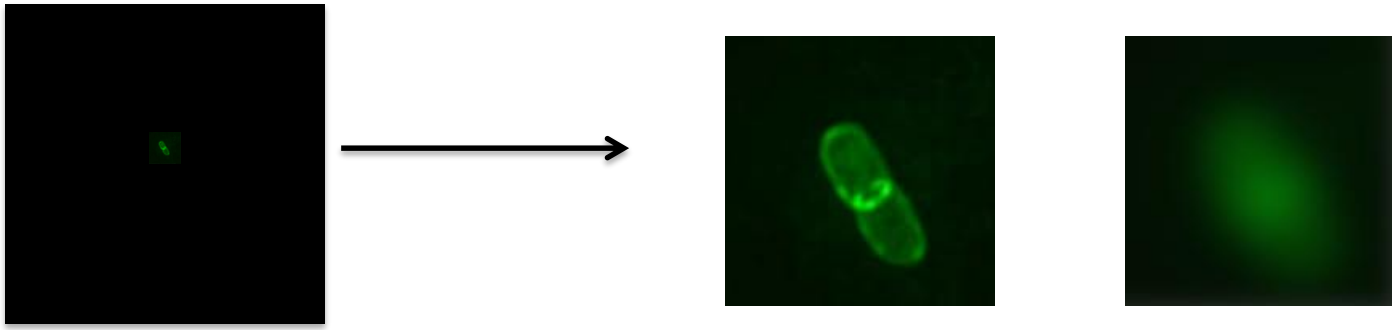
Week 6-7

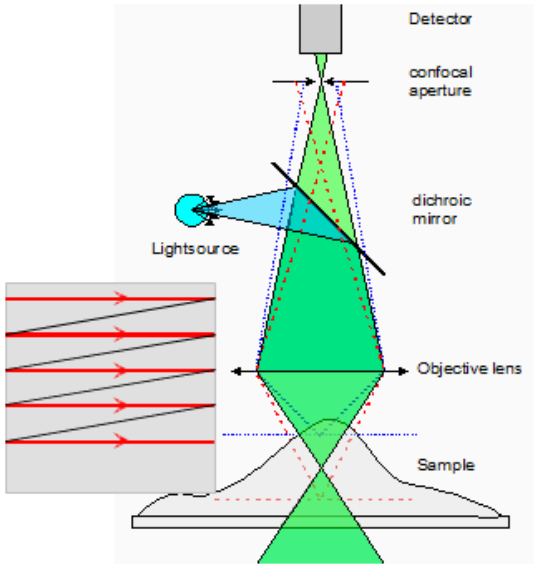
Fluorescence and FRET



Diffraction limited high resolution microscope Methods

1. Laser scanning confocal
2. Spinning disc confocal
3. Multi-photon confocal microscopy

Point Scanning Confocal



Spinning Disk Confocal

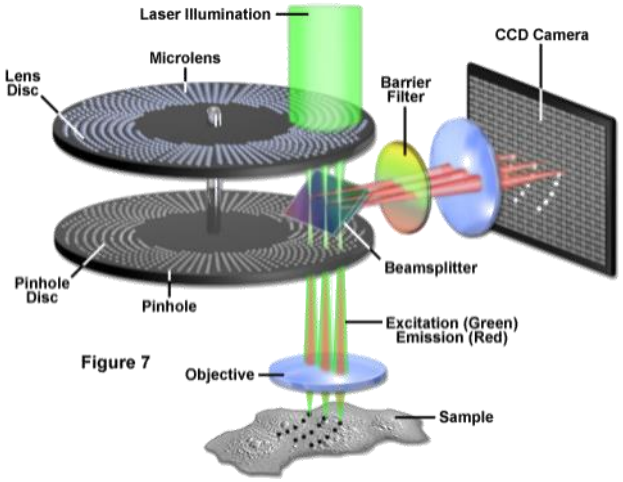
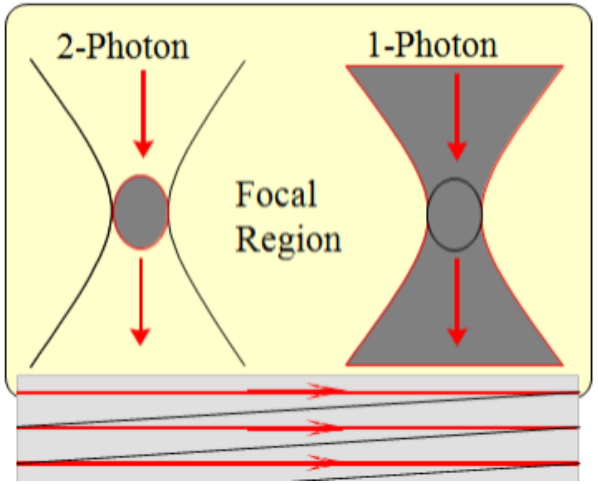
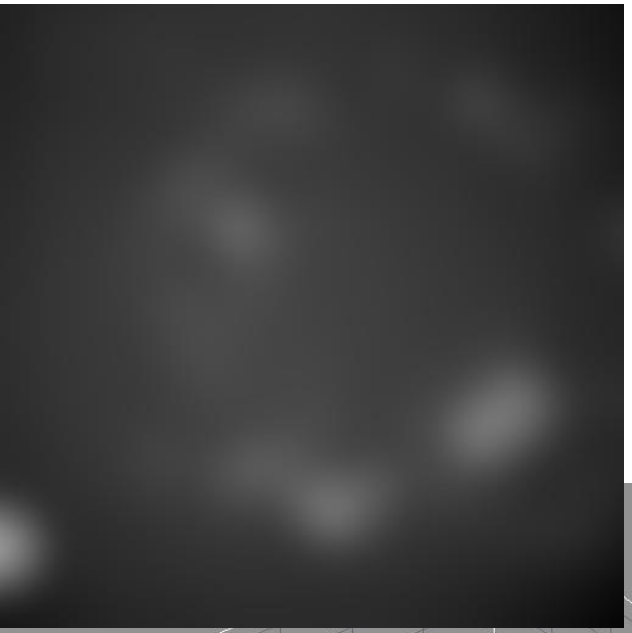


Figure 7

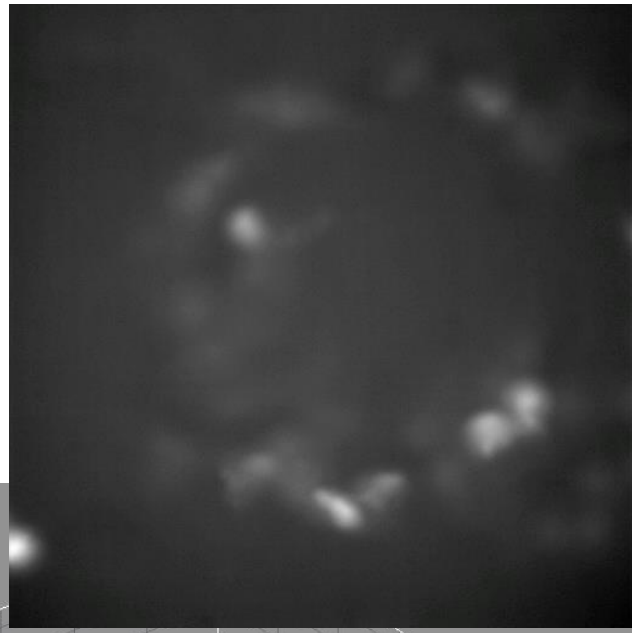
Multiphoton

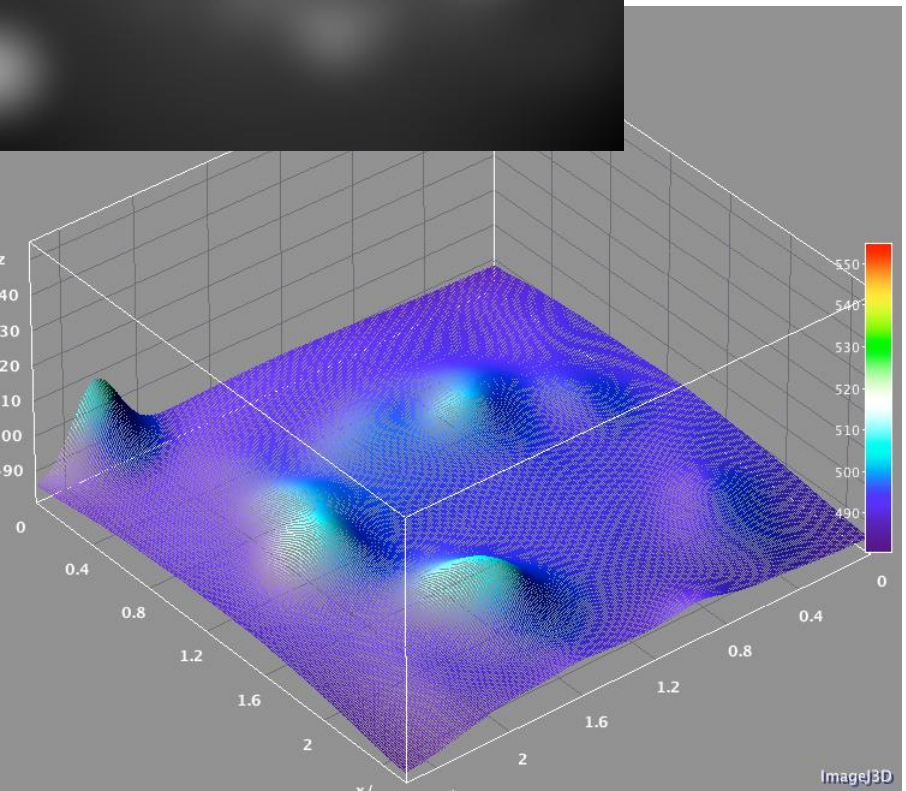


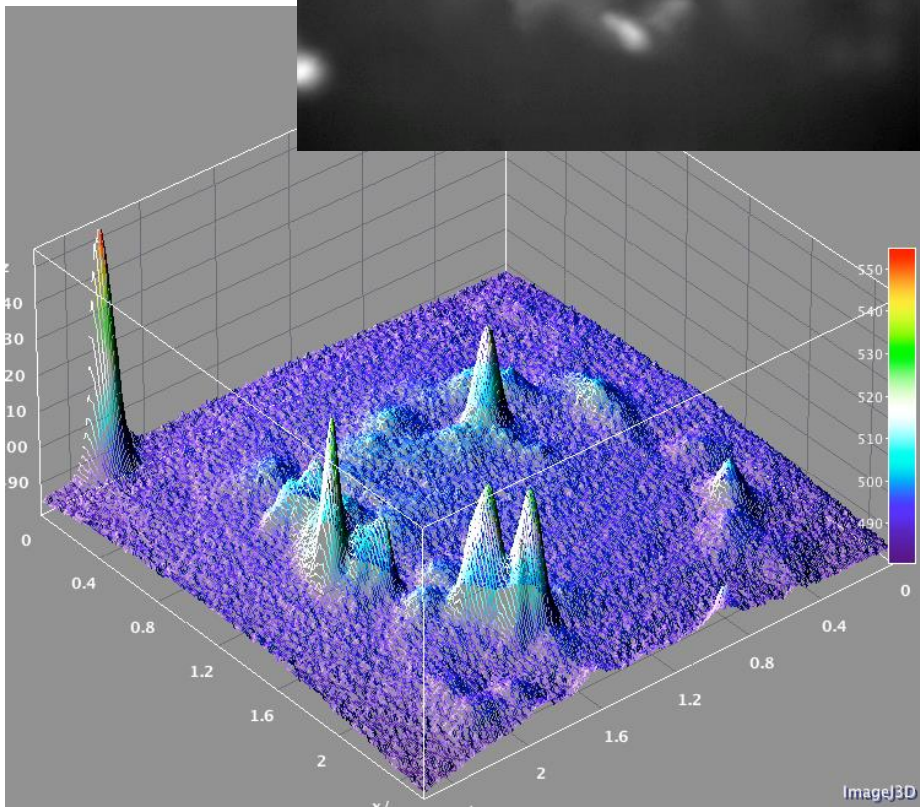
Point Spread Function



GFP expressing cells

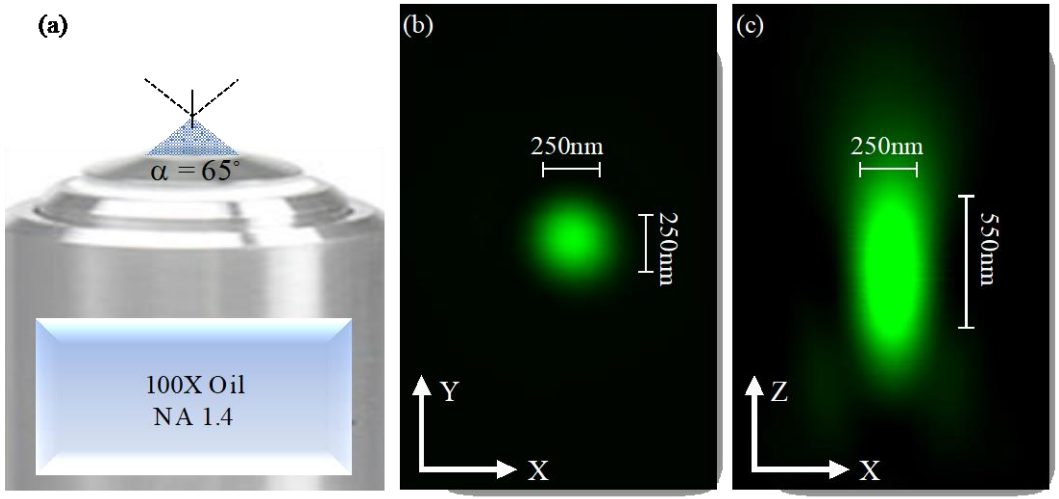




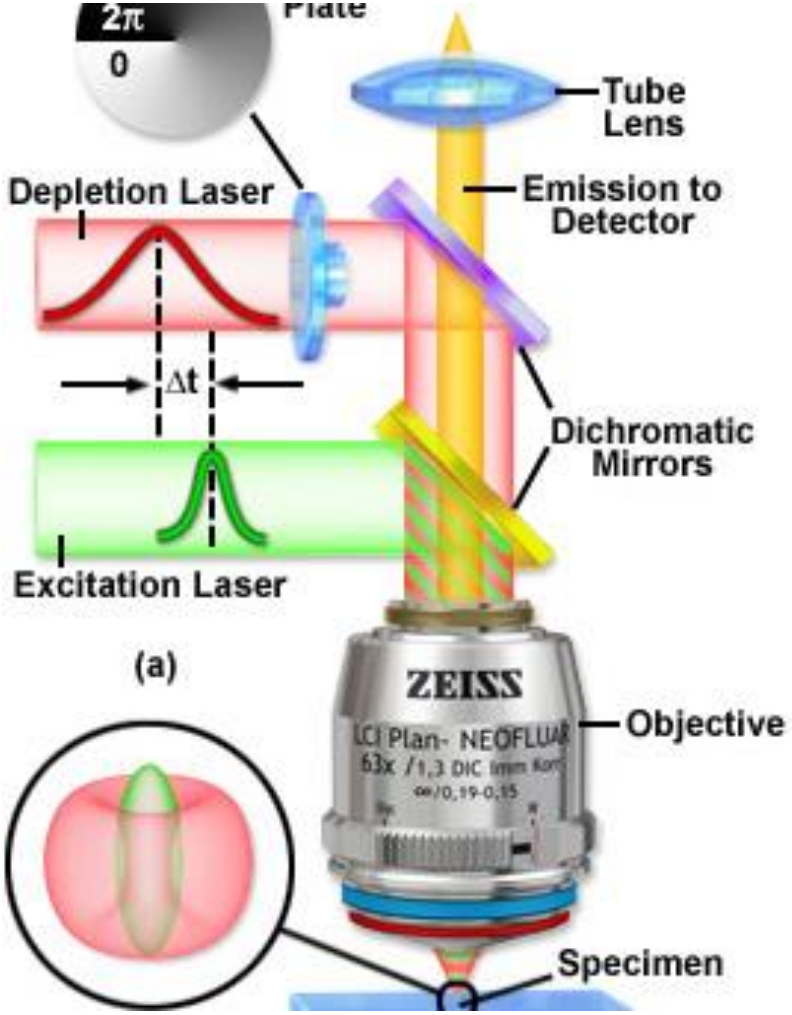


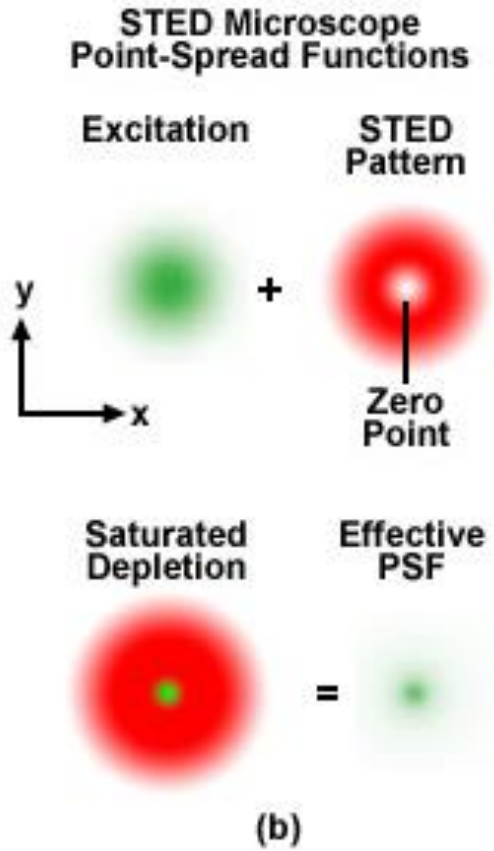
Super Resolution Microscopy

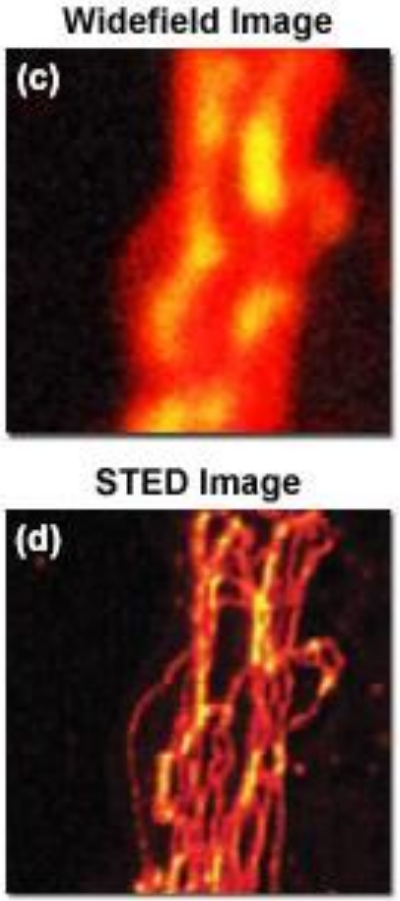
- STORM : Stochastic Optical Reconstruction Microscope
- PALM : Photo activated Localization Microscope
- STED: Stimulated Emission Depletion



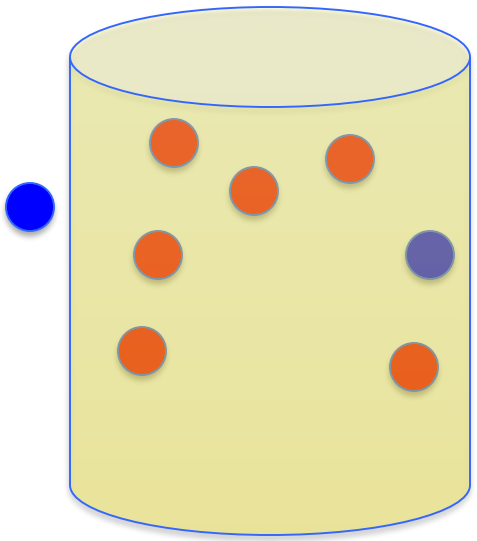
Can we reduce the size of the point spread function?
What other solutions are possible?


2π
0
Plate
Tube Lens
Depletion Laser
Emission to Detector
Δt
Dichromatic Mirrors
Excitation Laser
(a)
ZEISS
Objective
Specimen


STED Microscope
Point-Spread Functions
Excitation
STED Pattern
y
x
+
Zero Point
Saturated Depletion
Effective PSF
=
(b)


Widefield Image
(c)
STED Image
(d)

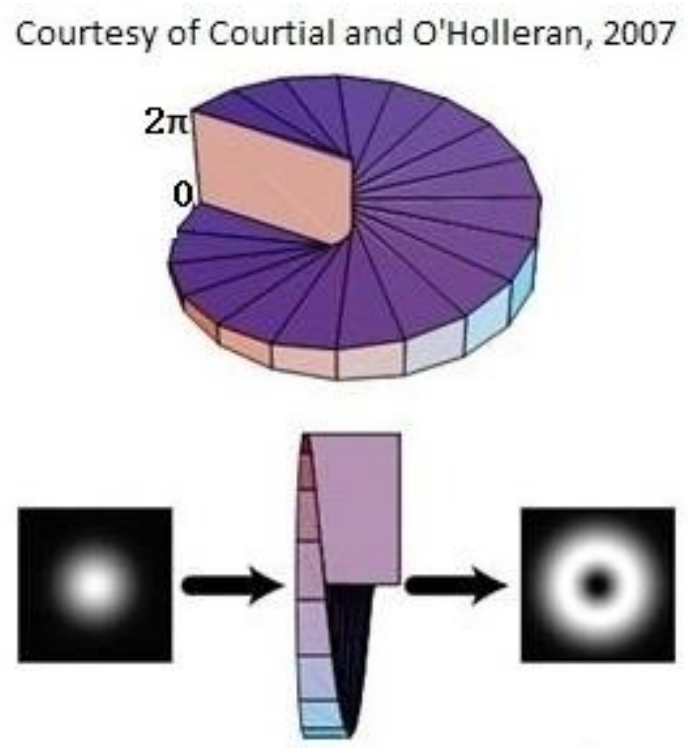
Many dye molecules are excited that reduce any good resolution. As the light of the different dye molecules can not be distinguished.



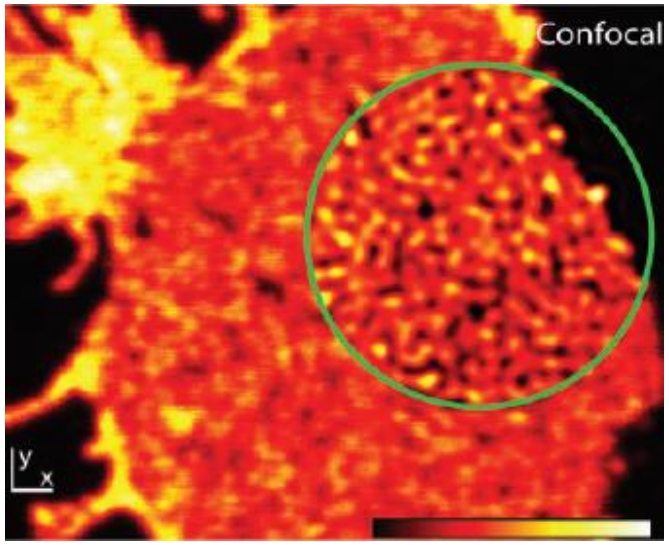
Many molecules are excited by a laser spot.

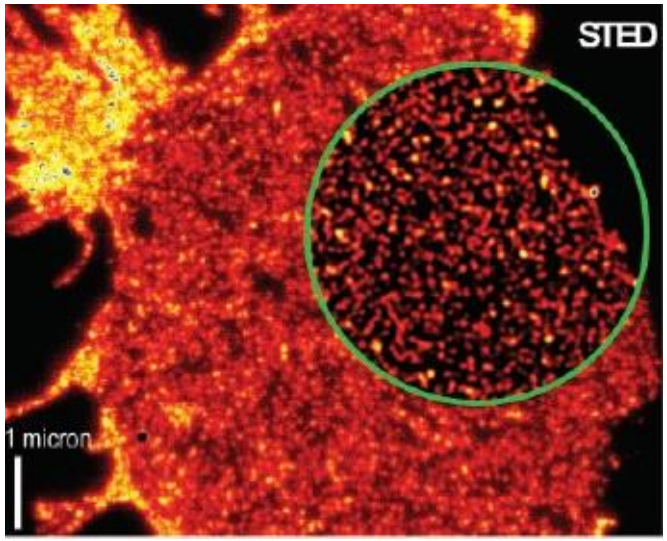
How to make a hole at the center of the beam?

- Optical vortex method can be used to make a twisted light with a center in the hole. Phase plate can be used to make optical vortex.
- Optical vortex has a zero of an optical field.

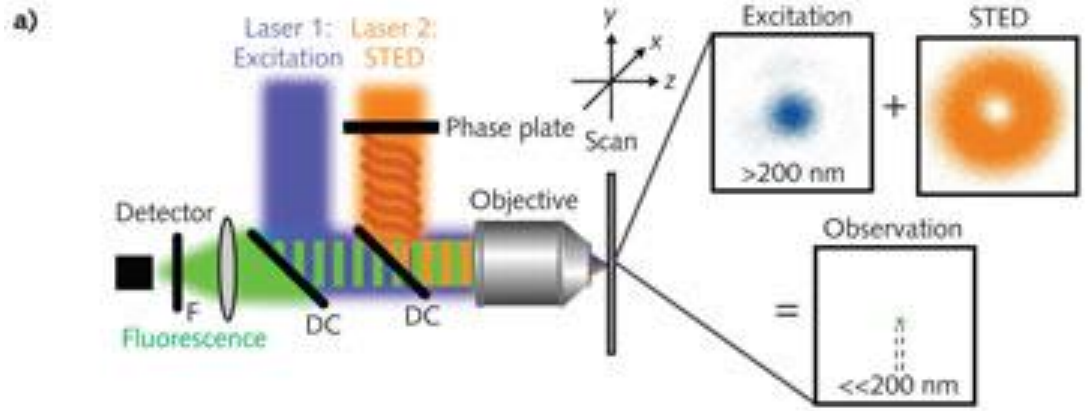


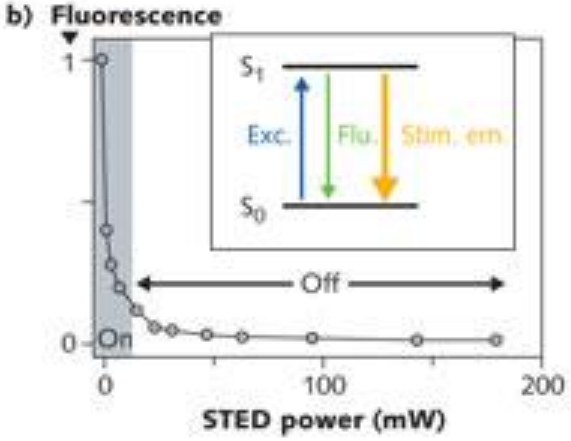
STED vs Confocal Microscope

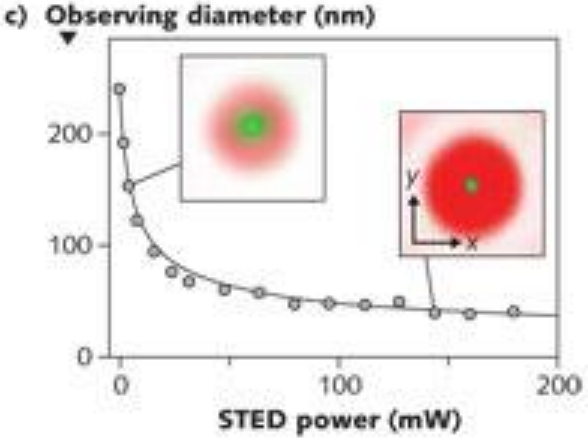


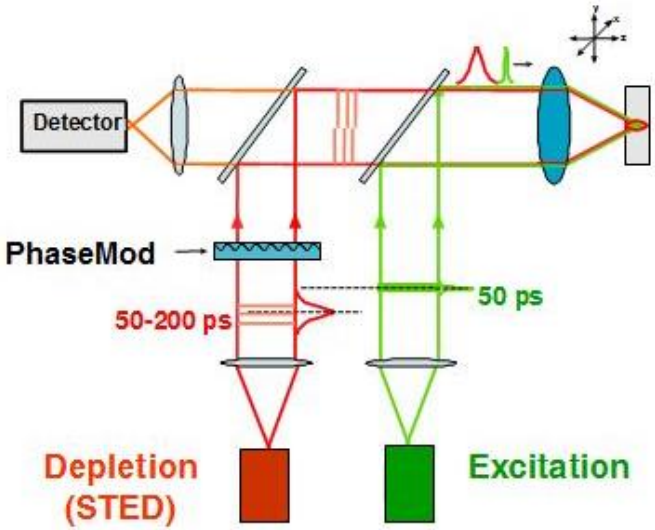


STED Setup

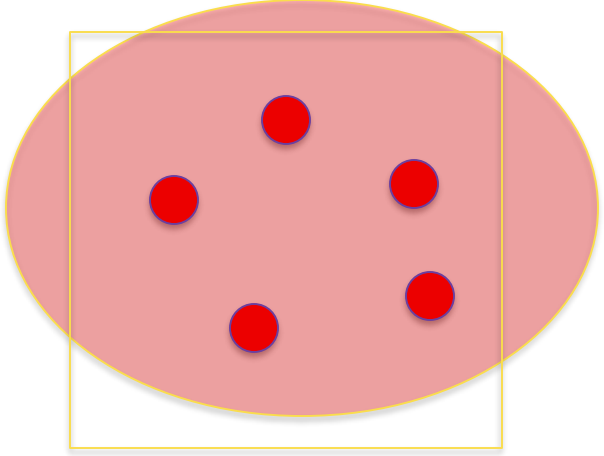






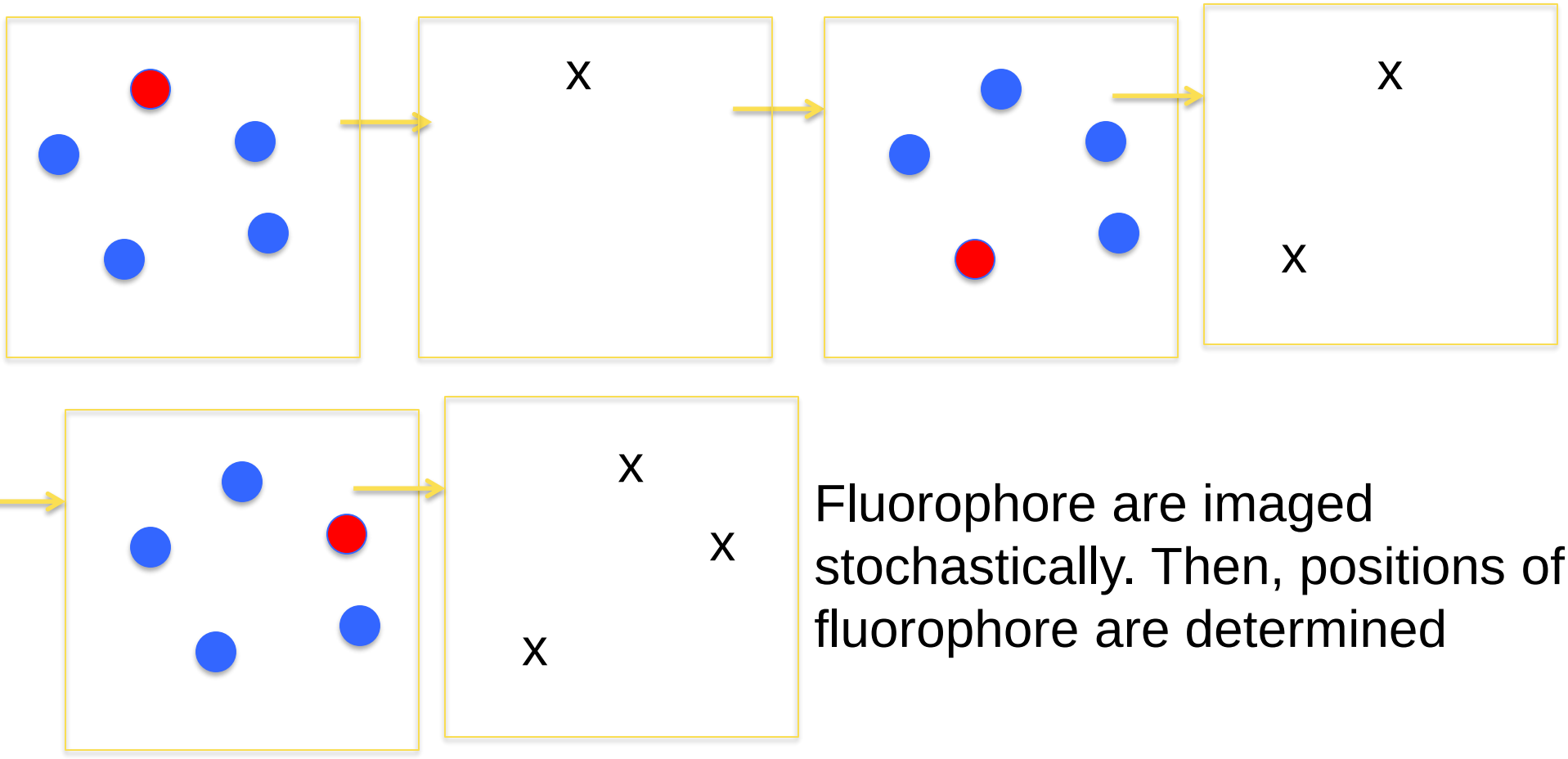


The power dependence delivers subdiffraction size observation volumes on the spot. The volume in which fluorescence emission is allowed (green, insets) but decreases with increasing STED laser power.



Confocal Spot illuminates many fluorophore distance less than diffraction limit

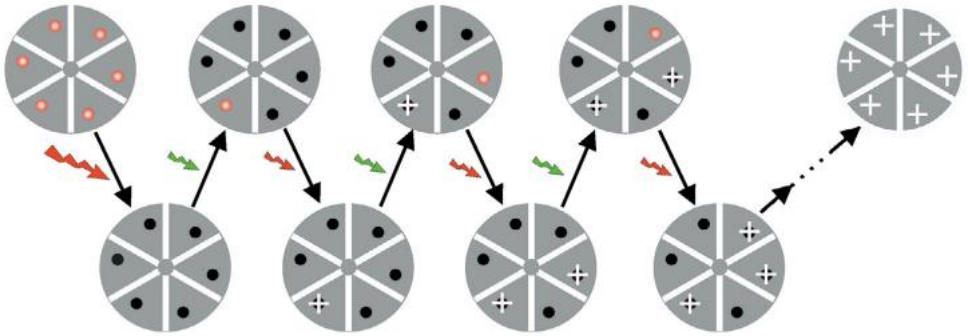
STORM IMAGING



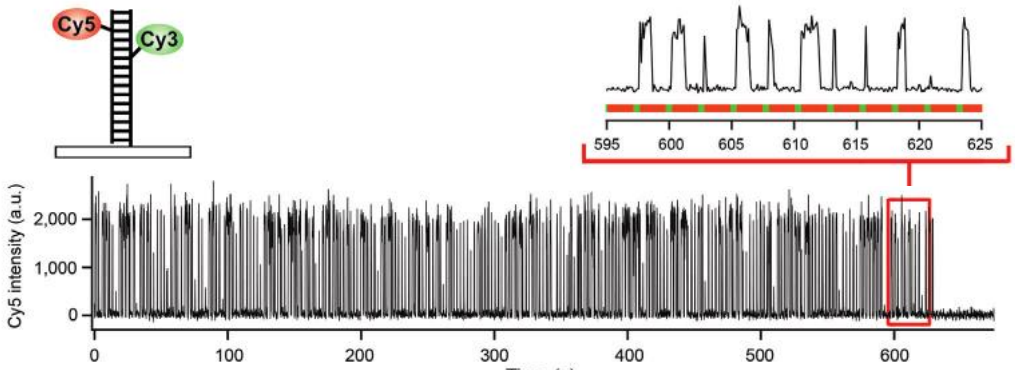
Fluorophore are imaged stochastically. Then, positions of fluorophore are determined

Each point are imaged stochastically

a



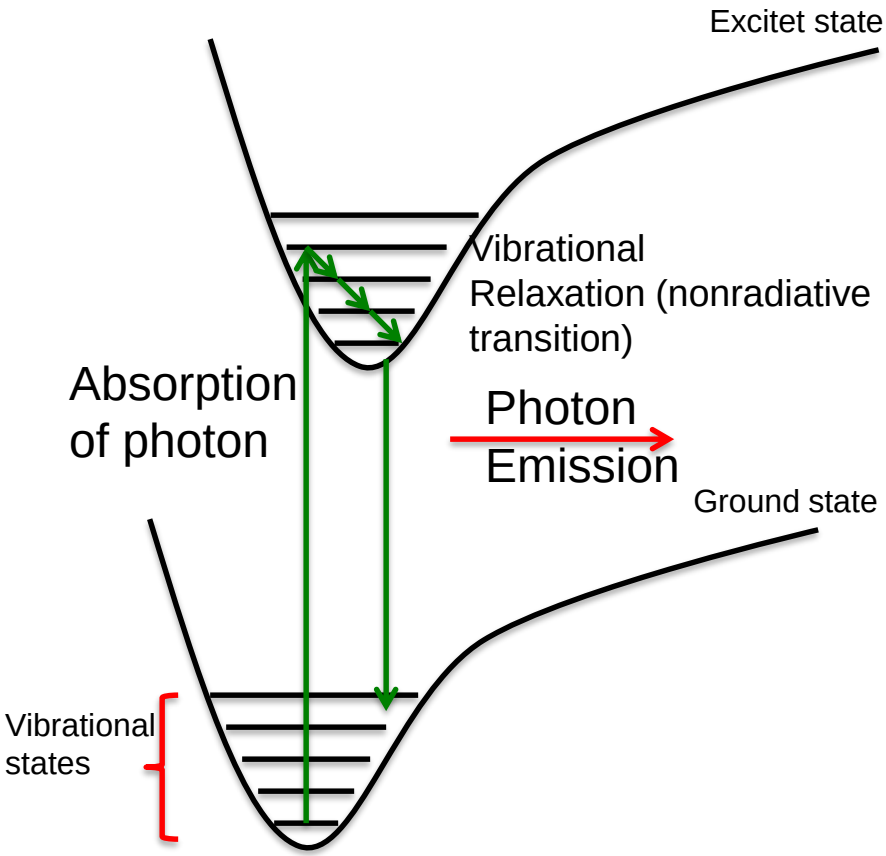
b

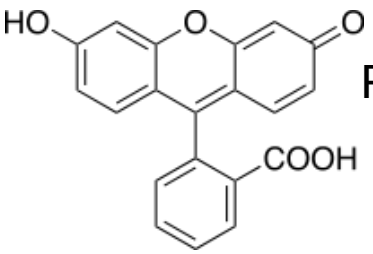


Fluorescence Resonance Energy Transfer (FRET)and its applications

Fluorescence

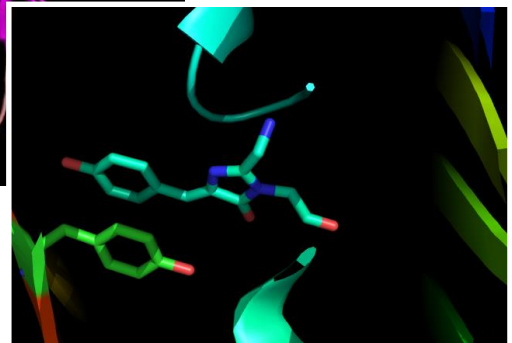
Electronic transition during fluorescence





Fluorescein

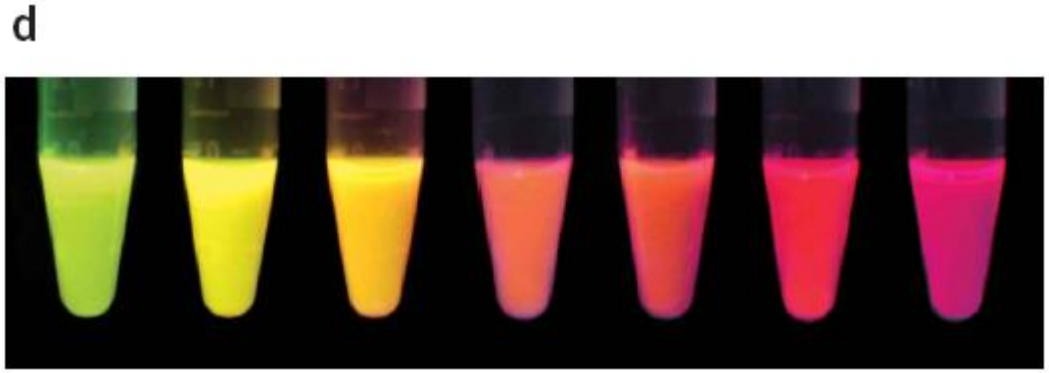
Green
Fluorescent
Protein



Different “GFPs”

Absorption

Fluorescence



mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry, mCherry

Shaner, Tsien,Nat. Bio., 2004

Förster resonance energy transfer (FRET)

RADIATION RESEARCH SUPPLEMENT 2, 326–339 (1960)

Transfer Mechanisms of Electronic Excitation Energy

TH. FÖRSTER

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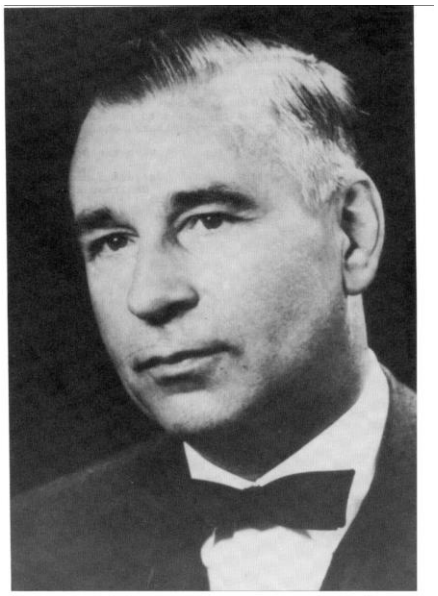
I. EXPERIMENTS ON ENERGY TRANSFER IN BIOLOGICAL SYSTEMS

For many years, special mechanisms of *energy transfer* have been discussed in relation to biological systems. It was thought necessary to assume that energy liberated at one molecule or at one distinct unit of a complex protein can be effective at another molecule or at another unit of the same protein molecule well-separated from the first one.

In order to account for such transfer, quite different mechanisms have been proposed, such as a successive shift of protons over internal hydrogen bridges (*1*) connecting different amino acids in a protein, or the free motion of electrons supposed to be common to the entire protein (*2*). These mechanisms are not considered in detail in the following, as this has been done well enough by other authors [cf. Bücher (*3*) or Vladimirov and Konev (*4*)]. Instead, discussed below are those experiments which, in my opinion, unequivocally show the existence of energy transfer in biological systems. From these experiments, and from the general properties of the systems involved, an attempt is made here to draw certain conclusions on the possible transfer mechanisms and to evaluate these mechanisms in detail.

Energy transfer has been discussed in relation to oxidative metabolism. In this instance, however, it seems from recent investigations, that the energy resides in energy-rich molecules of low molecular weight which are free to move from one enzyme to another. Energy transfer has been considered further in connection with the

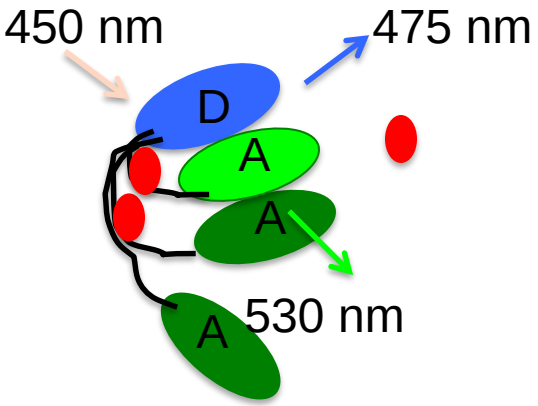


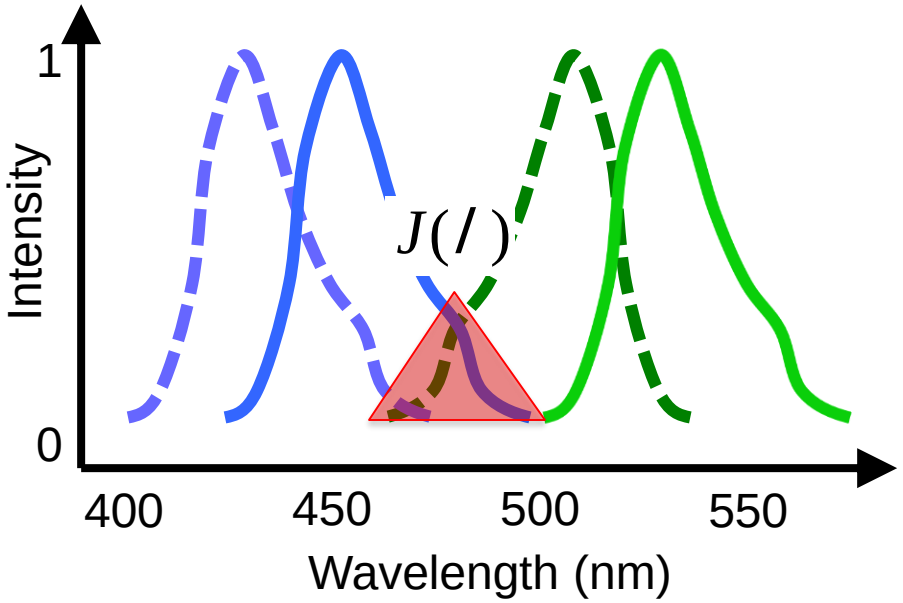
Theodor Förster
1910 - 1974

Theodor Förster

Fluorescence Resonance Energy Transfer (FRET)

- Excitation of donor fluorescent probe
- Emission of donor fluorescent probe
- Excitation of acceptor fluorescent probe
- Emission of acceptor fluorescent probe



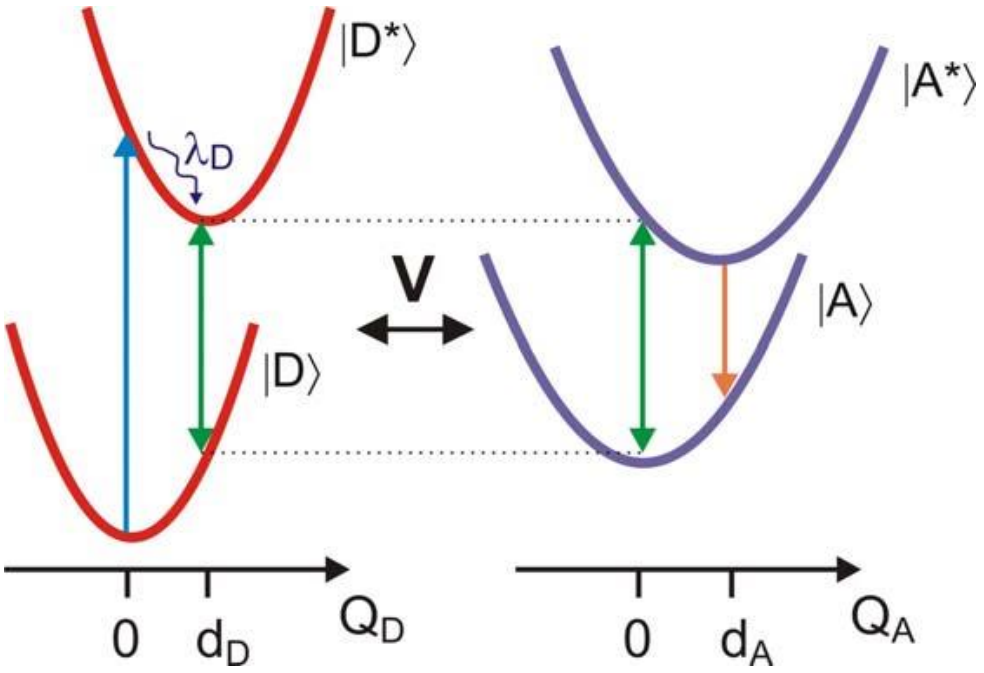


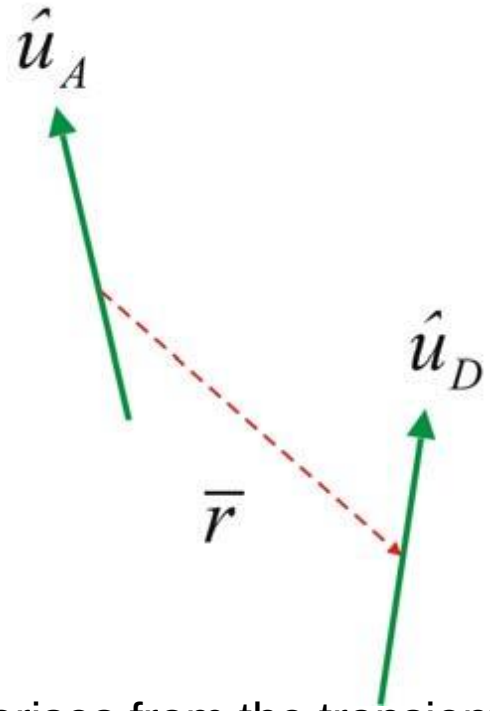
$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

$$R_0 = (\kappa^2 \cdot J(\lambda) \cdot n^{-4} \cdot Q)^{1/6}$$

$J(\lambda)$ = Degree of Spectral Overlap

Nonradiative energy transfer between two particles





FRET arises from the transient dipole interaction of donor and acceptor.

FRET is a nonradiative energy transfer from donor to acceptor.

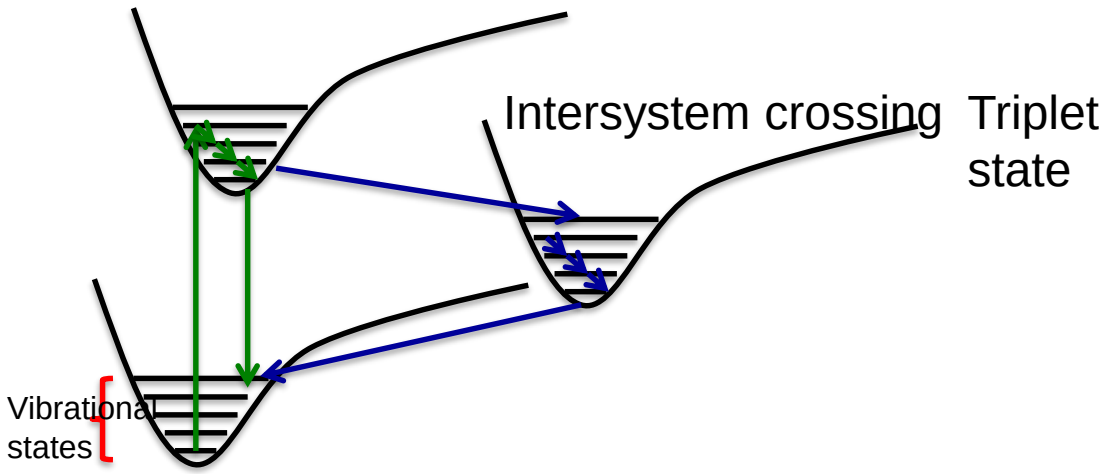
Quantum yield of fluorophore

The fluorescence quantum yield (Φ_F) is the ratio of photons absorbed to photons emitted through fluorescence.

$$F = \frac{numberofphotonsemittted}{numberofphotonsabsorbed}$$

$$F = \frac{k_f}{k_f + k_{nr} + k_{isc}}$$

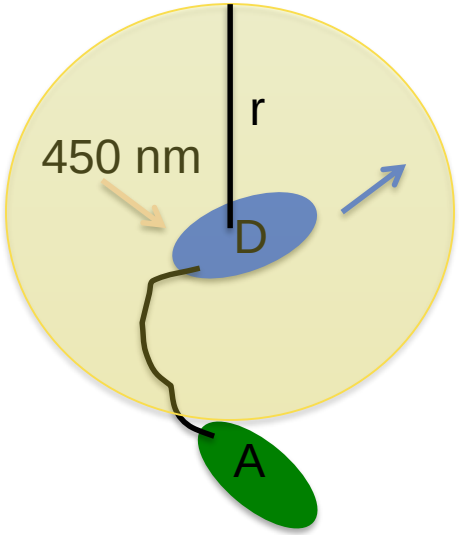
k_f = rate of fluorescence
k_i = rate of nonradiative decay
k_{isc} = rate of intersystem crossing



Distance and spectral overlap effect FRET

The distance which can be probed by FRET is limited typically 1-10nm.

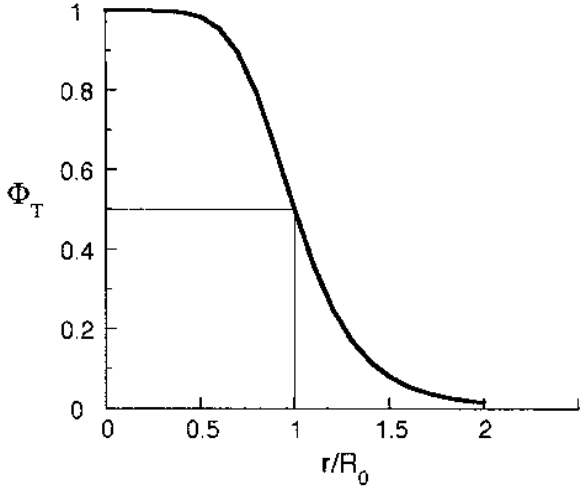
$$E = \frac{1}{1 + \left(\frac{r}{R_0} \right)^6}$$



Due to the $1/R^6$ distance dependence, distances in the range 0.5-1.5 R_0
Efficieny in the range 0.98-0.10, are suitable for FRET measurements.

Distance effect between donor and acceptor

Transfer efficiency is 50% when the donor-acceptor distance is equal to the Förster critical radius.



$$r = \left(\frac{1}{\Phi_T} - 1 \right)^{1/6} R_0$$

Tab. 8.4. Examples of Förster critical radii[a)]

Donor	*Acceptor*	*Ro* (nm)[b)]
Naphthalene	Dansyl	2.2
Anthracene	Perylene	3.1
Pyrene	Perylene	3.6
Phenanthrene	Rhodamine B	4.7
Fluorescein	Tetramethylrhodamine	5.5
Fluorescein-5-isothiocyanate	Eosin maleimide	6.0
Rhodamine 6G	Malachite Green	6.1
Europium (III) complex	Cy5 (carboxymethylindocyanine-N-hydroxysuccinimidyl ester)	7.6
Europium (III) complex	CdSe/ZnS nanocrystals (quantum dots)	8.4 – 9.6
Terbium (III) complex	CdSe/ZnS nanocrystals (quantum dots)	8.5 – 9.8
Tryptophan	Dansyl	2.1
Tryptophan	ANS	2.3
Tryptophan	Anthroyl	2.5
Tryptophan	Pyrene	2.8
Pyrene	Pyrene	1.0
2-Ethylnaphthoate	2-Ethylnaphthoate	1.4
Anthracene	Anthracene	2.2
Perylene	Perylene	3.8
Rhodamine 101	Rhodamine 101	5.8

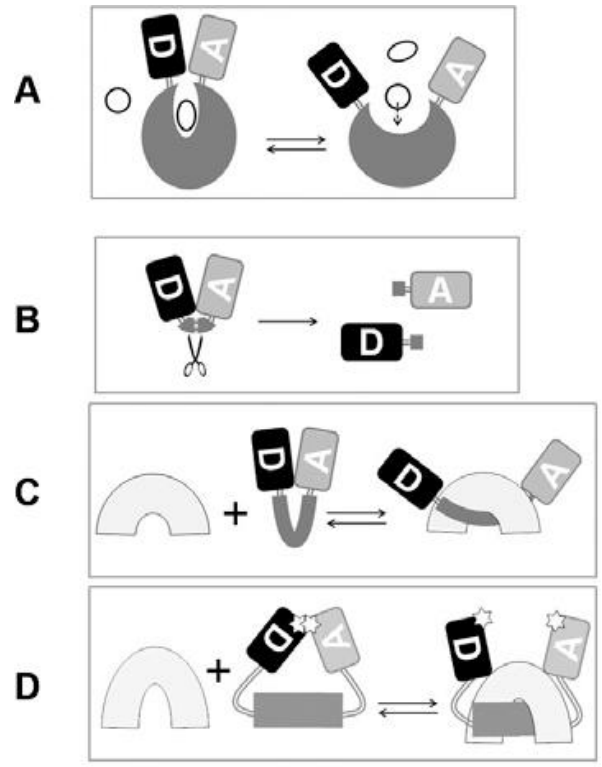
FRET pair examples

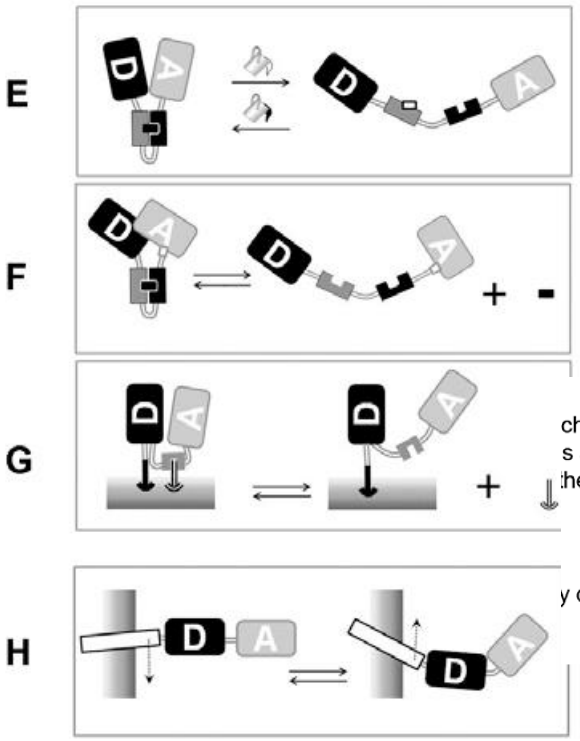
Laser	Donor	Acceptor	Donor Ex Acceptor Em
Violet	Alexa Fluor 405	Alexa Fluor 430	405/541
Argon	Cy2	Cy3	488/566
Argon	Cy3	Cy5	488/666
Argon	FITC	TRITC	488/577
Argon	PE	APC	488/660
Argon	Alexa Fluor 488	Alexa Fluor 514	488/541
Argon	Alexa Fluor 488	Alexa Fluor 532	488/553
Argon	Alexa Fluor 488	Alexa Fluor 546	488/572
Argon	Alexa Fluor 488	Alexa Fluor 610	488/626
R-HeNe	Alexa Fluor647	Alexa Fluor 680	633/700
R-HeNe	Alexa Fluor647	Alexa Fluor 700	633/720
R-HeNe	Alexa Fluor647	Alexa Fluor 750	633/780

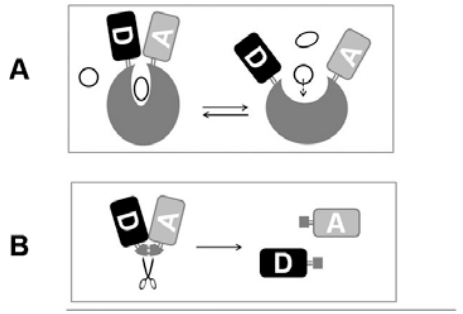
FRET Pair Fluorescent Proteins

Laser	Donor	Acceptor	Donor Ex Acceptor Em
Violet	CFP	YFP	405/526
Violet	Cerulean FP	YFP	405/526
Argon	GFP	YFP	488/526
Argon	GFP	mRFP	488/579

FRET assays







Structural cl
interactions
termini of th

B. Sensory

A. Full-length protein (enzyme) as a sensory domain

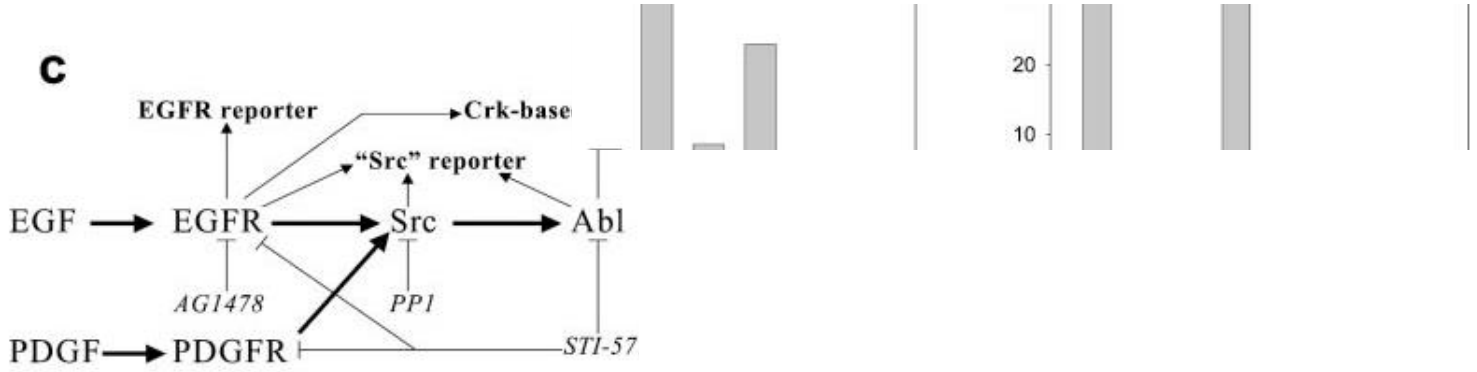
changes of a full-length protein which are concomitant with its function a
s are signaled by changes of FRET between D and A attached at the op
he protein [45].

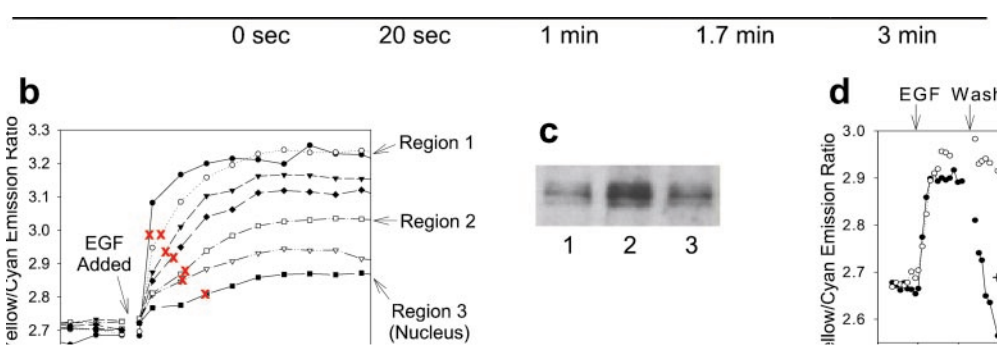
B-D: Single sensory domain

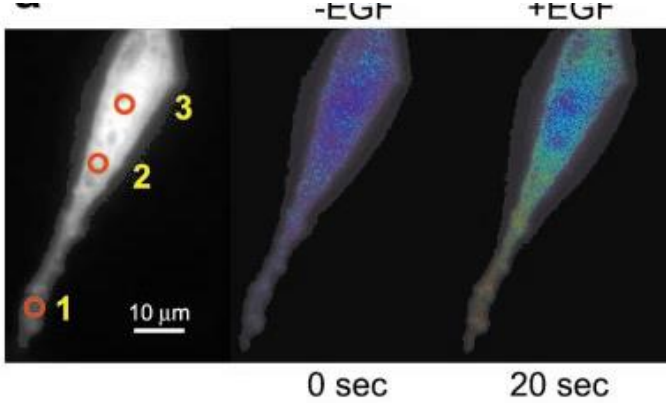
y domain with a protease cleavage site [47,48].

Soderholm et all. Methods, 2010, 220

FRET Assay to monitor kinase activity in cells

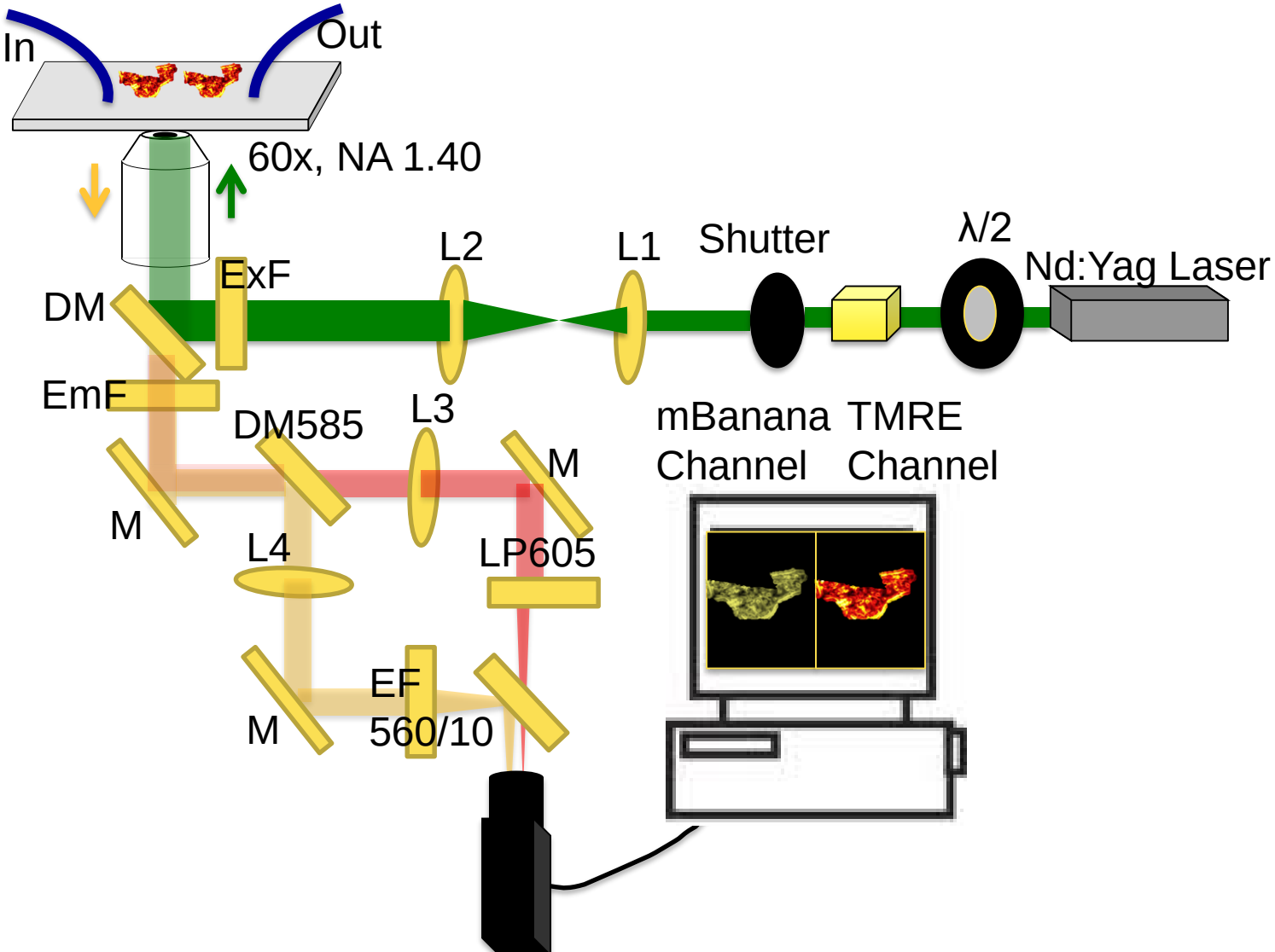






Alice Ting et. al. PNAS, 2001, 98, 15003

Dual color imaging for FRET

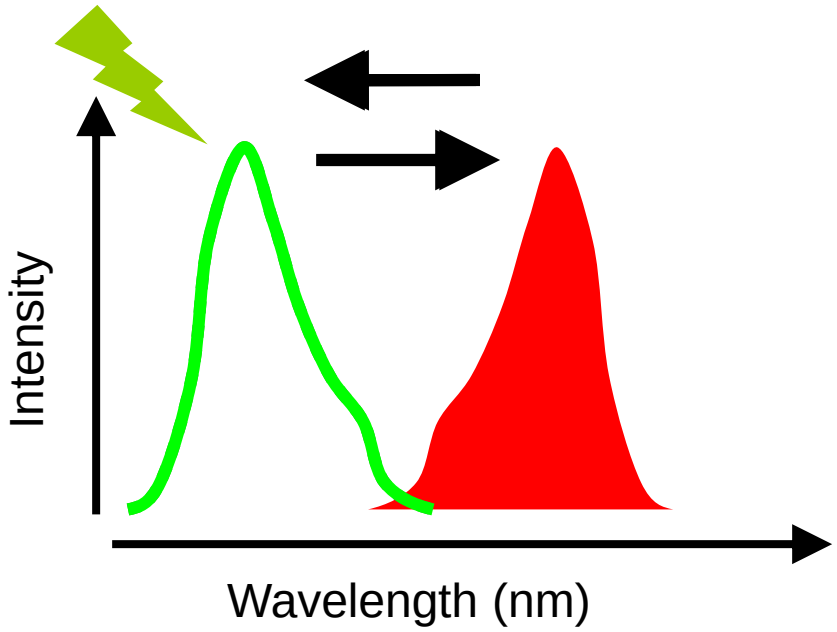


Theory of Photochromic FRET for sensing protein dynamics

Ground state absorption of membrane protein

Intermediate state absorption of membrane protein

Emission of fluorescent probe (donor)

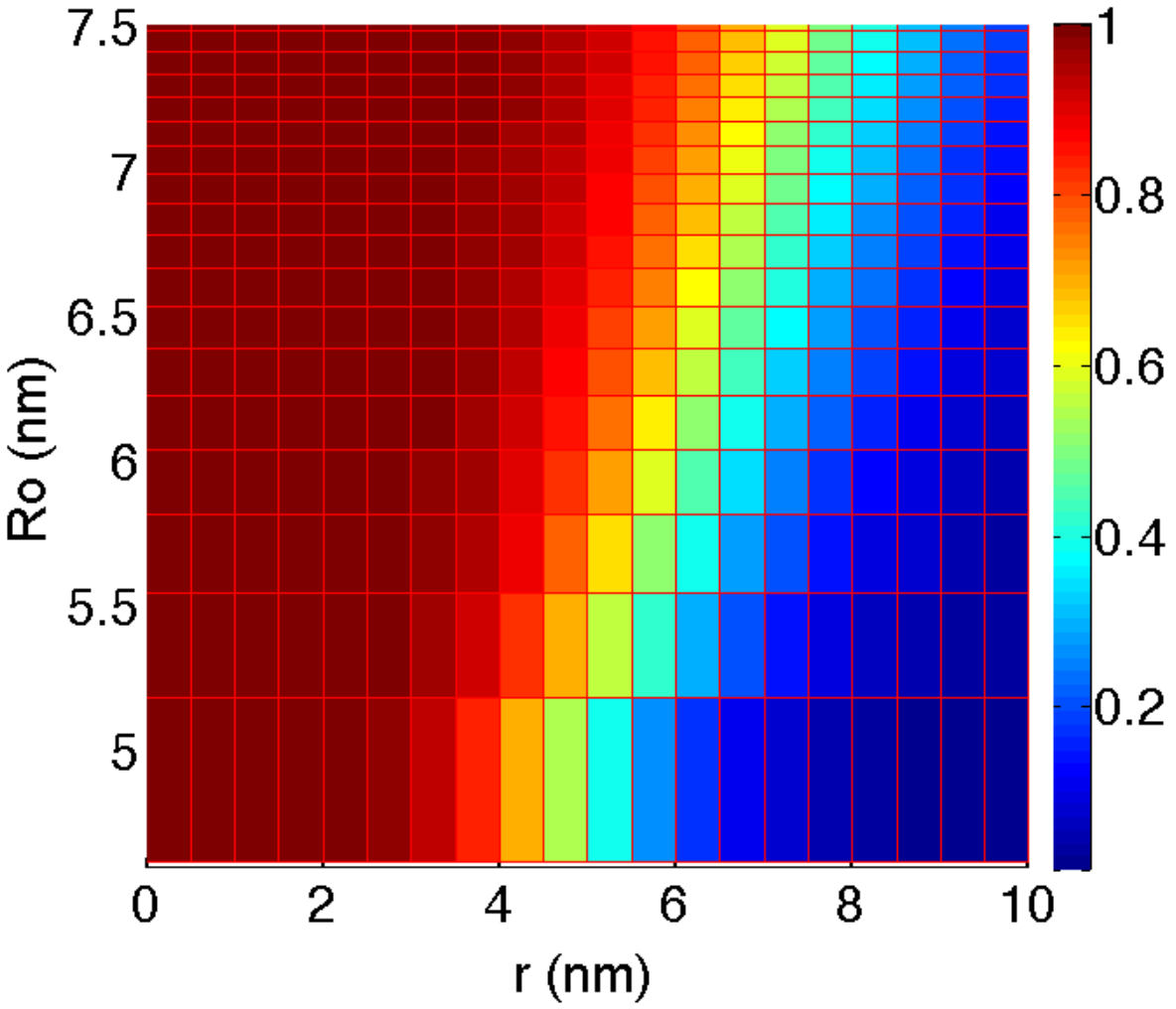


$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

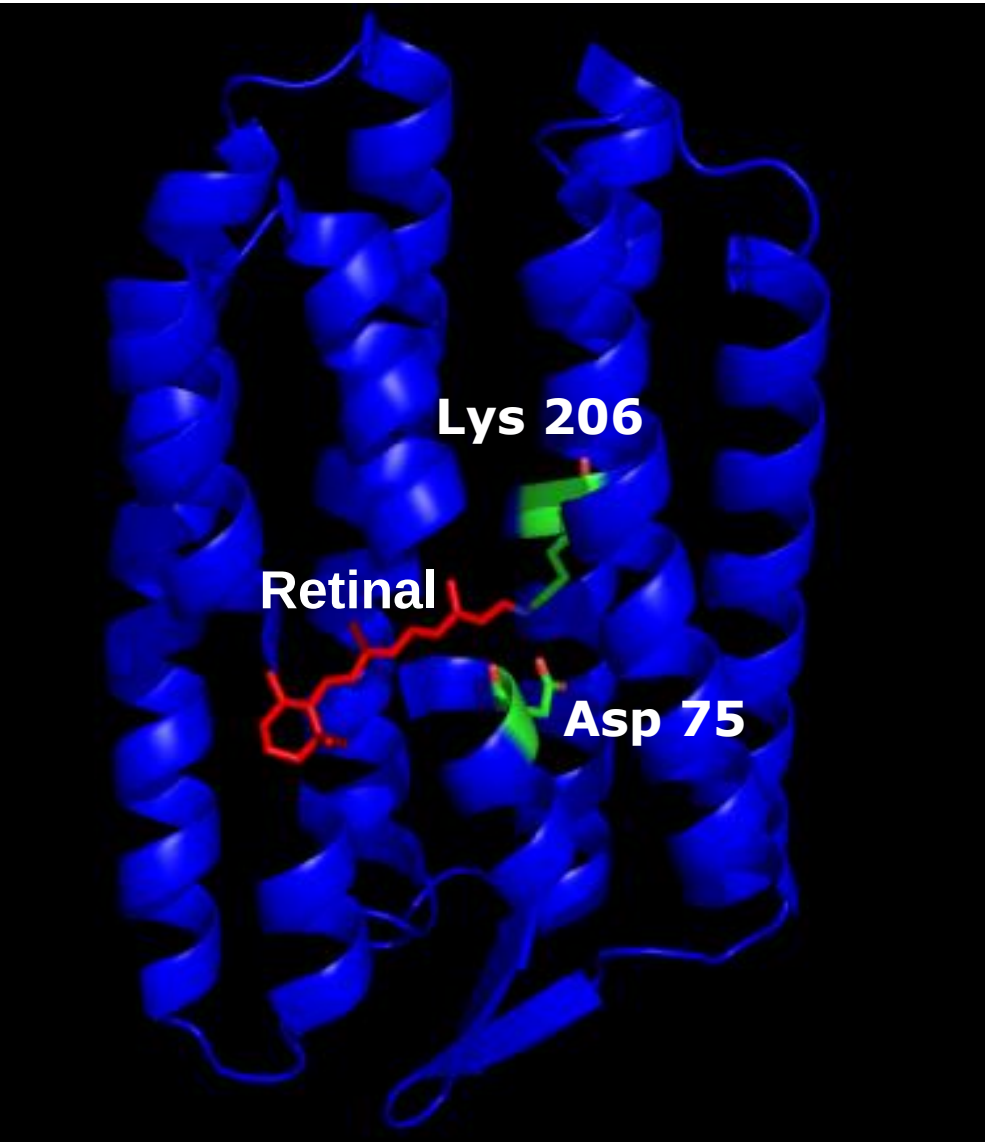
$$R_0 = (\kappa^2 \cdot J(\lambda) \cdot n^{-4} \cdot Q)^{1/6}$$

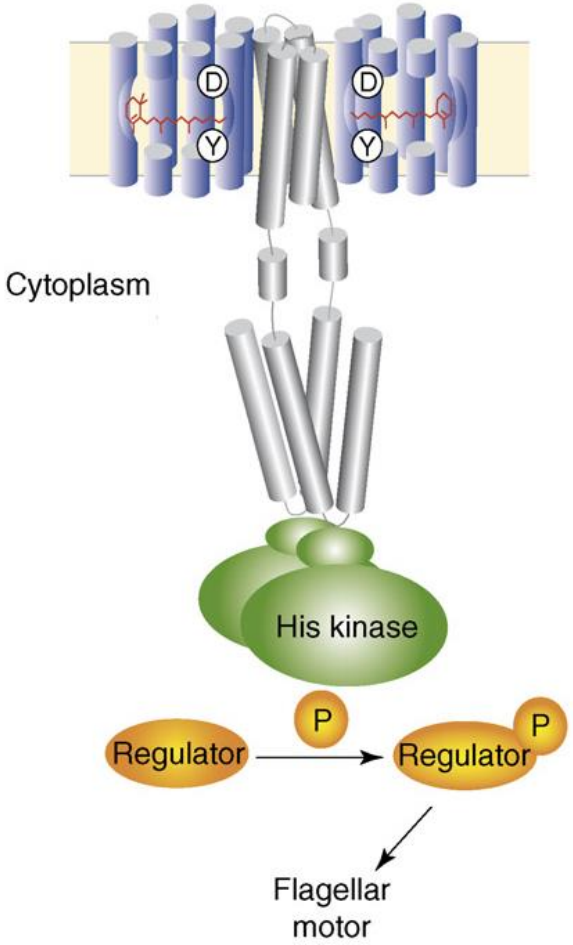
$J(\lambda) =$ Degree of Spectral Overlap

pcFRET efficiency



Crystal structure of sensory rhodopsin II





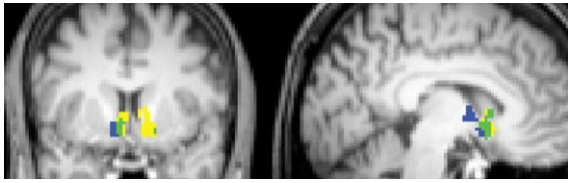
Royant et. al., PNAS, 2001, 98, 10131

Studying photosensitive membrane proteins

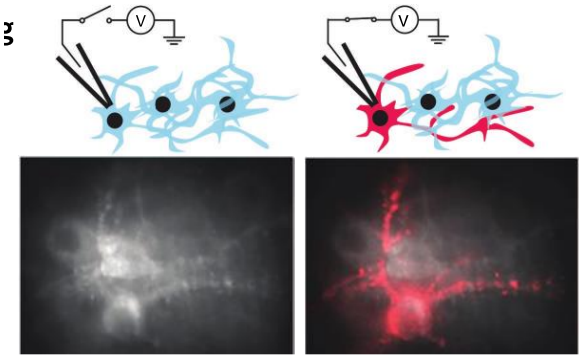
- Dynamic studies of photoactive membrane proteins are currently limited by absorption.
- Can we measure the photocyle of membrane protein in the cell? What does protein work in the cell membrane?
- How does the photocycle of a single membrane protein differ from the bulk?
- How different is the photocyle among single membrane proteins?

Optogenetics

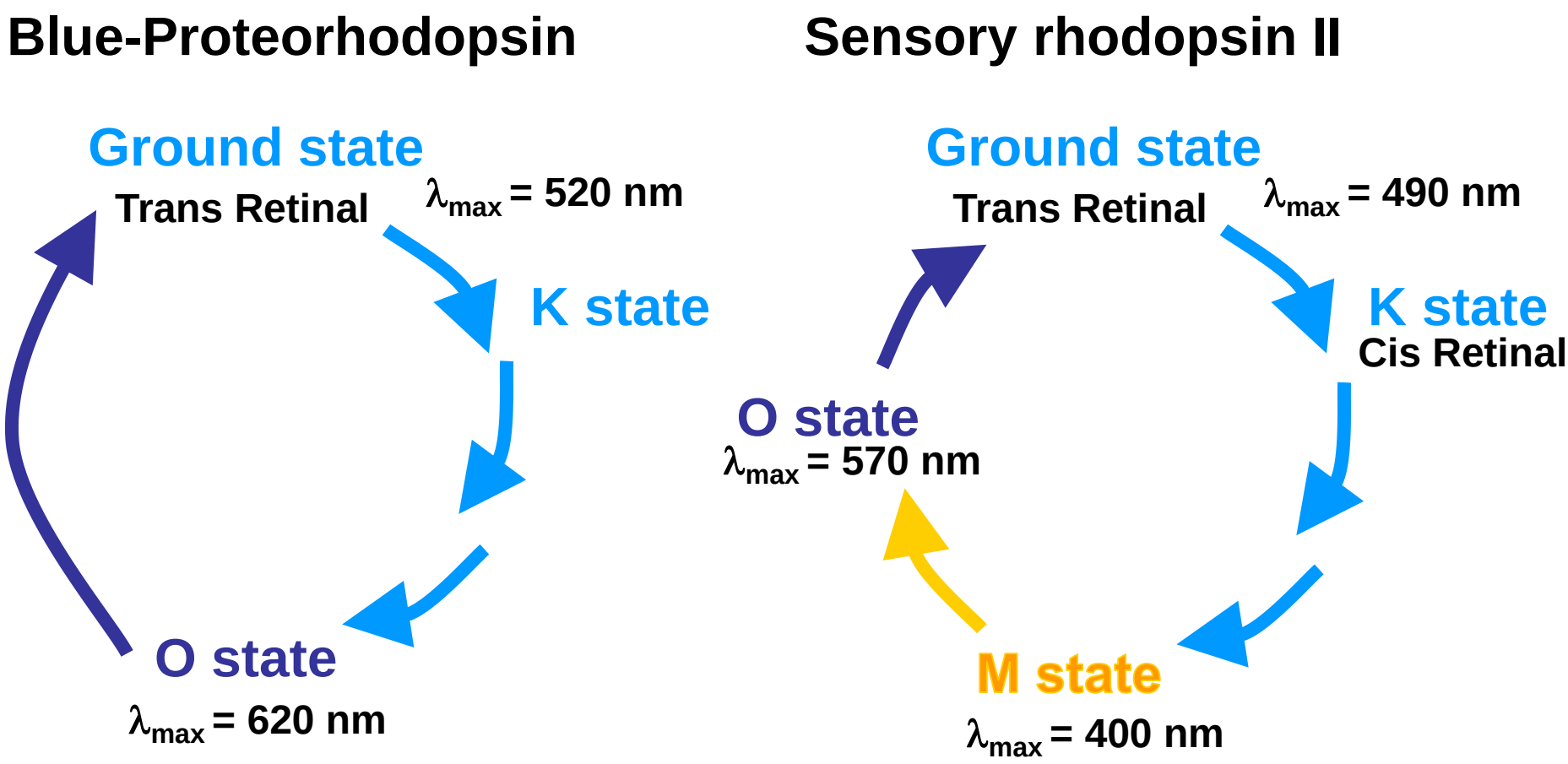
- Optical control of neuronal activity



- Optical probes for membrane environment

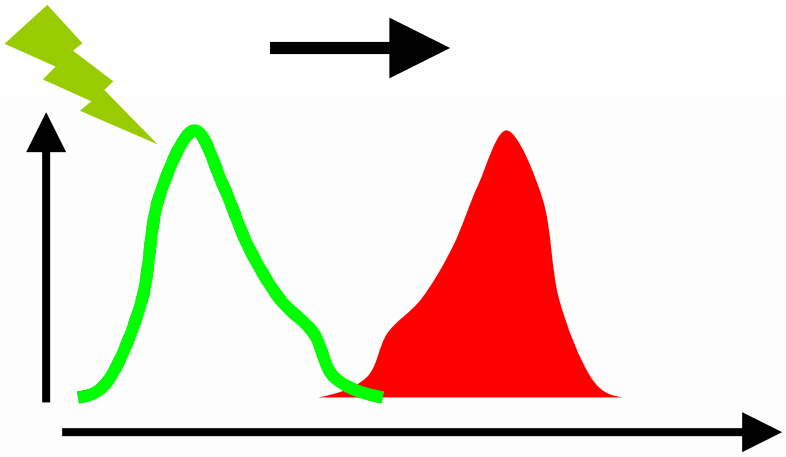


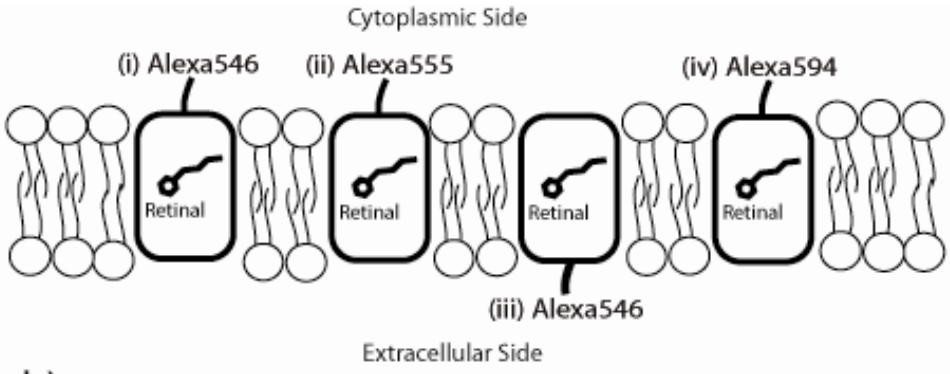
Photocycle of photoactive membrane proteins

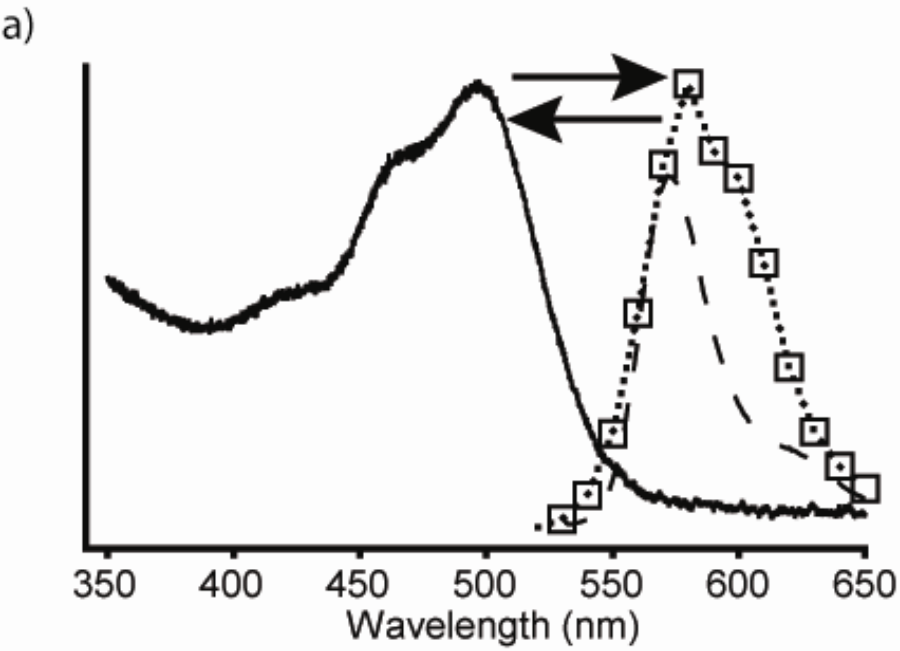


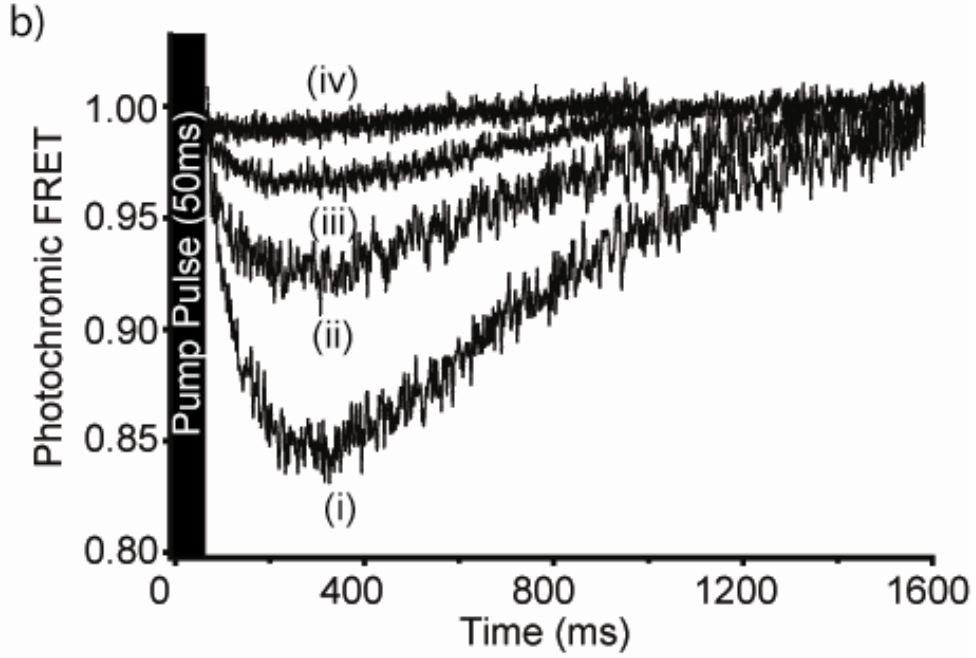
Spudich et. al.Trends Microbiol. 14: 480-487.

Photochromic FRET for SRII



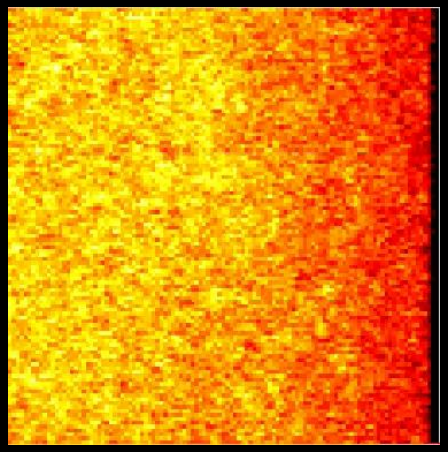




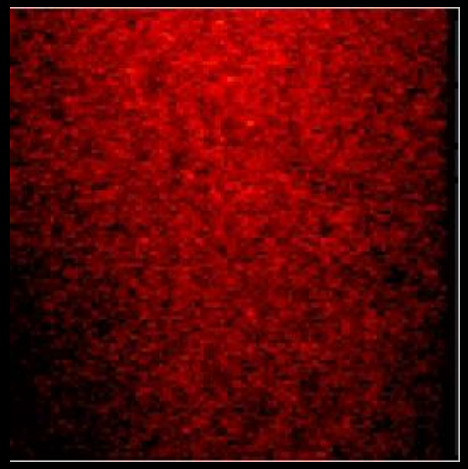


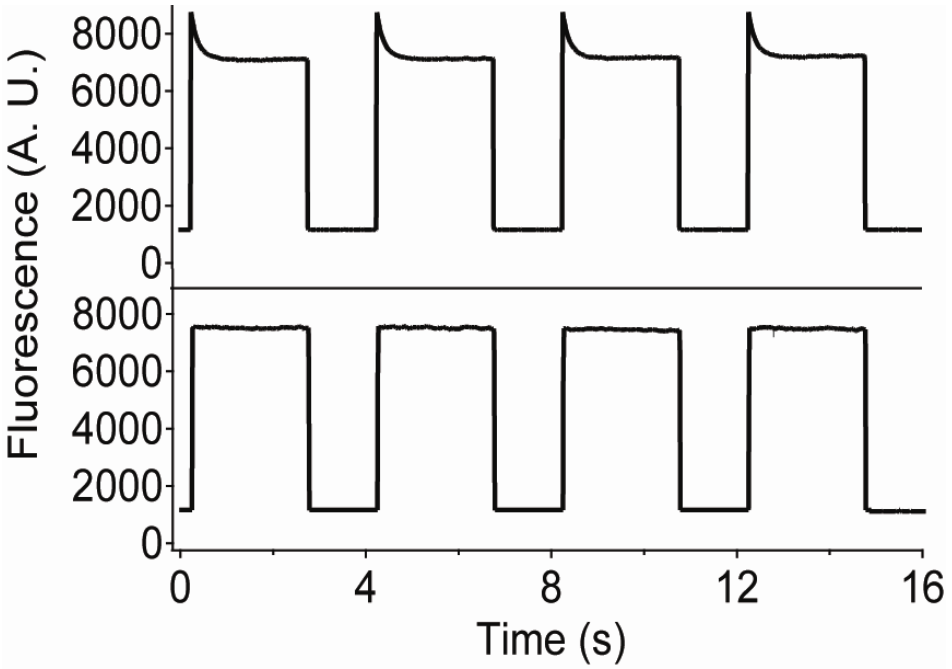
O Intermediate state formation in SRII photocycle

SRIIAlexa546 (+Retinal)

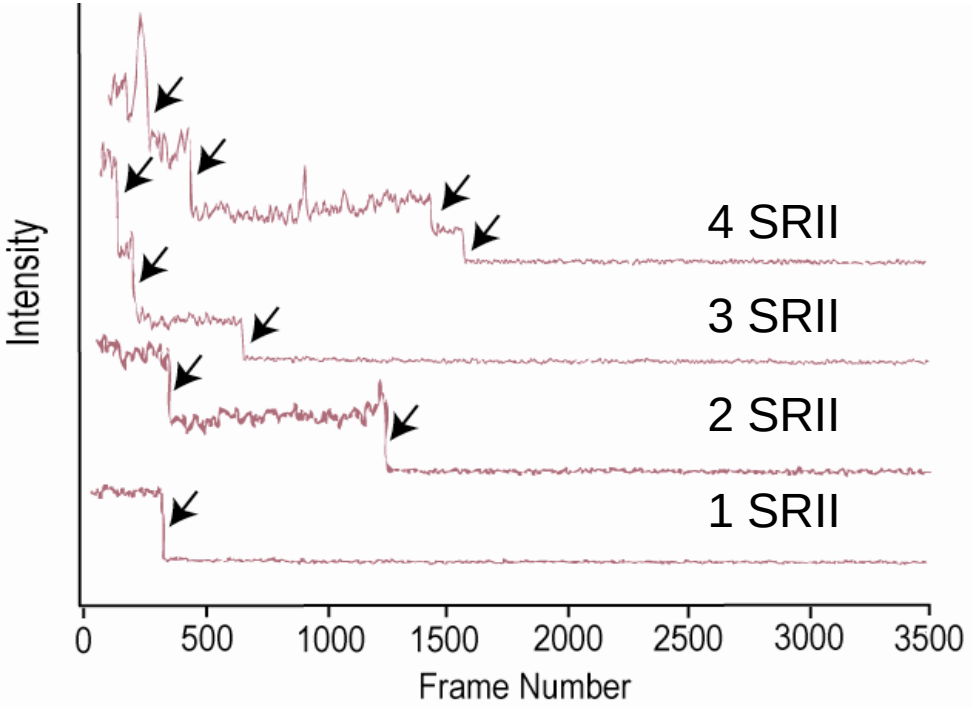


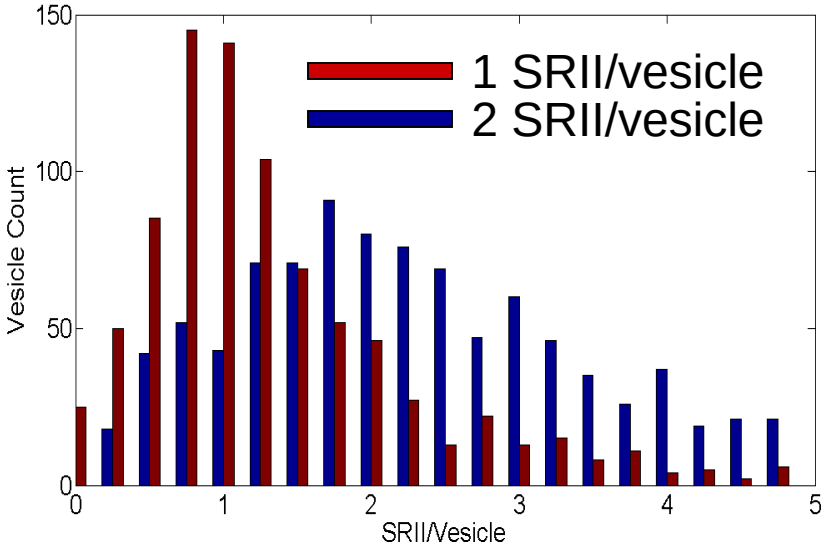
Control = SRIIAlexa546 (-Retinal)





Distribution of number of SRII in vesicles



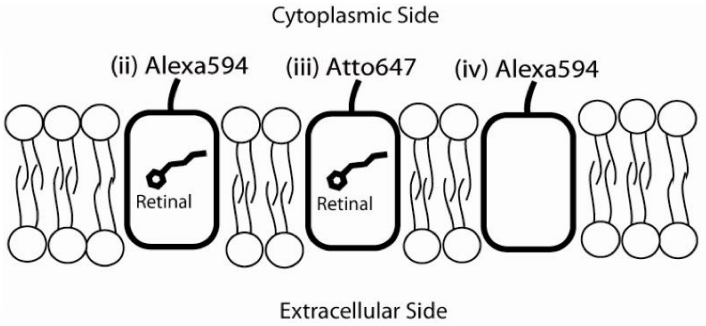


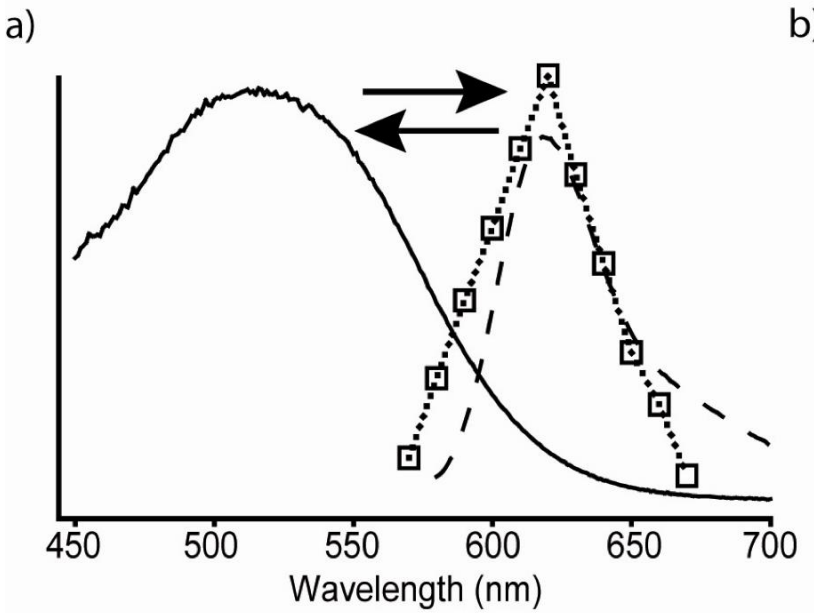
Photochromic FRET for Blue-Proteorhodopsin

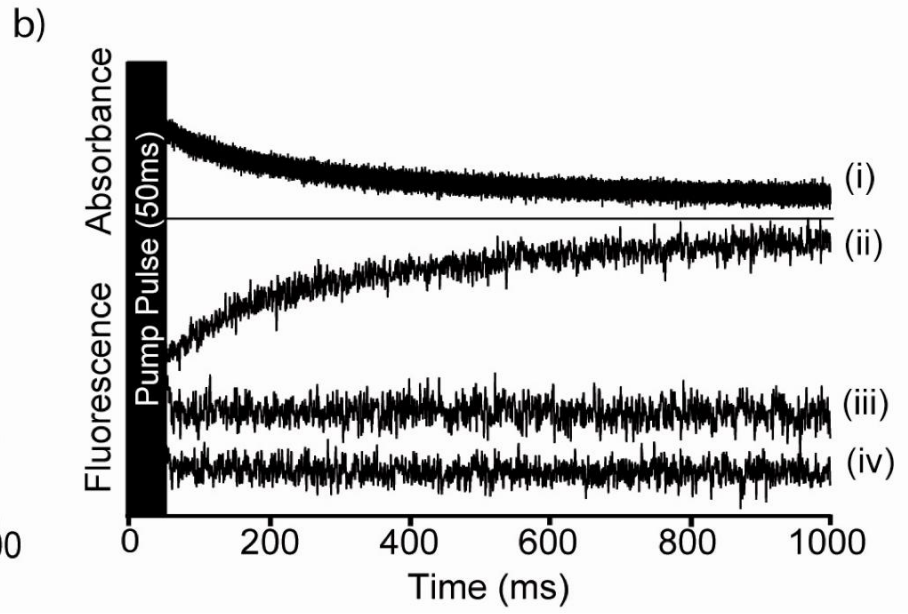
——Ground state absorption

. . . . . Intermediate state absorption

- - - - - Emission of Alexa 594

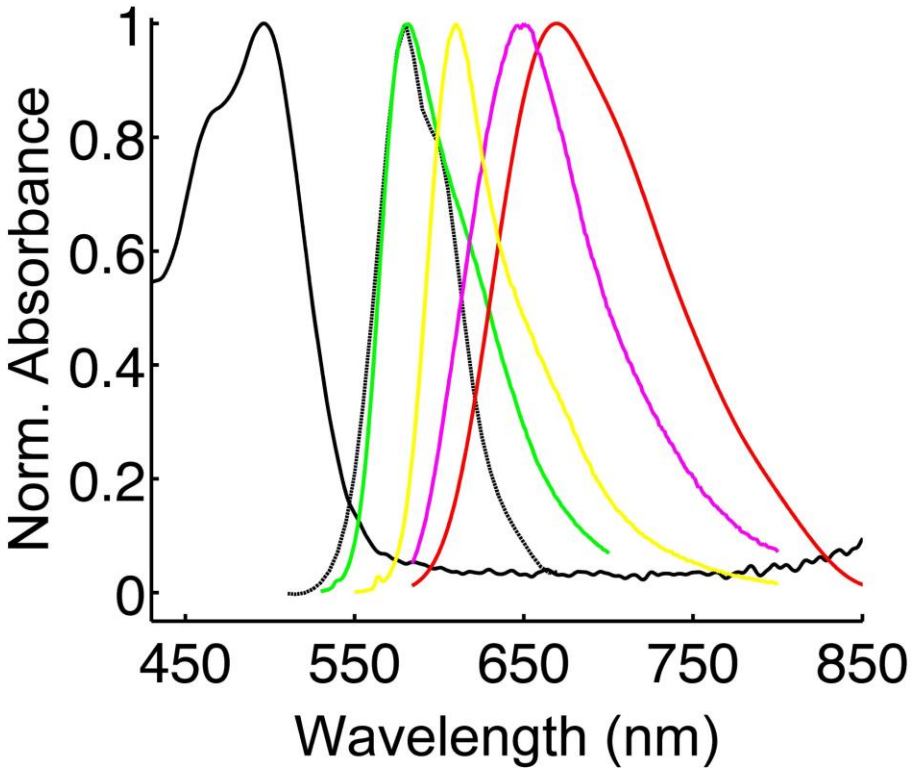




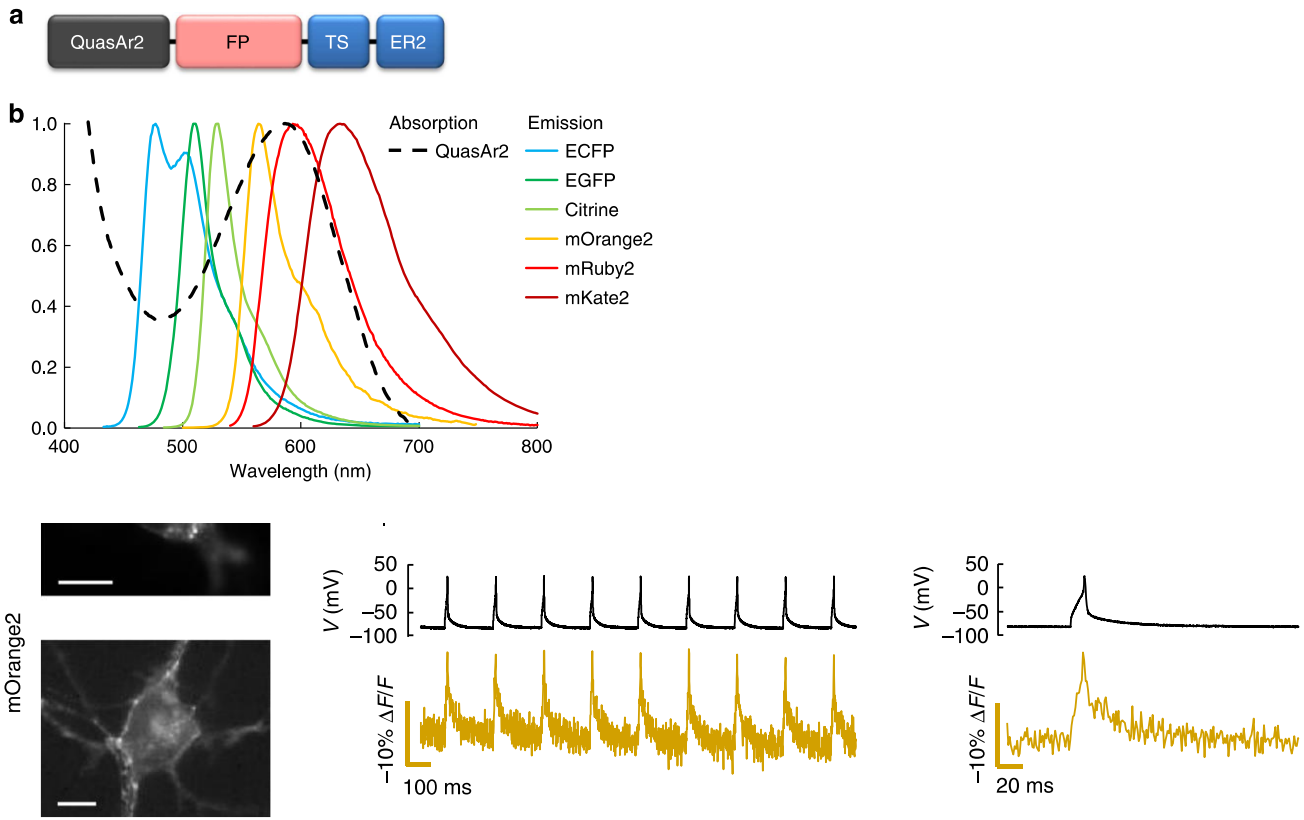


Photo&Photoboiology 2012, 88, 90

(A)


Norm. Absorbance
1
0.8
0.6
0.4
0.2
0
450
550
650
750
850
Wavelength (nm)

Measuring Action Potential with FRET

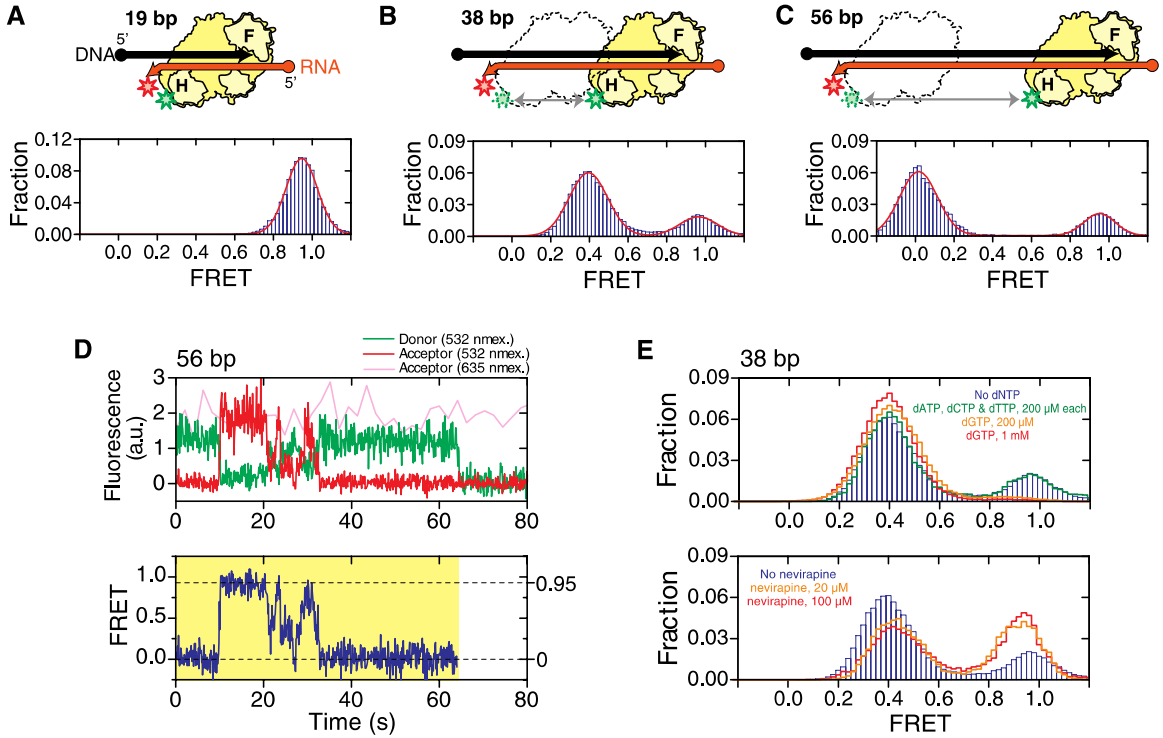


Nature Communication 2014

Measuring molecular mechanics by using FRET

Slide into Action: Dynamic Shuttling of HIV Reverse Transcriptase on Nucleic Acid Substrates

Shixin Liu,[1] Elio A. Abbondanzieri,[1] Jason W. Rausch,[4] Stuart F. J. Le Grice,[4] Xiaowei Zhuang[1,2,3]*

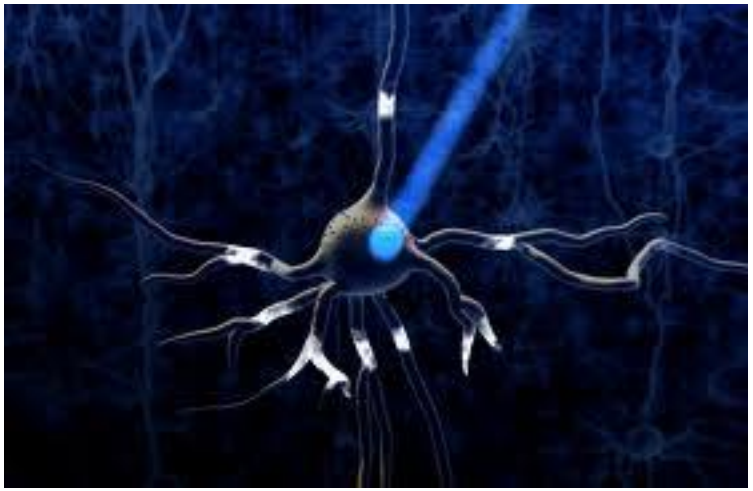


What tools do we need to advance biology in the future?

Computational Tools

1. Advance programs

Biological Tools



1. New biological fluorescent reporters
2. Optigenetic switch
3. Optical and imaging methods

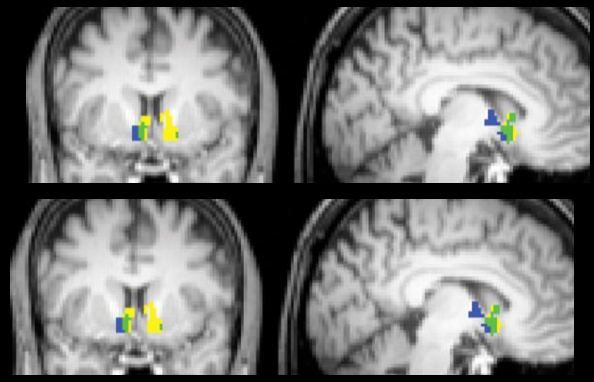
Biological tool (optogenetics) to understand brain

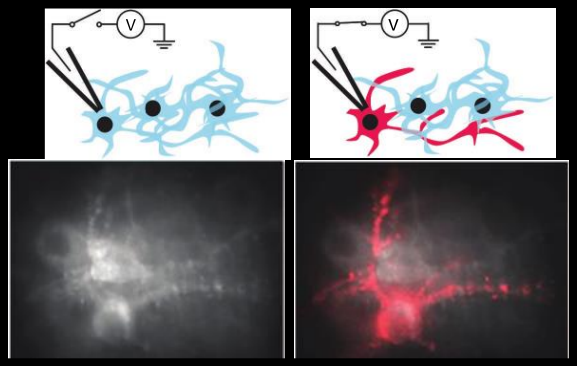
- Optical control of neuronal activity

Prof. Karl Diesseroth-Stanford
Prof. Ed Boyden-MIT

- Optical probes for membrane environment

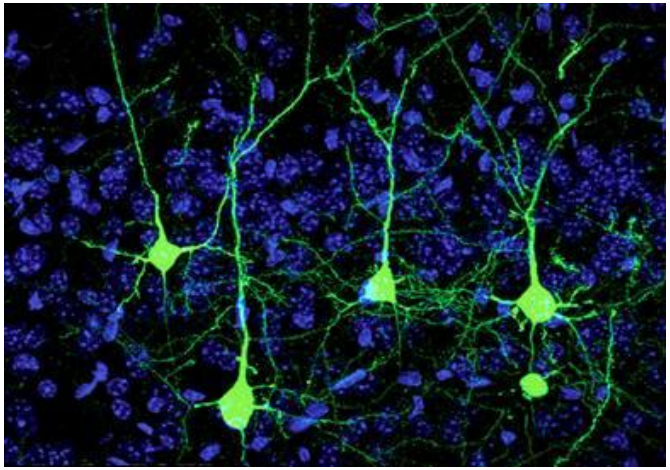
Prof. Adam Cohen-Harvard





Kralj et al. Nature Methods 2012, 9, 95

New optical tools and probes are needed to increase spatiotemporal resolution.

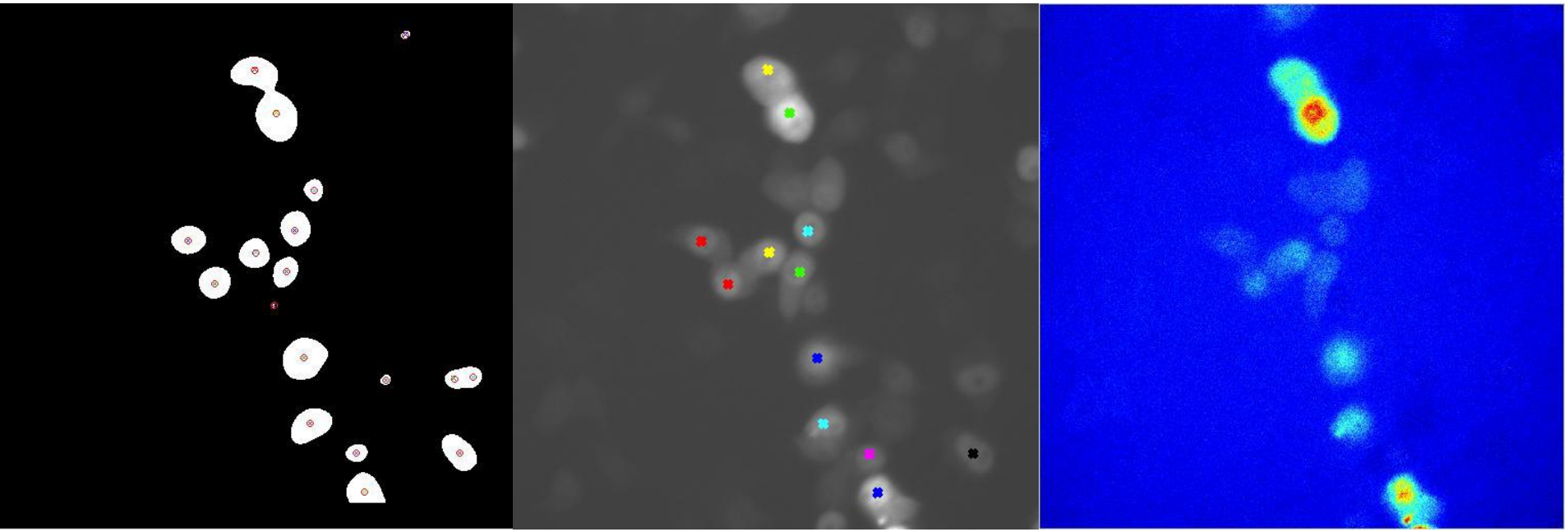


Static Imaging of cell function
Low spatiotemporal resolution

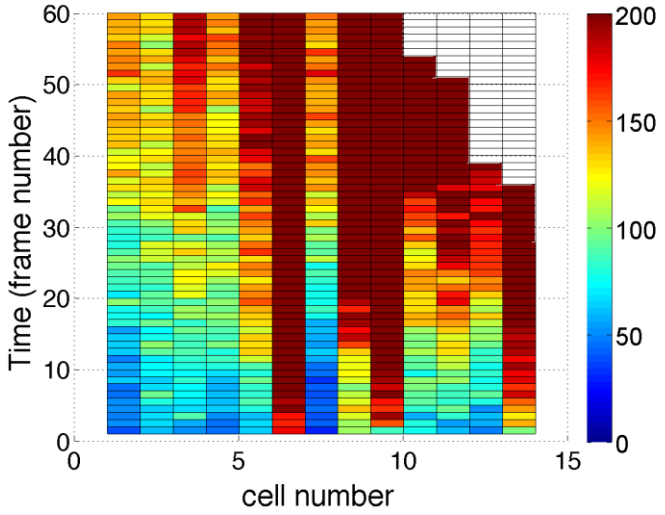
→

Dynamic Imaging of cellular function
High spatiotemporal resolution

Optogenetic tools to understand Cancer



We need advance computational tools and biological probes to solve problems in cancer

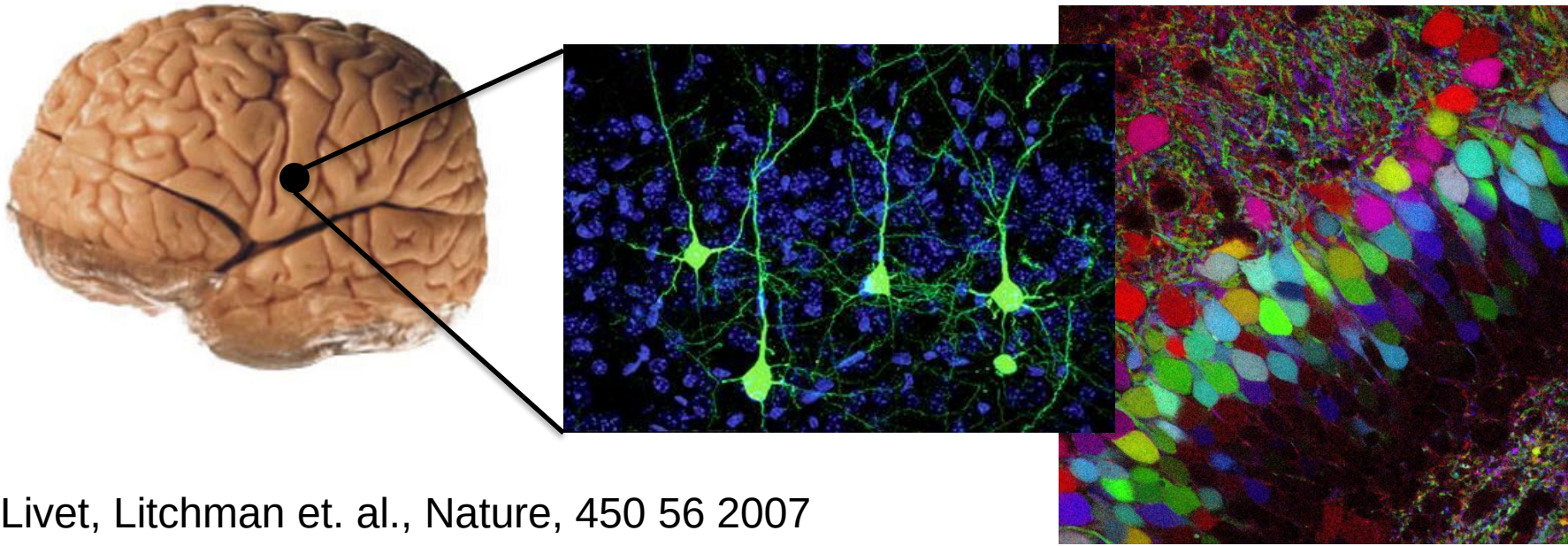


Data and Video Courtesy - Z. Kaya, N. Lack, H. Bayraktar

Advance Problems:

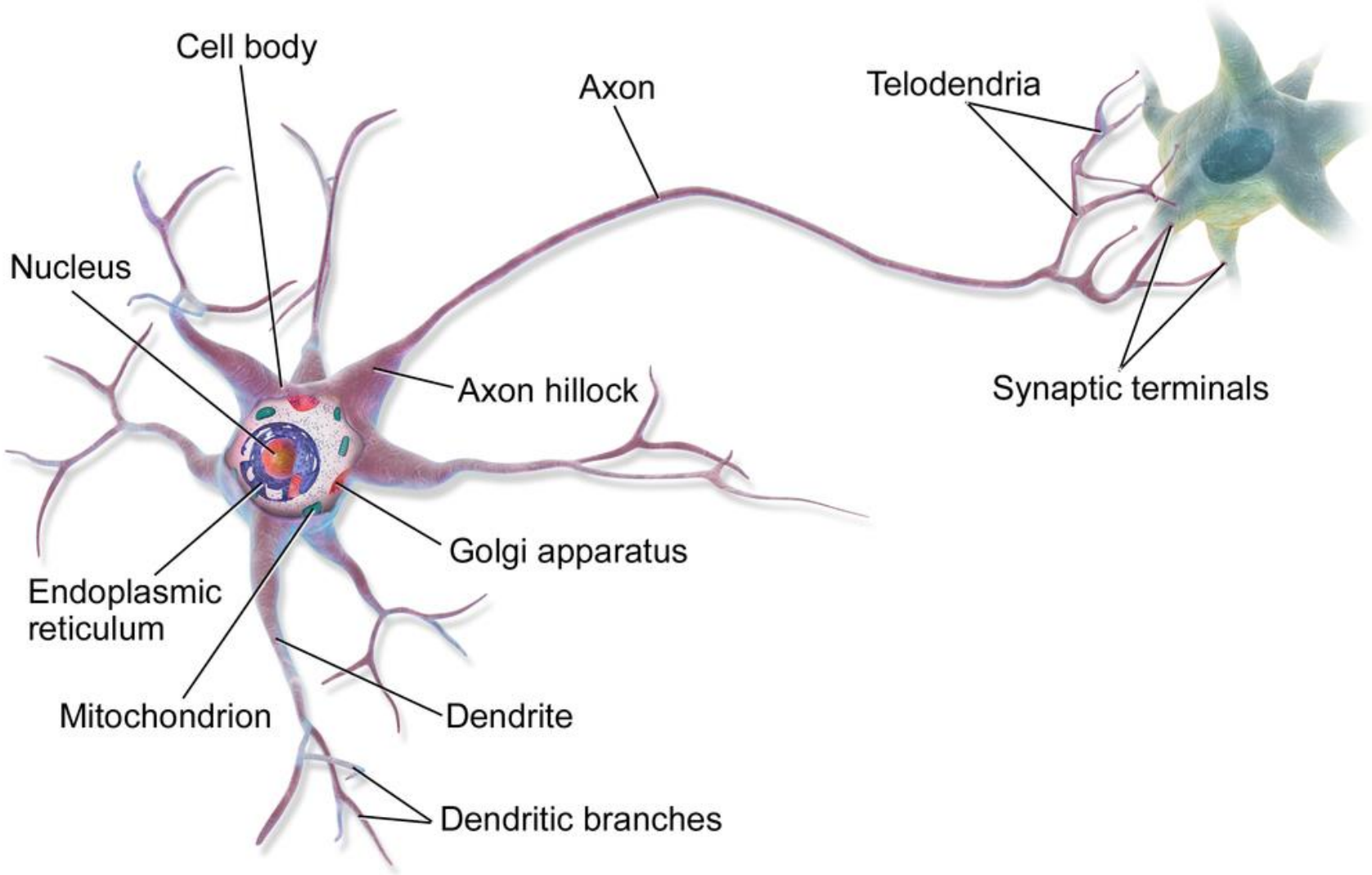
Brain is a very complex organ

- Millions of neurons forms a highly complex architecture.
- Neurons are specialized to control certain function.
- A neuron subset are localized with other subsets so distinguish them are very difficult.

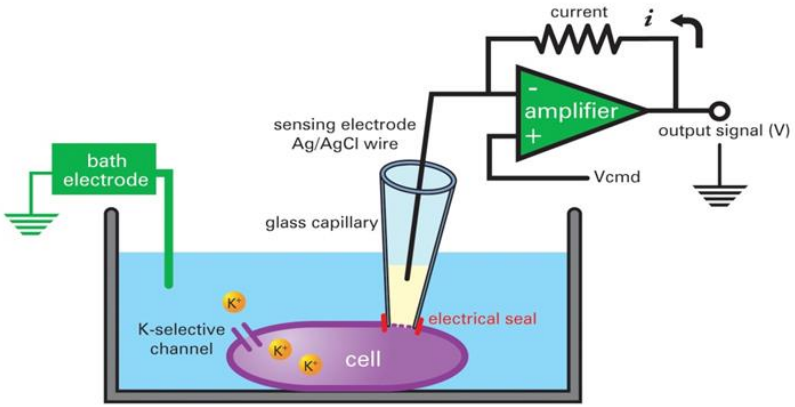


Livet, Litchman et. al., Nature, 450 56 2007

Neuron

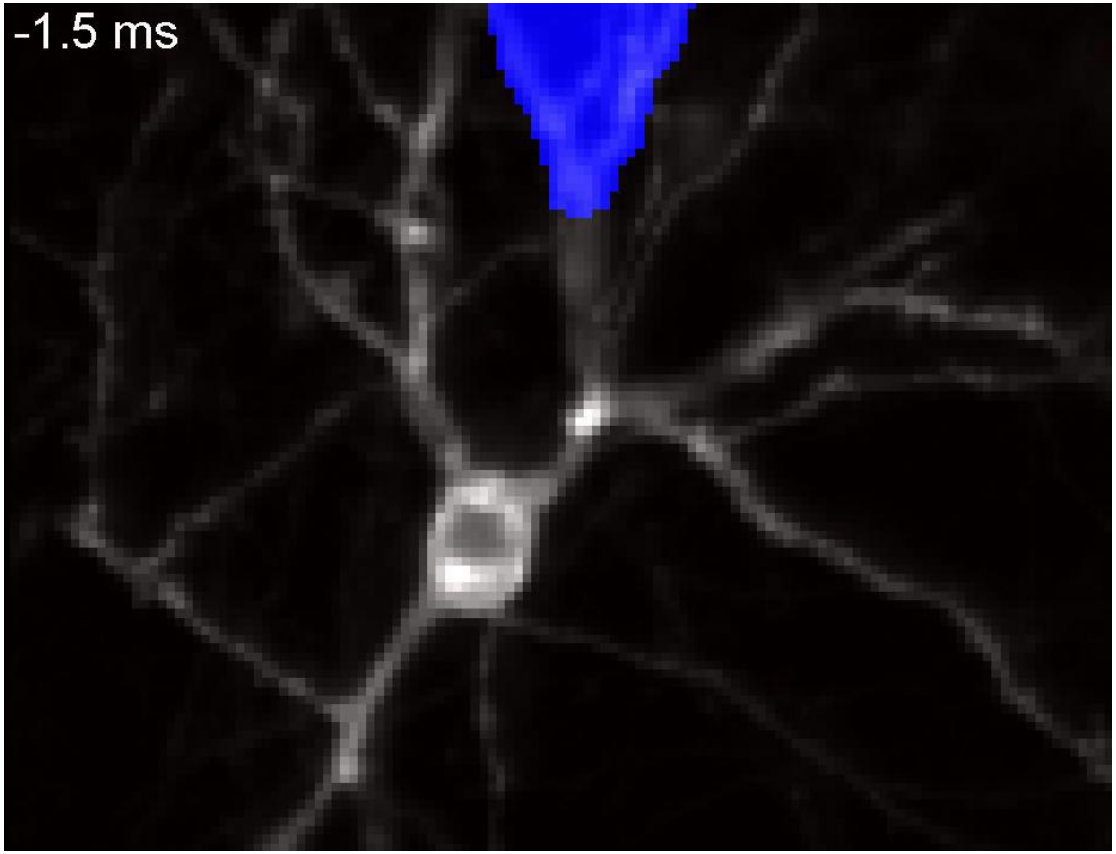

Cell body
Axon
Telodendria
Nucleus
Axon hillock
Synaptic terminals
Golgi apparatus
Endoplasmic reticulum
Mitochondrion
Dendrite
Dendritic branches

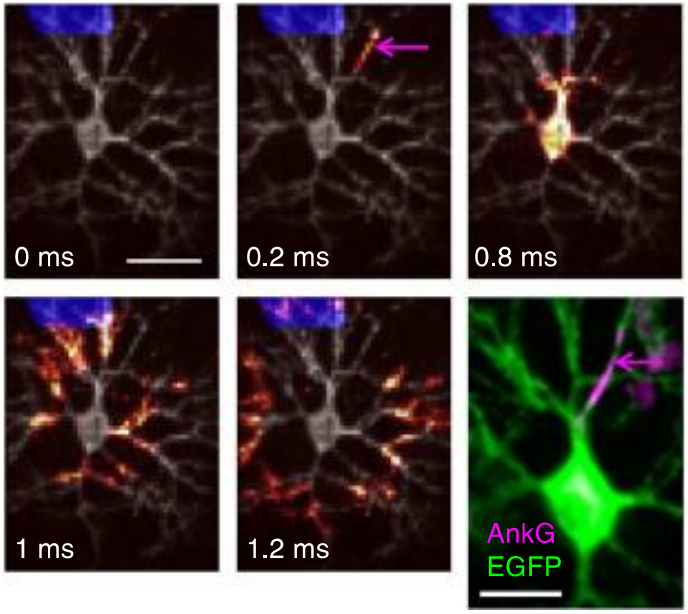
Conventional Methods to measure membrane potential



80 years old conventional method

All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins

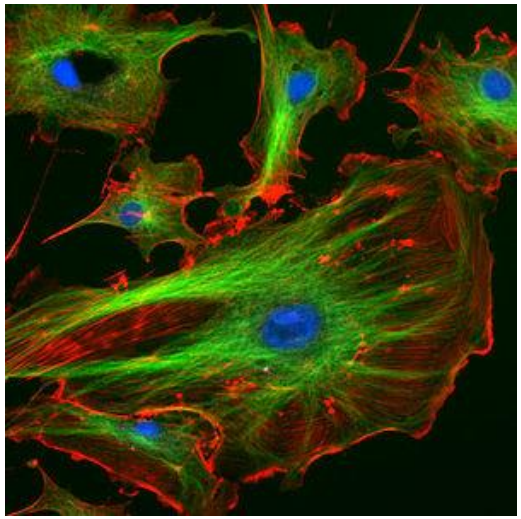




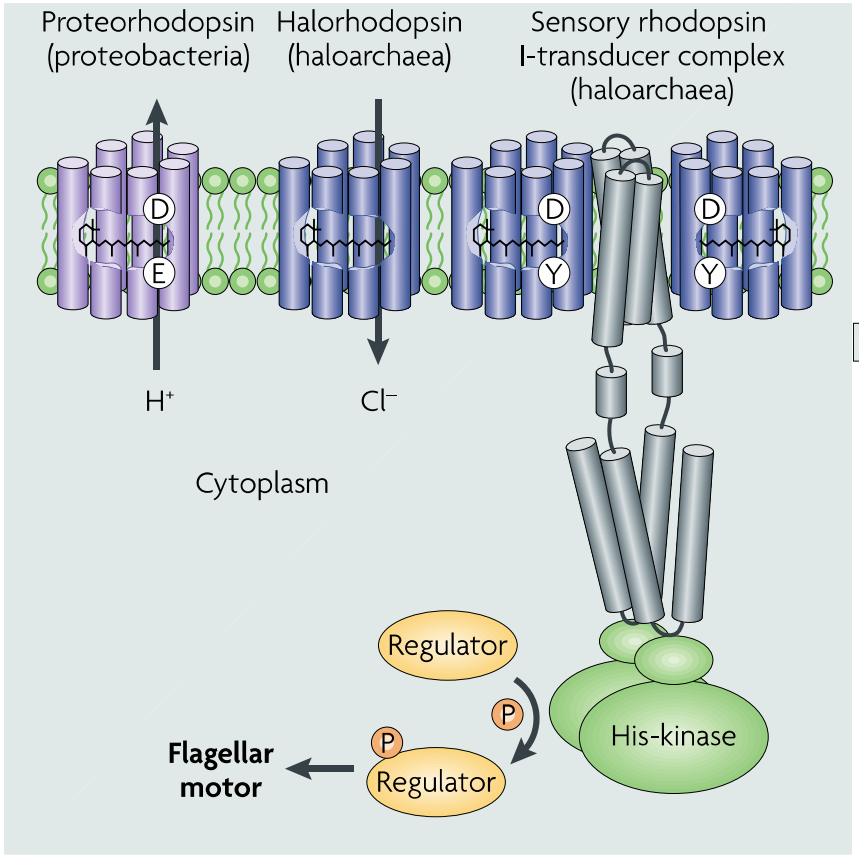
Video Courtesy Adam Cohen Harvard University
Nature Methods , 2014

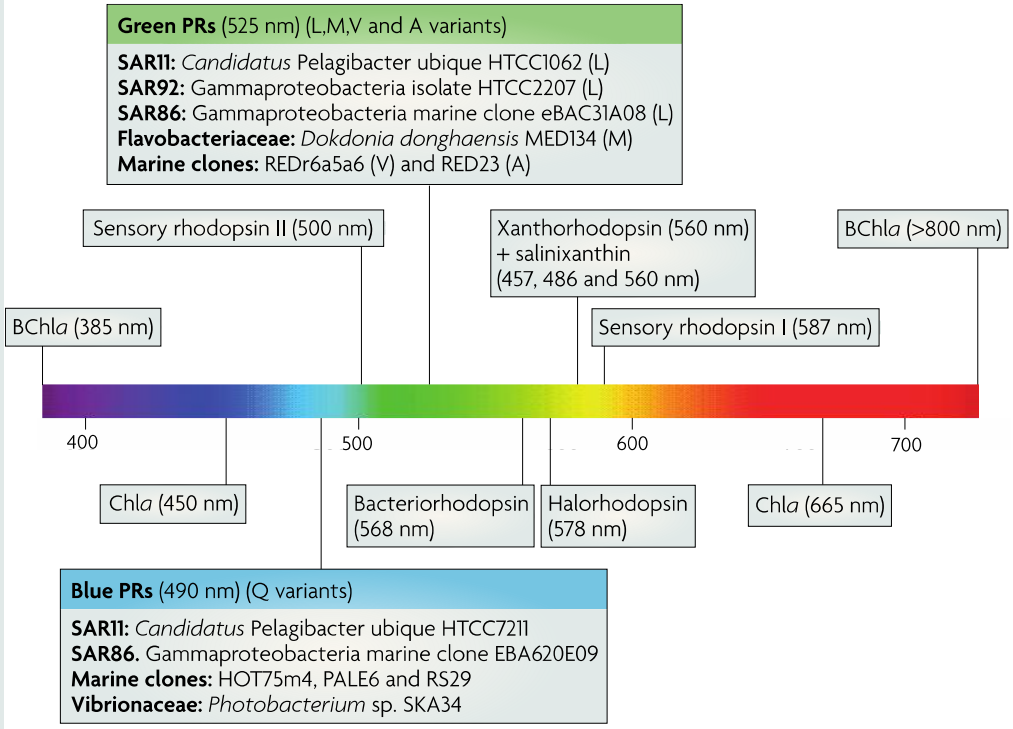
Optic + genetic = Optogenetic

Optogenetic is the combination of genetic and optical methods to control and to monitor specific events in targeted cells of living tissue.



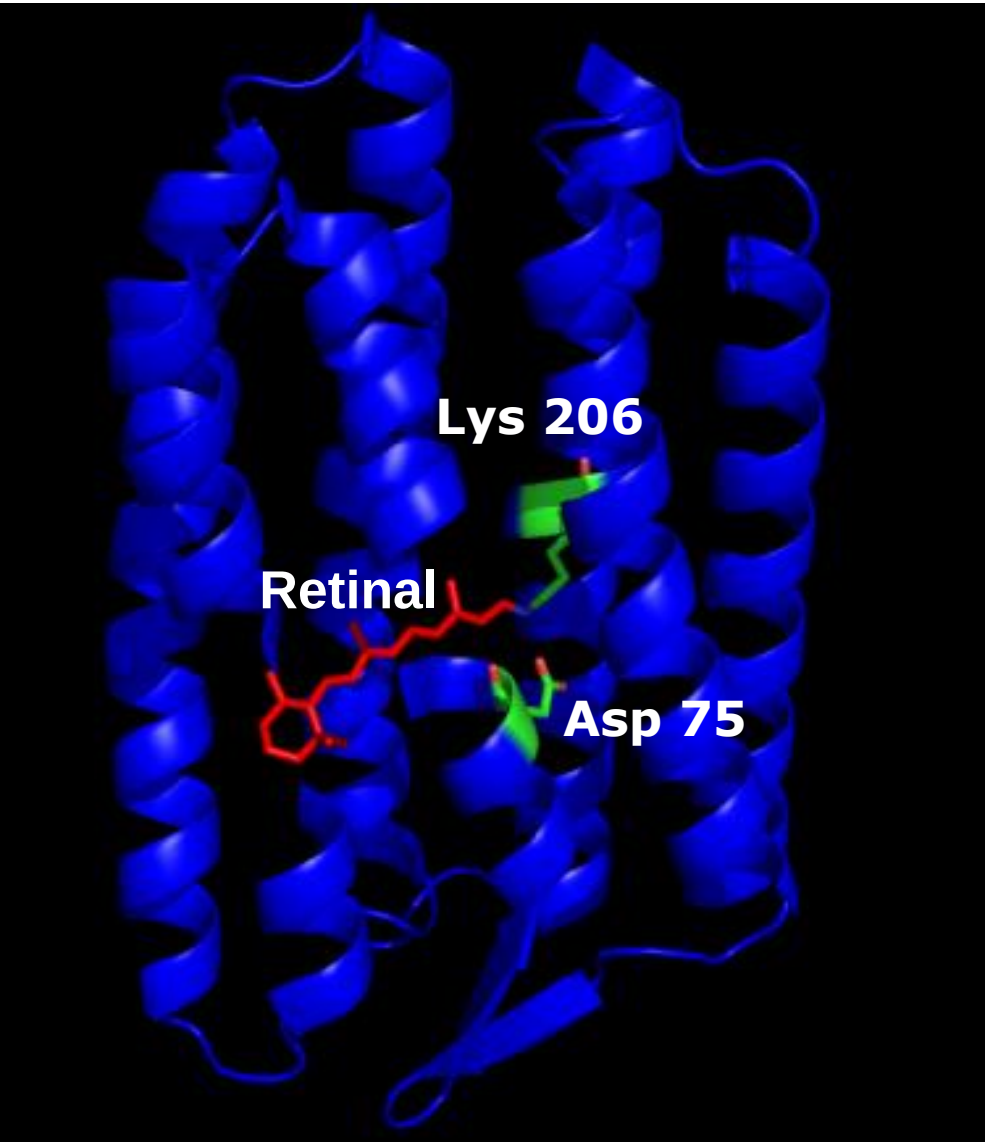
Photoactive membrane proteins





Stingl et. al., Nature Microbiology Reviews, p488, 2008

Channel rhodopsin

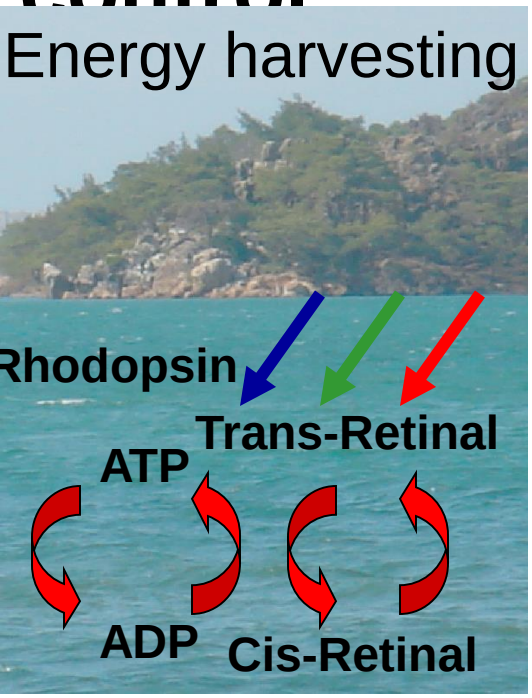


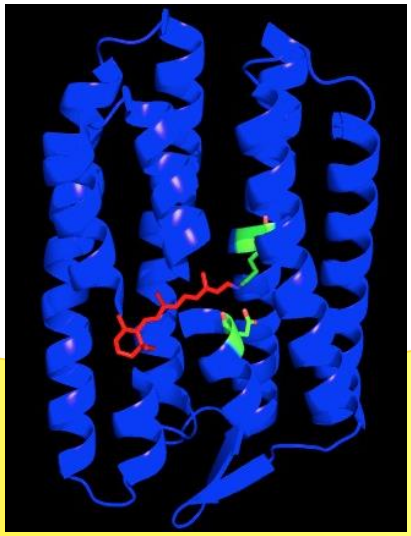
Light gated Ion channel
It control phototaxis in algae.

ChR2 absorbs blue light with A max wavelength 490 nm

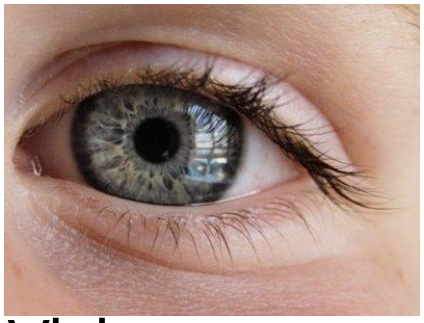
Royant et. al., PNAS, 2001, 98, 10131

Photoactive membrane proteins: energy harvesting, phototaxis, vision and optical control

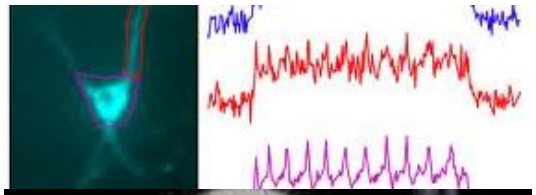




PHOTOACTIVE RHODOPSIN MEMBRANE PROTEIN



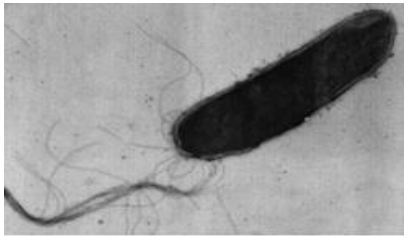
Vision



Optogenetic Applications

- Optical control of neuronal activity
- Optical probes for membrane potential

Phototaxis

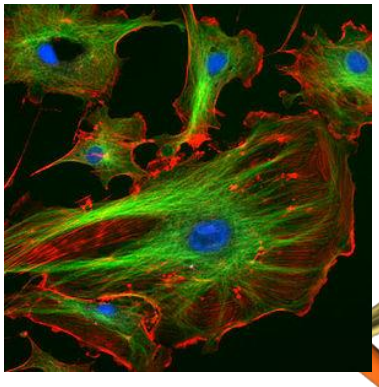


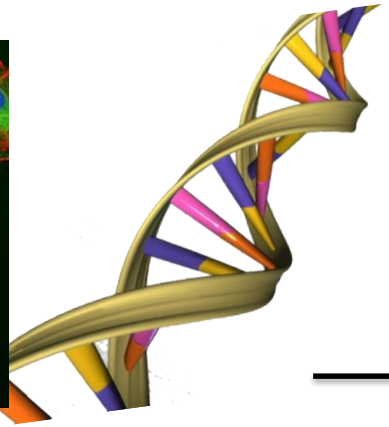
Halobacterium salinarum

The a photon is absorbed by the retinal. The *trans*-retinal complex change its conformation from trans to cis.

The emerge of optogenetic methods

“Optogenetics is the combination of genetic and optical methods to control and to monitor specific events in targeted cells of living tissue.” Resource : Wikipedia



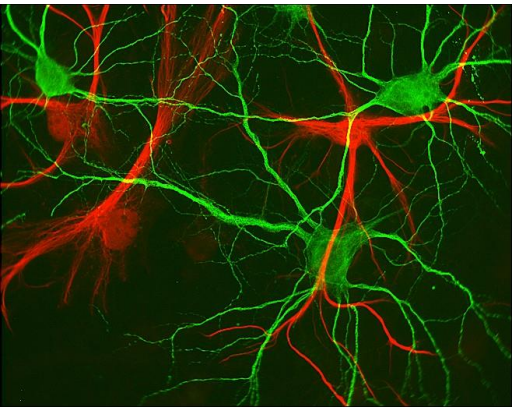


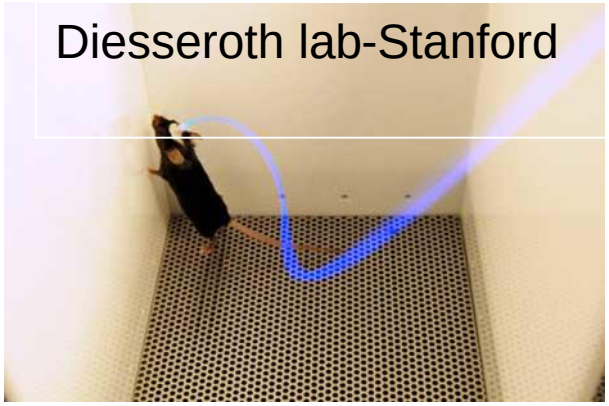
Dr. Karl Deisseroth Lab - Stanford

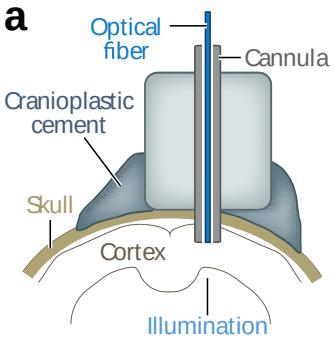
Adamantidis, Deisseroth et. al, Nature, 450, 420, 2007

1. Optical control of cellular functions

A) Control of cell potential by activating Rhodopsin membrane proteins using lasers

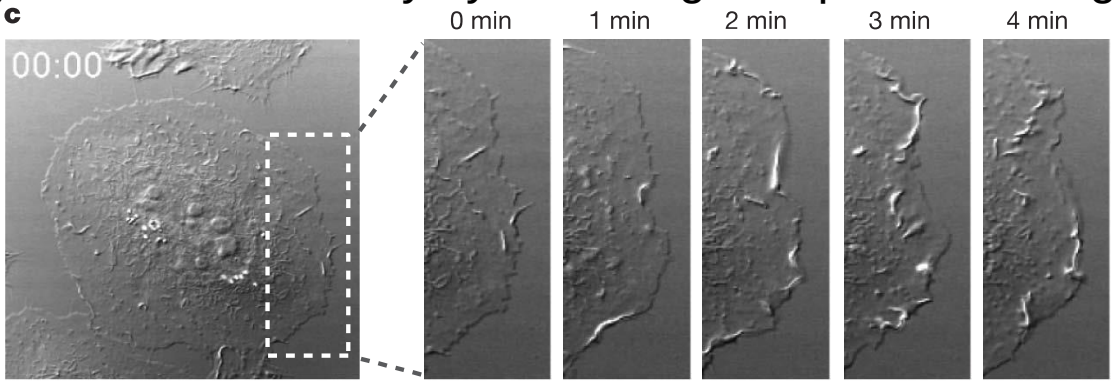


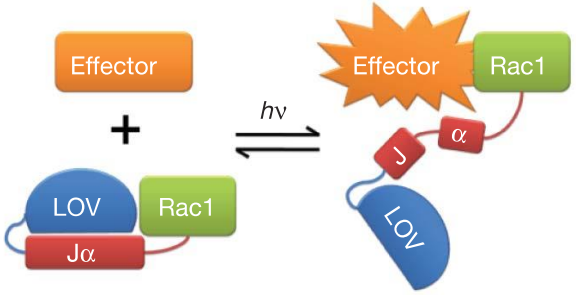




Hippocampus : Controls movement

B) Control cell motility by activating Rac proteins using lasers





Wu et. al. Nature 461, 104

How to modulate neurosignalling?

Small molecules can be used to affect the synapse activity

They are
Noepinephrine
Acethycholine
Dopamine
Serotonin

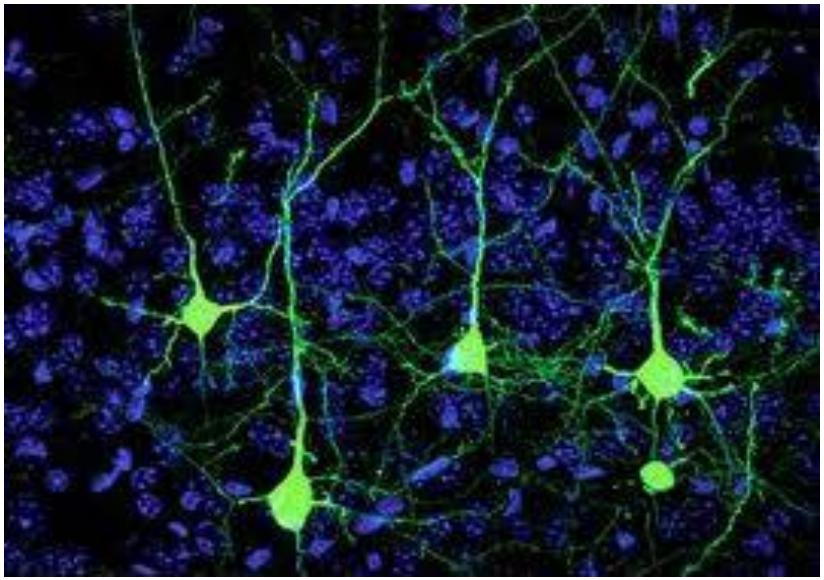
Here is the problem:

- Neurons system is composed by a large subset of specialized neurons.
- If a neurons needs to be studied, it has be modulated without modulating other things belongs to a different set of speciazed cells
- Small molecules will be localized in all cells because of the diffusion and affect all neurons.

- The question is can we modulate individual neurons? If how?

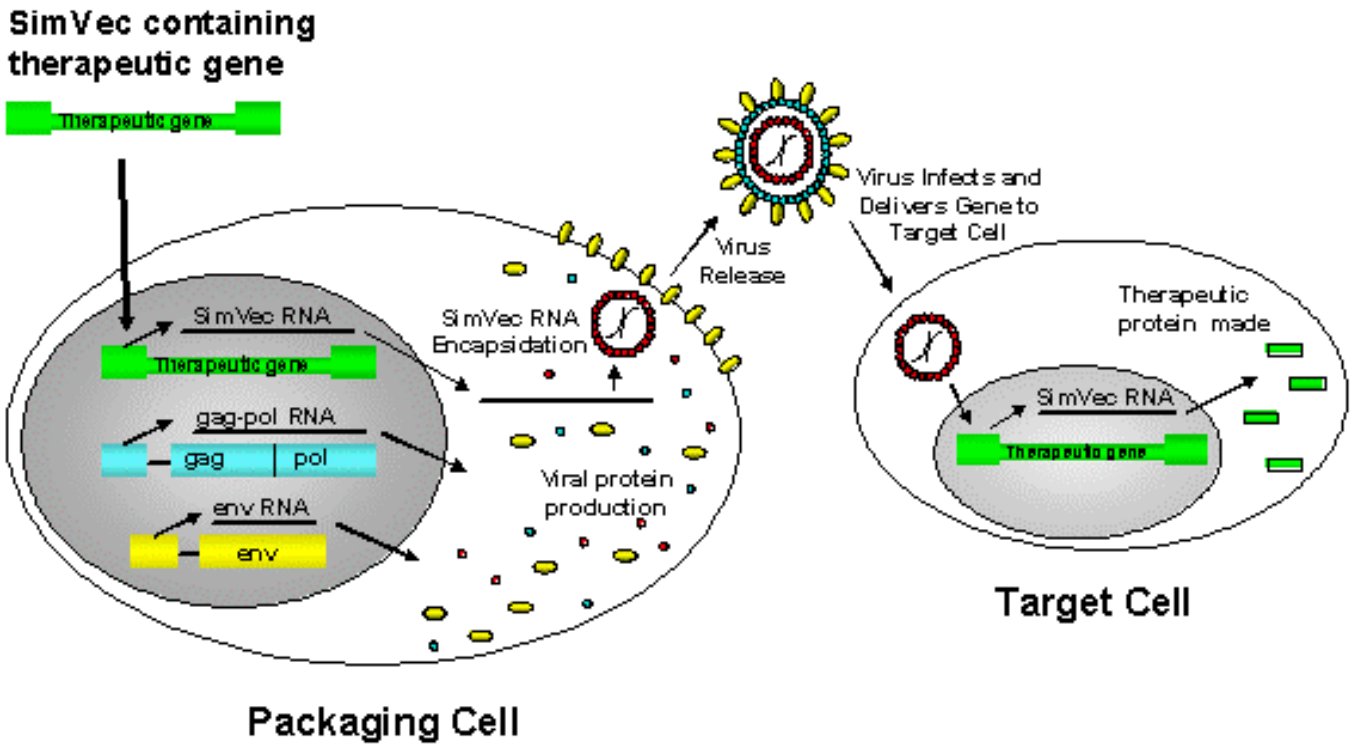
a) Optic methods
b) Genetic methods

Lasers to modulate neurons

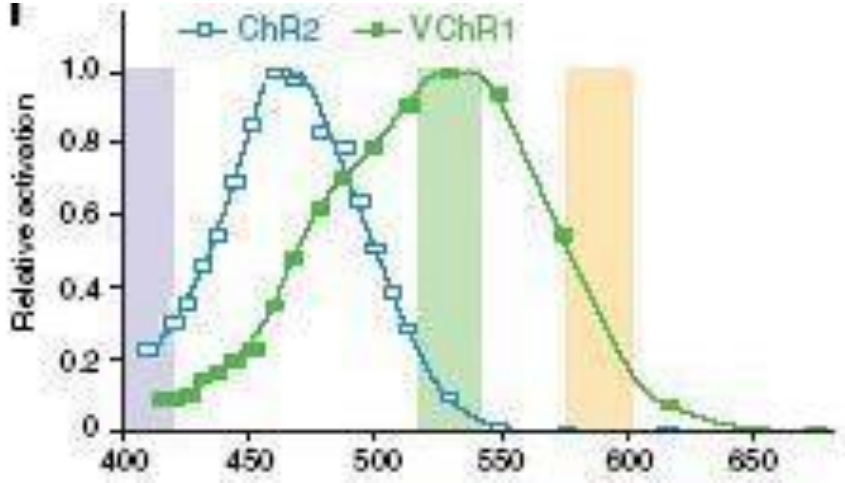


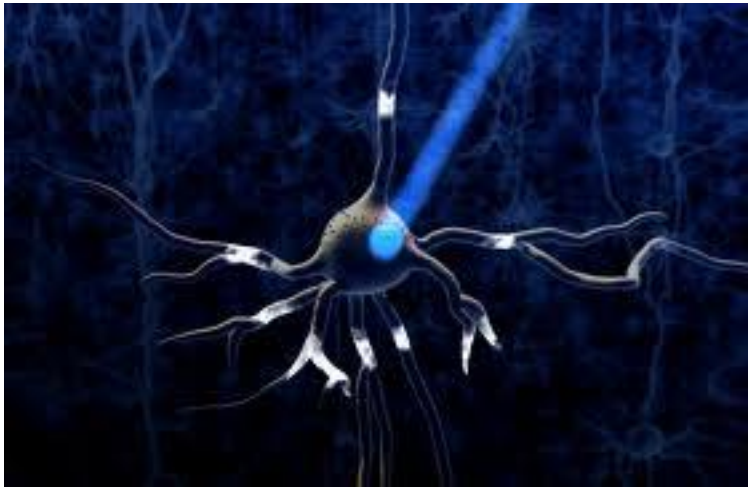
Lentivirus for transferring genes

293Ft cells can be used for producing lentivirus.

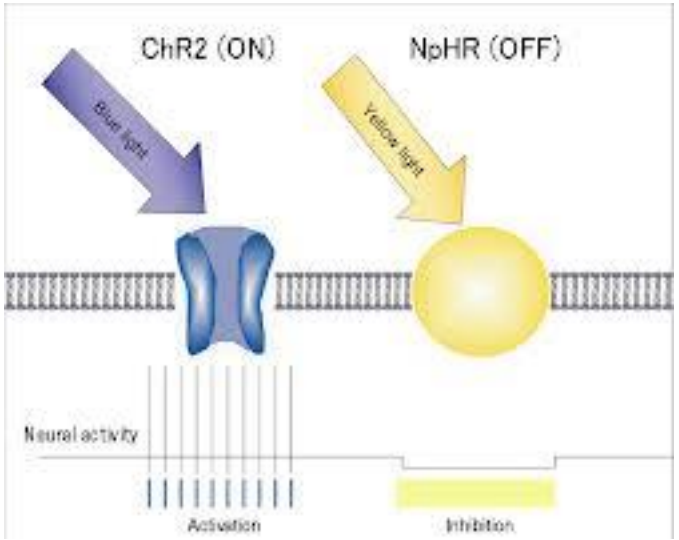


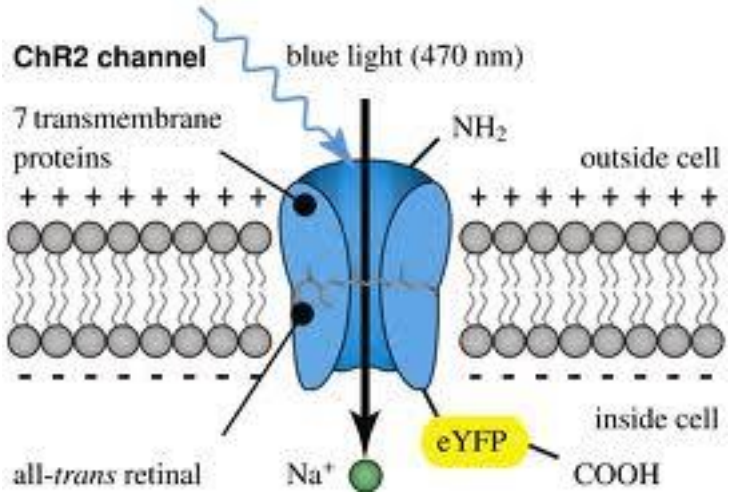
Channel Rhodopsin

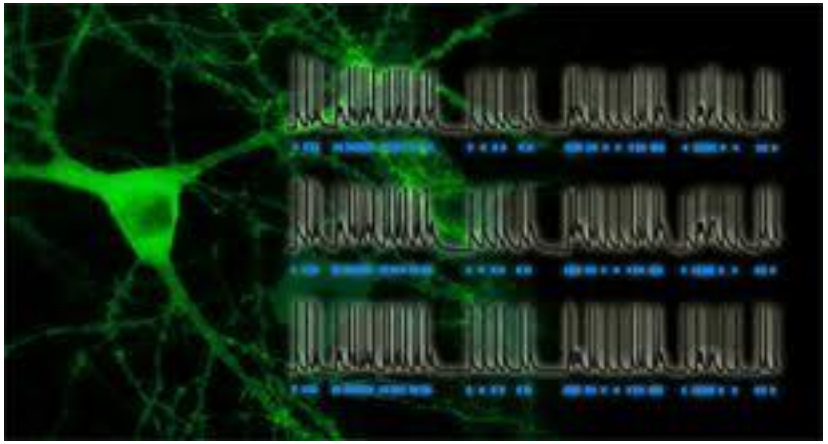




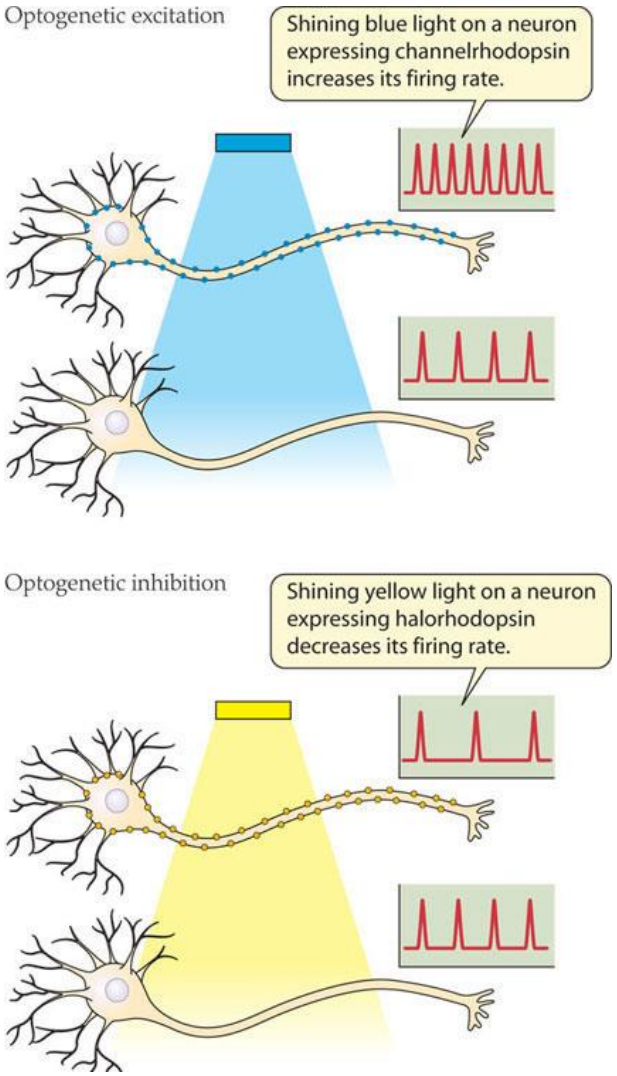
How Channel Rhodopsin Activates Neurons?





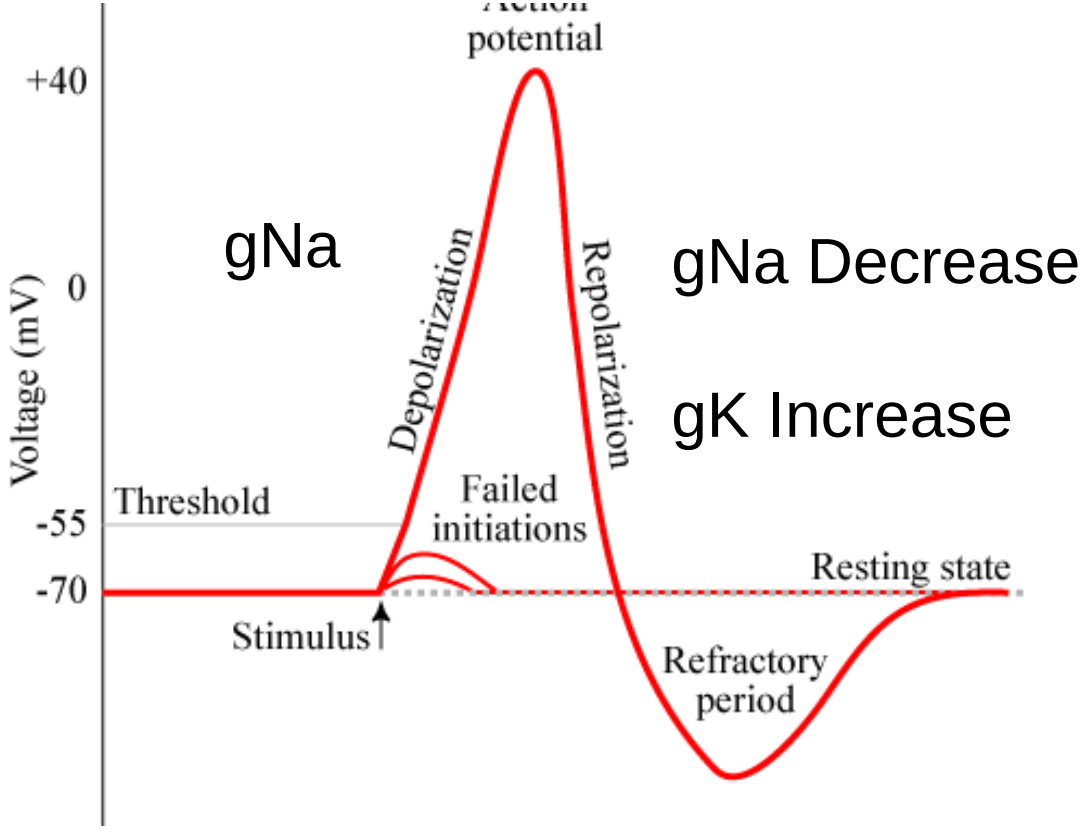


Rate of firing can be modulated



Frequency of spiking in a given time interval. When the brain for your hand holding a paper, the motor neurons controls the muscles by increasing firing rate.

Action Potential



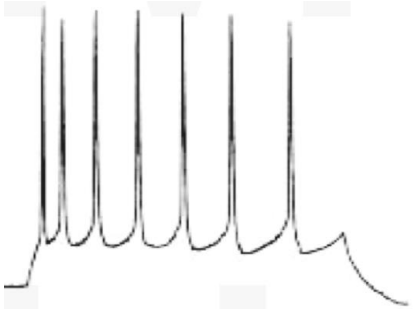
gNa

gNa Decrease

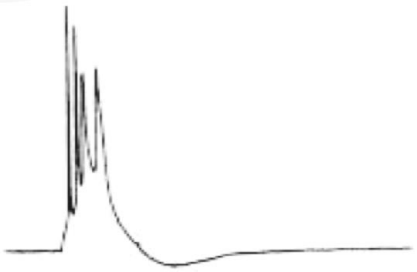
gK Increase

Ion channel diversity leads to a neuronal diversity

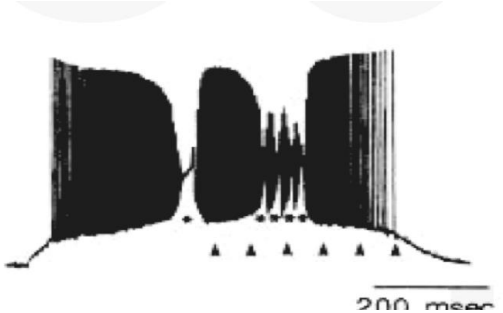
Regular Firing



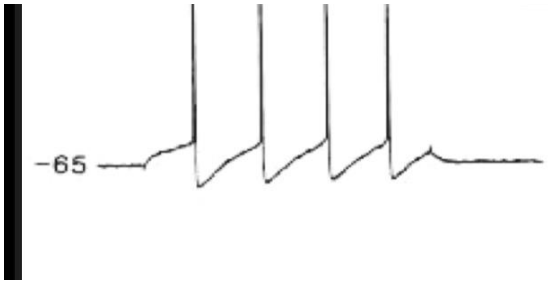
Burst Firing



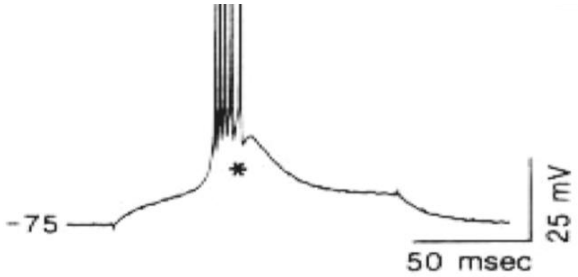
Cerebellar Purkinje Cell



Cornical Pyramidal Cell

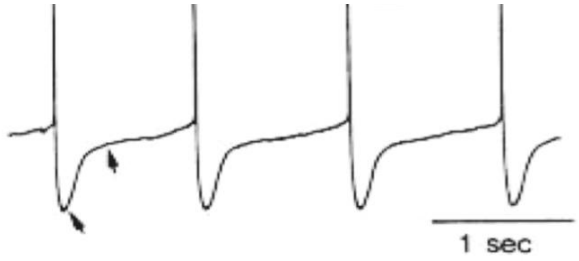


Transfer mode



Burst mode

Medial Habenular Cell



Chemical signaling is OK for short distances

Time constant for one dimensional diffusion:

$$\tau = \frac{x^2}{2D}$$

For a small molecule
($D \sim 10^{-5}$ cm^2 /s)

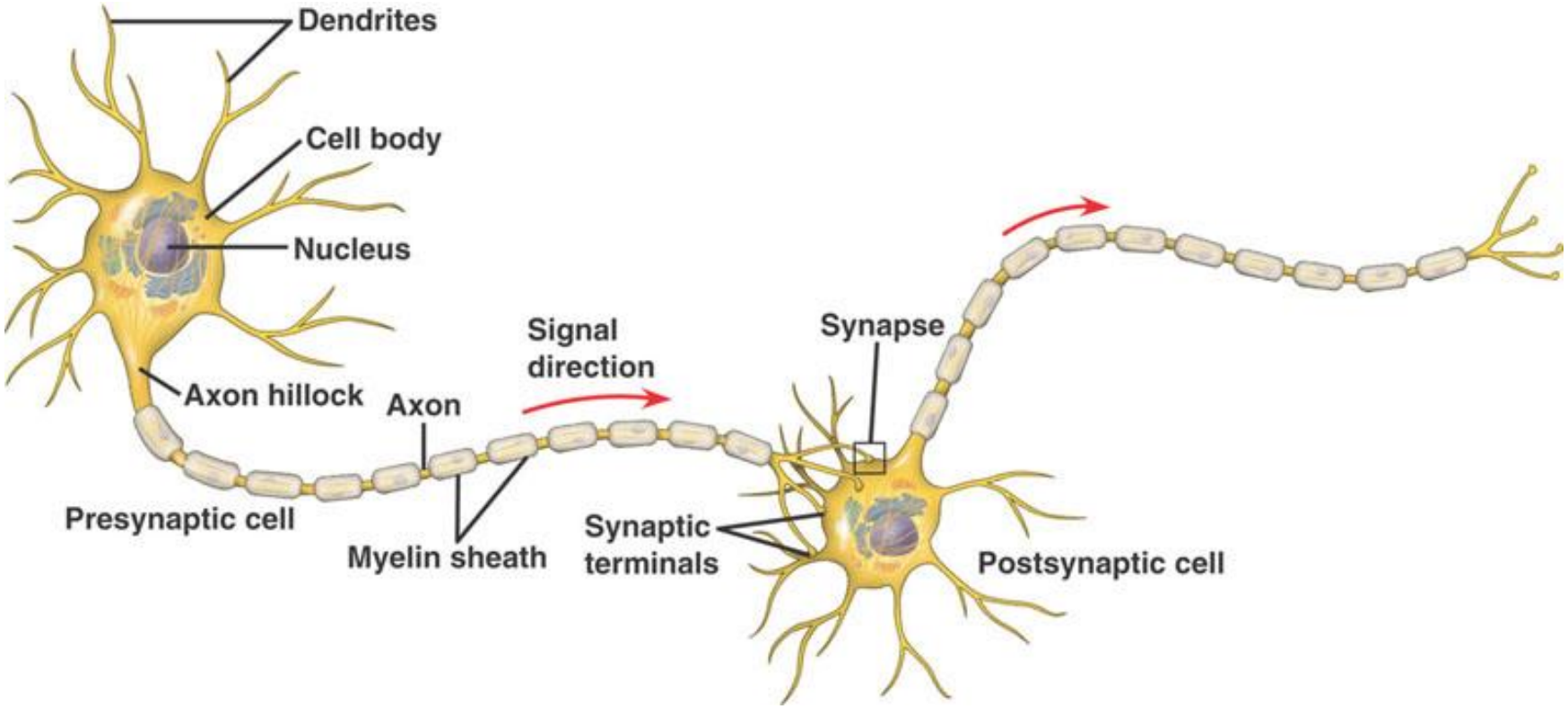
where, x is the displacement in one dimension
D is the diffusion coefficient

x	t
1 μm	0.5 ms
100 μm	5 s
1 cm	13.9 hrs
1 m	16 years

Motor proteins move at only 10 μm/s, or 27.8 hrs for 1m.
In contrast, for electrical signals it takes 10ms to travel 1m.
Electrical signals tend to be faster over long distances.

On dendrites, the analog signal is received through synapse

Signal is transmitted by action potential inside the neuron and exchange it across synapses



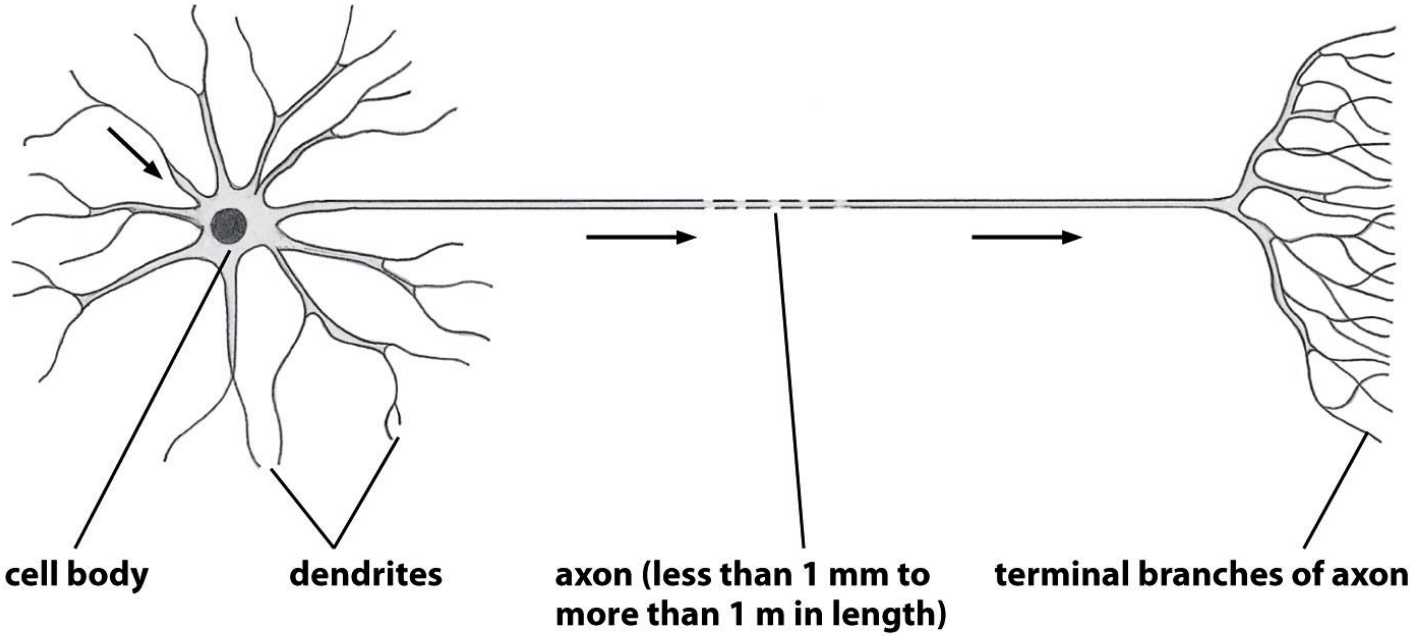
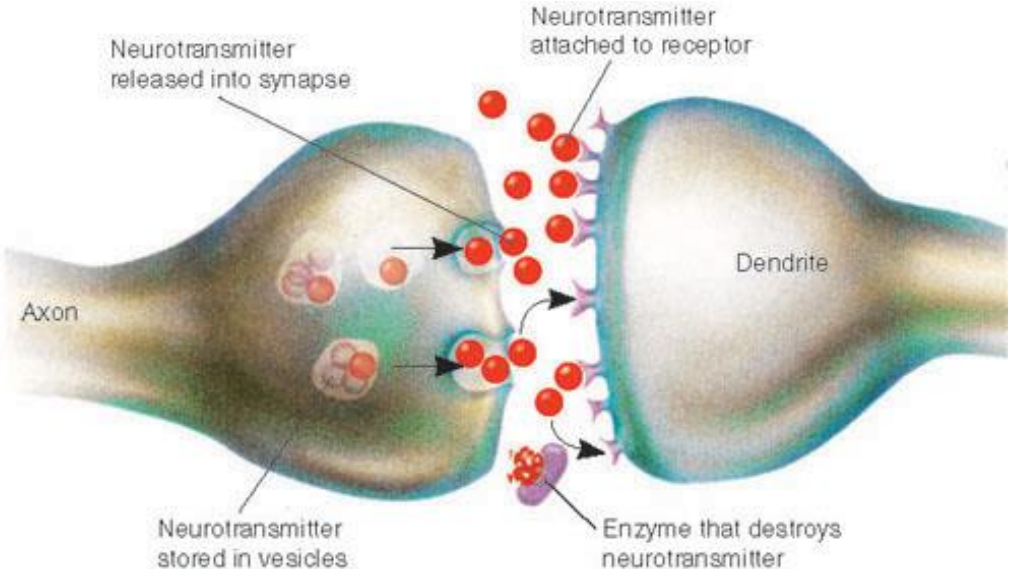


Figure 17.1 Physical Biology of the Cell (© Garland Science 2009)



The Axon,

Generally one per neuron

Many microtubules and neurofilaments are presents

A few micron to meter length

Transmit signal from some of the cell to the neuron synapses

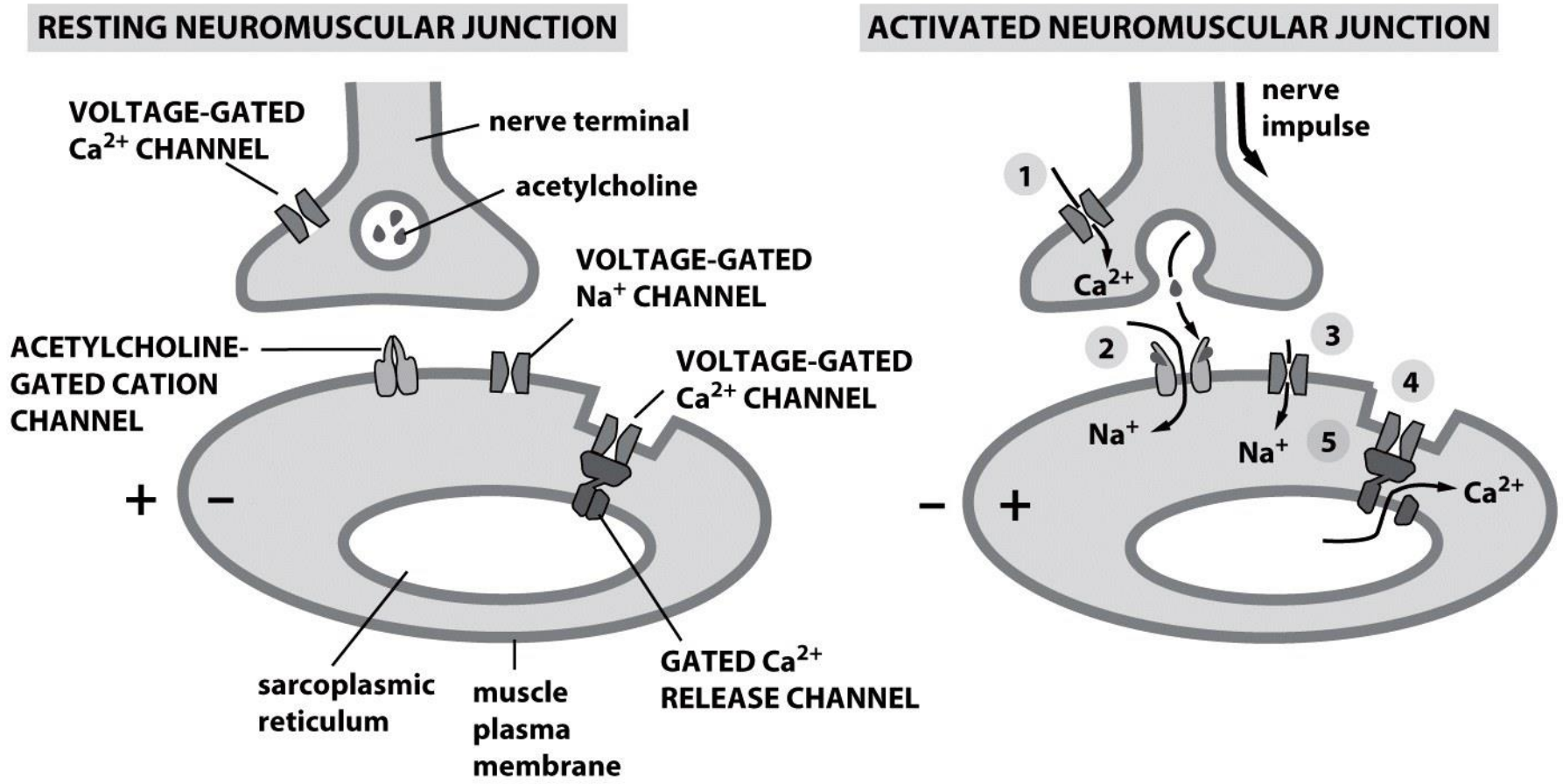


Figure 17.9 Physical Biology of the Cell (© Garland Science 2009)

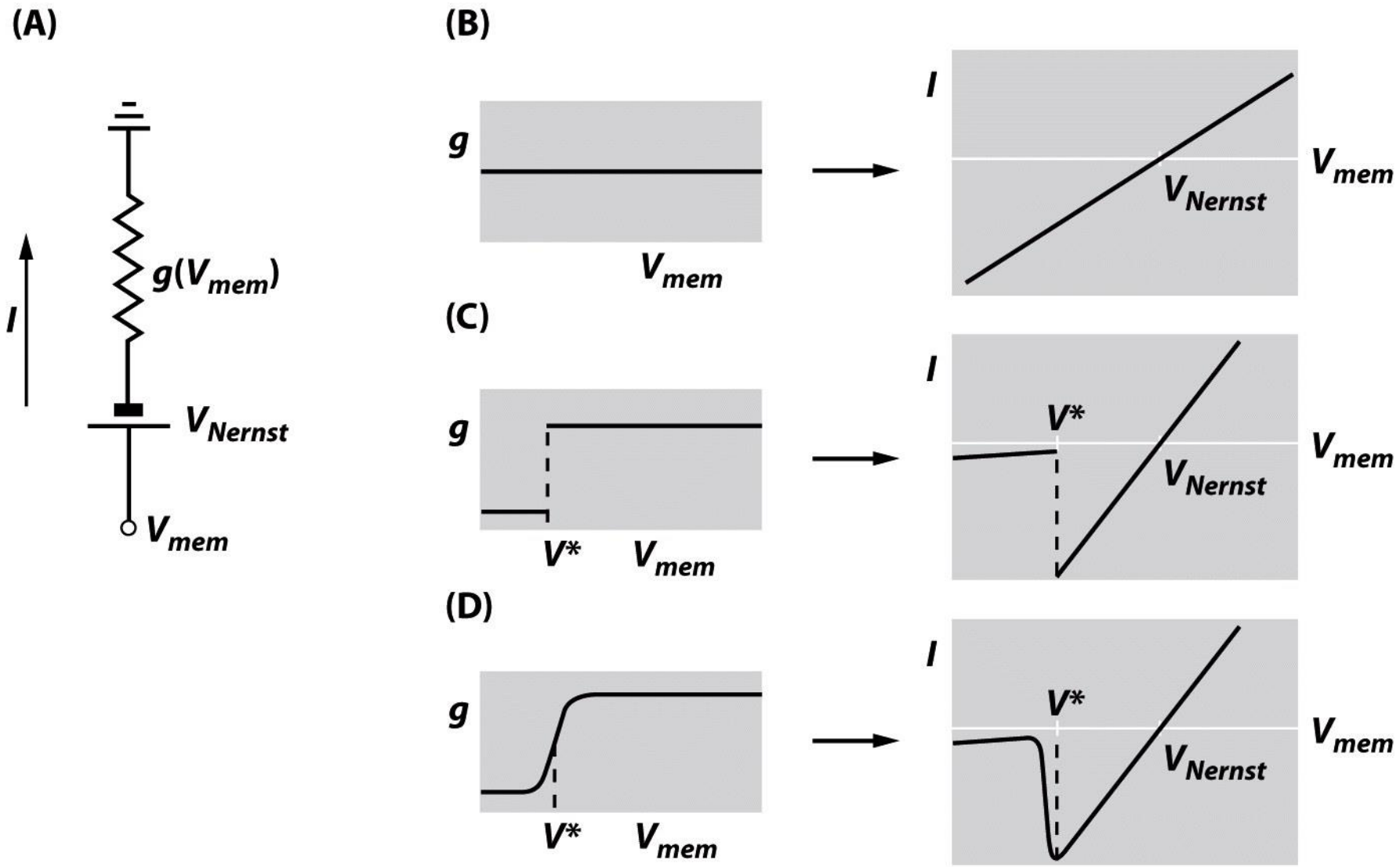
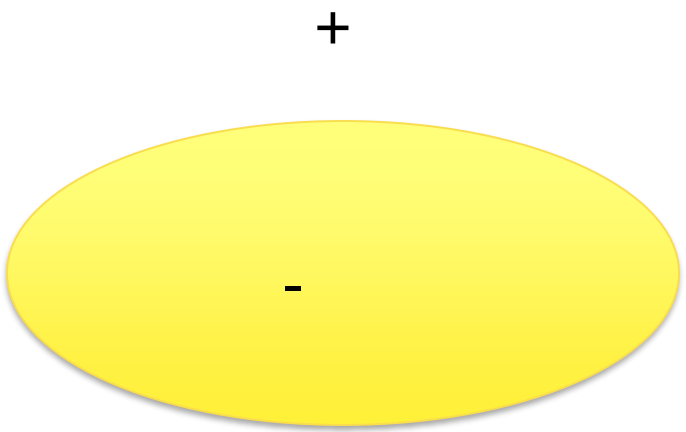


Figure 17.14 Physical Biology of the Cell (© Garland Science 2009)

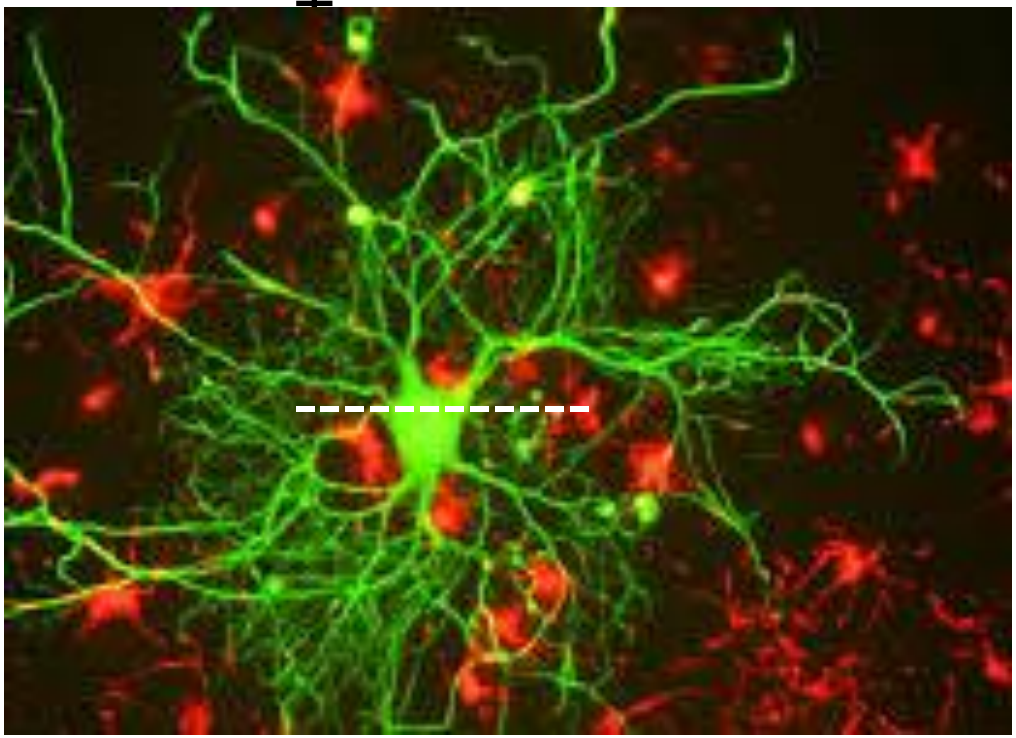
Membrane potential of -70 mV means that the inside of the cell is negative compared to the outside (the outside of the cell is **always** of the opposite charge of what is inside).



Membrane potential of -70 mV means that the inside of the cell is less negative compared to the

+++++++

+



The Nernst Equation and Concentration Dependence of the Emf

$$aA + bB \longrightarrow cC + dD \qquad Q = \frac{a_C^c a_D^d}{a_A^a a_B^b}$$

$$\Delta G = \Delta G^\circ + RT \ln Q$$

$$\Delta G = -nF\mathcal{E} \qquad \Delta G^\circ = -nF\mathcal{E}^\circ$$

$$-nF\mathcal{E} = -nF\mathcal{E}^\circ + RT \ln Q$$

Nernst equation

$$\mathcal{E} = \mathcal{E}^\circ - \frac{RT}{nF} \ln Q$$

At 25ºC (298.15 K),

$$\mathcal{E}(25^\circ\text{C}) = \mathcal{E}^\circ - \frac{0.025693\ \text{V}}{n} \ln Q$$

At equilibrium. no net transfer of electrons, so $\mathcal{E}^\circ_{\text{cell}} = 0$, $Q = K$.

$$\mathcal{E}^\circ_{\text{cell}} = \frac{RT}{nF} \ln K$$

Nerst Equation is used to calculate the equilibrium potential

There is a high negative charge outside of the cell
155 mM inside while the outside concentration of K is typically about 4 mM.
Remember that negative charges attract positive ones such as K^+ ions.

$$E = - \frac{RT}{nF} \ln \frac{K_i}{K_o}$$

$$E = -59mV \log \frac{K_i}{K_o}$$

n is the elementary charges
F is faraday constant
T is temperature
R is gas constant

$E_K = -59 \log(155/4)$

$E_K = -98$ mV

If the membrane were permeable only to K, E would be -90 mV. Since the electrical force exactly balanced with the diffusion force when the membrane potential was -92 mV.

If the cell membrane is only permeable to Na ions. Then what is the membrane potential?

$$E_K = -59 \log \frac{Na_i}{Na_o}$$

If $Na_{outside}$ = 145 mM Na_{inside} = 12 mM

Then,

E = -59mV log(12/145)

E = +67 mV

Summary:

Vm = -90 to -70 mV

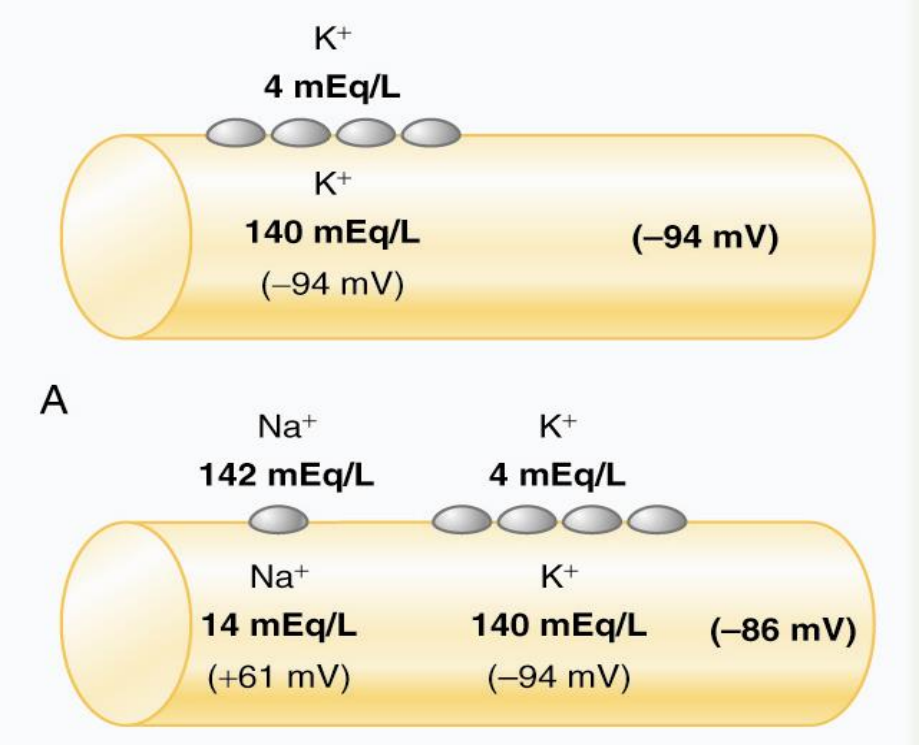
E_K = -98 mV
E_{Na} = +67 mV

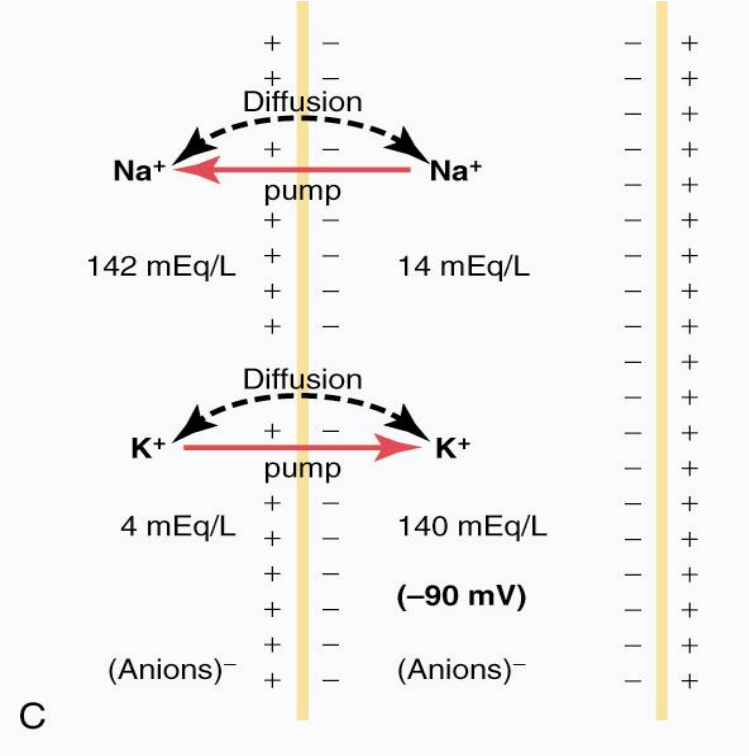
Since the membrane is more permeable to K than Na, The Vm is more close to E_K than E_{Na}

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

Table 17.1 Physical Biology of the Cell (© Garland Science 2009)

Resting Membrane Potential Summary

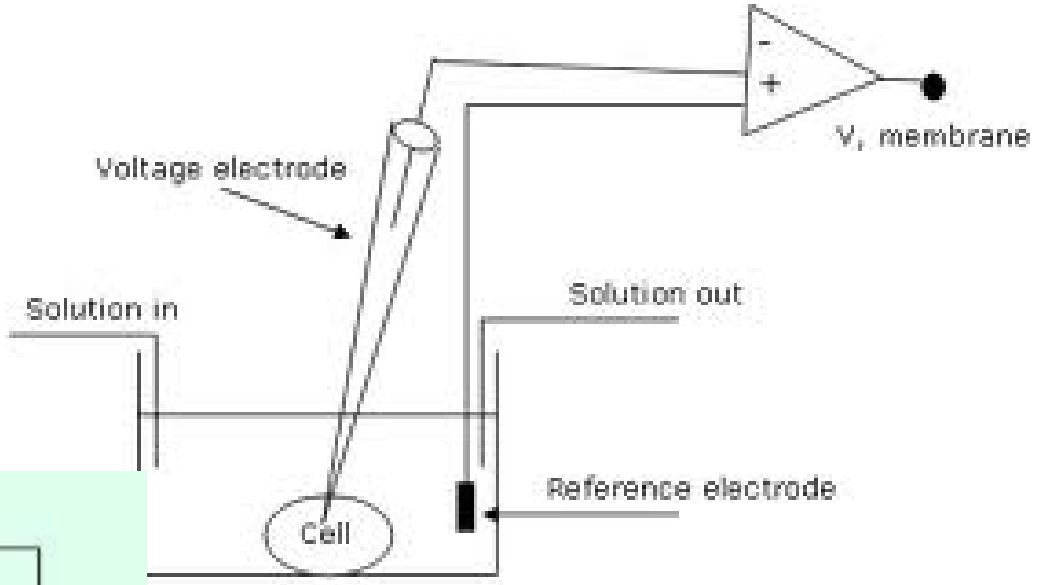


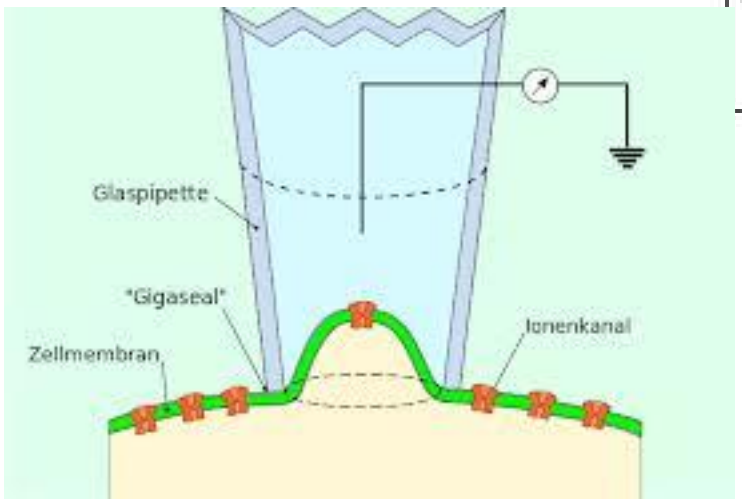


How the neuron activity can be measured?

Patch Clamp

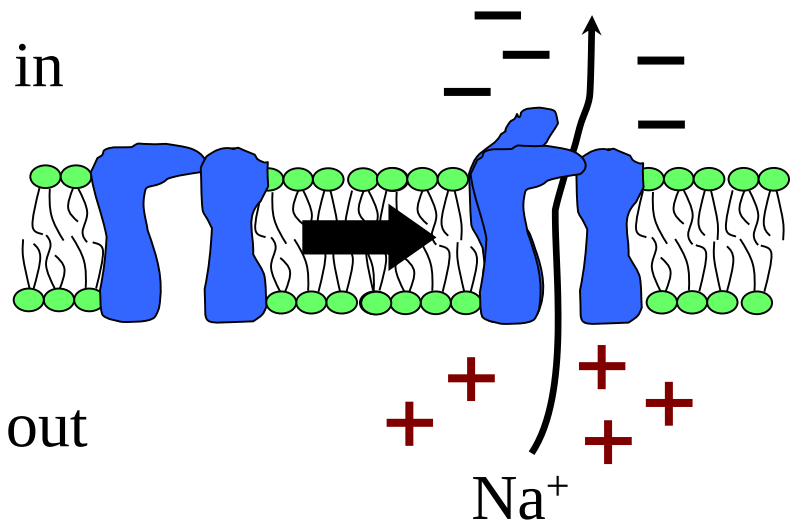
Nobel Prize in Physiology & Medicine -1991





Extracellular

Ion Channels



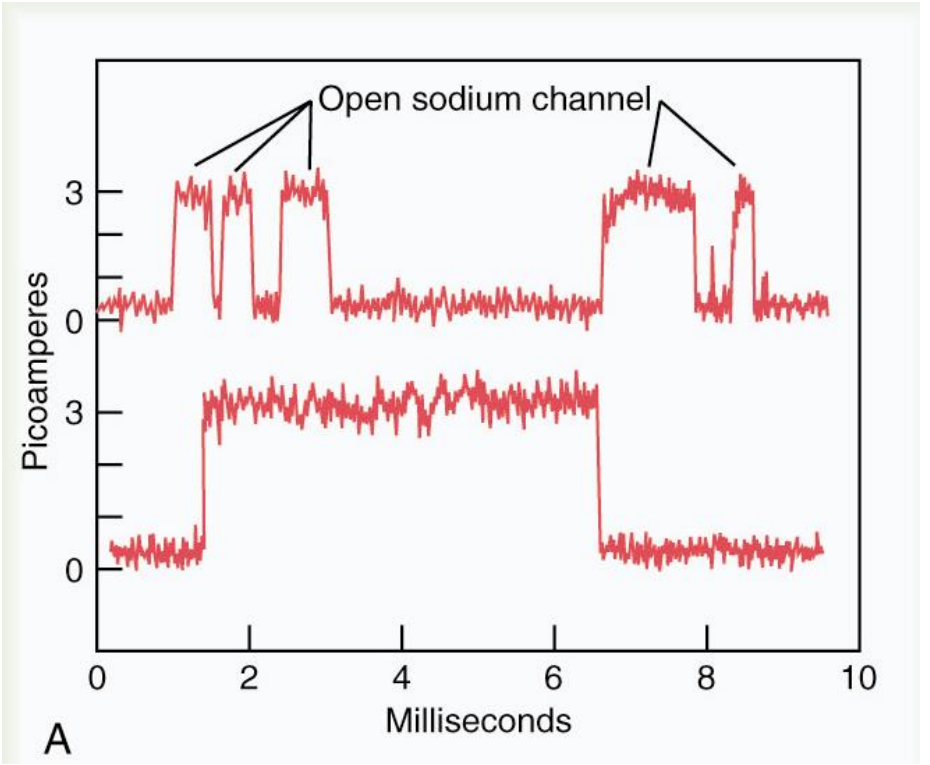
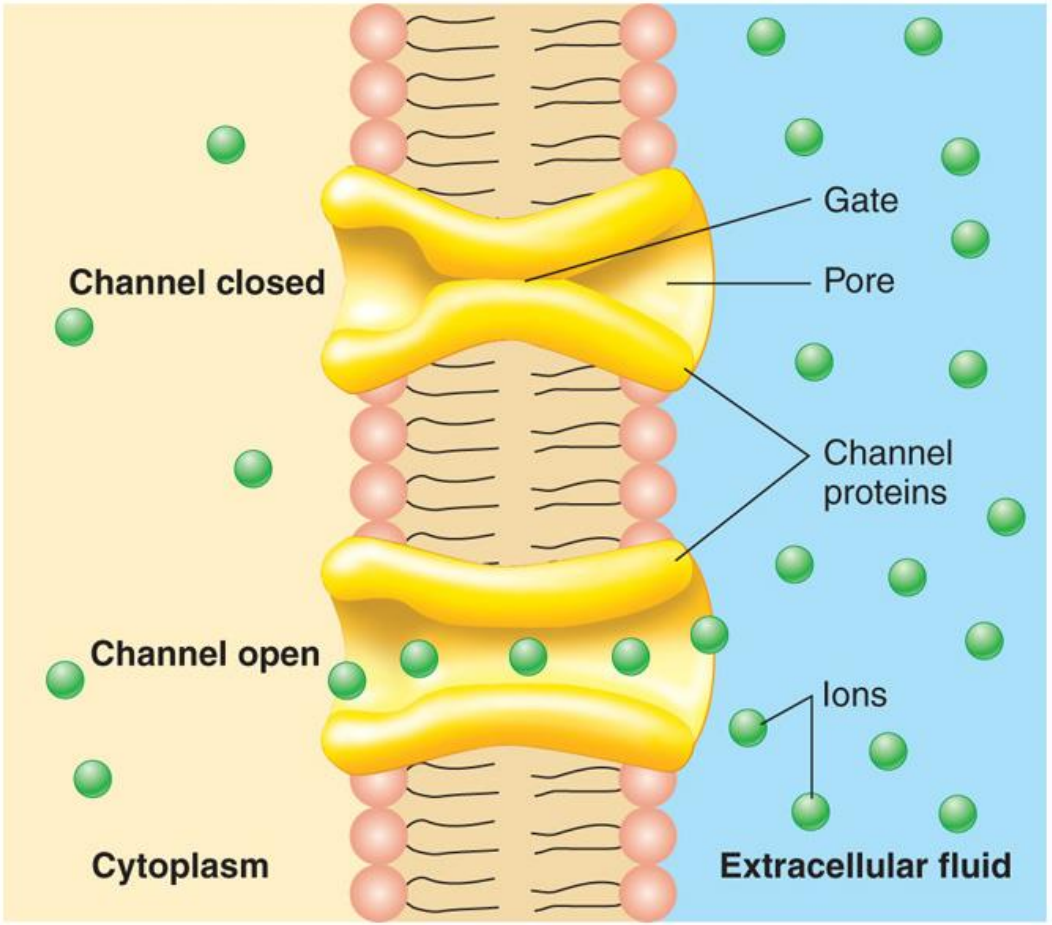


Figure 4-5; Guyton & Hall

Diffusion continued

- **Cell membranes are impermeable to charged and most polar compounds**
  - Charged molecules must have an ion channel or transporter to move across membrane



*(** across an artificial lipid bilayer)*