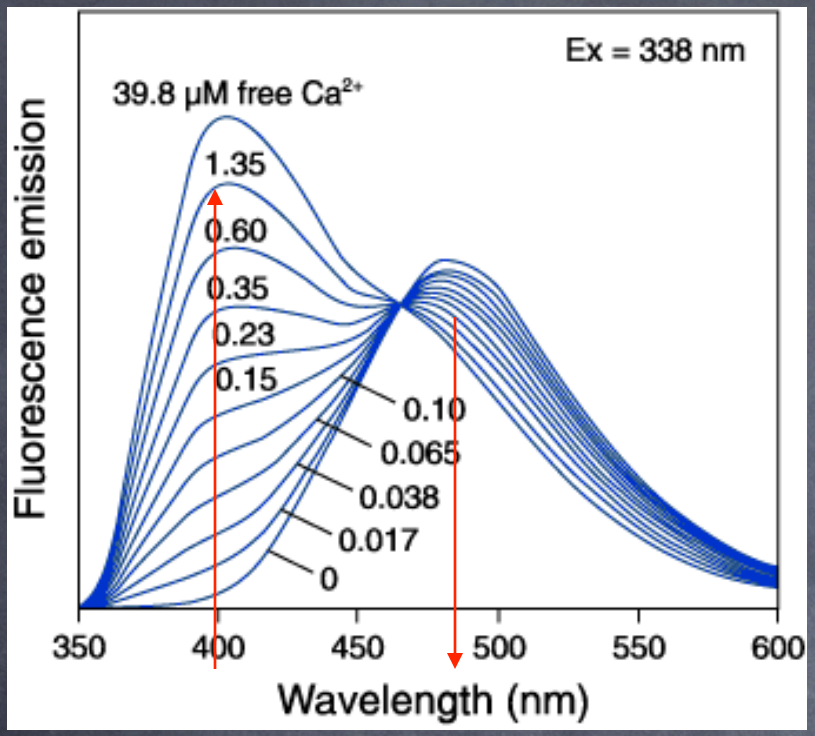
QUANTITATIVE measurements

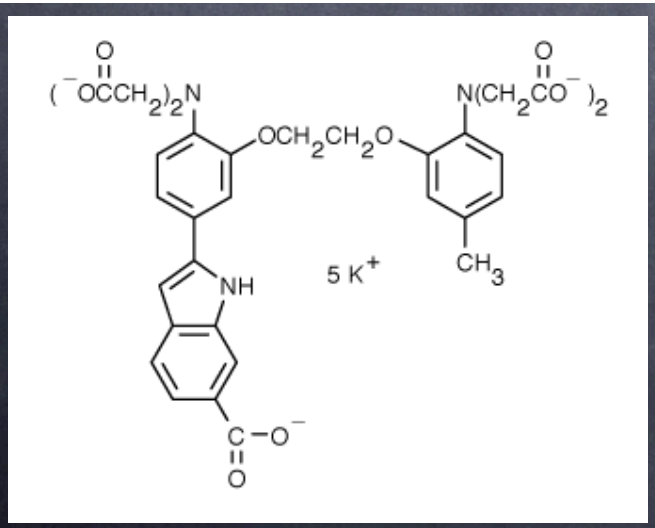
Fura-2



- Excitation at 340 and 380 nm.
- emission at 510 nm
- $[Ca^{2+}]$ is a function of Ex340/ Ex380, independently from fura-2 concentration and other environmental factors

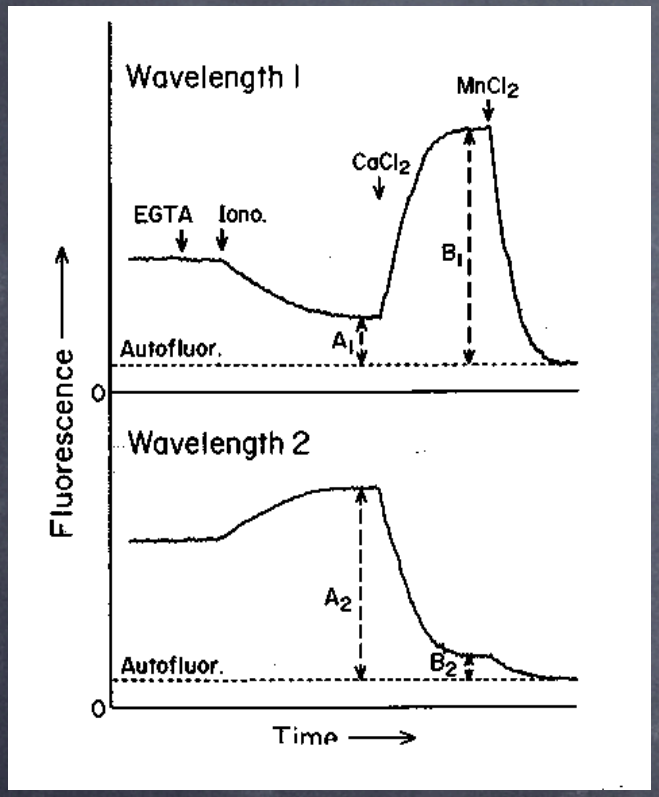
Indo-1





- excitation at 380 nm
- double emission (400 and 475 nm)
- [Ca^{2+}] = Em400 / Em475 ratio

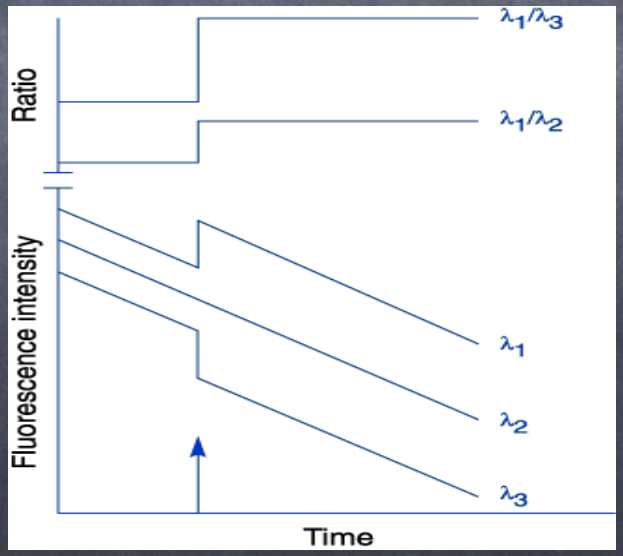
Calibration



$$[Ca^{2+}] = K_d \frac{R - R_{\min}}{R - R_{\max}} \frac{S_{f2}}{S_{b2}}$$

$$R_{\min} = \frac{A_1}{A_2} \qquad R_{\max} = \frac{B_1}{B_2}$$

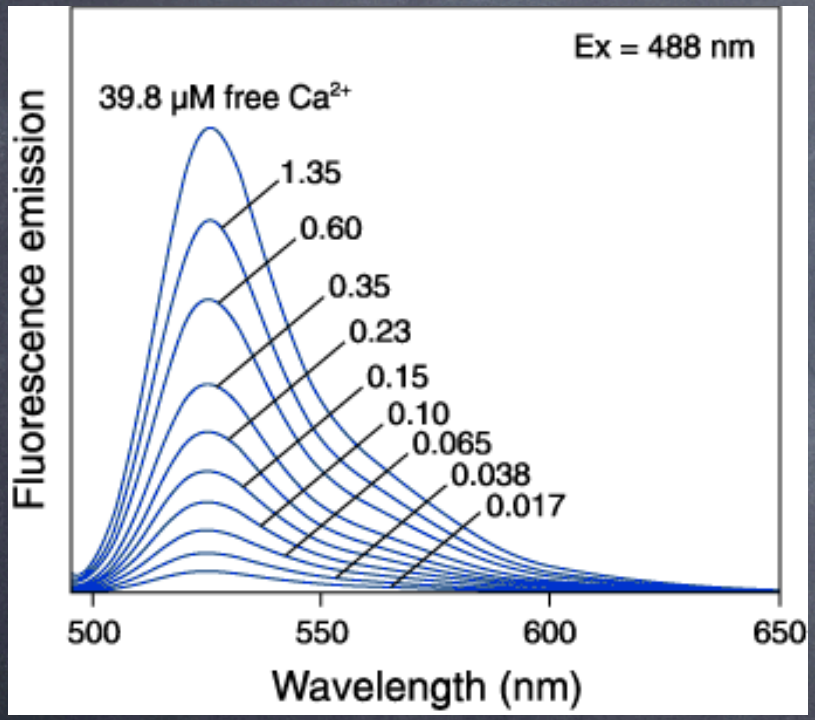
$$\frac{S_{f2}}{S_{b2}} = \frac{A_2}{B_2}$$



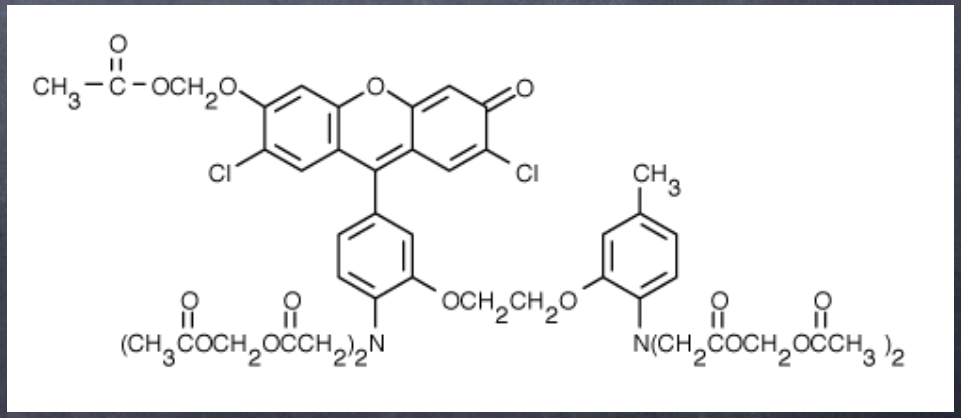
Visible light-emission probes: advantages

- Confocal microscopy
- Less autofluorescence
- Less damage and scattering
- More absorbance
- Caged compounds

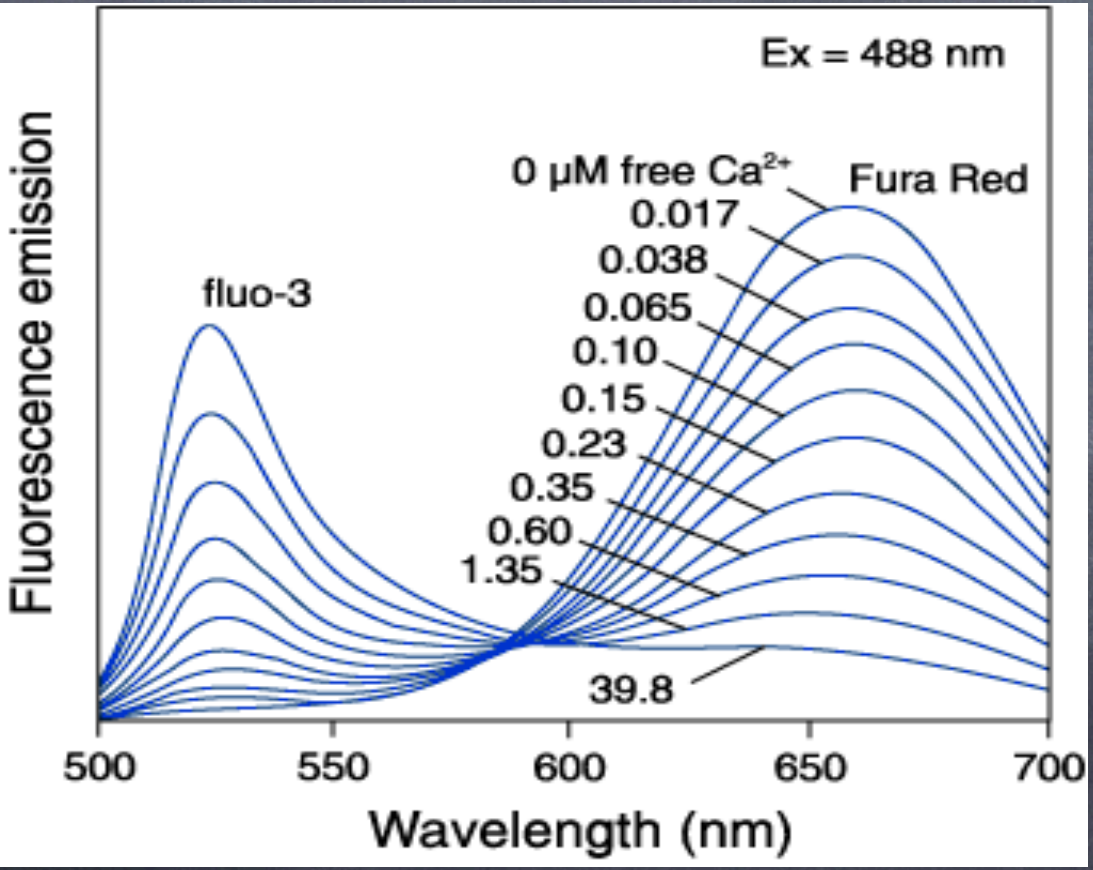
Fluo-3



- Excitation at 488 nm
- Emission at 525 nm
- Non ratiometric

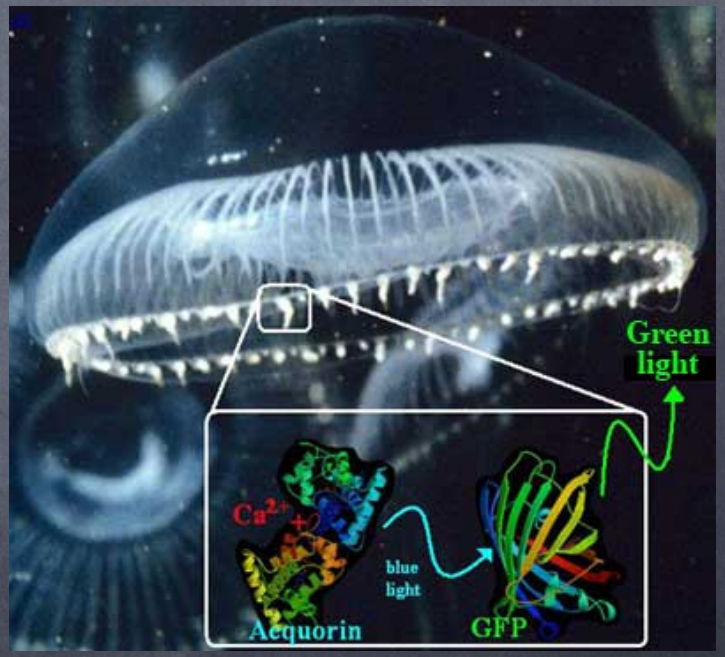


A nice trick:
how to 'mimic' a ratiometric measurement using visible-excited probes (Fluo-3 e Fura Red)



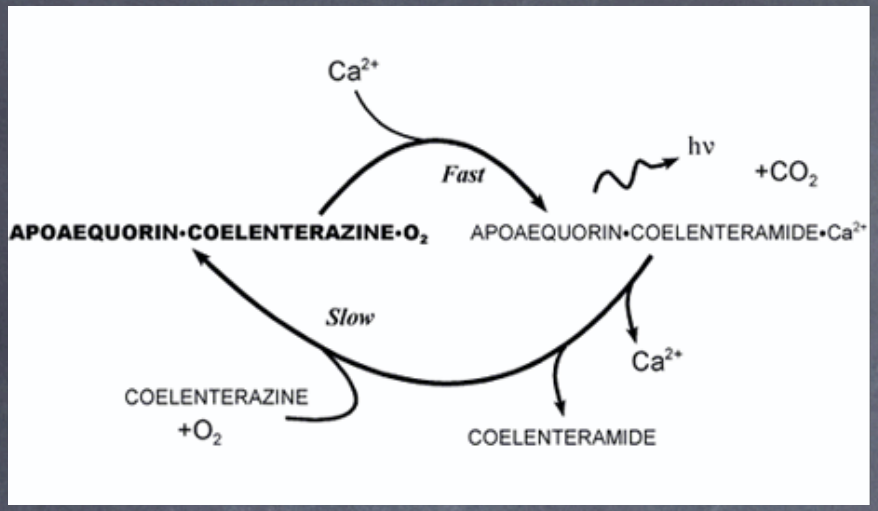
BIOLUMINESCENCE

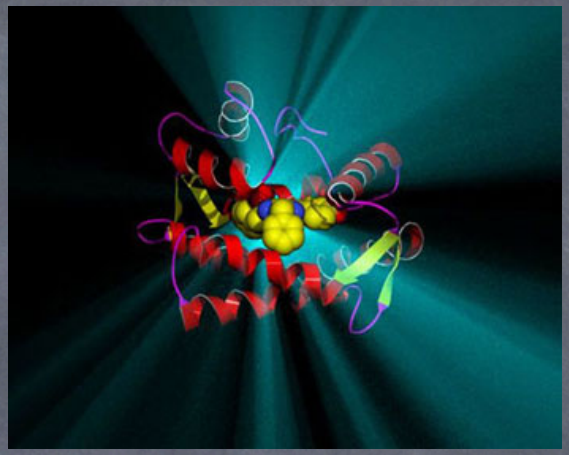
- Bioluminescence is light emission by photoproteins in some organisms
- Product of a chemical reaction
- No excitation required
- Emission usually low
- Aequorin is a photoprotein extracted from Aequoria Victoria

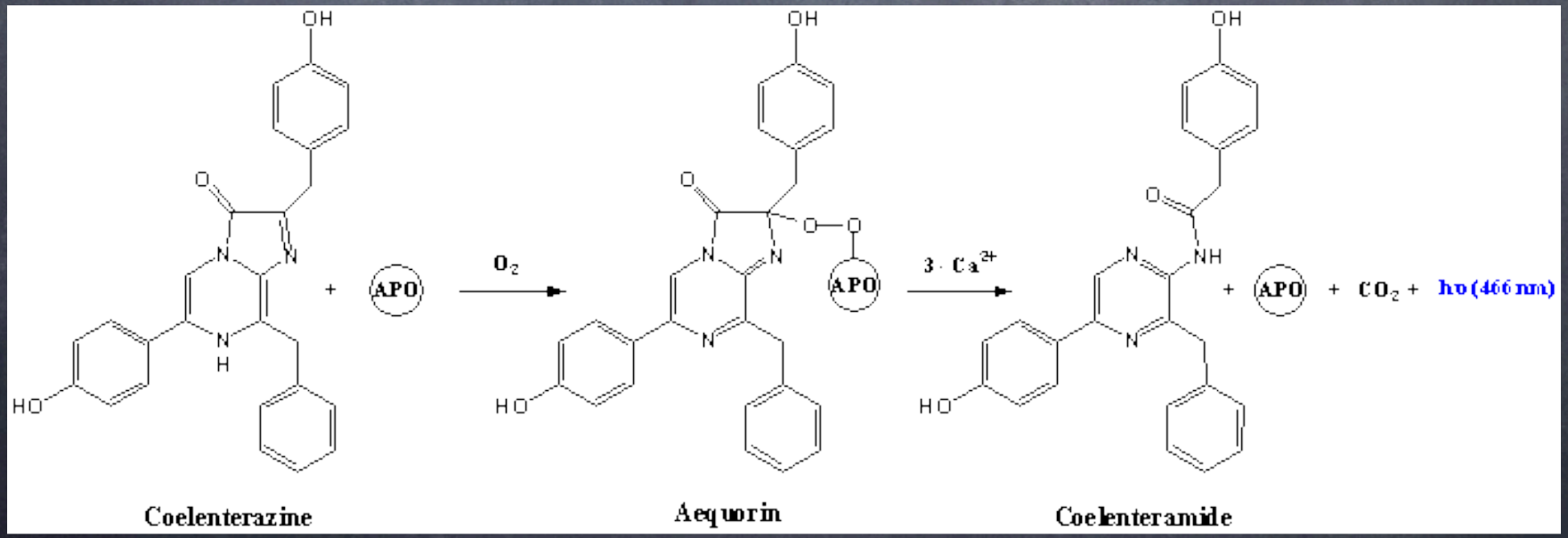


Aequoria Victoria

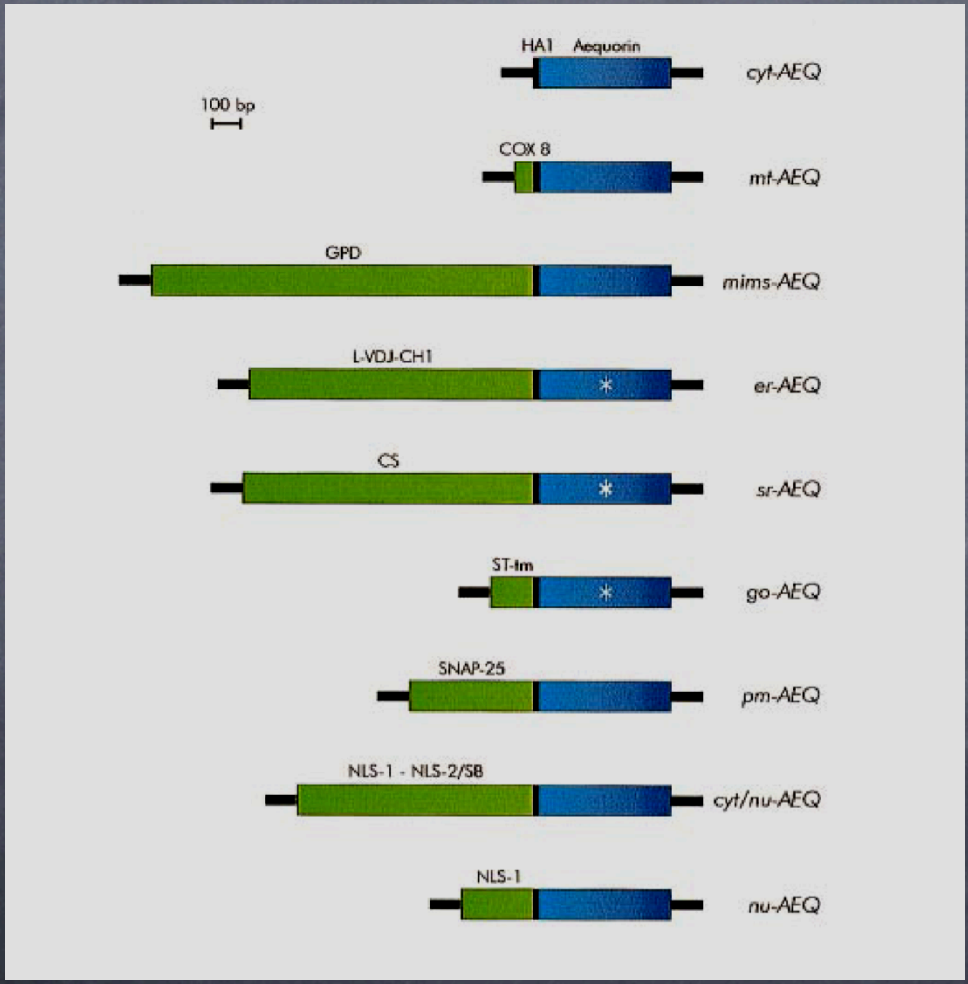
! Sometimes we can use natural devices and processes to setup new techniques !


Ca2+
Fast
hν
+CO2
APOAEQUORIN•COELENTERAZINE•O2
APOAEQUORIN•COELENTERAMIDE•Ca2+
Slow
Ca2+
COELENTERAZINE
+O2
COELENTERAMIDE




OH
O
N
N
N
H
HO
+
APO
O2
O
O
O
N
N
N
APO
HO
3 · Ca2+
O
NH
N
N
+
APO
+
CO2 +
hν (466 nm)
HO
Coelenterazine
Aequorin
Coelenteramide

Recombinant Aequorin can be selectively targeted in different intracellular compartments



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PROTOCOL

Subcellular calcium measurements in mammalian cells using jellyfish photoprotein aequorin-based probes

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The jellyfish *Aequorea victoria* produces a 22-kDa protein named aequorin that has had an important role in the study of calcium (Ca^{2+}) signaling. Aequorin reacts with Ca^{2+} via oxidation of the prosthetic group, coelenterazine, which results in emission of light. This signal can be detected by using a special luminescence reader (called aequorinometer) or luminescence plate readers. Here we describe the main characteristics of aequorin as a Ca^{2+} probe and how to measure Ca^{2+} in different intracellular compartments of animal cells (cytosol, different mitochondrial districts, nucleus, endoplasmic reticulum (ER), Golgi apparatus, peroxisomes and subplasma-membrane cytosol), ranging from single-well analyses to high-throughput screening by transfecting animal cells using DNA vectors carrying recombinant aequorin chimeras. The use of aequorin mutants and modified versions of coelenterazione increases the range of calcium concentrations that can be recorded. Cell culture and transfection takes ~3 d. An experiment including signal calibration and the subsequent analyses will take ~1 d.

TABLE 1 | Summary of the most common techniques for intracellular Ca^{2+} detection.

Probe	Origin	Detection technique	Ref.
Aequorin	Genetically encoded	Luminometry	15
Berovin	Genetically encoded	Luminometry	22
Obelin	Genetically encoded	Luminometry	23
Cameleon	Genetically encoded	FRET microscopy	24
Troponin C biosensor	Genetically encoded	FRET microscopy	25
Camgaroo	Genetically encoded	Fluorescence microscopy	26
Ratiometric Pericam	Genetically encoded	Ratiometric fluorescence microscopy	27
GEM-GECO1	Genetically encoded	Ratiometric fluorescence microscopy	28
Calcium Green-1	Synthetic	Fluorescence microscopy	29
Fluo-3, Fluo-4	Synthetic	Fluorescence microscopy	29
Fura-2, Indo-1	Synthetic	Ratiometric fluorescence microscopy	30

TABLE 2 | Description of the compartment-specific aequorin chimeras available.

Intracellular localization	Acronym	Targeting strategy
Cytosol	CytAEQ	No targeting sequence is added to aequorin; the sequence of aequorin was modified only by adding the epitope tag HA1 (ref. 15)
Nucleus	NuAEQ	A fragment of rat glucocorticoid receptor, lacking the hormone-binding domain and the nuclear localization signal are fused with the HA1-tagged aequorin[31]
	MtAEQwt	Mitochondrial pre-sequence of subunit VIII of cytocrome *c* oxidase (COX) is fused to the HA1-tagged aequorin, for measurements of $[Ca^{2+}]$ up to 10–15 μM (ref. 32)
Mitochondrial matrix	mtAEQmut	The mutated version of mtAEQwt. Because of the cooperativity between the three Ca^{2+}-binding sites of aequorin, the point mutation (Asp119Ala)[13] that affects the second EF-hand domain, produces a mutated aequorin, which can be used to measure $[Ca^{2+}]$ in the range of 10–500 μM (ref. 33)
	mtAEQmut28,119	Double-mutated form (Asp119Ala and Asn28Leu) of mtAEQwt, which can be used to measure $[Ca^{2+}]$ in the millimolar range for long periods of time, without problems derived from aequorin consumption[14]
Mitochondrial intermembrane space	MimsAEQ	HA1-tagged aequorin is fused (sequence in frame) with glycerol phosphate dehydrogenase, an integral protein of the inner mitochondrial membrane, with a large C-terminal tail protruding on the outer side of the membrane, i.e., in the mitochondrial intermembrane space[34]
Plasma membrane	pmAEQ	The targeting of aequorin to the subplasmalemmal space was based on the construction of a fusion protein including the HA1-tagged aequorin and SNAP-25, a protein that is synthesized on free ribosomes and recruited to the inner surface of the plasma membrane after the palmitoylation of specific cysteine residues[35]
Endoplasmic reticulum	erAEQmut	The encoded polypeptide includes the leader sequence (L), the VDJ and CH1 domains of an Igg2b heavy chain (HC) and the HA1-tagged aequorin at the C-terminus. In this chimera, retention in the ER depends on the presence of the CH1 domain at the N terminus of aequorin. This domain is known to interact with the luminal ER protein BiP, thus causing the retention of the Igg2b HC in the lumen. In the absence of the immunoglobulin light chain, the polypeptide is retained in this compartment[36]
Sarcoplasmic reticulum	srAEQmut	Calsequestrin (CSQ), a resident protein of the sarcoplasmic reticulum, is fused to HA1-tagged aequorin. This chimera is used to measure $[Ca^{2+}]$ in the sarcoplasmic reticulum, the specialized muscle compartment involved in the regulation of Ca^{2+} homeostasis[37]
Golgi apparatus	goAEQmut	Fusion of the HA1-tagged aequorin and the transmembrane portion of sialyltransferase, a resident protein of the Golgi lumen[18]
Secretory vescicles	vampAEQmut	Mutated AEQ (AEQmut; Asp119Ala) is fused to the vesicle-associated membrane protein (vamp)2/synaptobrevin (a vesicle-specific SNARE with a single transmembrane-spanning region) allowing intravesicular $[Ca^{2+}]$ to be monitored[38]
Peroxisomes	peroxAEQ	HA1-tagged wild-type and Asp119Ala mutant aequorins were fused with a peroxisomal targeting sequence[17]

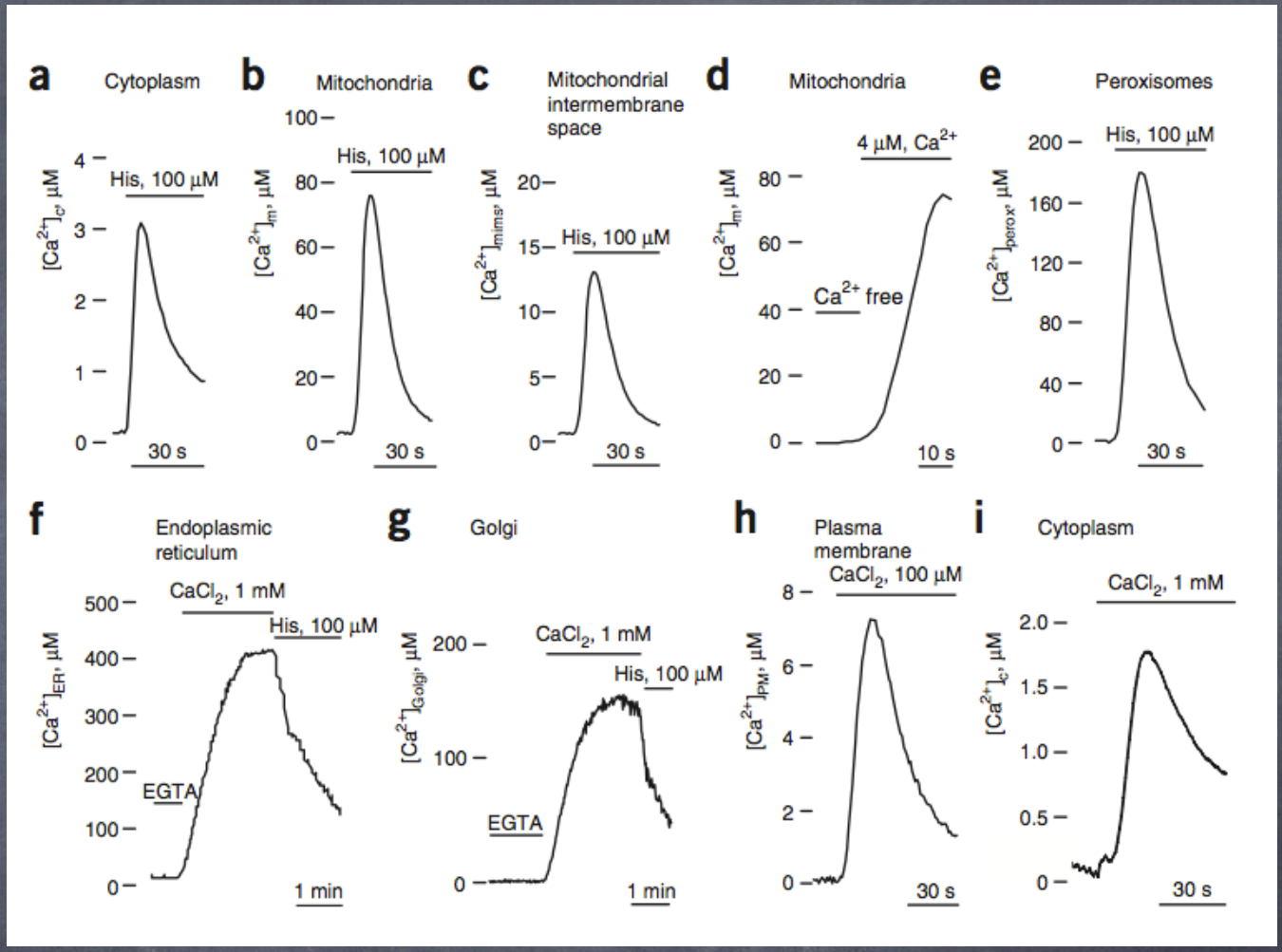

Figure 4 | Measurements of Ca^{2+} in different cellular compartments. (**a**–**i**) Representative Ca^{2+} kinetics measured with aequorin in cytoplasm (**a**), mitochondria (**b**), mitochondrial intermembrane space (**c**), mitochondria from permeabilized cells (**d**), peroxisomes (**e**), endoplasmic reticulum (**f**), Golgi apparatus (**g**), subplasmalemma (**h**) and cytoplasm during capacitative influx stimulation (**i**) in HeLa cells.

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Bioluminescence imaging of mitochondrial Ca^{2+} dynamics in soma and neurites of individual adult mouse sympathetic neurons

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online data

Two distinct calcium pools in the endoplasmic reticulum of HEK-293T cells

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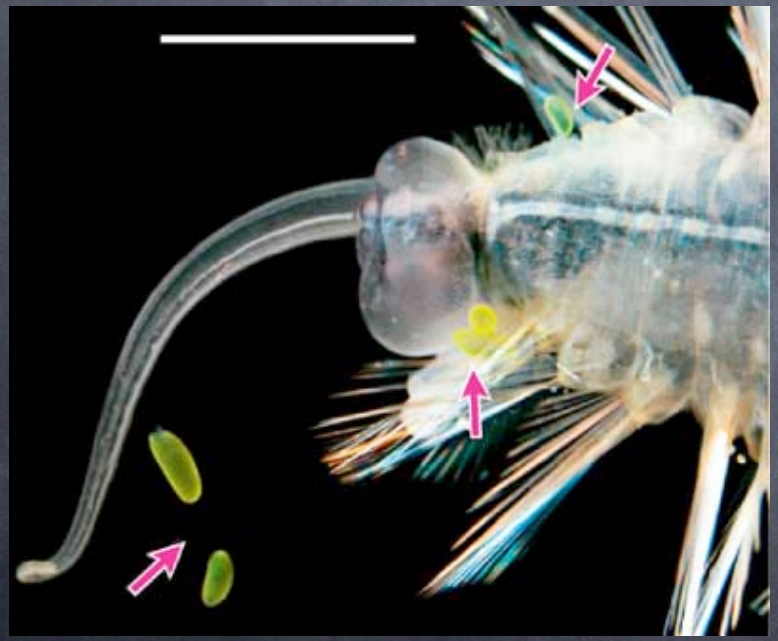
Glowing Marine Worms



Deep-Sea, Swimming Worms with Luminescent "Bombs"

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