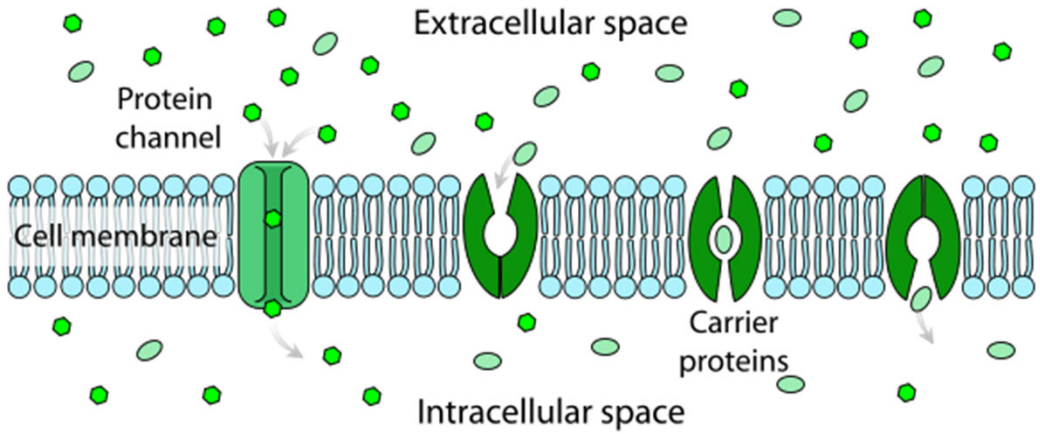
Effects of compartmentalization

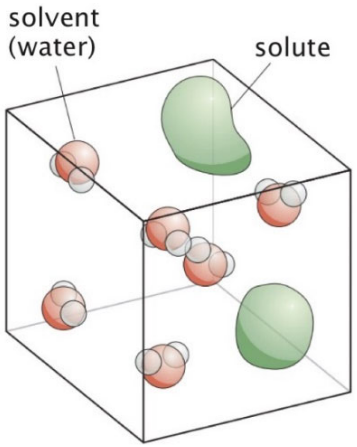
Concentration gradients

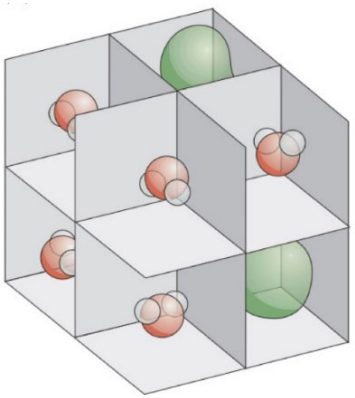


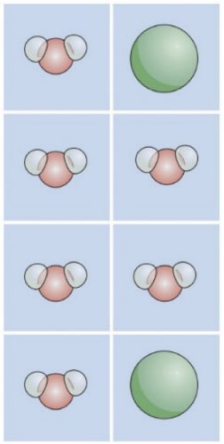
osmotic pressure
charge separation

regulation
signaling
chemical work

Chemical potential: The free energy of dilute solutions





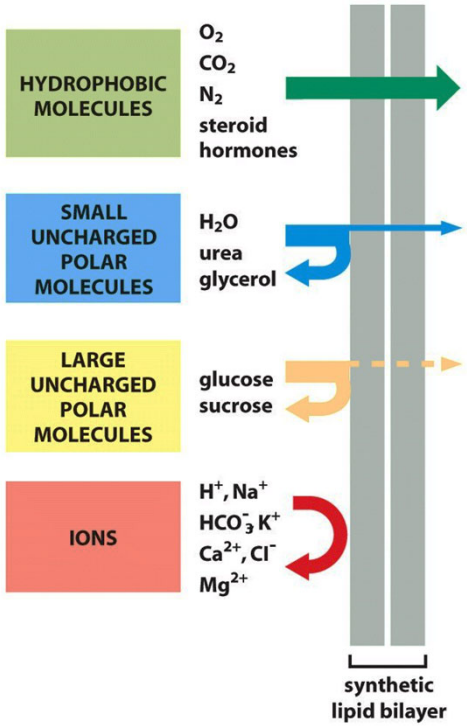


Lattice models:
allow counting of molecules
N_{H2O}, N_S

configuations can be evaluated

cont. chem. pot. &
osmotic pressure

Membranes are impermeable for ions



Membrane permeability depends on **hydrophobicity** (octanol partition coefficient logP)

Permeable to small uncharged molecules or hydrophobic molecules

impermeable to **charged, polar molecules** and **ions**

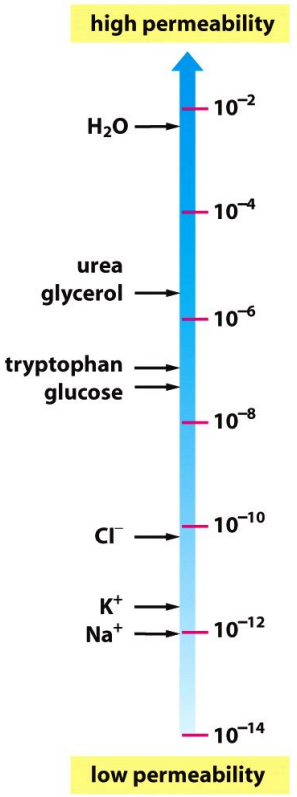
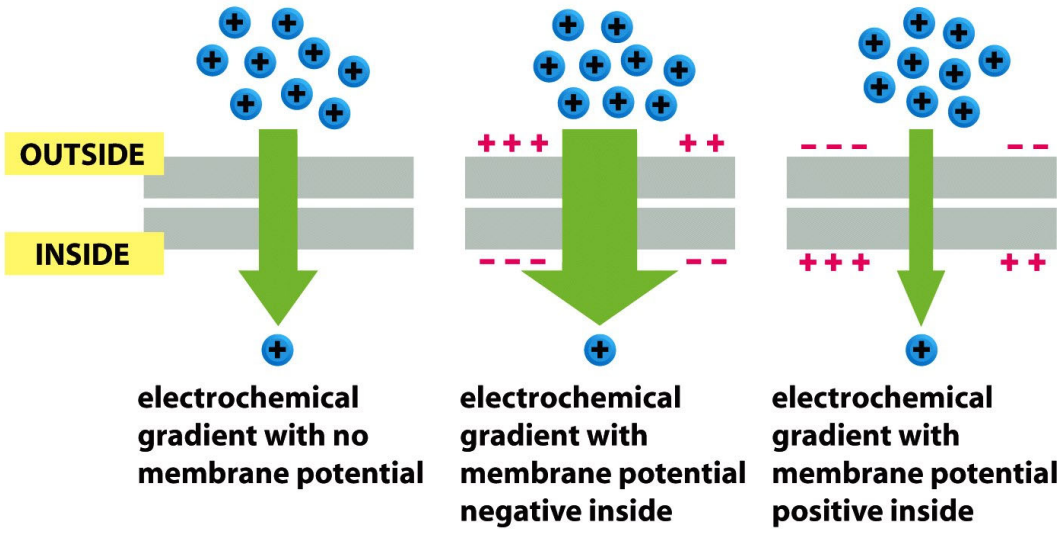


Figure 11-1 *Molecular Biology of the Cell* (© Garland Science 2008)

Controlling the electrical potential across membranes



to understand, how cells regulate the membrane potential:

- effect of charges, ionic solutions
- effect of membranes in charge separation
- mechanism of channel function

Figure 11-4b *Molecular Biology of the Cell* (© Garland Science 2008)

cont. membrane potential.

Nernst potential in cells

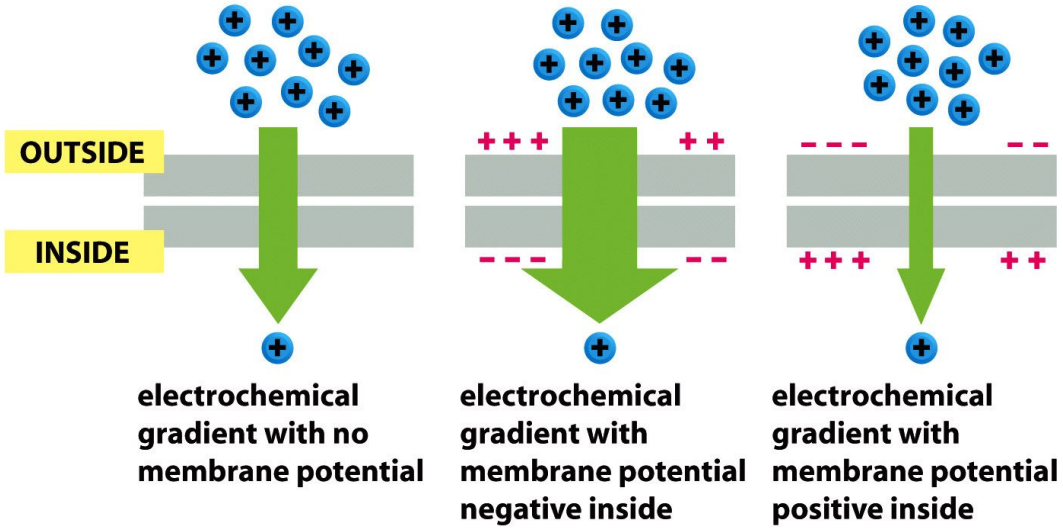


Figure 11-4b *Molecular Biology of the Cell* (© Garland Science 2008)

Nernst potential

$$V_2 - V_1 = \frac{k_B T}{ze} ln \frac{c_1}{c_2}$$

$$k_B T/e \approx 25mV$$

most excitable cells have membrane potentials in the range of 10-100 mV

→ **thermal energy plays a significant role**

Ion concentrations and Nernst potential in cells

Ion species	Intracellular concentration (mM)	Extracellular concentration (mM)	Nernst potential (mV)
K^+	155	4	−98
Na^+	12	145	67
Ca^{2+}	10^{-4}	1.5	130
Cl^-	4	120	−90

Physical biology of the cell

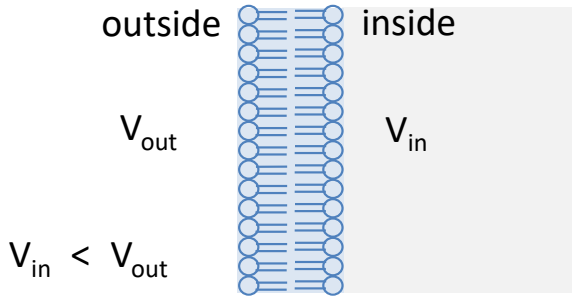
Nernst potential

$$V_2 - V_1 = \frac{27\ mV}{ze} ln \frac{c_{out}}{c_{in}}$$

at 37° C

Surplus positive charge within cells → buffered by neg. charge on DNA, proteins

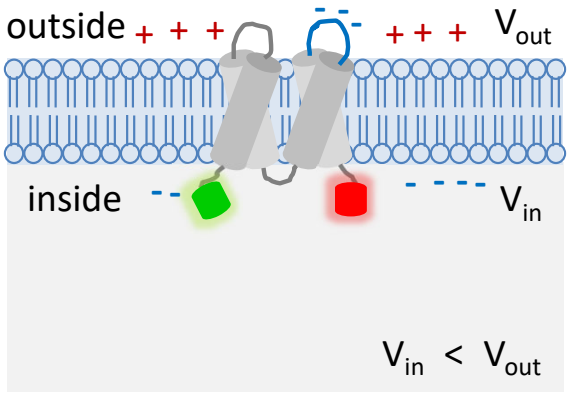
Resting potential across membrane:
- 90 mV (muscle cell)



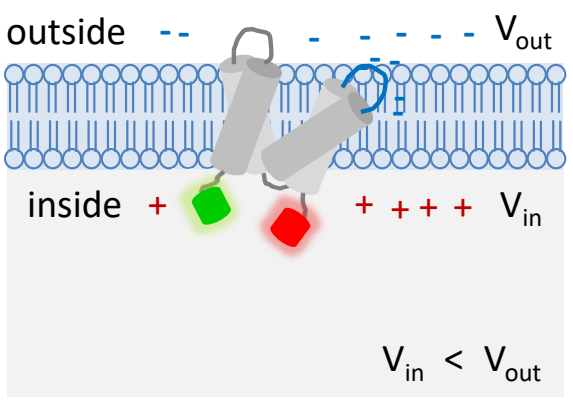
Question:
Based on the membrane potential – what are the expected ion concentrations? Is this found, and why?

Spectroscopic imaging of membrane potential

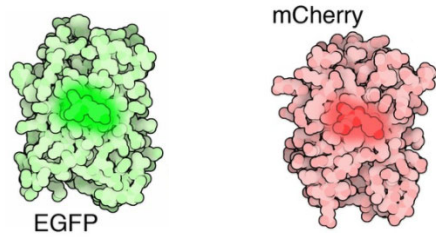
voltage-sensitive fluorescent protein (**VSFP**)



fluorescent proteins: distance far



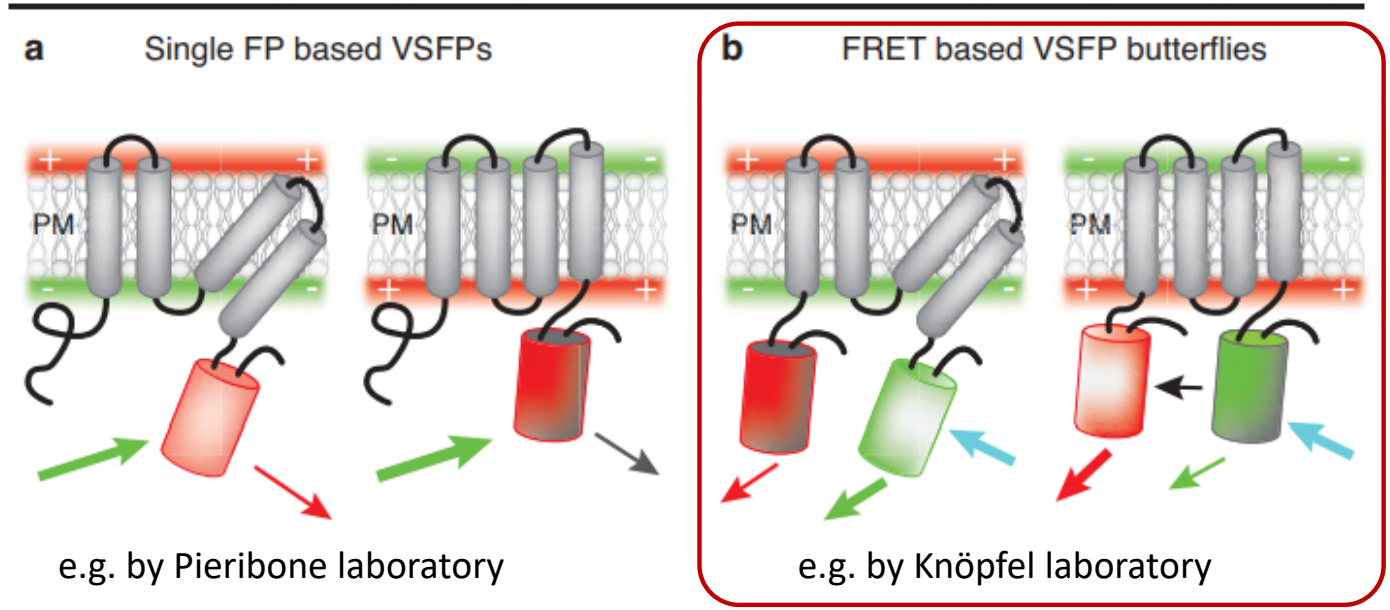
fluorescent proteins: distance close



Fluorophores: Fluroescent proteins (drawing D. Goodsell)

Spectroscopic imaging of membrane potential

voltage-sensitive fluorescent protein (**VSFP**)

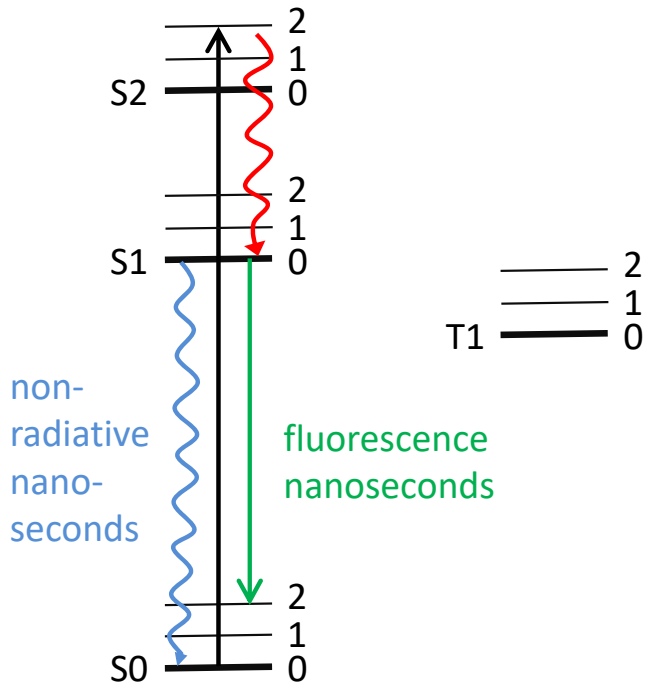


e.g. by Pieribone laboratory

e.g. by Knöpfel laboratory

Nina Vogt, Nat. Meth. 2015

Fluorescence emission



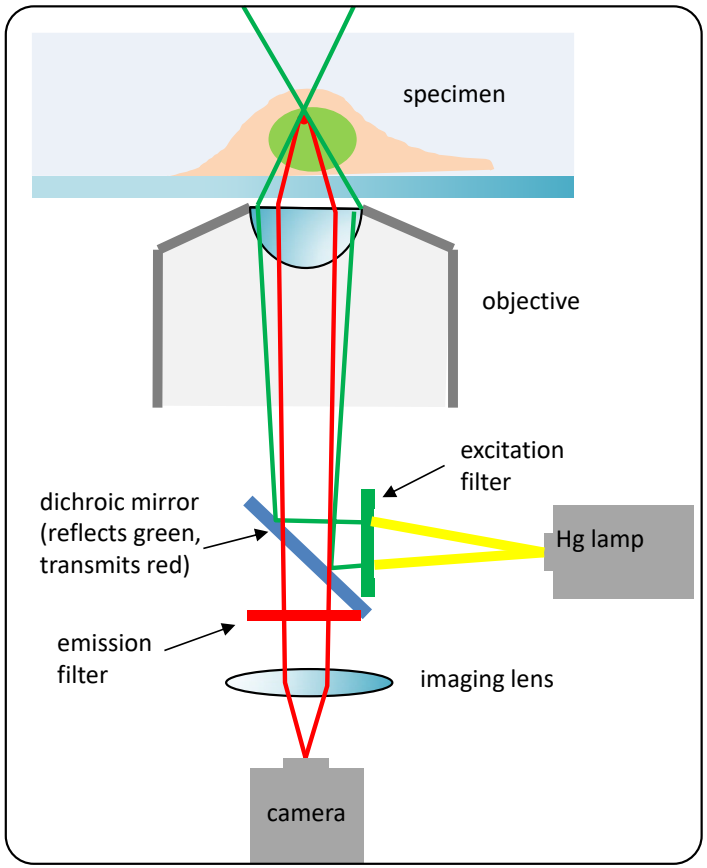
Fluorescence

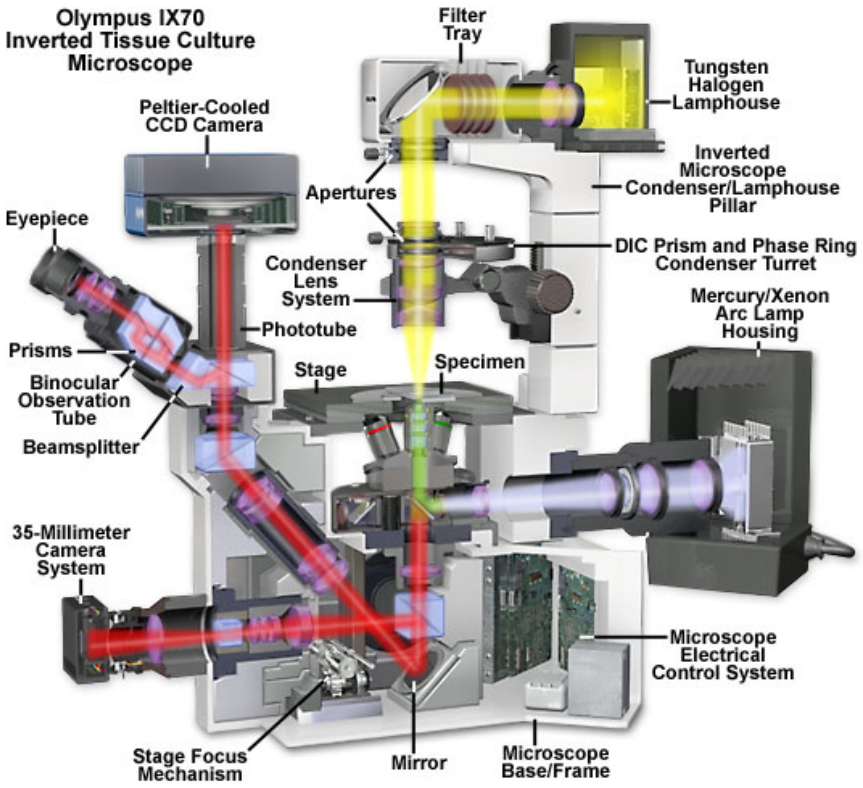
time: 10^{-8} - 10^{-10} s

Nonradiative, internal conversion

time: 10^{-8} - 10^{-10} s

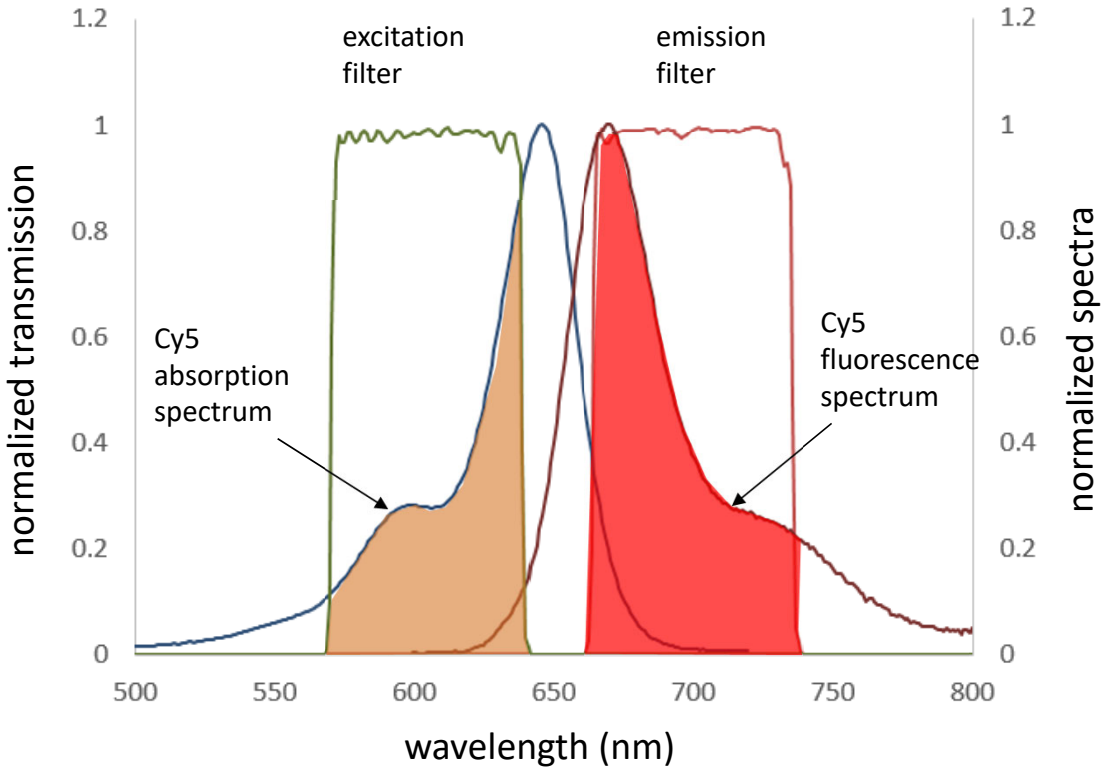
Wide field fluorescence microscope

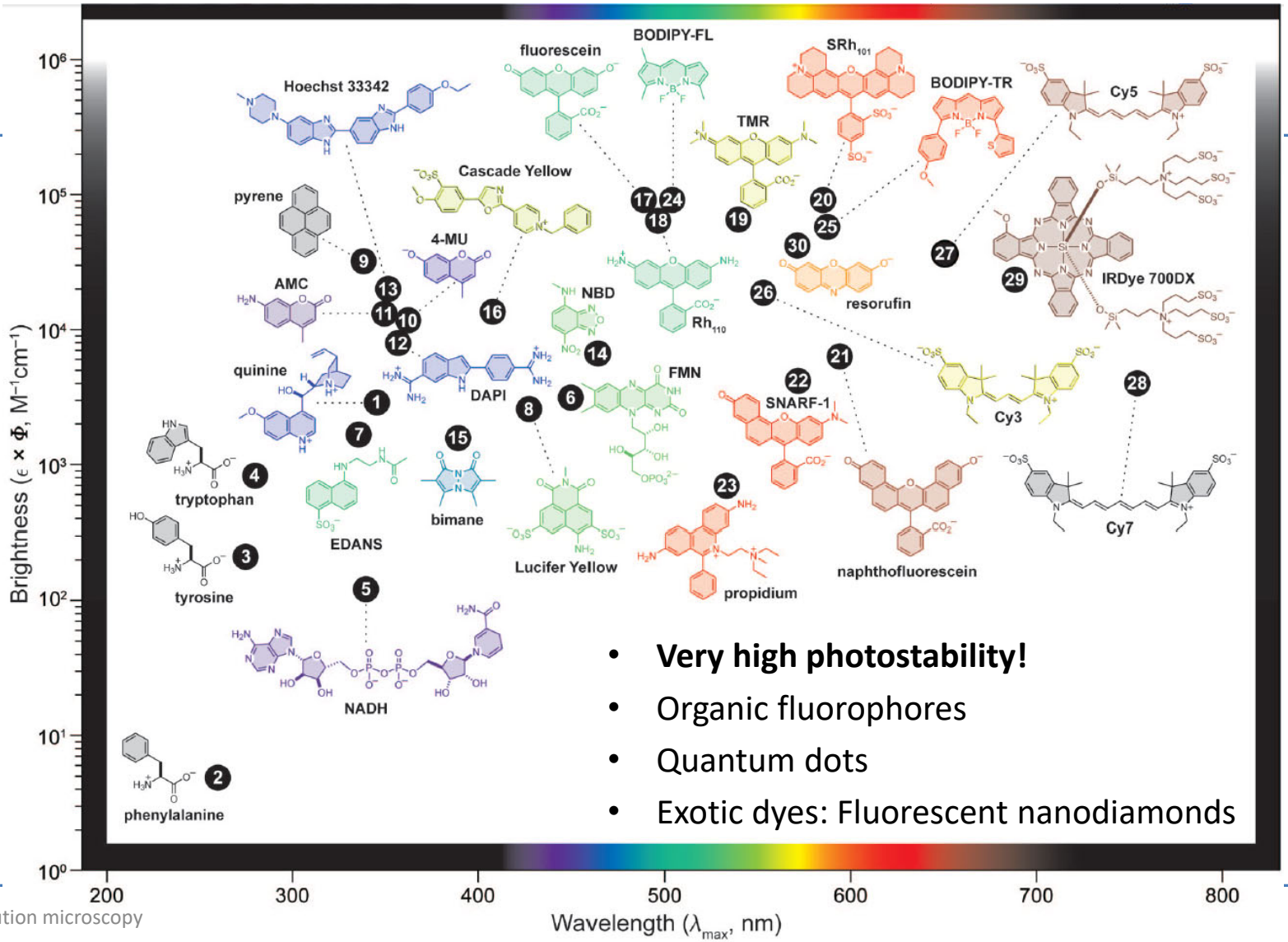




https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/fluorescence/ix70fluorescence/

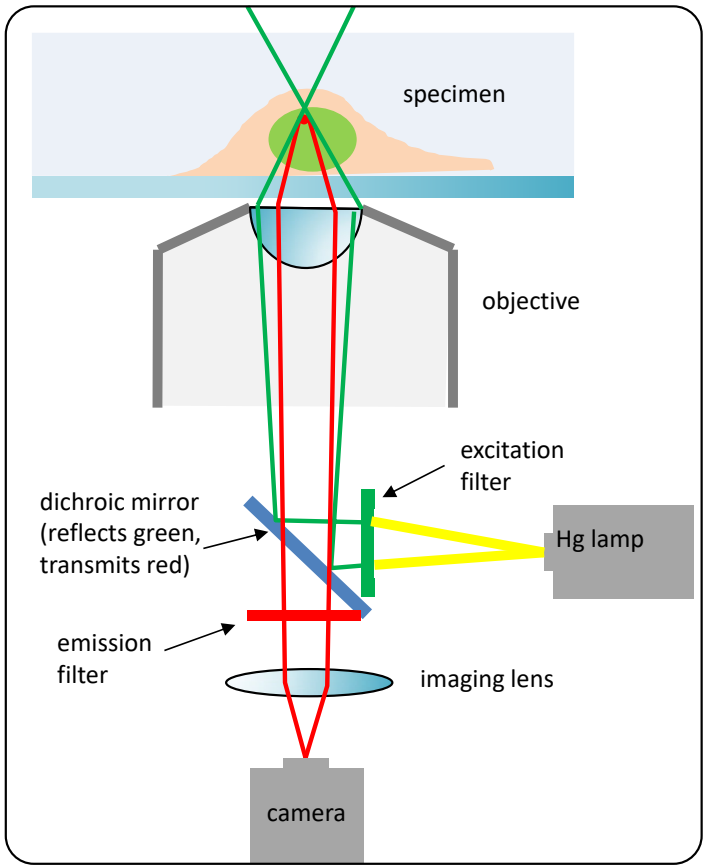
Filters allow separating emission from excitation light

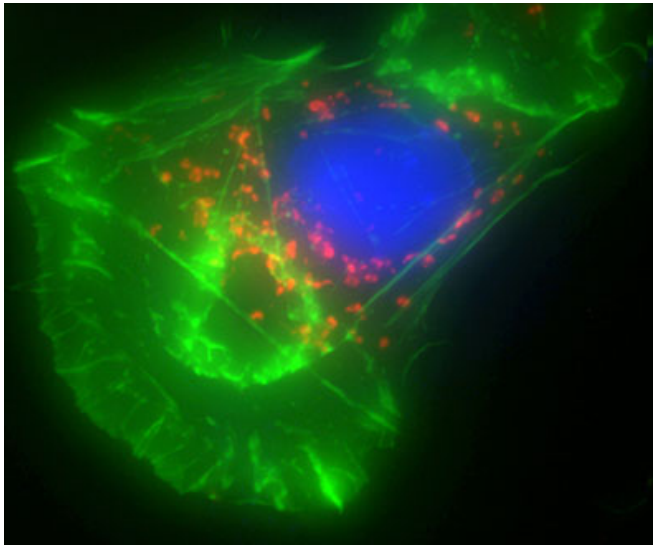




- **Very high photostability!**
- Organic fluorophores
- Quantum dots
- Exotic dyes: Fluorescent nanodiamonds

Wide field fluorescence microscope: Background fluorescence from out-of-focus sample

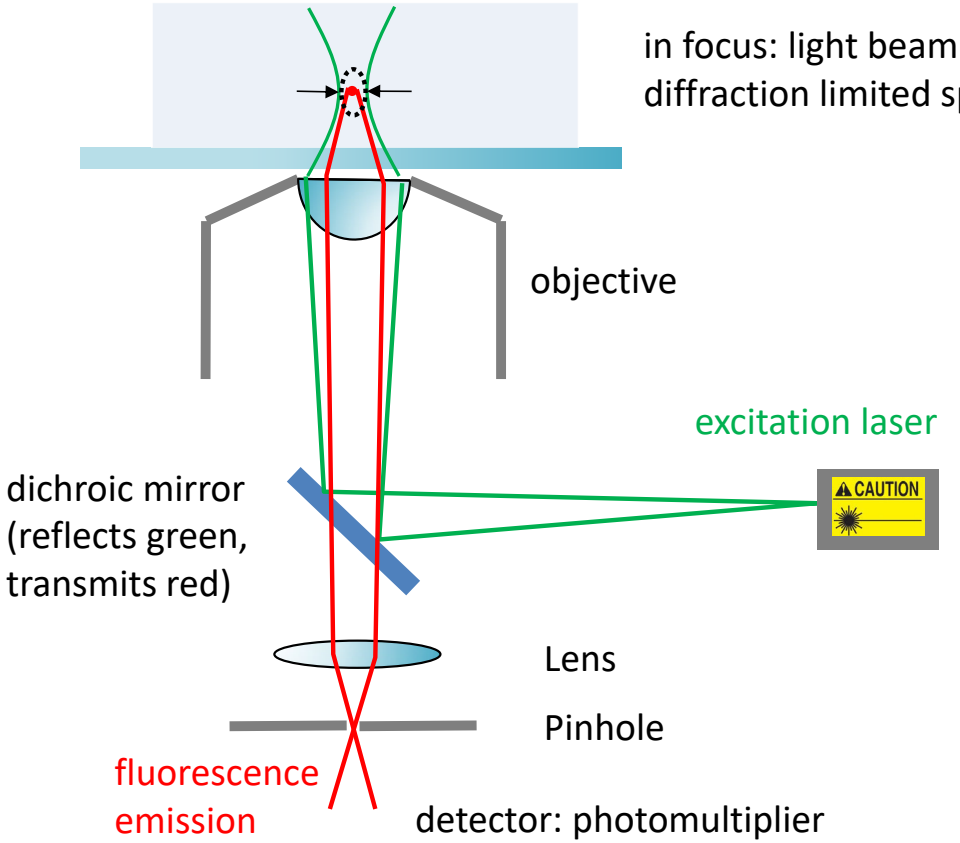




https://navigator.innovation.ca/en/facility/mcgill-university/advanced-bioimaging-facility-abif

Cell in a wide field fluorescence microscope
blue: DNA, green: actin filaments, red: mitochondria

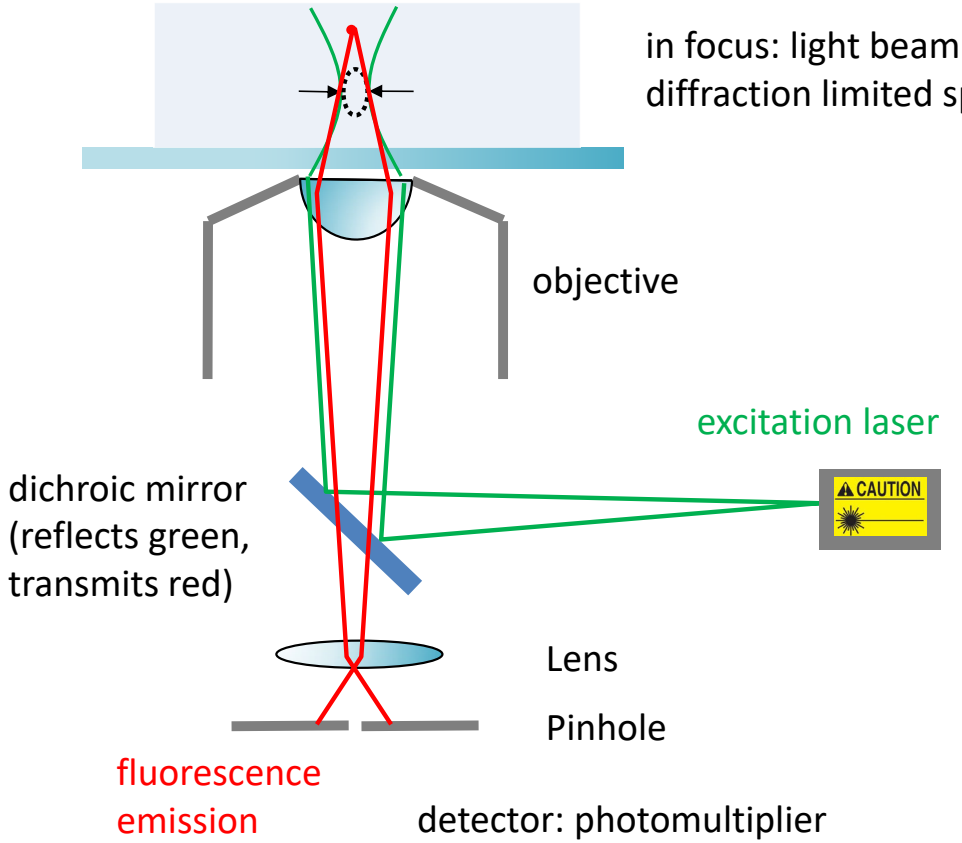
In a confocal microscope, out-of-focus light is rejected



fluorescence emission from molecules **within** the confocal volume reach the detector through a pinhole

confocal volume: dashed circle, with a diameter of 250 nm and height 700 nm

In a confocal microscope, out-of-focus light is rejected



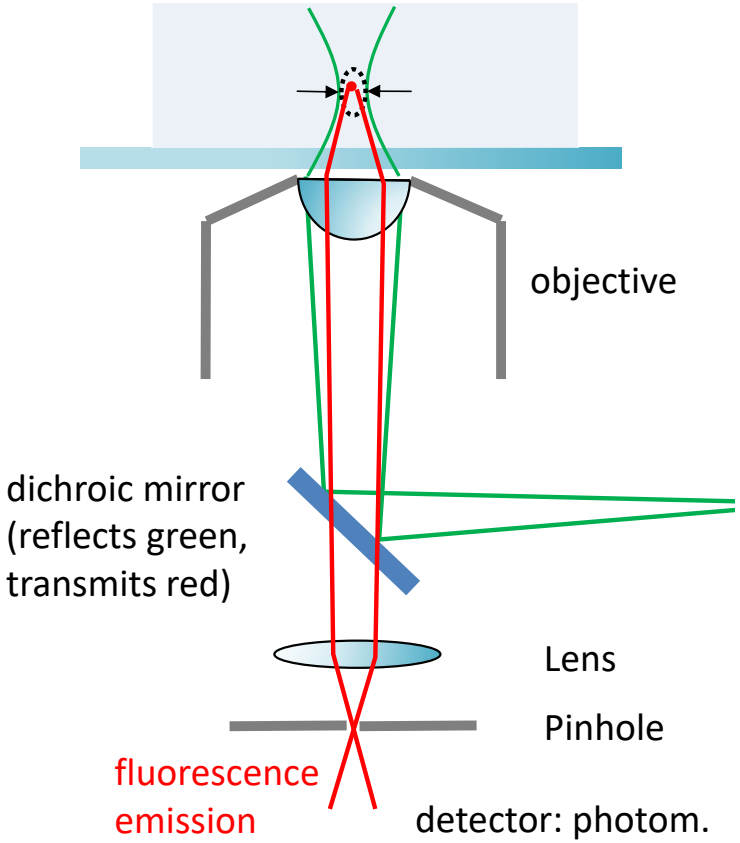
in focus: light beam is focused on a diffraction limited spot (d = 250 nm)

fluorescence emission from molecules **outside** the confocal volume are **blocked** as they do not pass the pinhole

Result: only molecules within the very small confocal volume are observed

→ imaging by scanning in 3 dimensions

Confocal imaging: The confocal volume



Confocal imaging of molecules:

Only light from a small volume is recorded, all other light from the sample is blocked by a **pinhole**.

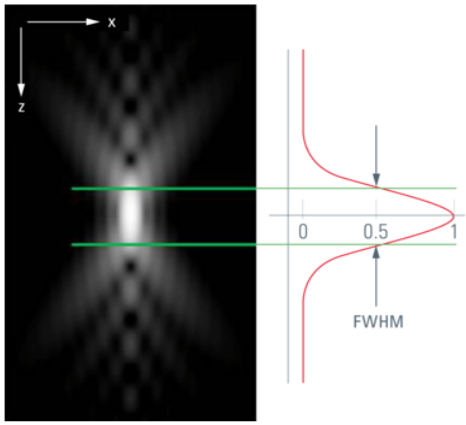
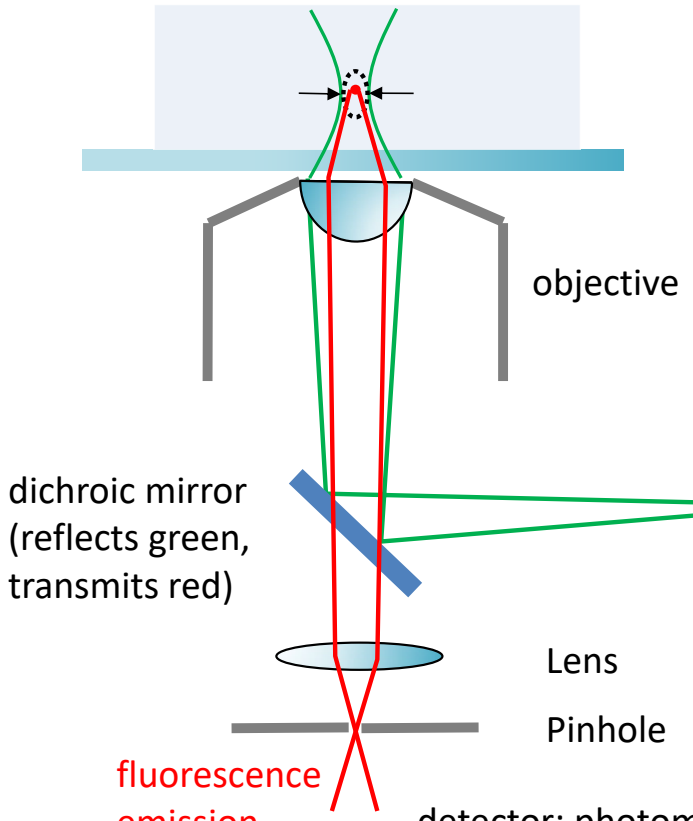
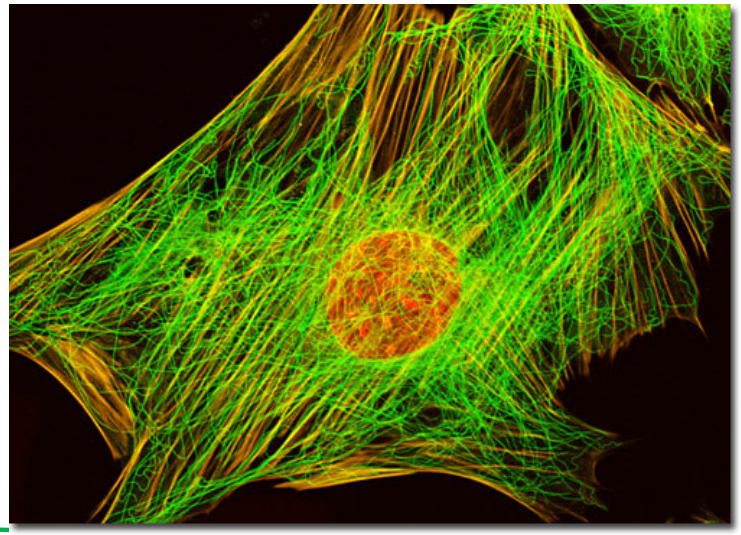


Fig. 1: Full width half maximum (FWHM) of the intensity profile in z direction is used as a measure of optical sectioning performance. The point spread function – generated by a sufficiently small latex bead – is recorded by collecting the intensity in an xz profile section. The intensity profile in the center of the diffraction pattern is displayed (here in red) and the distance in z between the 50 % values is measured (green indicators).

www.leica-microsystems.com

Confocal Imaging of biological samples requires rapid scanning



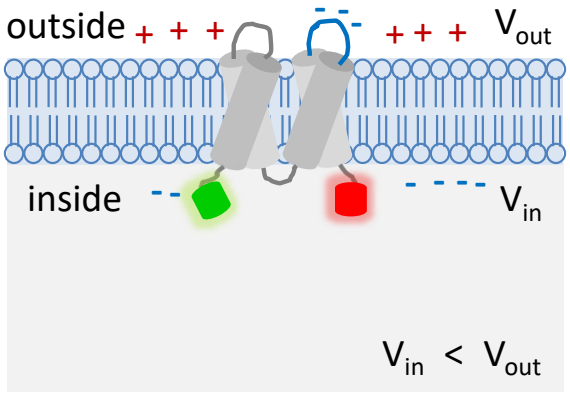


confocal imaging allows 3D sectioning of samples by **scanning** the confocal volume through the sample and recording with different excitation / emission wavelengths
(3T3 cell stained for: orange: actin, green: tubulin, red: DNA)
http://www.olympusfluoview.com/gallery/cells/3t3/3t3large.html

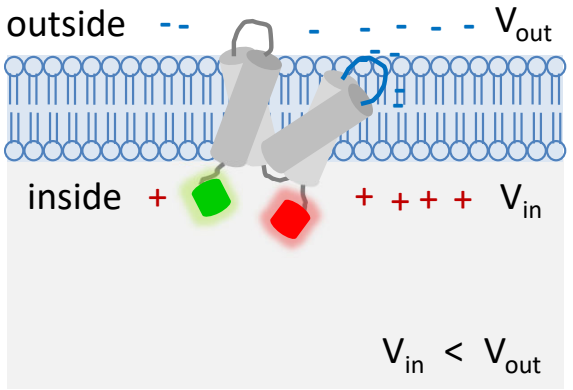
Quiz: How can confocal microscopy be used for 3D imaging of a 'thick' specimen? What is the major limitation?

Measuring FRET to detect membrane potential

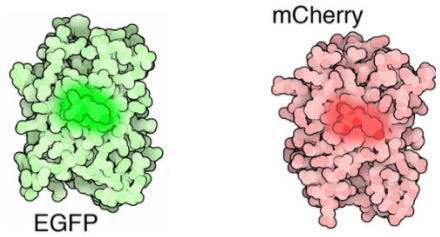
voltage-sensitive fluorescent protein (**VSFP**)



fluorescent proteins: distance far



fluorescent proteins: distance close

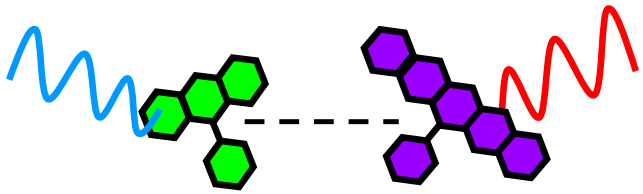


Fluorophores: Fluroescent proteins (drawing D. Goodsell)

Fluorescence resonance energy transfer (FRET)

Theodor Förster
1910 - 1974

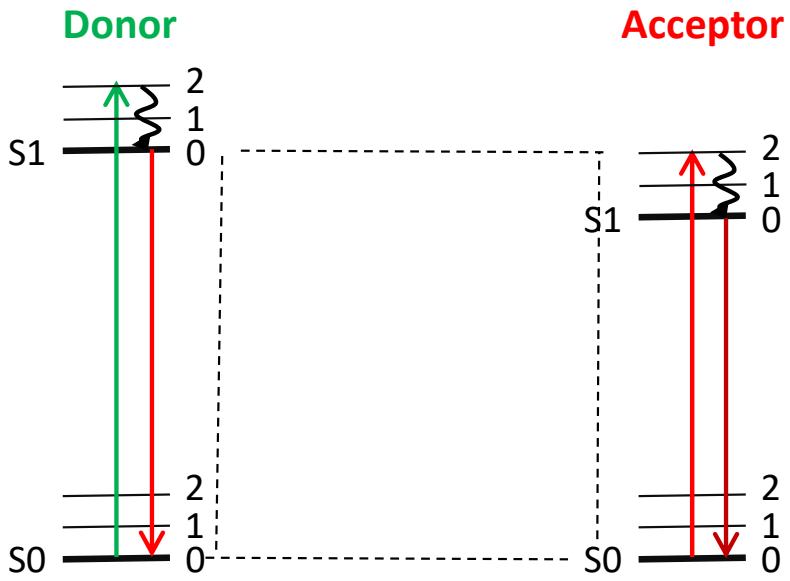
Energy transfer between two chromophores through a **resonance mechanism**



Described and theory developed
by Theodor Förster, 1946 at MPI Göttingen
for ET reactions in photosynthesis

Fluorescence resonance energy transfer

Singlet-singlet energy transfer

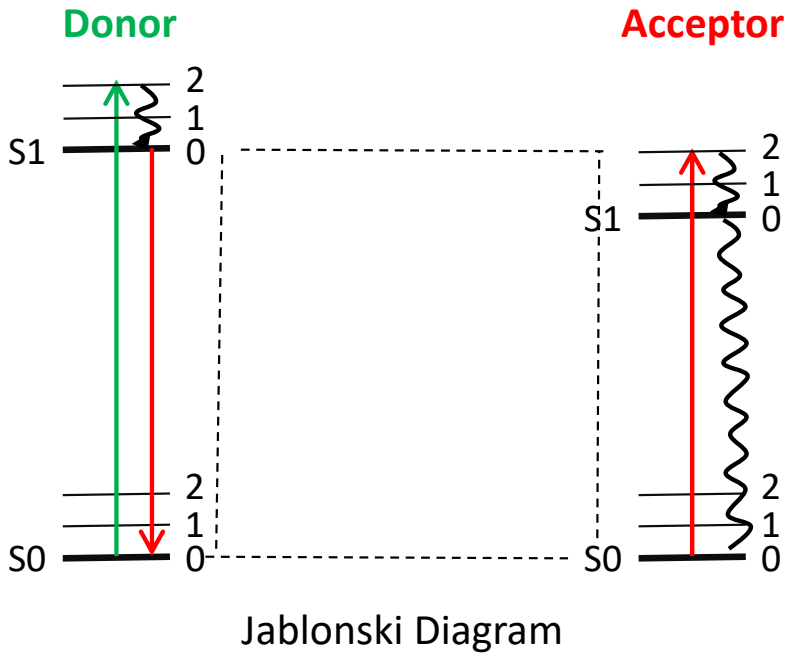


Jablonski Diagram

- **Dipole-dipole interaction through space.**
- No orbital overlap
- No electrons exchanged
- No **photons emitted or absorbed**

Comparison of FRET to quenching

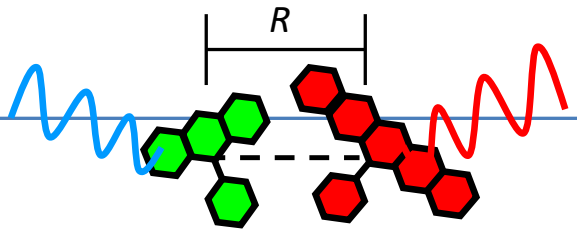
Fluorescence quenching



Jablonski Diagram

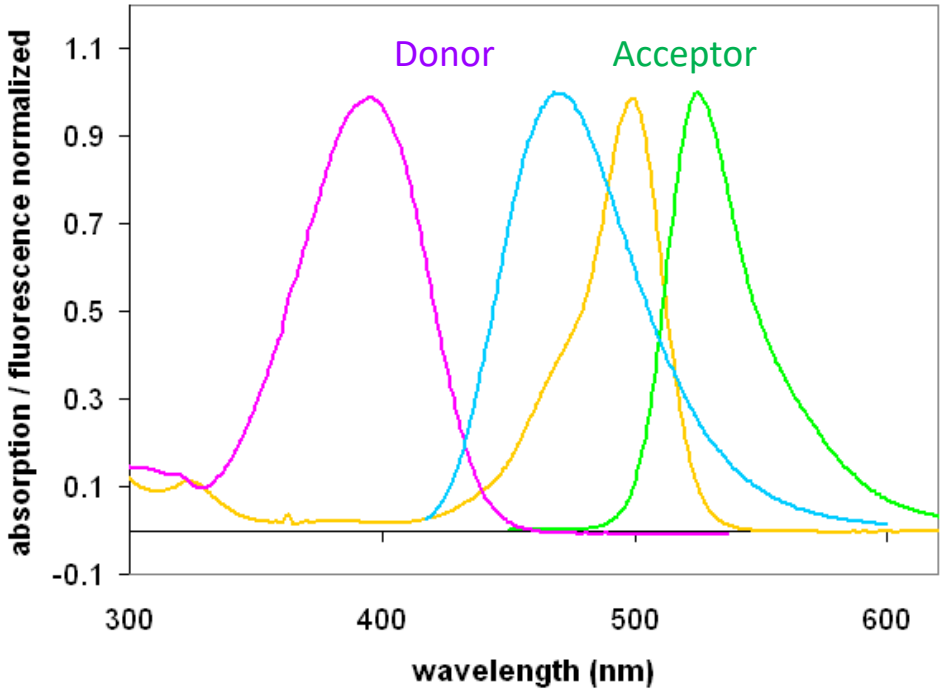
- **Quenching:**
- energy transfer through contact (e- exchange) **or** through dipole-dipole interaction → $1/r^6$ distance dependence
- no emission (acceptor relaxes through internal conversion, generates heat)

Spectral effects of FRET

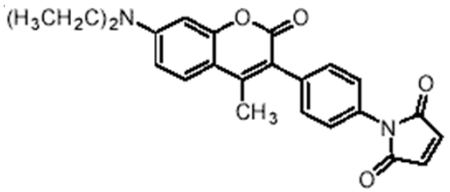


Combined Absorption and Fluorescence Spectra

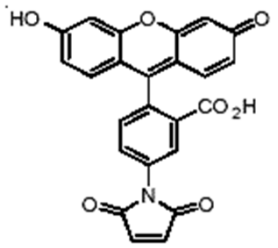
1M GmdCl, 20mM tris, 1mM EDTA, 1mM DTT, 22.5°C, 2mm pathlength
(ex), 1cm pl (em), ex wl 390nm, int t 0.5s, slits 1/1



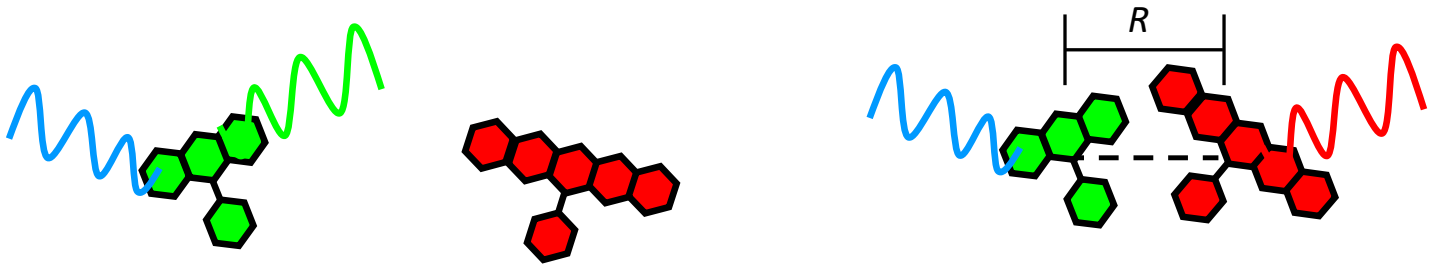
Donor



Acceptor



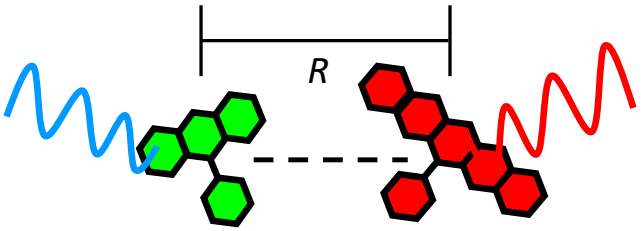
Efficiency of energy transfer



FRET efficiency depends on:

- Chromophore distance
- Excitation and emission spectra of donor and acceptor chromophores
- Alignment of donor and acceptor transition dipole moments

Efficiency of energy transfer



"Molecular ruler"

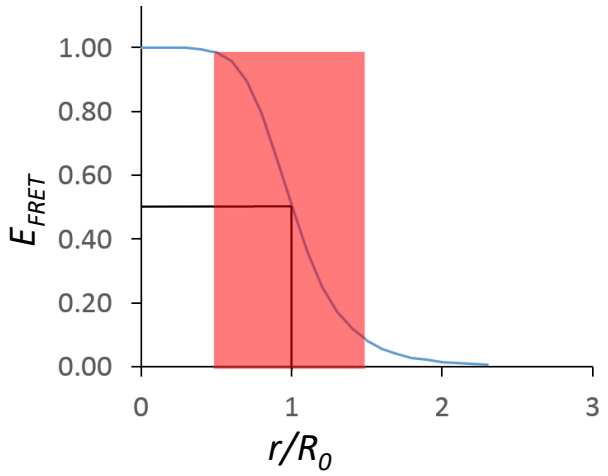
Rate of energy transfer: $k_{FRET} = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$

τ_D: fluorescence lifetime of the donor
Exponent of r^{-6} arises from the square of the dipole-dipole coupling (scales with exponent of -3)

Transfer efficiency:

$$E_{FRET} = \frac{R_0^6}{R_0^6 + r^6} = \frac{k_{FRET}}{k_{FRET} + \frac{1}{\tau_D}}$$

high sensitivity region



Experimental verification of 1/R^6 dependence of FRET efficiency

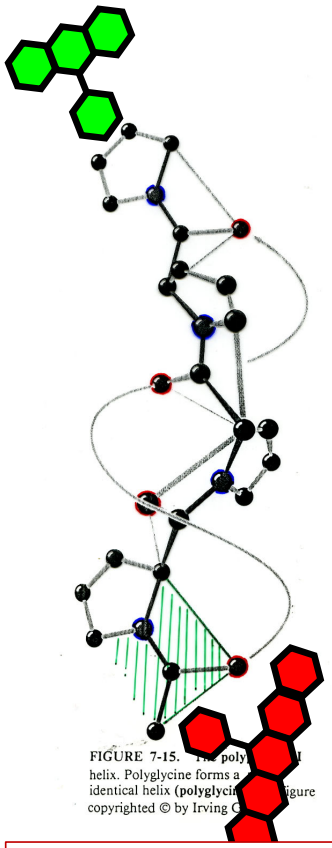
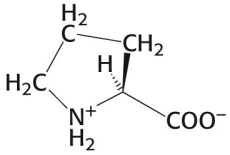


FIGURE 7-15. The poly… helix. Polyglycine forms a … identical helix (**polyglyci**… figure copyrighted © by Irving C…



Proline (Pro, P)

$$E_{FRET} = \frac{R_0^6}{R_0^6 + r^6}$$

$$R_0^6 = \frac{9000(ln10)\kappa^2 Q_D}{128\pi^5 N n^4} J(\lambda)$$

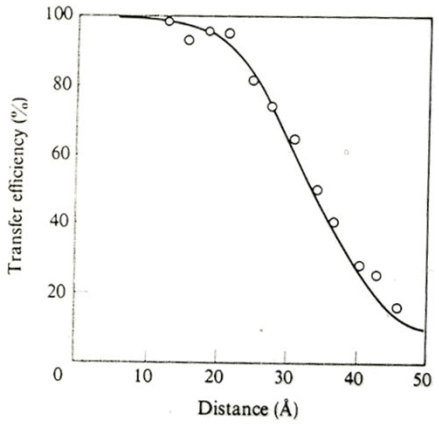


Figure 8-20

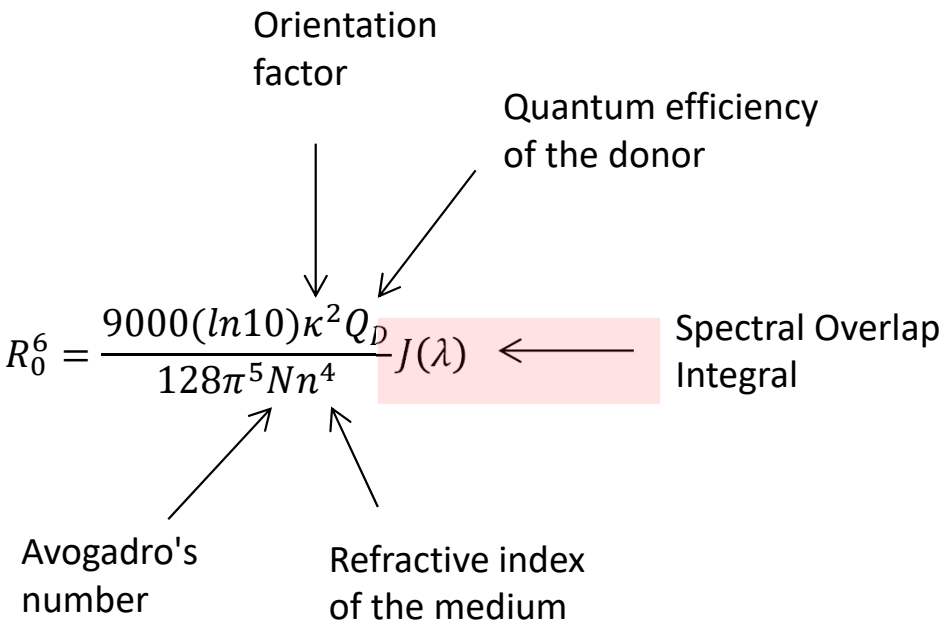
Efficiency of energy transfer as a function of distance in dansyl-(L-prolyl)$_n$-α-naphthyl semicarbazide oligomers with n = 1 to 12. The curve was fit to the data with Equation 8-57. [From L. Stryer and R. P. Haugland, *Proc. Natl. Acad. Sci. USA* 98:719 (1967).]

Why did they use proline peptides for this experiment

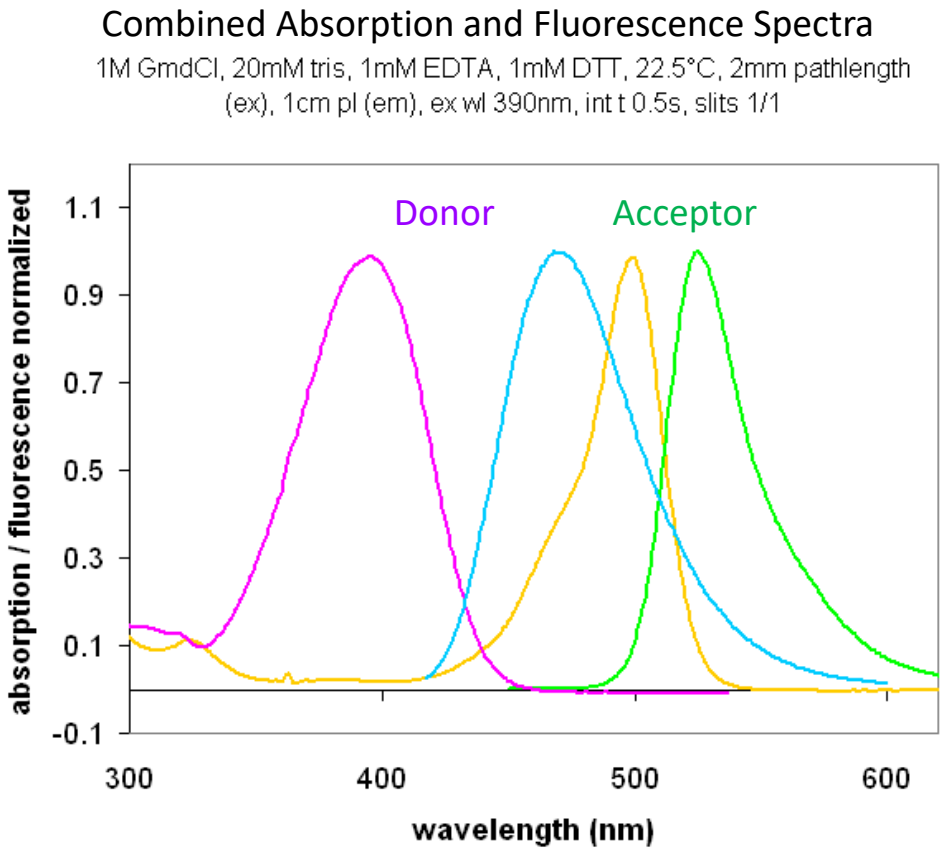
What is the R_0 of the used dye pair in this experiment?

Förster radius R_0

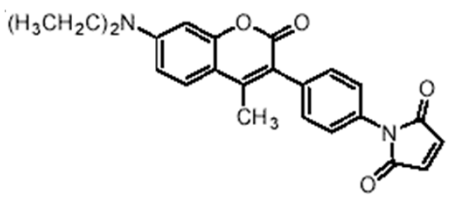
Distance of half-maximal FRET efficiency



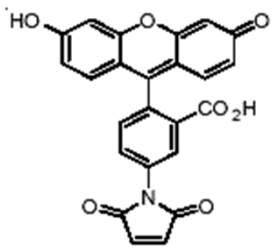
Spectral overlap integral



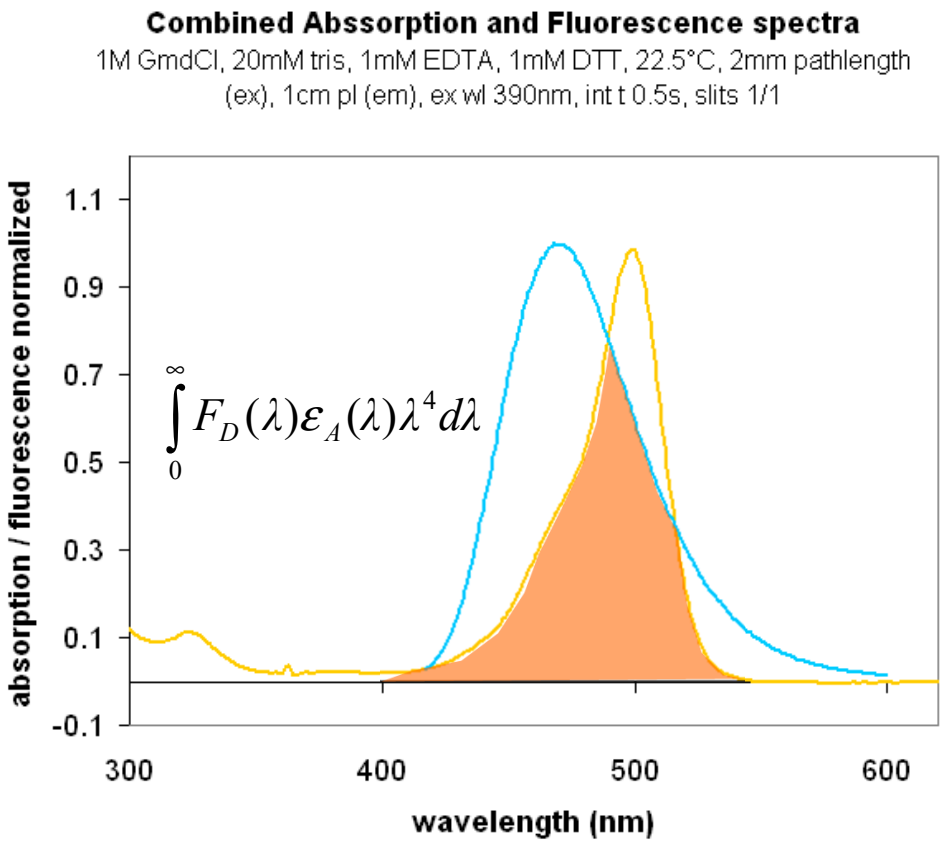
Donor



Acceptor



Spectral overlap integral

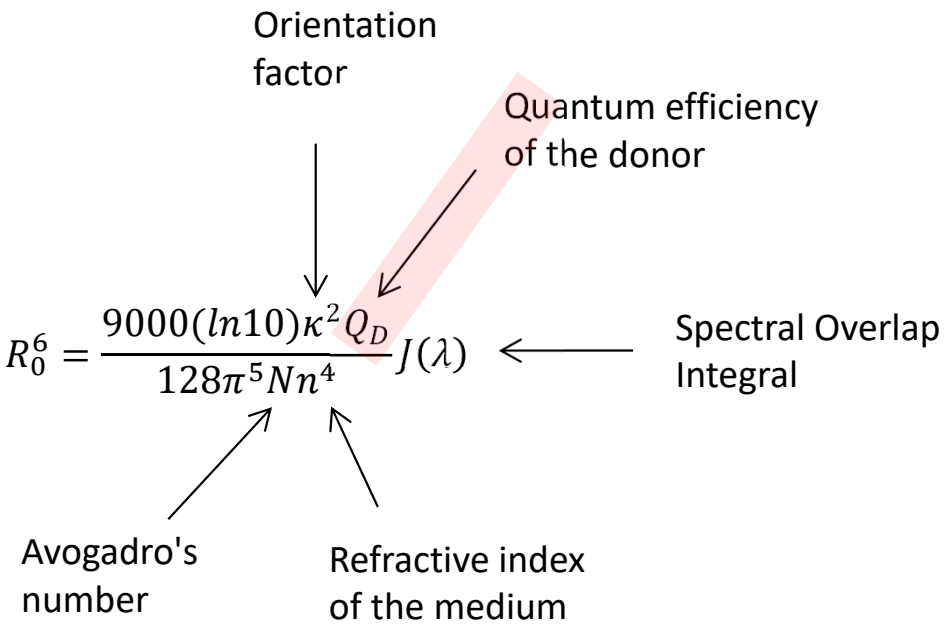


Required for FRET:

Spectral overlap between donor emission and acceptor excitation spectrum

Förster radius R_0

Distance of half-maximal FRET efficiency



Revision: Quantum yield

$$N(t) \begin{cases} \xrightarrow{k_F} N_{GS} + hv \\ \rightarrow N_{GS} + heat \\ \rightarrow N_{GS} + \ldots \end{cases}$$

competing pathways:

- fluroescence
- internal conversion
- quenching
- FRET

$$N(t) \xrightarrow{k=\sum ki} N_{GS}$$

here, k contains all non-radiative transitions in a single kinetic constant

$$N(t) = \mathrm{N}(0)\,\mathrm{e}^{-(k+k_F)t}$$

$$\tau = 1/(k + k_F)$$

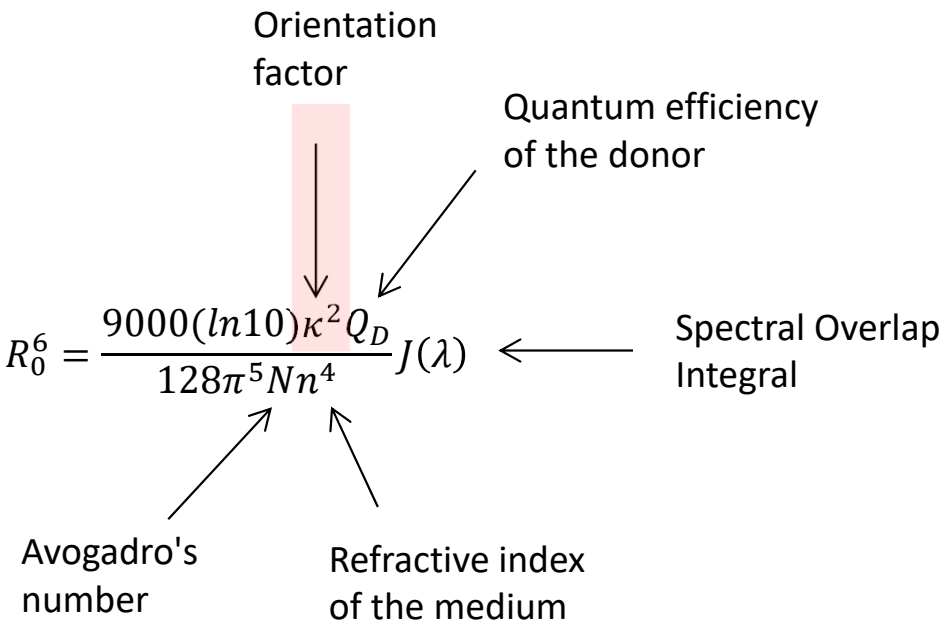
lifetime of the excited state with non-radiative transitions included

Quantum yield:

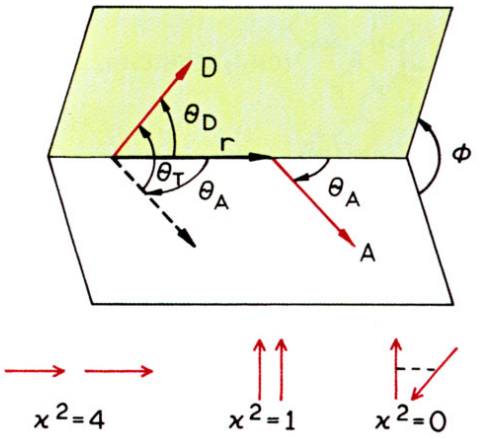
$$Q_F = \frac{k_F}{k + k_F} = k_F \cdot \tau$$

Förster radius R_0

Distance of half-maximal FRET efficiency



The effect of the orientation factor



$$x^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2$$

$$x^2 = (\sin\theta_D \sin\theta_A \cos\phi - 2\cos\theta_D \cos\theta_A)^2$$

Lakowicz, Principles of fluorescence spectroscopy

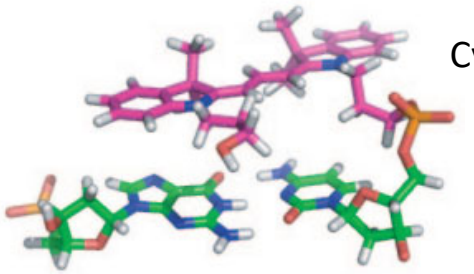
For two freely rotating chromophores:

$$\langle\kappa^2\rangle = 2/3$$

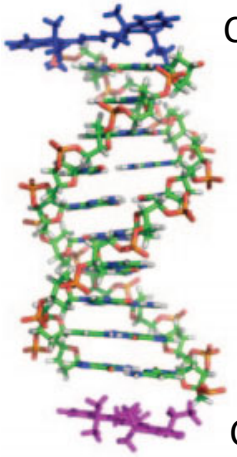
Accurate distance calculation by FRET:

Fluorescence anisotropy measurements → control that this conditions is fulfilled!

Orientation dependence of FRET

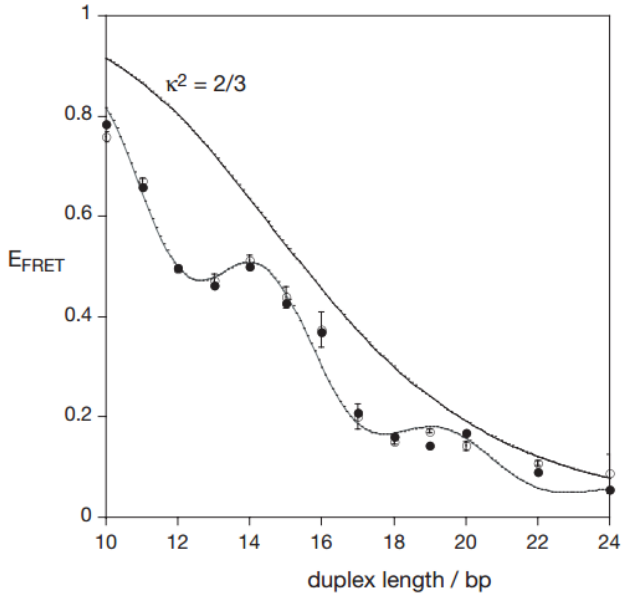


Cy3 on DNA



Iqbal et al. 2008, PNAS

$$R_0^6 = \frac{9000(ln10)\kappa^2 Q_D}{128\pi^5 N n^4} J(\lambda)$$



Summary: Förster radius

$$R_0^6 = \frac{9000(ln10)\kappa^2 Q_D}{128\pi^5 N n^4} J(\lambda)$$

The Förster radius is a property of:

- the fluorophore pair: J(λ), Q_D
- the labeled proteins: κ^2, Q_D

It has thus to be determined for each new protein sample!

Which of the two dye pairs is expected to have a larger R_0:
Cy3 - Cy5 or **Cy3 - Cy7** ?

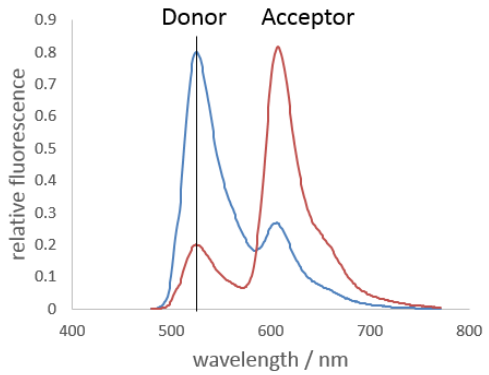
Table 13.3. Representative Förster Distances for Various Donor–Acceptor Pairs[a]

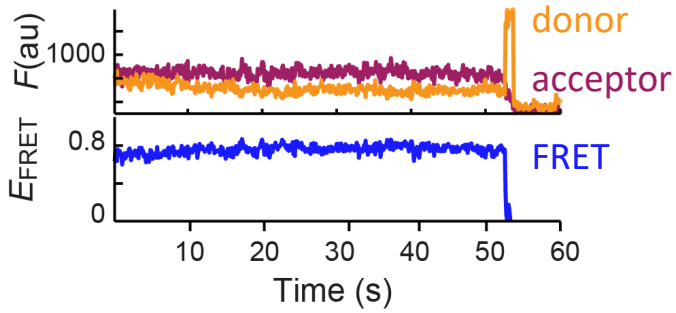
Donor	Acceptor	R_0 (Å)
Naphthalene[14]	Dansyl	22
Dansyl[95]	FITC	33–41
Dansyl[14]	ODR	43
ε-A[14]	NBD	38
IAF[14]	TMR	37–50
Pyrene[14]	Coumarin	39
FITC[14]	TMR	49–54
IAEDANS[14]	FITC	49
IAEDANS[14]	IAF	46–56
IAF[14]	EIA	46
CF	TR	51
Bodipy[25]	Bodipy	57
BPE[14]	Cy5	72
Terbium[96]	Rhodamine	65
Europium[94]	Cy5	70
Europium[97]	APC	90

[a]Dansyl, 5-dimethylamino-1-napthalenesulfonic acid. ε-A, 1-N^6-ethenoadenosine; APC, allophycocyanin; Bodipy, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; BPE, B-phycoerythrin; CF, carboxylfluorescein, succinimidyl ester; Cy5, carboxymethylindocyanine-N-hydroxysuccinimidyl ester; EIA, 5-(iodoacetamido) eosin; FITC, fluorescein-5-isothiocyanate; IAEDANS, 5-(2-0((iodocetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; IAF, 5-iodoacetamidofluorescein; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; ODR, octadecylrhodamine; TMR, tetramethylrhodamine; TR, Texas Red.

Lakowicz, Principles of fluorescence spectroscopy

Measuring the efficiency of energy transfer





FRET efficiency from spectra:

$$E_{FRET} = 1 - F_{DA} / F_D$$

F_D : Donor emission in the absence of acceptor

F_{DA} : Donor emission in the presence of acceptor

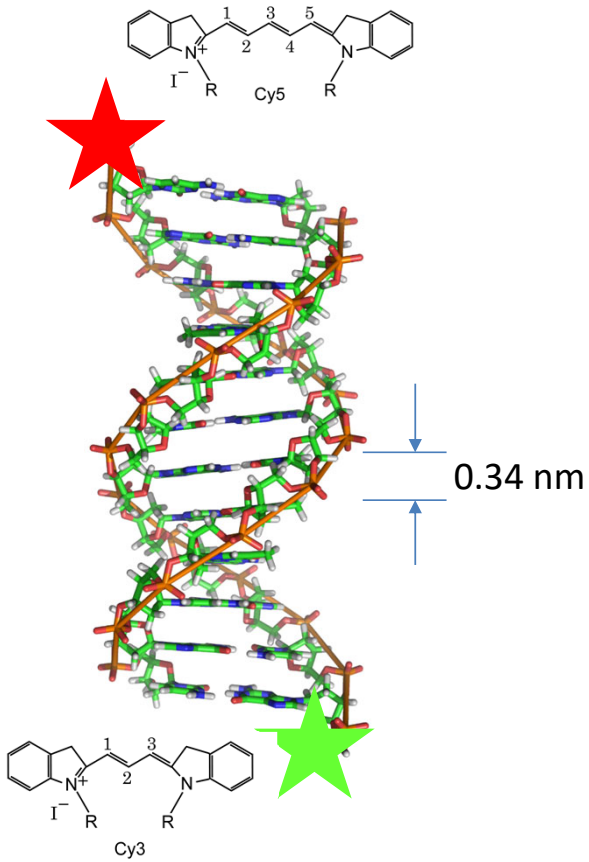
FRET efficiency from single-molecule traces:

$$E_{FRET} = F_A / (F_{DA} + F_A)$$

F_A : Acceptor emission when donor is excited

Short quiz

- you have a DNA oligonucleotide (12 base pairs) labeled with two FRET dyes, Cy3B and Cy5 (R_0 = 5 nm)
  - what is the ex**pected FRET efficiency**, assuming freely rotatable dyes?
- Cy3B has a fluorescence lifetime of 2.8 ns
  - what is the expected fluorescence lifetime of Cy3B in this DNA sample?



Problem of FRET experiments: Incomplete labeling

Largest source of error in FRET measurements: Incomplete labeling

$$E = 1 - \frac{F_{DA} - F_D(1 - f_A)}{F_D f_A} = \left(1 - \frac{F_{DA}}{F_D}\right)\frac{1}{f_A}$$

here: f_A is fraction labeled with donor

Myosin S-1 contains two reactive cysteines, randomly labeled

purple line: measured data – yielding E_{FRET} of 0.3 and DA distance of 5 nm

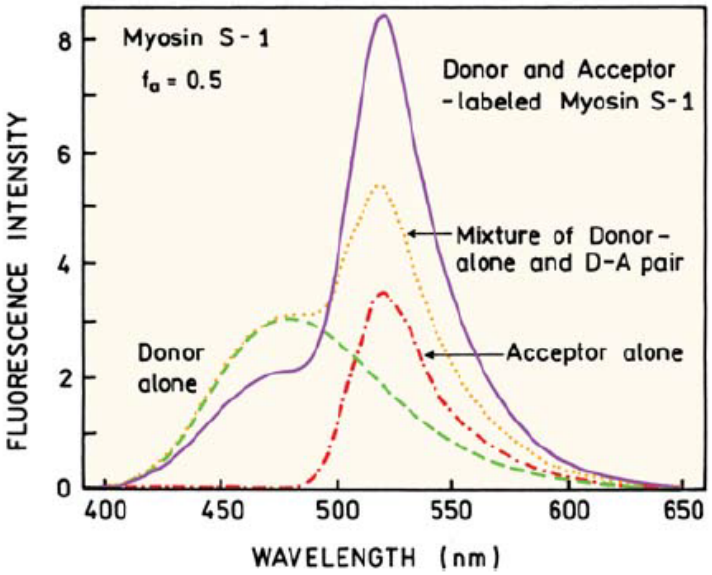
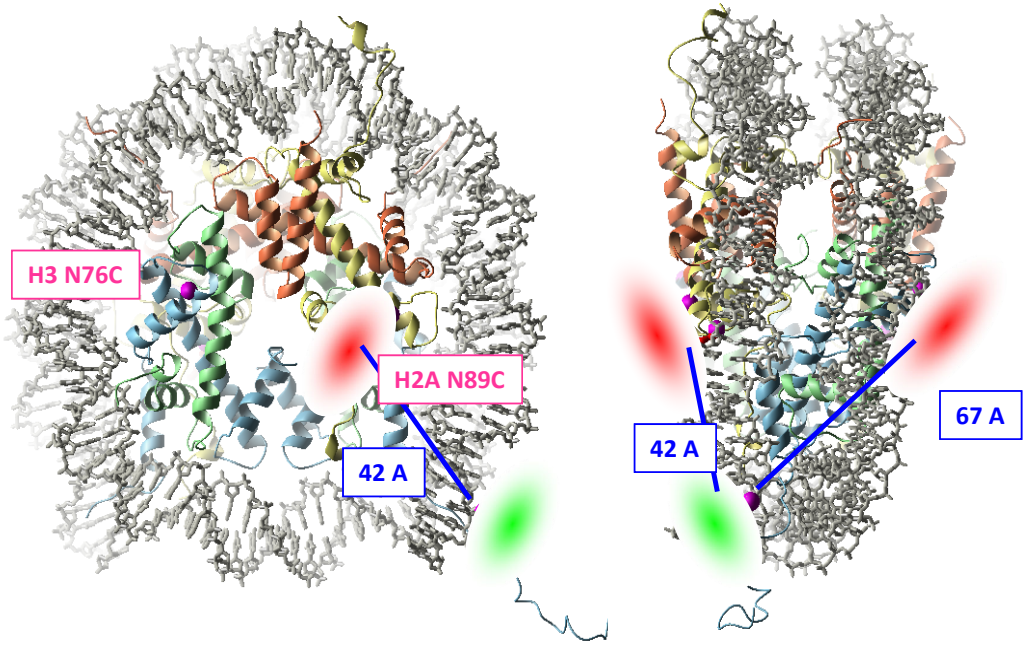


Figure 13.11. Emission spectra of labeled Myosin S-1. The donor is 1,5-IAEDANS and the acceptor is IAF. Revised from [34].

however, only 50% are labeled, thus E_{FRET} is 0.6 and DA distance of 4 nm

Application 1: FRET detection of nucleosome dissassembly

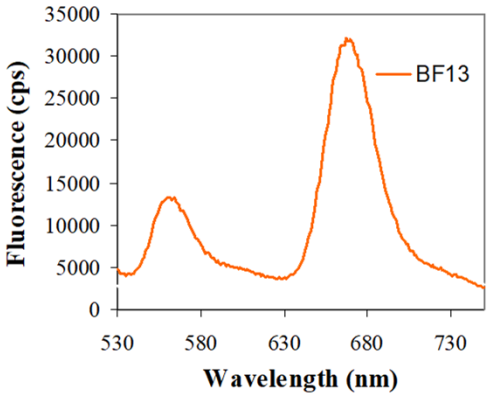


FRET is measured between donor (on DNA) and acceptor (on histone protein)

Nucleosome unfolding: Histone proteins dissocate from DNA, FRET is lost.

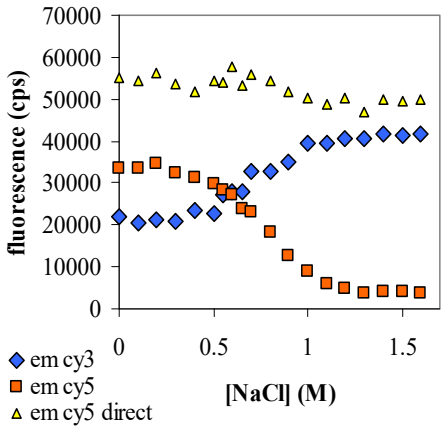
Studying nucleosome dissociation by FRET measurements

spectra



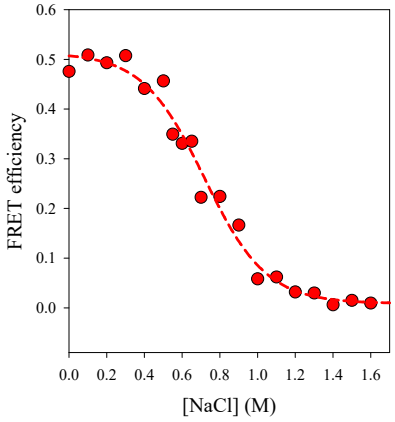
start of the experiment, FRET indicates nucleosome is formed

donor and acceptor fluroescence



Addition of salt: Nucleosome dissociates and FRET is lost

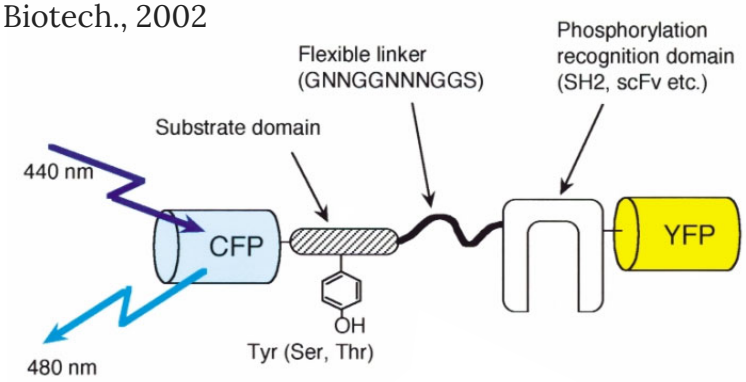
FRET efficiency 1-D/DA

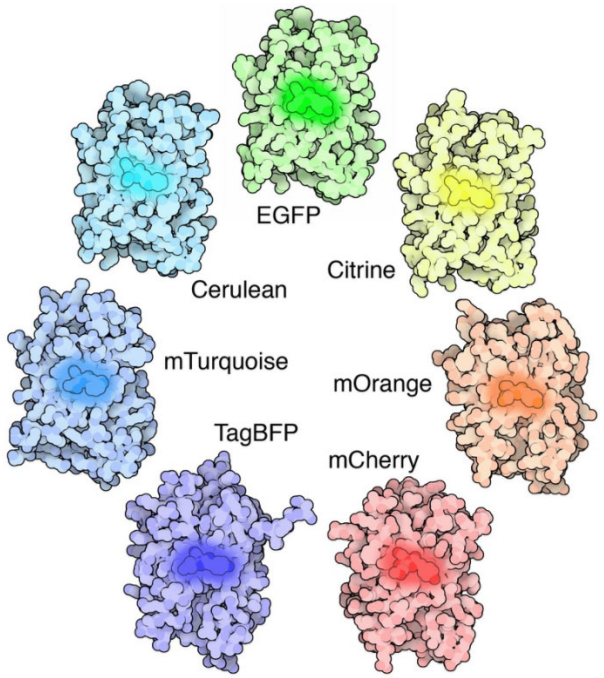


At **700 mM NaCl**, half of all nucleosomes are disociated.

Application 2: A FRET sensor to detect phosphorylation *in vivo*

Fluorescent indicators for imaging protein phosphorylation in single living cells, Sato et al. Nat. Biotech., 2002

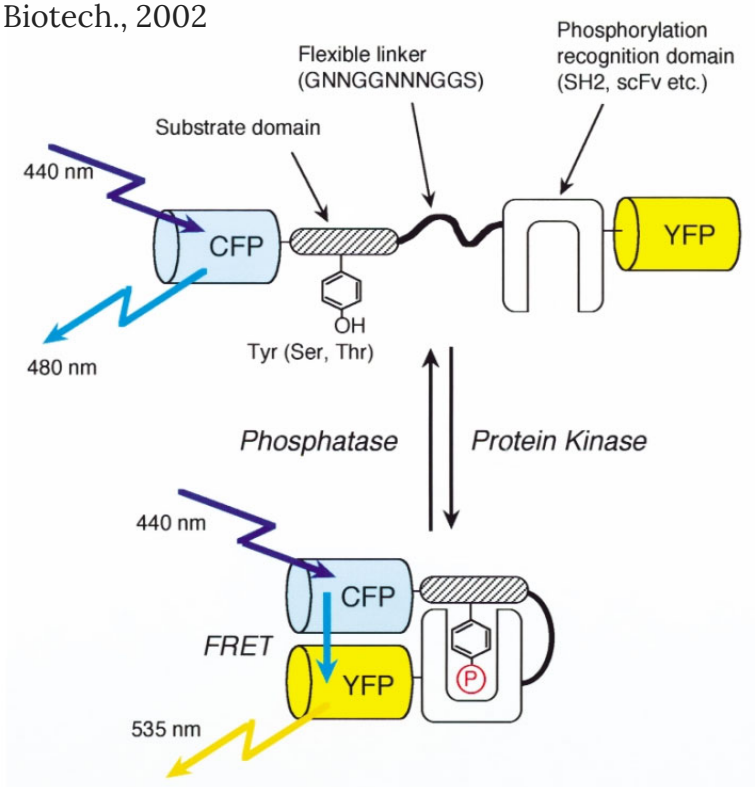


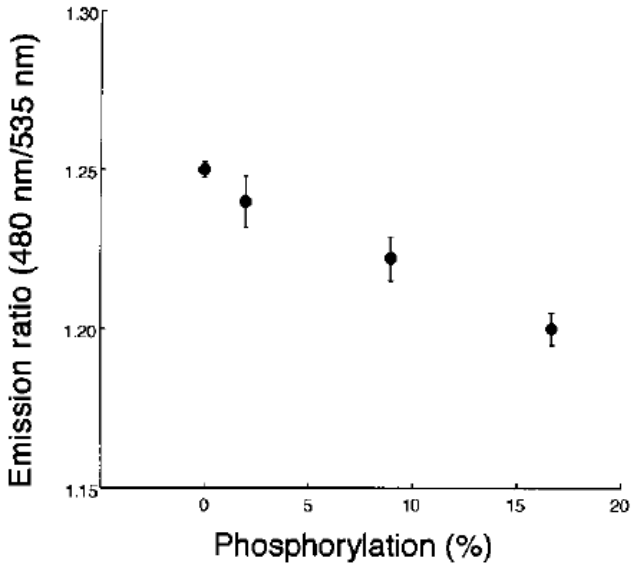


Fluorophores: Fluroescent proteins (drawing D. Goodsell)

Application 2: A FRET sensor to detect phosphorylation *in vivo*

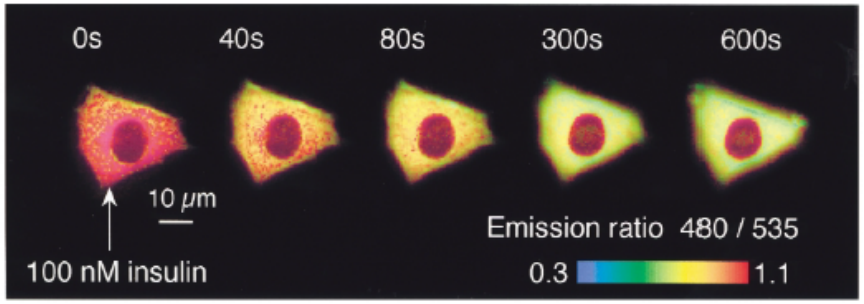
Fluorescent indicators for imaging protein phosphorylation in single living cells, Sato et al. Nat. Biotech., 2002



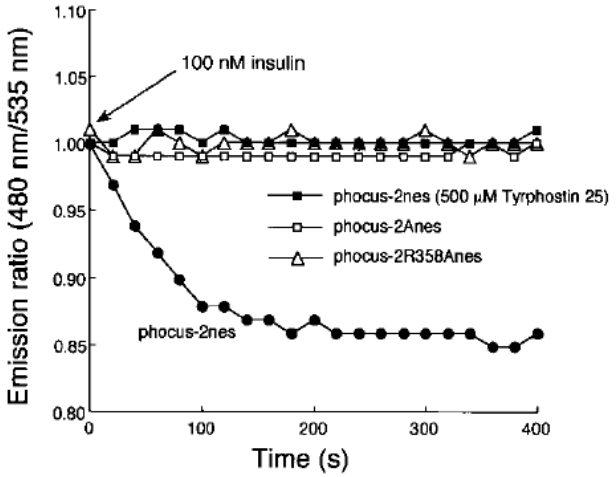


In vitro phosphorylation assay of purified phocus-2. The CFP/YFP emission ratio seen after excitation at 440 ± 10 nm was measured under a fluorescence microscope.

Single-cell detection of protein phosphorylation due to insulin stimulation



Pseudocolor images of the CFP/YFP emission ratios after the addition of 100 nM insulin, obtained from the CHO-IR cells expressing phocus-2nes.



Time courses of the cytosolic emission ratio from phocus-2nes (●), that from phocus-2nes pretreated with 500 μM tyrophostin 25 (▪), that from phocus-2Anes (□), and that from phocus-2R358Anes (Δ), each when stimulated with 100 nM insulin at 25°C.

FRET applications

- **Protein-protein interactions**

  Contact between proteins results in FRET

- **Protein dynamics**

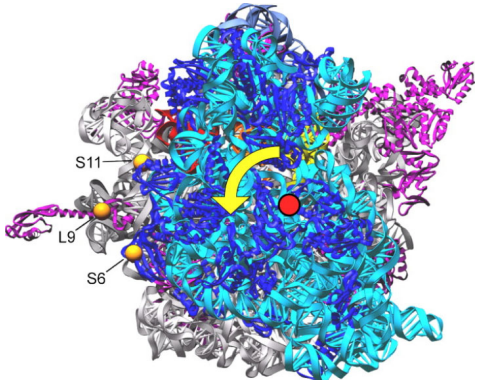
  Structural changes alter FRET efficiency

- **Structure determination**

  FRET based distance measurements

- **Sensors**

  encoded FRET sensors



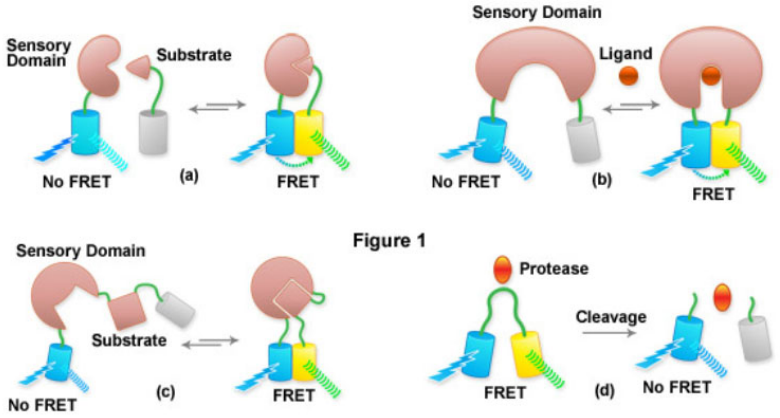
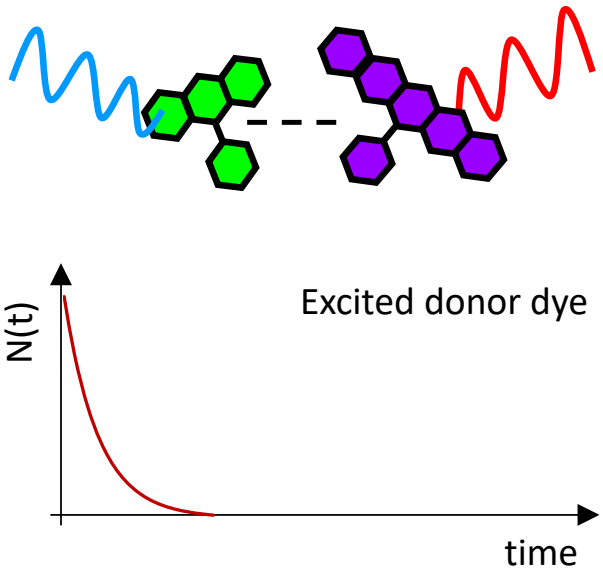
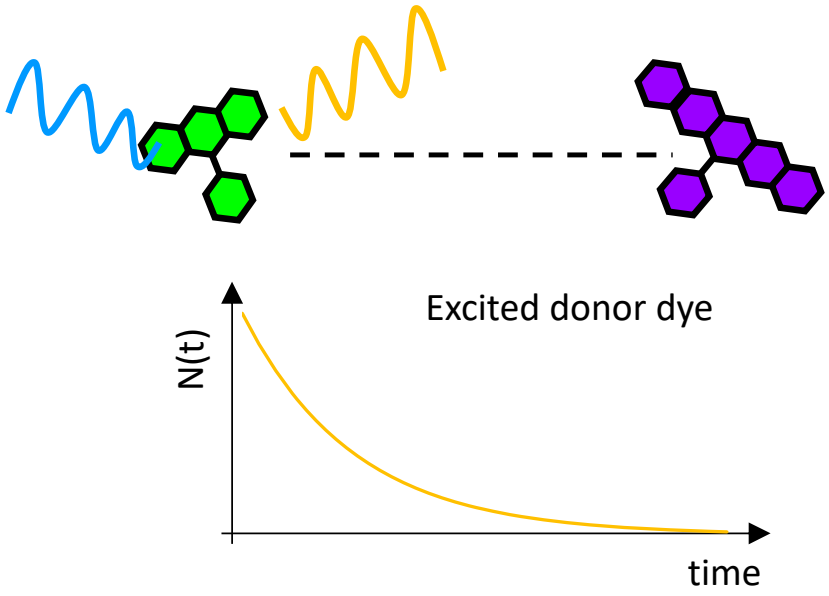


Figure 1

FRET-Fluorescence lifetime imaging (FLIM)

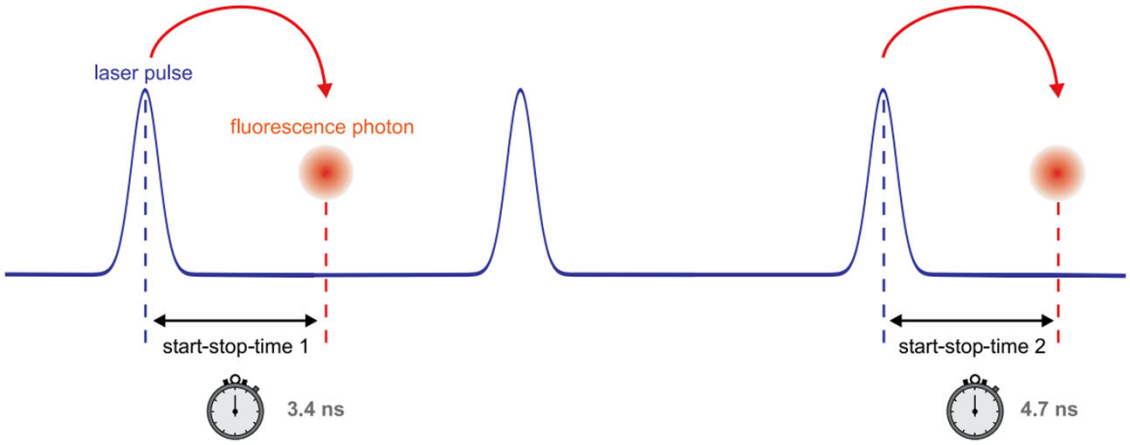


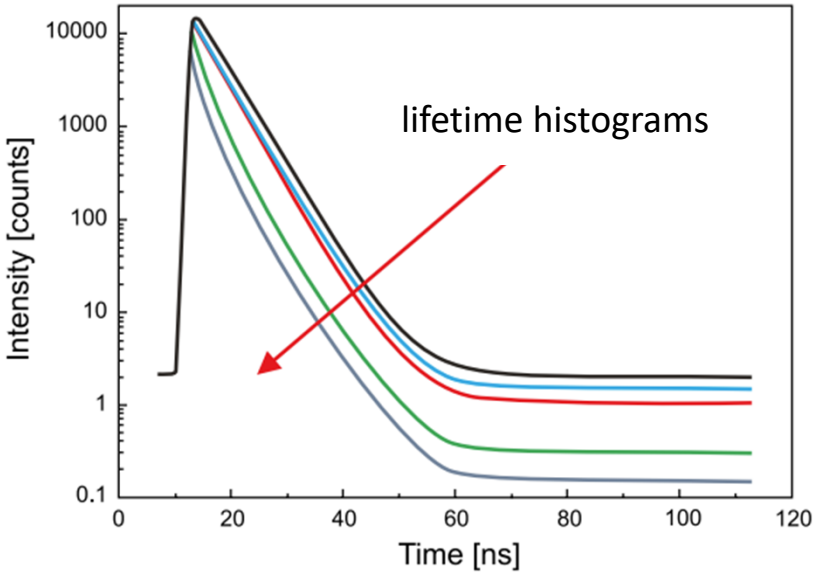


FRET results in reduction of donor lifetime

$$k_{eff} = k_F + k_{FRET} + k$$

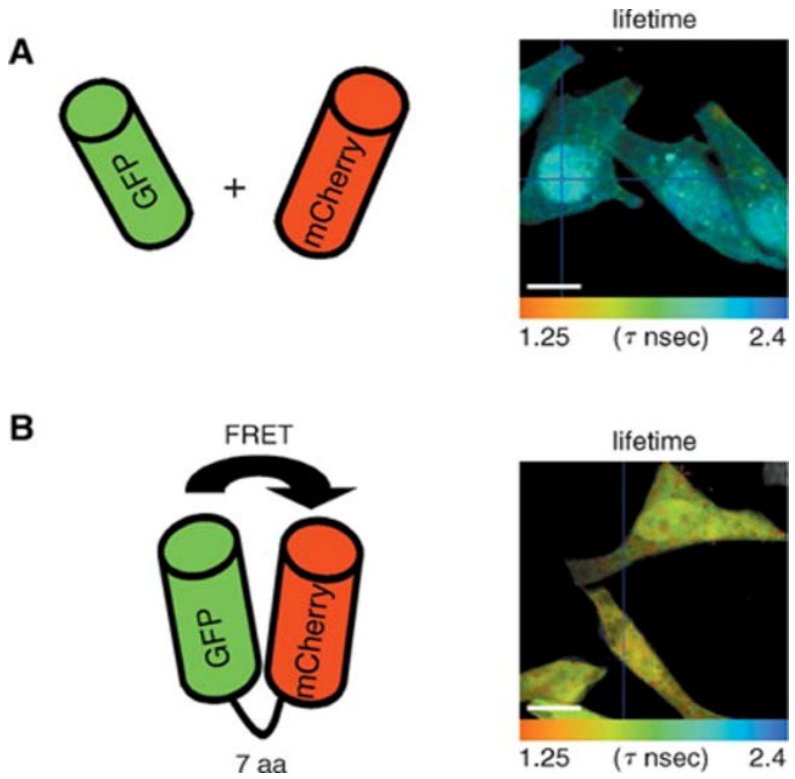
Detection of fluorescence lifetime – Time correlated single photon counting





- Pulsed excitation laser source
- Detection of the arrival time for a single photon for each pulse
- generation of lifetime histogram

FRET-Fluorescence lifetime imaging (FLIM)



FRET-FLIM is highly useful for *in cellulo* FRET measurements:

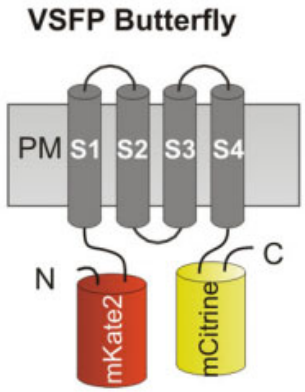
- does not rely on ratiometric measurements of donor- and acceptor fluorescence
- is insensitive/less sensitive to different donor and acceptor dye concentration
- results in accurate FRET detection

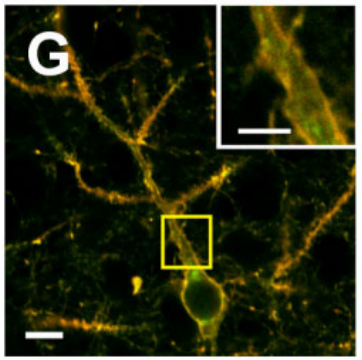
Complex equipment required:

- confocal microscope with fluorescence lifetime detection

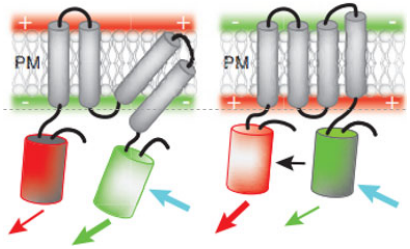
CP Cytometry, Volume: 42, Issue: 1, Pages: 12.10.1-12.10.19, First published: 01 October 2007, DOI: (10.1002/0471142956.cy1210s42)

Measuring membrane voltage by FRET



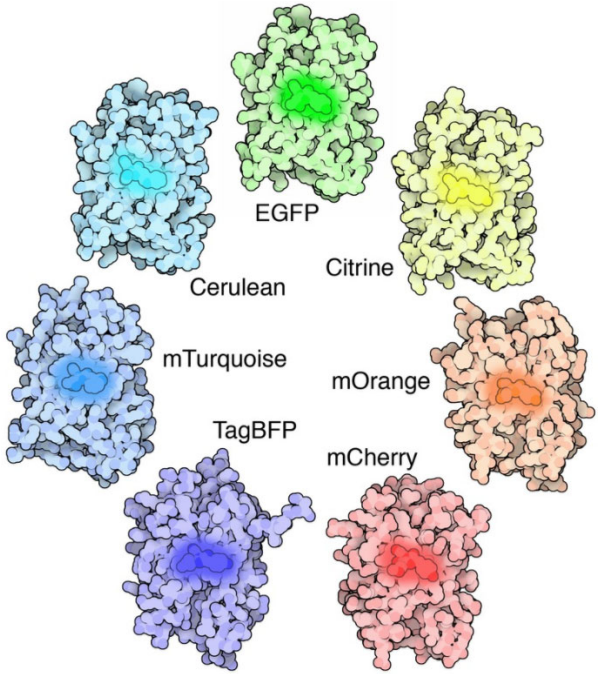


expression in neurons



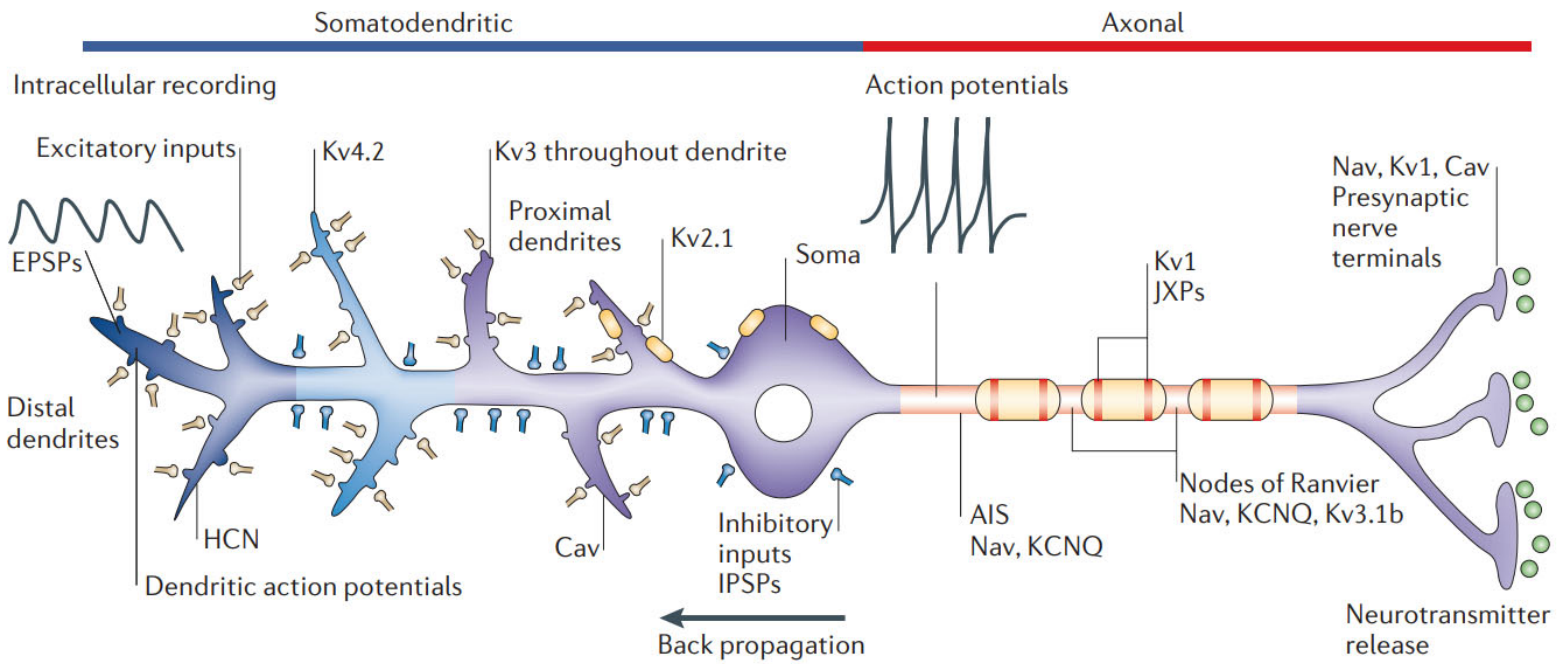
Design: FRET sensor using voltage sensitive fluroescent protein (VSFP), named butterfly.

Akemann et al, *J Neurophysiol* 2012



Fluorophores: Fluroescent proteins (drawing D. Goodsell)

Dynamics: Neurons generate electical action potentials for signaling



Action Potential - Detail

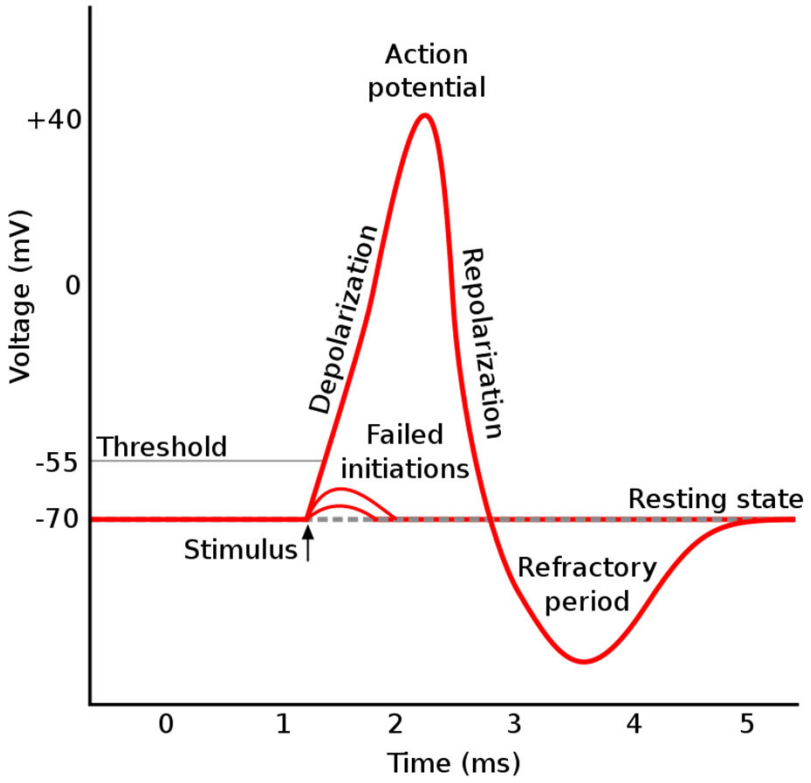
Increase in membrane potential (> 15 mV)

- Na+ ion channels open, Na+ ions enter the cell

→ **Depolarization** (potential of the cell is higher than the cell's resting potential)

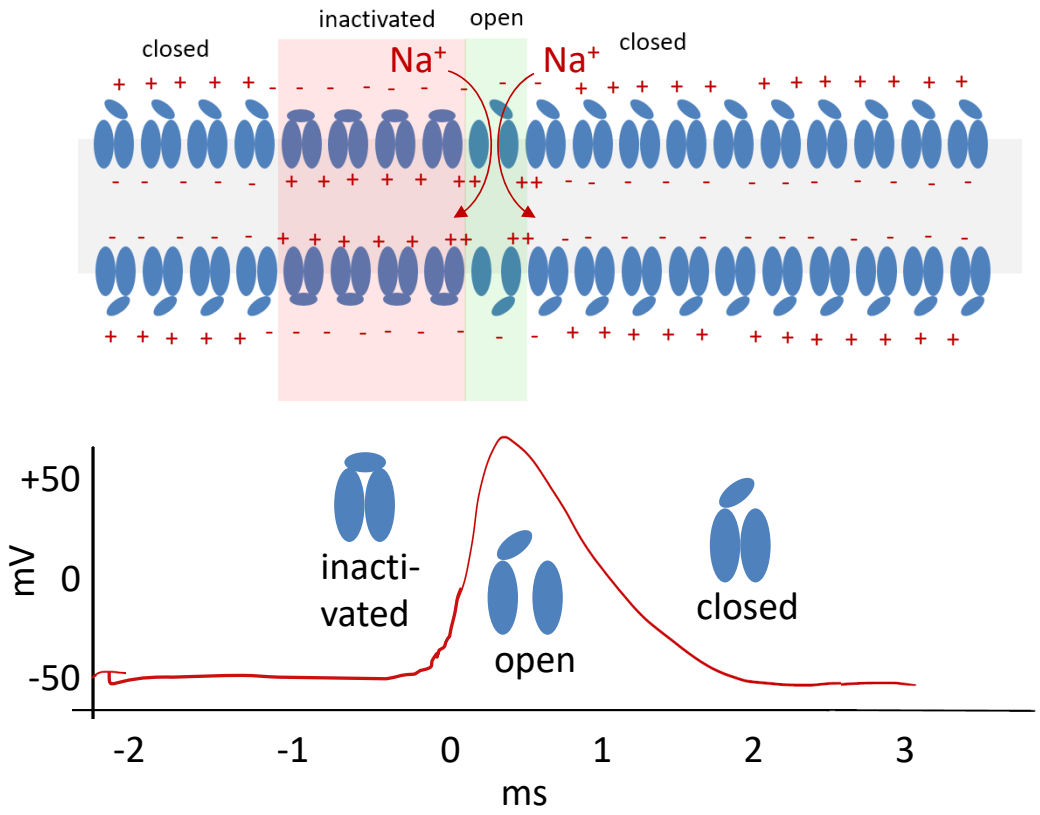
- Na+ channels close at the peak of the action potential
- Opening of K+ ion channels, K+ ions exit from the cell

→ **Hyperpolarization** (refractory period)

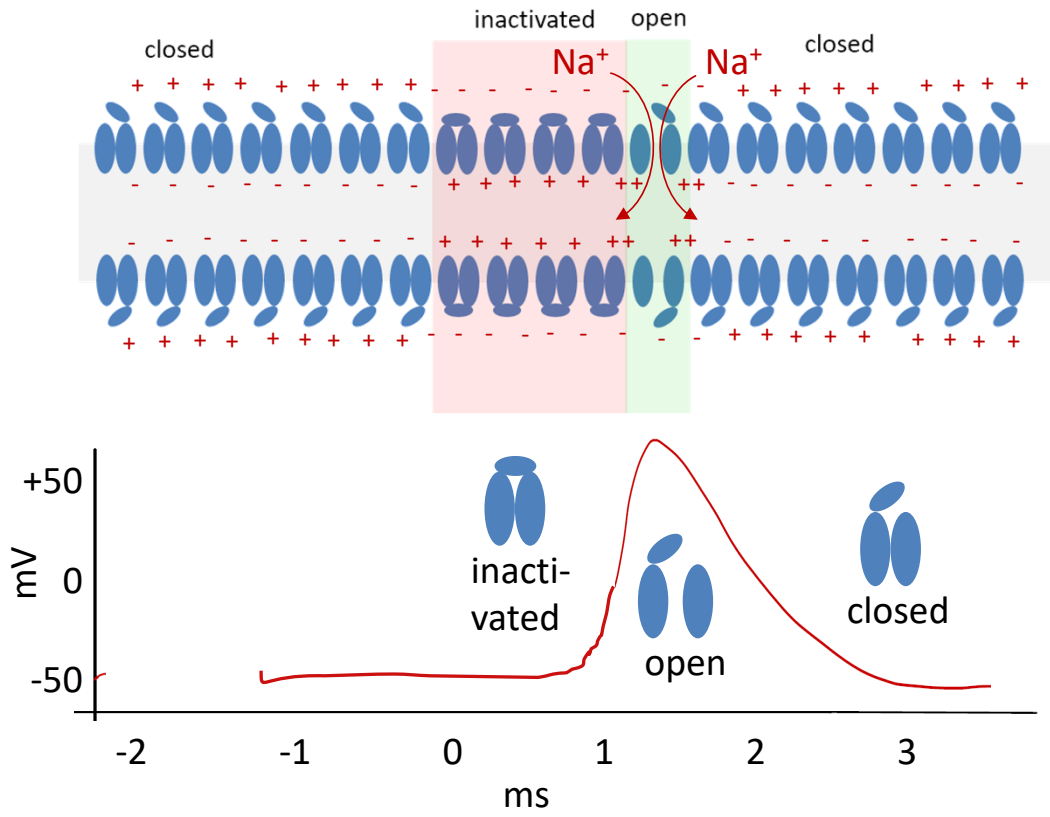


Source: https://en.wikipedia.org/wiki/Action_potential#/media/File:Action_potential.svg

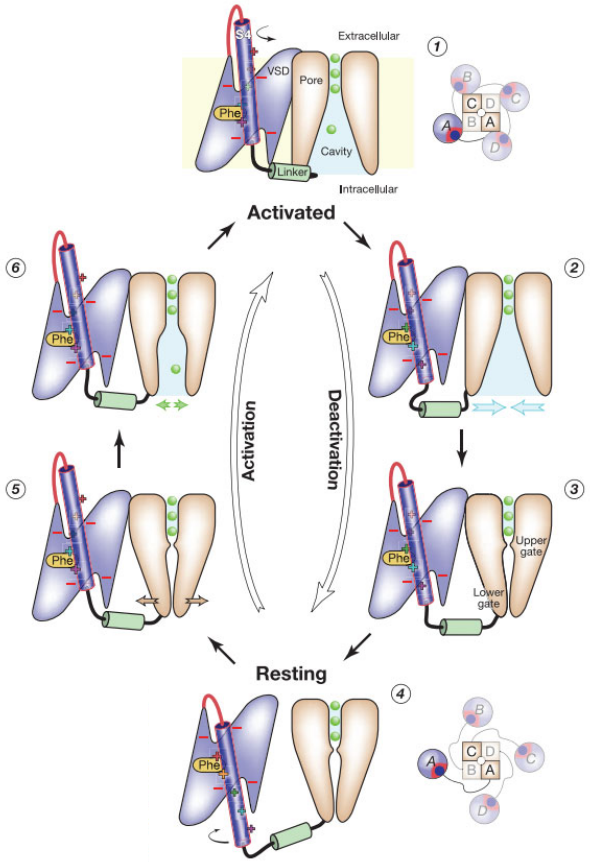
Action potential propagation

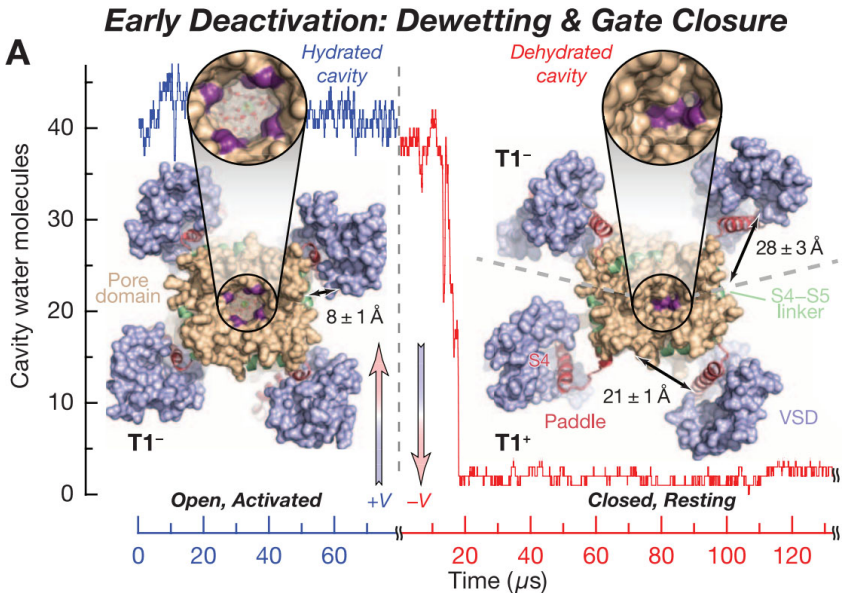


Action potential propagation



Control of ion flux in cells – voltage gating in ion channels



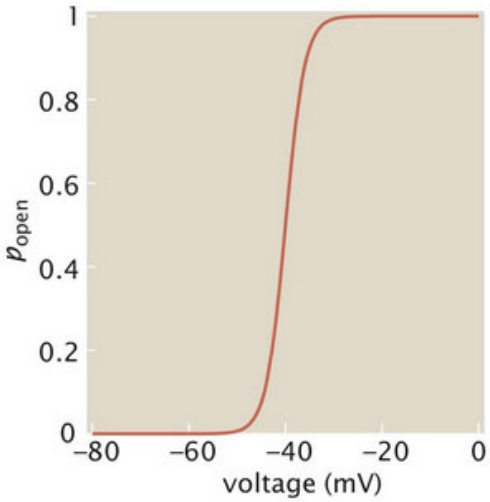


Movement of voltage sensing domain (VSD) controls channel opening and closing

Shaw lab, *Scie*

voltage gating

Voltage gating – model expectation



open probability

V* = -40 mV
q = 12 e^- (12 charges that move across membrane)

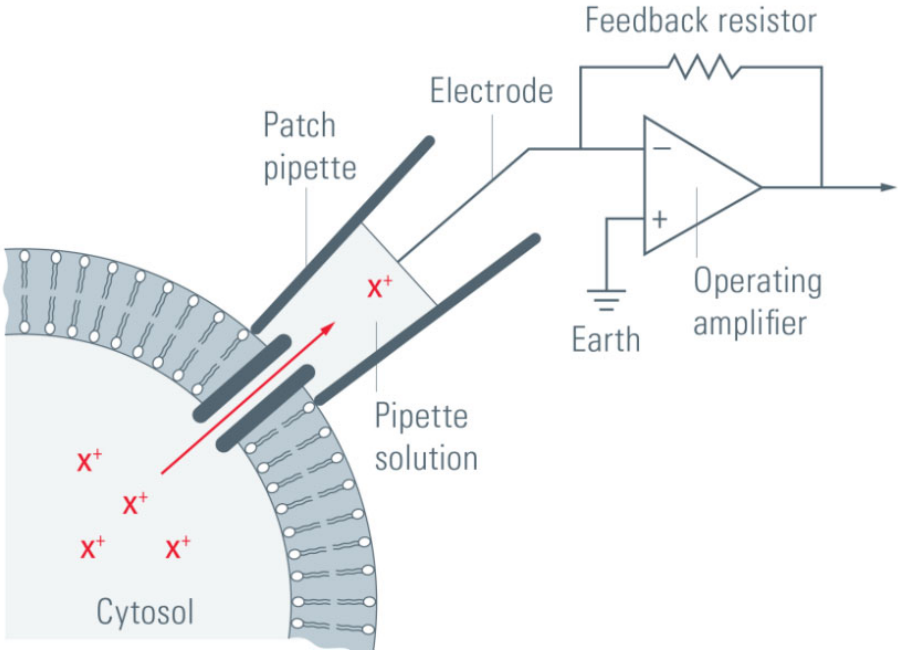
Question:

- what happens when the pathlength (f) increases?
- What, when the gating charge is inverted (+ to -)?

→ **how can the channel behavior be experimentally determined?**

Physical biology of the cell

Measuring membrane potential: Electrophysiology



https://www.leica-microsystems.com/science-lab/the-patch-clamp-technique/

Glass micropipette containing an electrode makes contact with cell membrane

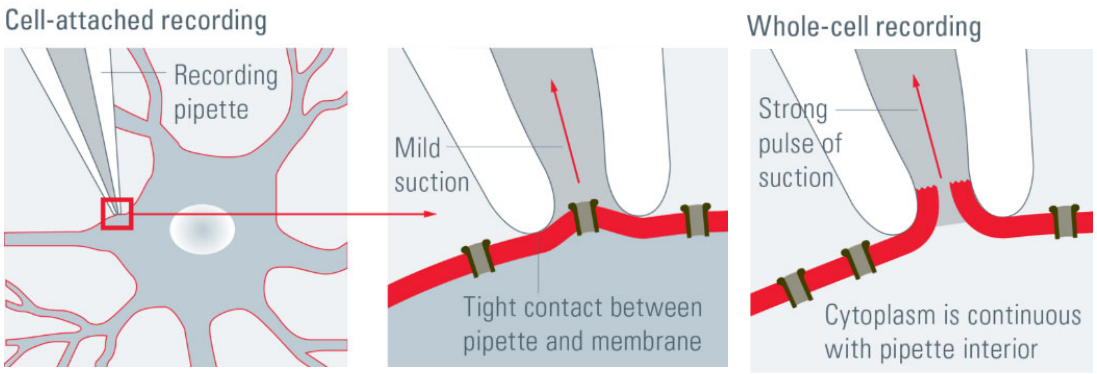
Tight seal (MΩ) is formed (high electrical resistance, due to loss of ion flux)

If an **ion channel** is caught with the pipette: ions may still flow through

→ the current can be measured

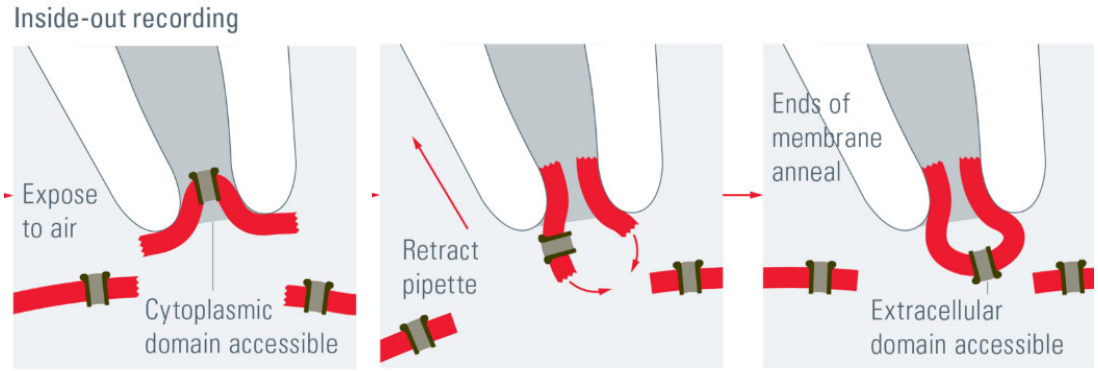
→ recordings are taken from few or even a single channel with high accuracy

Different patch clamp measurements



Recording at the membrane of a cell, or over a **whole cell**

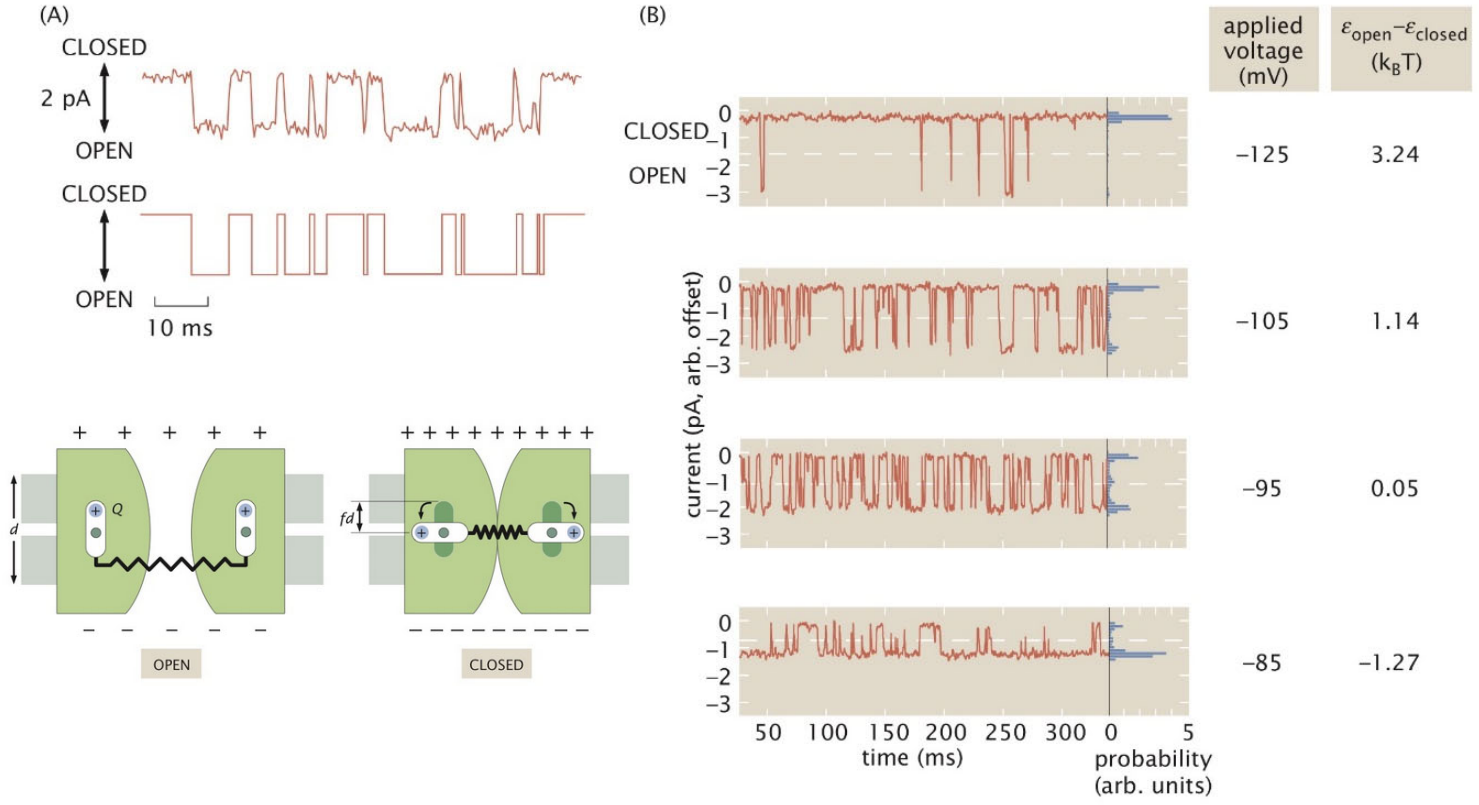
Measurement of the membrane potential directly



Recording single channels or over membrane patches obtained from cells

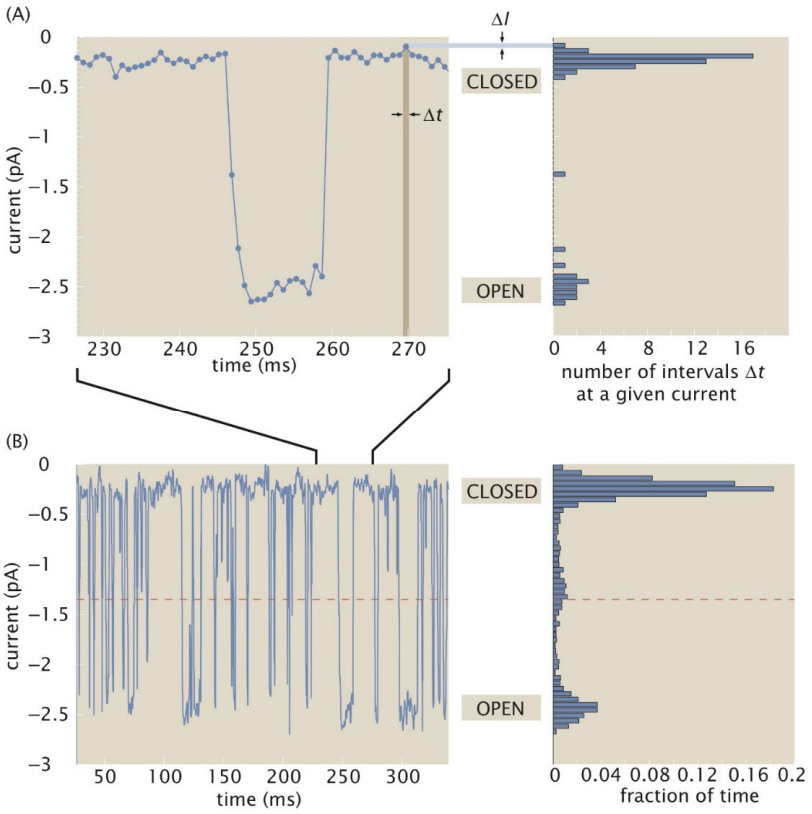
→ such arrangements can also be reconstituted from **purified proteins** and **synthetic lipids**

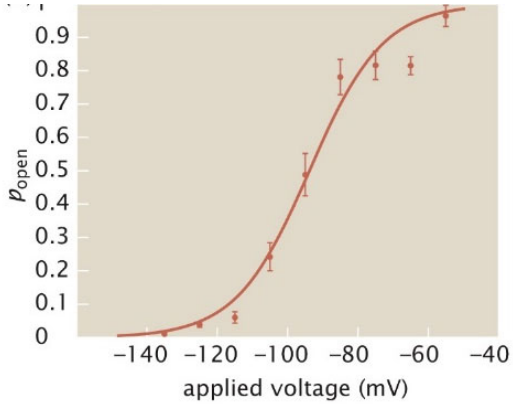
A true single-molecule experiment



Physical biology of the cell

Calculating open probabilites from kinetic data

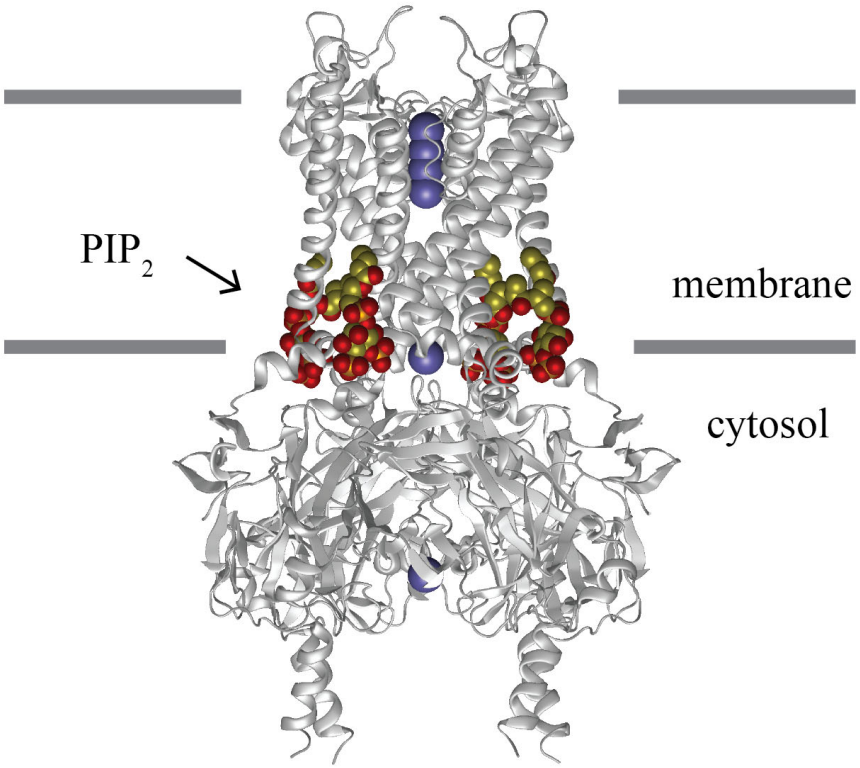




$$p_{open} = \frac{T_{open}}{T_{open} + T_{closed}}$$

Physical biology of the cell

Observing channel gating by single-molecule FRET



wikipedia

Inward Rectifier Potassium (Kir) channel

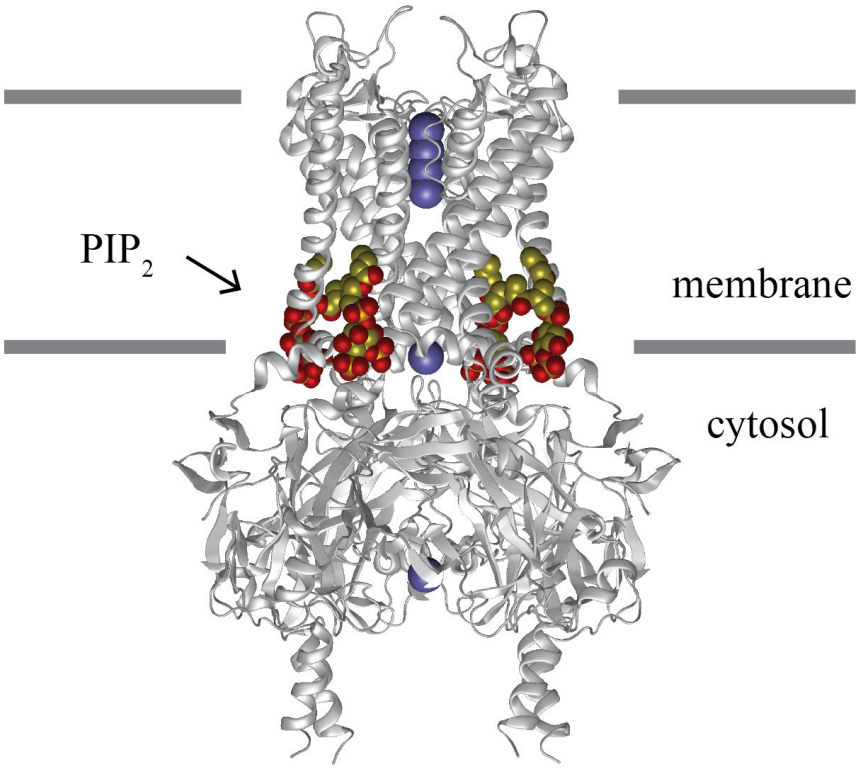
- Voltage gated channel
- Phosphatidylinositol 4,5-bisphosphate (PIP2) is a gating ligand
- universal activatory ligand
- Stabilizes the open structure

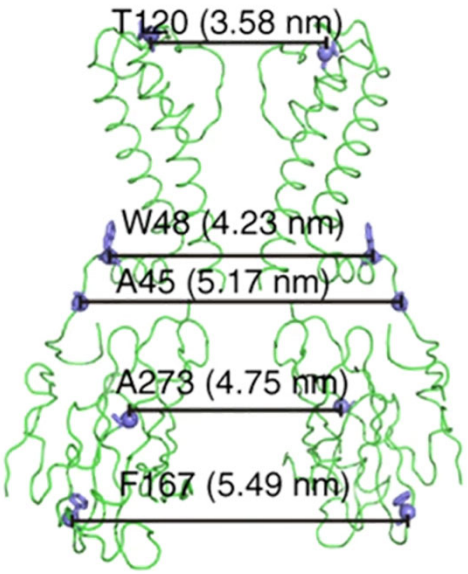
Function of Kir channels

- Shape the action potentials of cardiomyocytes
- Modulate membrane potentials of nerve and glial cells
- Control electrical activity of endothelial and smooth muscle cells

Wang et al.
NSMB 2016

Observing channel gating by single-molecule FRET





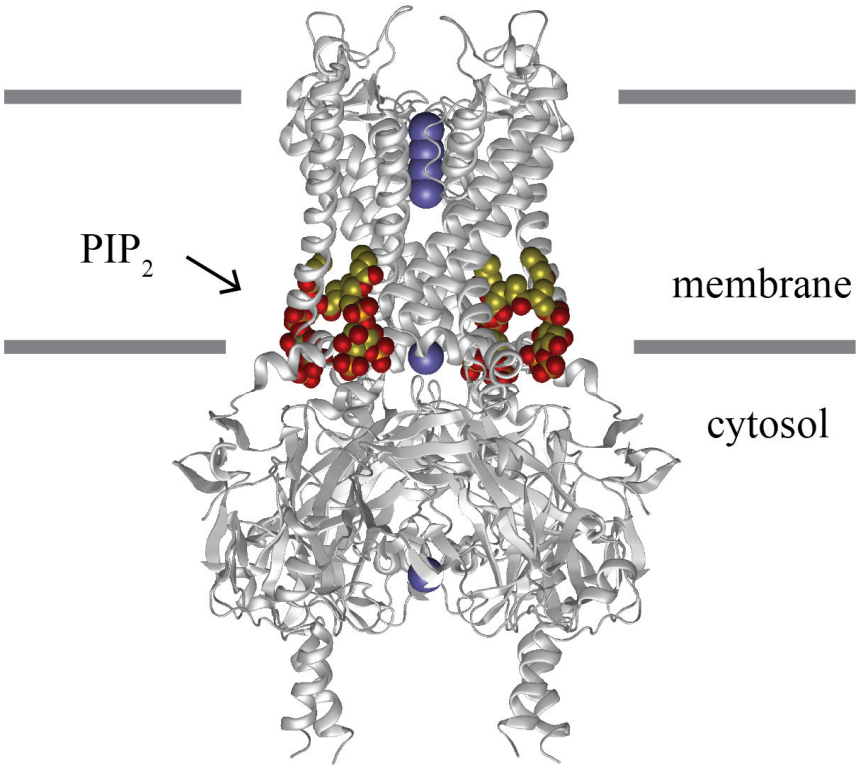
Fluorescent labeling of the channel:

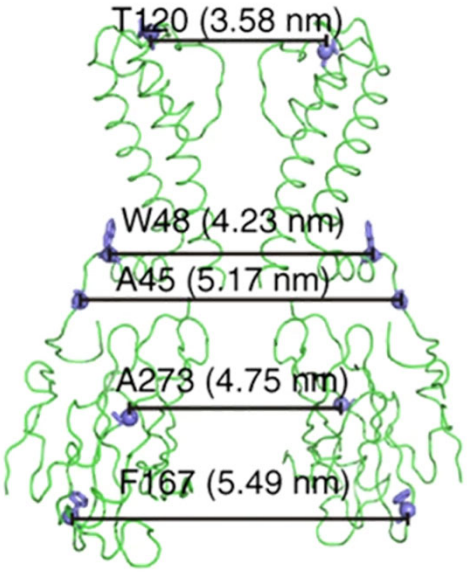
- use of a well characterized bacterial homolog

- introduction of defined cysteines into the protomers of the **tetrameric channel**

what is the main problem?

Observing channel gating by single-molecule FRET





- defined labeling with donor and acceptor dye possible

Fluorescent labeling of the channel:

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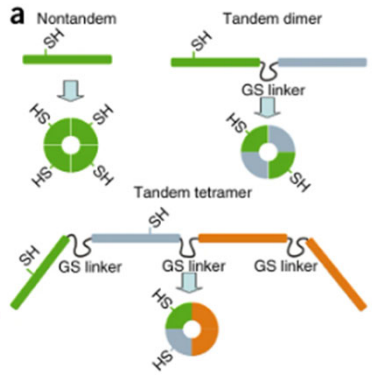
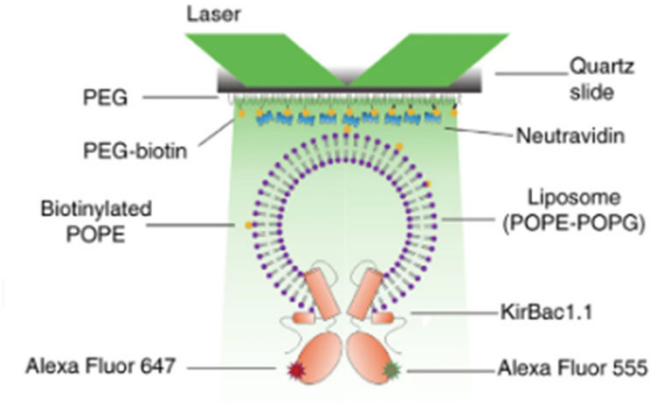
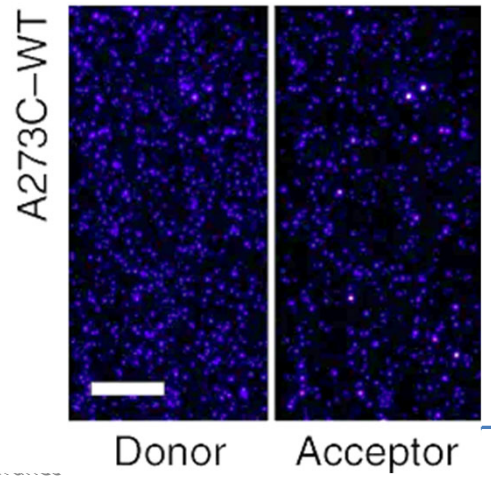
- introduction of defined cysteines into the protomers of the tetrameric channel



Observing channel gating by single-molecule FRET

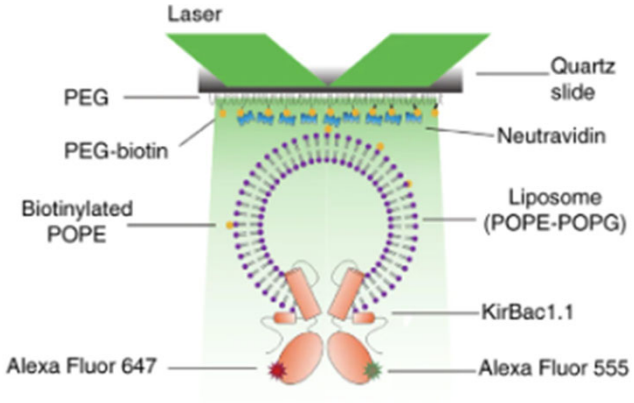




Single molecule imaging:

- individual channels are incorporated into liposomes and immobilized
- **Total internal reflection fluorescence** (TIRF) illumination
- Donor and acceptor fluorescence emission is collected
- changing conditions, e.g. PIP2 addition

Observing channel gating by single-molecule FRET



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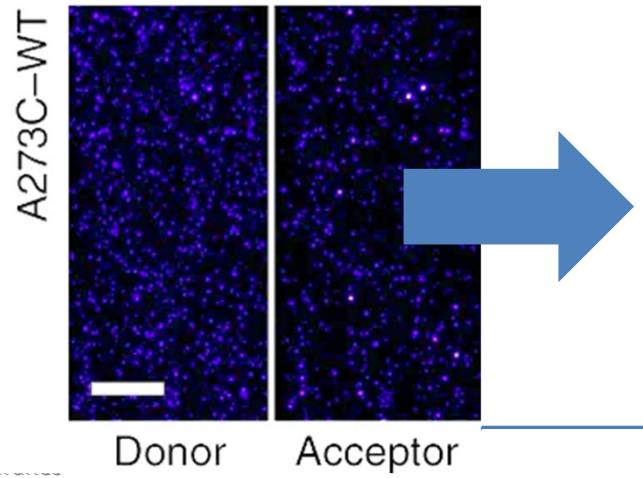
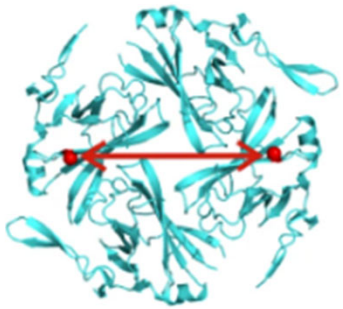
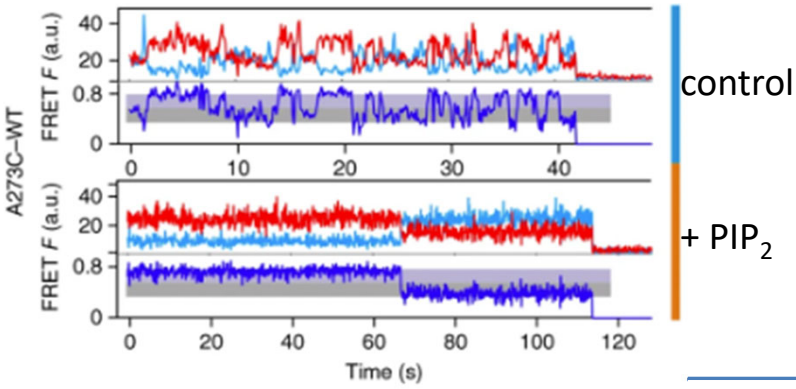
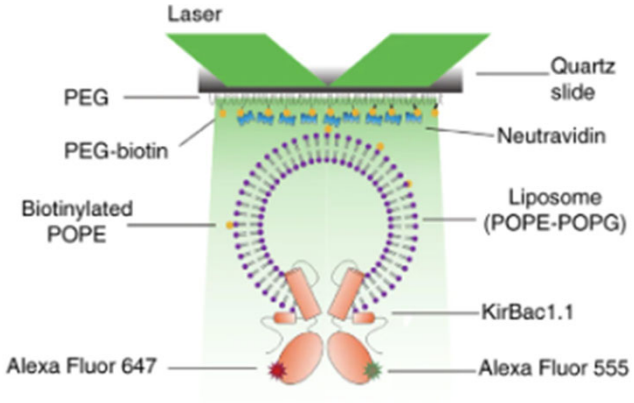
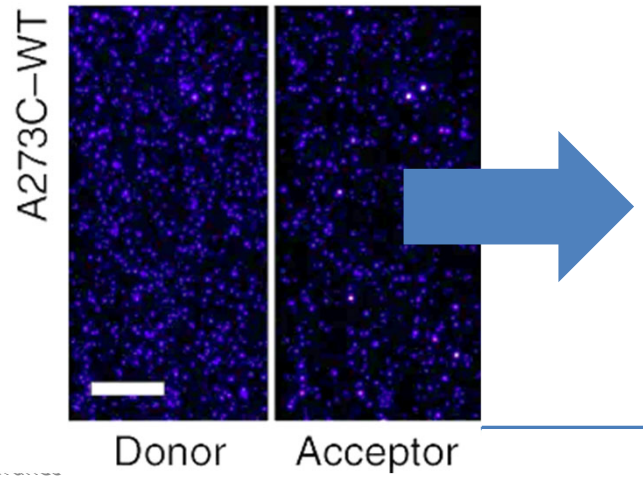


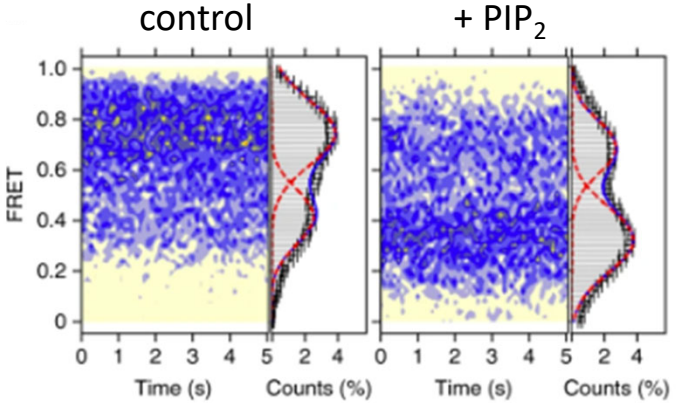




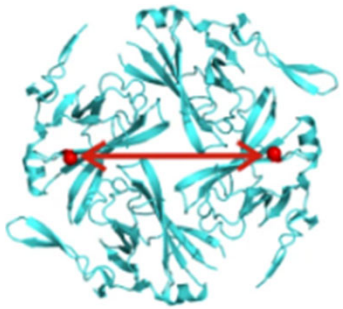
Observing channel gating by single-molecule FRET

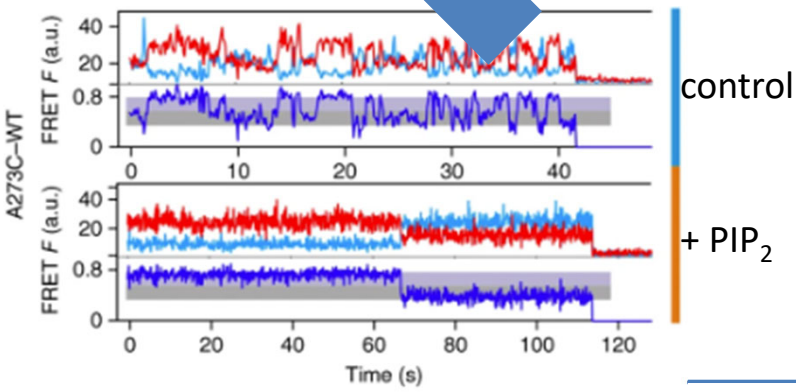




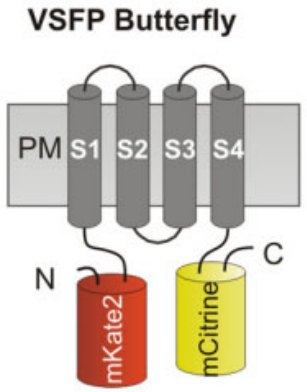


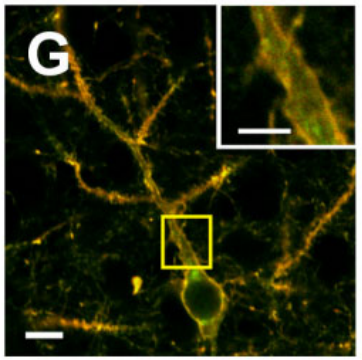
- dynamic gating observed in real time
- conformational changes
- PIP2 action: open channel stabilized



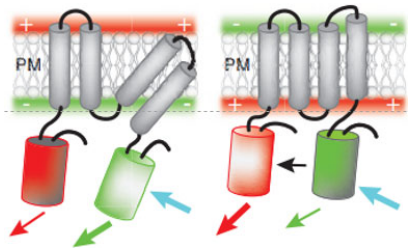


Measuring membrane voltage by FRET





expression in neurons

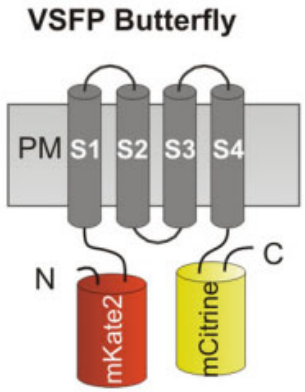


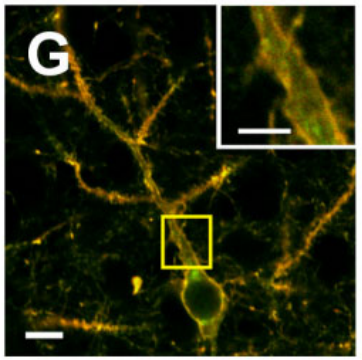
Design: FRET sensor using voltage sensitive fluroescent protein (VSFP), named butterfly.

Akemann et al, *J Neurophysiol* 2012

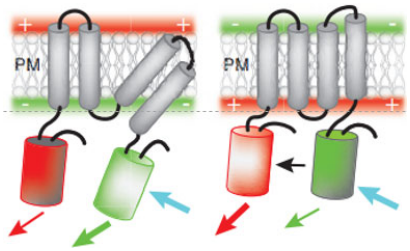
Can we observe dynamic processes now?

Measuring membrane voltage by FRET





expression in neurons



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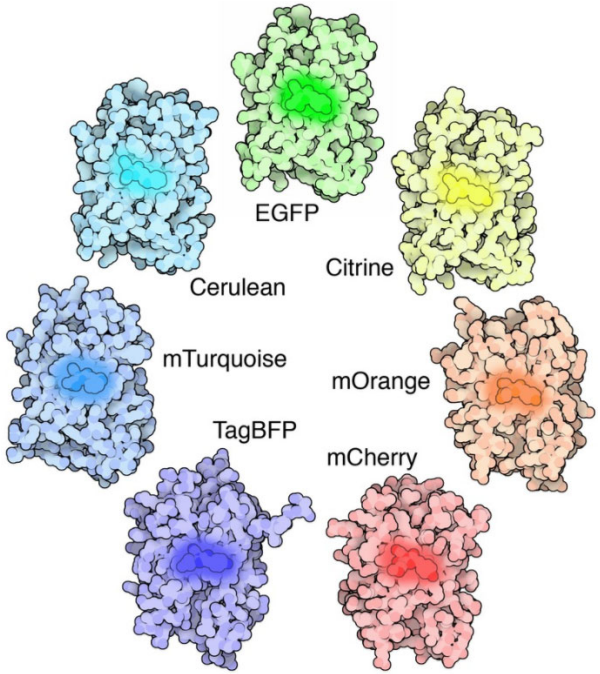
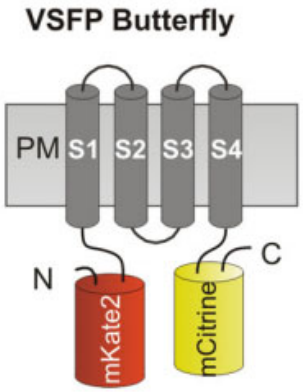
Akemann et al, *J Neurophysiol* 2012



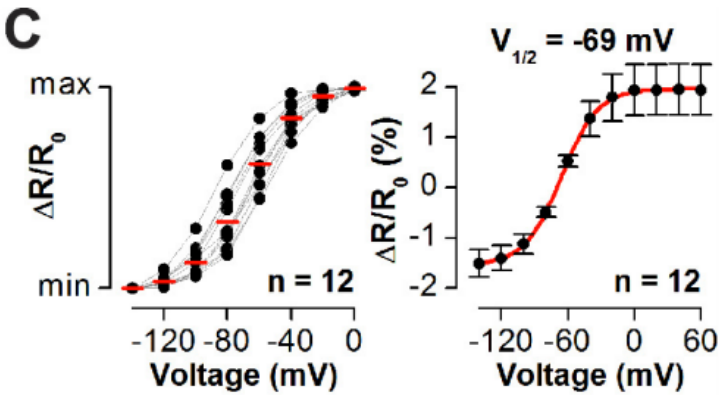
Fluorophores: Fluroescent proteins (drawing D. Goodsell)

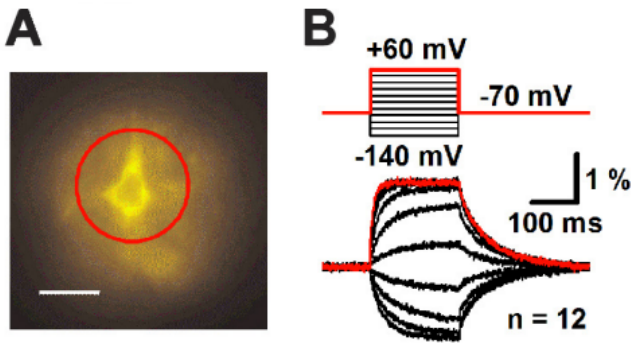
Measuring membrane depolarization in real time using fluorescent voltage sensors

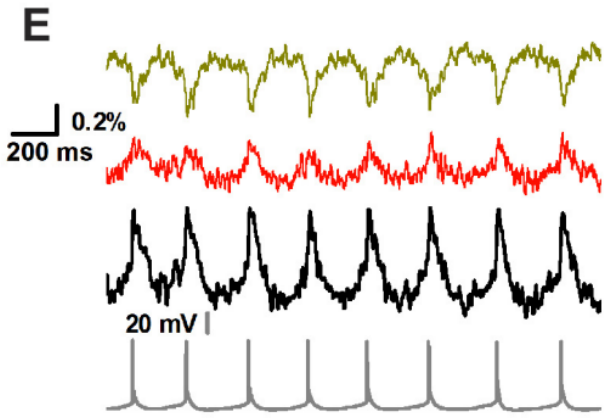


patch-clamp control of membrane potential

-> results in FRET change

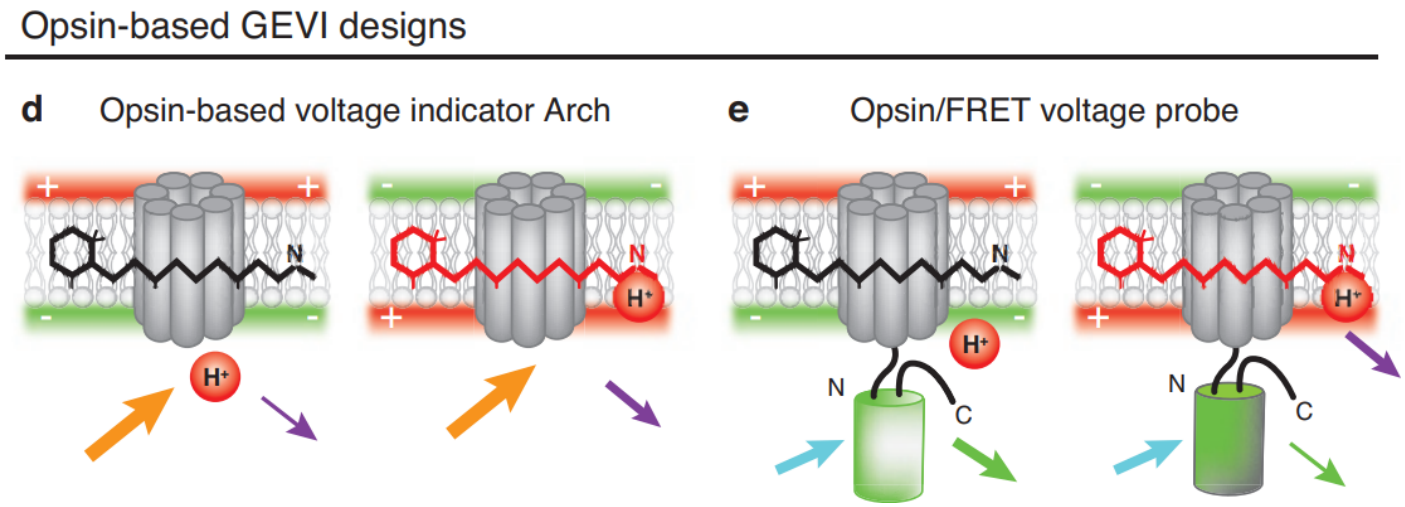






Akemann et al, *J Neurophysiol* 2012

Other sensors provide higher S/N and response time



e.g. by Cohen laboratory

Nina Vogt, Nat. Meth. 2015

All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins

nature methods
2014

Daniel R Hochbaum[1,16], Yongxin Zhao[2,16], Samouil L Farhi[3], Nathan Klapoetke[4–7], Christopher A Werley[3], Vikrant Kapoor[8], Peng Zou[3], Joel M Kralj[3], Dougal Maclaurin[9], Niklas Smedemark-Margulies[3], Jessica L Saulnier[10], Gabriella L Boulting[10], Christoph Straub[10], Yong Ku Cho[4–7], Michael Melkonian[11], Gane Ka-Shu Wong[12–14], D Jed Harrison[2], Venkatesh N Murthy[8], Bernardo L Sabatini[10,15], Edward S Boyden[4–7,17], Robert E Campbell[2,17] & Adam E Cohen[3,9,15]