Neurofunctional Techniques

Lessons 3 & 4

7, 9 October 2024

1) **Ca^{2+} indicators**
2) **Ca^{2+} binding**
3) **Diffusion**

Calendar

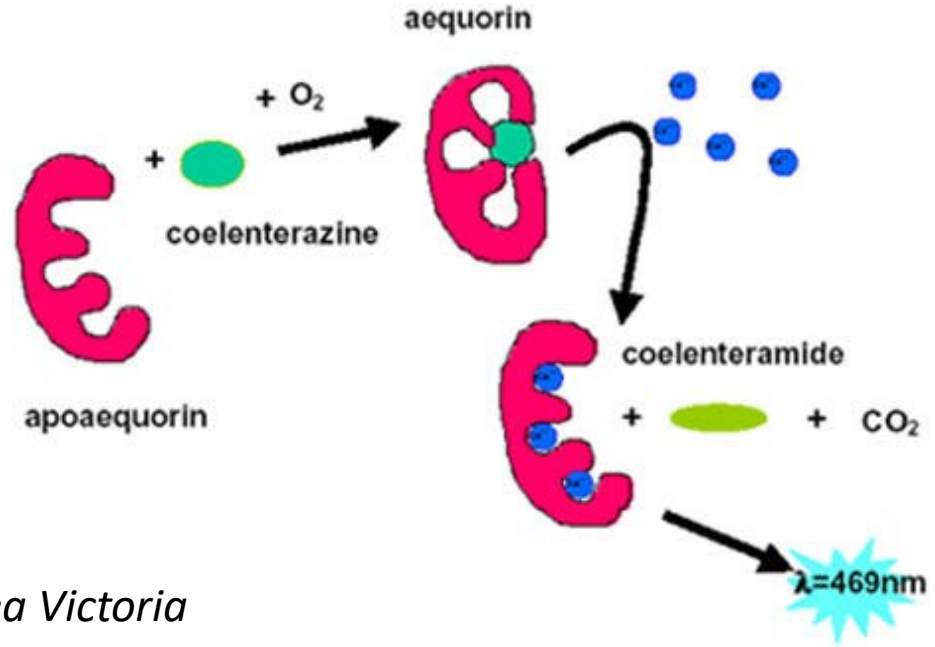
- **M 30 Sept:** Course introduction
- **W 2 Oct:** Functional imaging
- **F 4 Oct:** Statistics (Cesca)
- **M 7 Oct:** Functional imaging
- **W 9 Oct:** Biophysics of diffusion
- **F 11 Oct:** Statistics (Cesca)
- **M 14 Oct:** Functional imaging
- **W 16 Oct:** General introduction to the papers for the presentations
- **F 18 Oct:** Statistics (Cesca)
- **M 21 Oct:** Modeling in neuroscience
- **W 23 Oct:** Practical exercises on the first part of the course
- **F 25 Oct:** Statistics (Cesca)
- **F 25 Oct:** Laboratory (14:00- 18:00) to be confirmed!
- **M 28 Oct:** Molecular approaches in modern neuroscience
- **W 30 Oct:** Genome editing in neuroscience (Dr. Jaudon)
- **M 4 Nov:** Optogenetics
- **W 6 Nov:** Papers assignment to the groups; introductions to the specific papers
- **T 12 Nov:** X-genetics + Practical exercises on the second part of the course
- **W 13 Nov:** Introductions to the specific papers
- **M 18 Nov:** Introductions to the specific papers

- **9, 10, 11 Dic (15:00-19:00):** Paper presentation (all 3 days!)
- **Week 16 Dic: Test (to be confirmed!)**

Ca^{2+} imaging

- Review Ca^{2+} signaling
- Ca^{2+} indicators
  - Small molecule indicators (SMIs)
  - Genetically-encoded Ca^{2+} indicators (GECIs)
- Ca^{2+} binding
- Ca^{2+} diffusion
- Ca^{2+}-dependent fluorescence properties
- Simplified models of Ca^{2+} dynamics
- Imaging devises
- What can we do with it

A bit of history: Aequorin bioluminescence



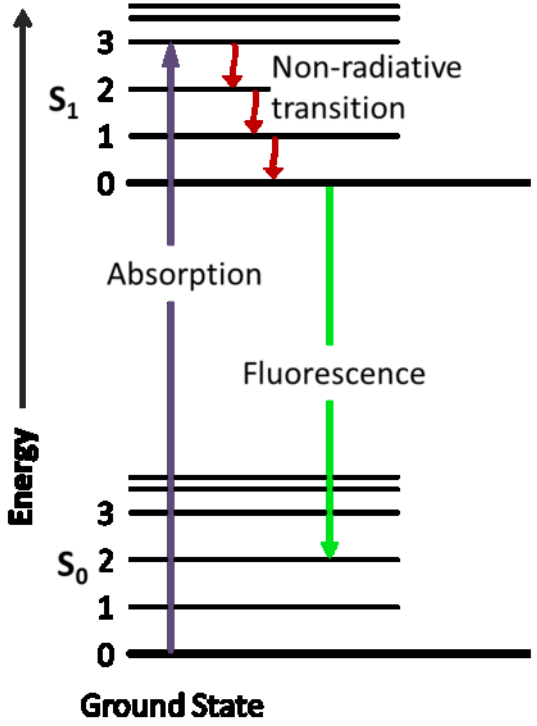
Aequorin derived from the jellyfish *Aequorea Victoria*

Bioluminescence is the production of light by a living organism, for example using a chemical reaction. Bioluminescence is different from fluorescence as it does not require external illumination.

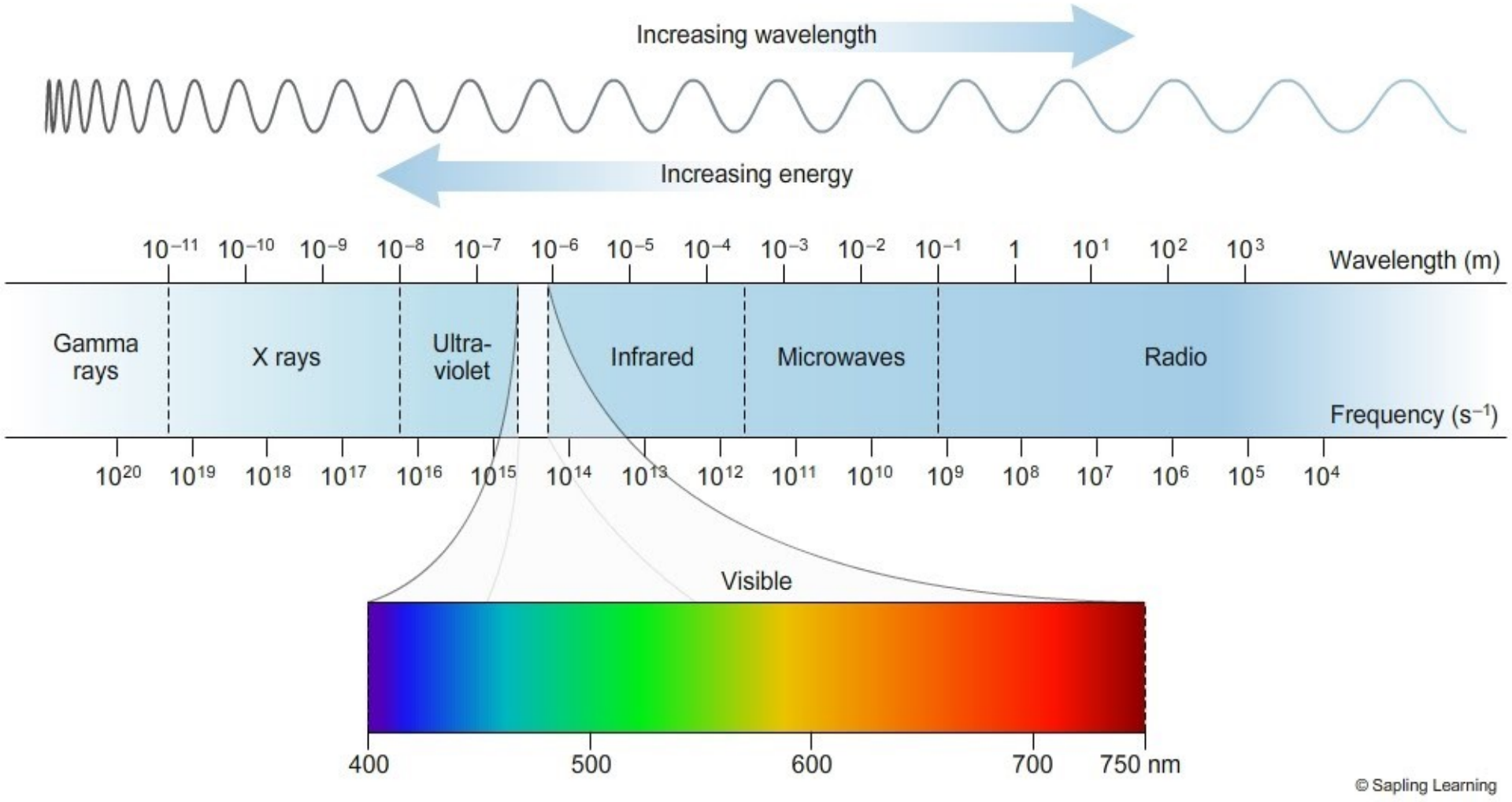
Advantages: there are no problems of phototoxicity, photobleaching, autofluorescence.
Disadvantages: each molecule performs only one emission cycle and recharging the coelenterazine is a **slow process.**

Fluorescence

Fluorescence is not produced by the organism itself; fluorescent molecules absorb photons of light (preferentially of a specific wavelength), which temporarily excite electrons to a higher energy level, giving light off as a waste product. The emitted light has a longer wavelength, and therefore lower energy, than the absorbed light.



Electromagnetic spectrum



An example: GFP (green fluorescent protein)

https://www.chroma.com/spectra-viewer?fluorochromes=10392

Getting Started Getting Started Come iniziare

Visualizza informazioni sul sito

Spectra Viewer – EGFP

Legend Fluorochromes Sets Parts

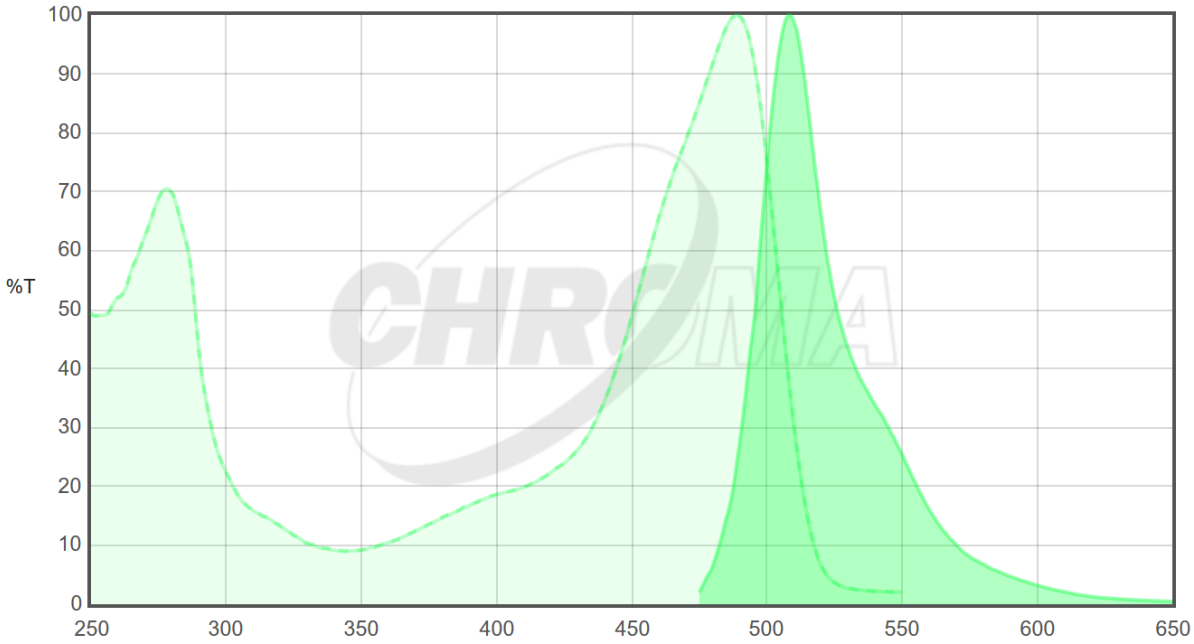
Fluorochromes

EGFP Hide Individual Filters x

PLOT	FILTERS	TYPE	%T-OD	AOI	DATA
☑	EGFP Excitation Spectra	EX	49.06 T at 255nm	0°	ASCII
☑	EGFP Emission Spectra	EM	0.00 T at 255nm	0°	ASCII

Reload Plot

View By: ◉ %T ○ OD Wavelength 250 to 650 update reset export



Attributes

Ex λ	Em λ
488	507

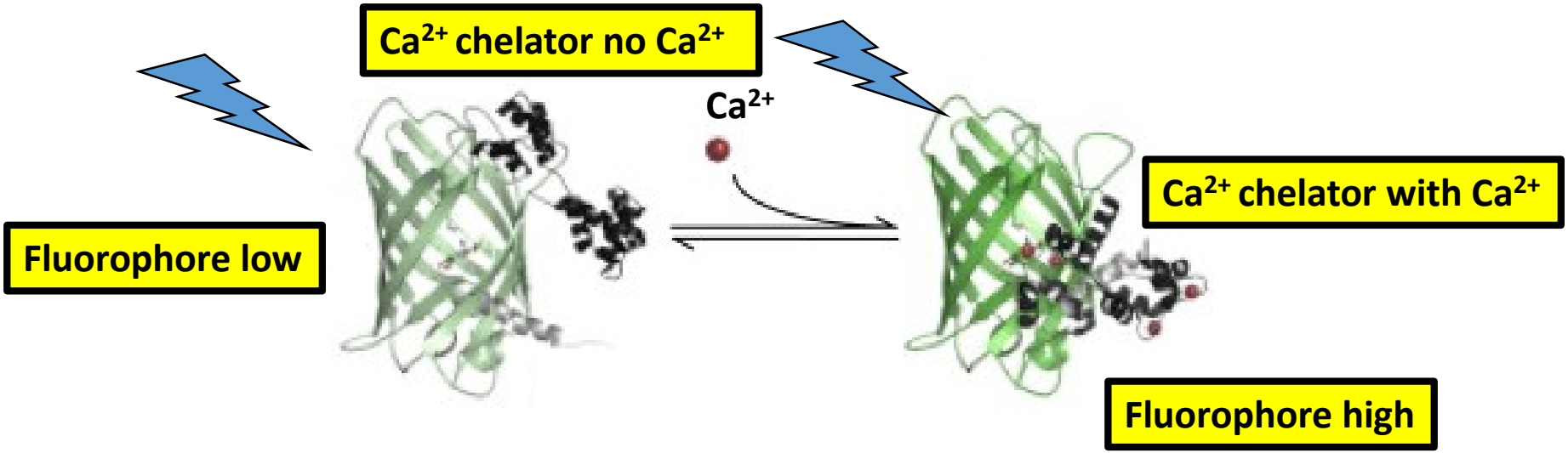
Fluorescent Ca^{2+} indicator

Fluorescent indicators are advantageous because even at low concentration they allow for high-contrast labeling

How do you make a fluorescent Ca^{2+} indicator?

It should have two moieties:

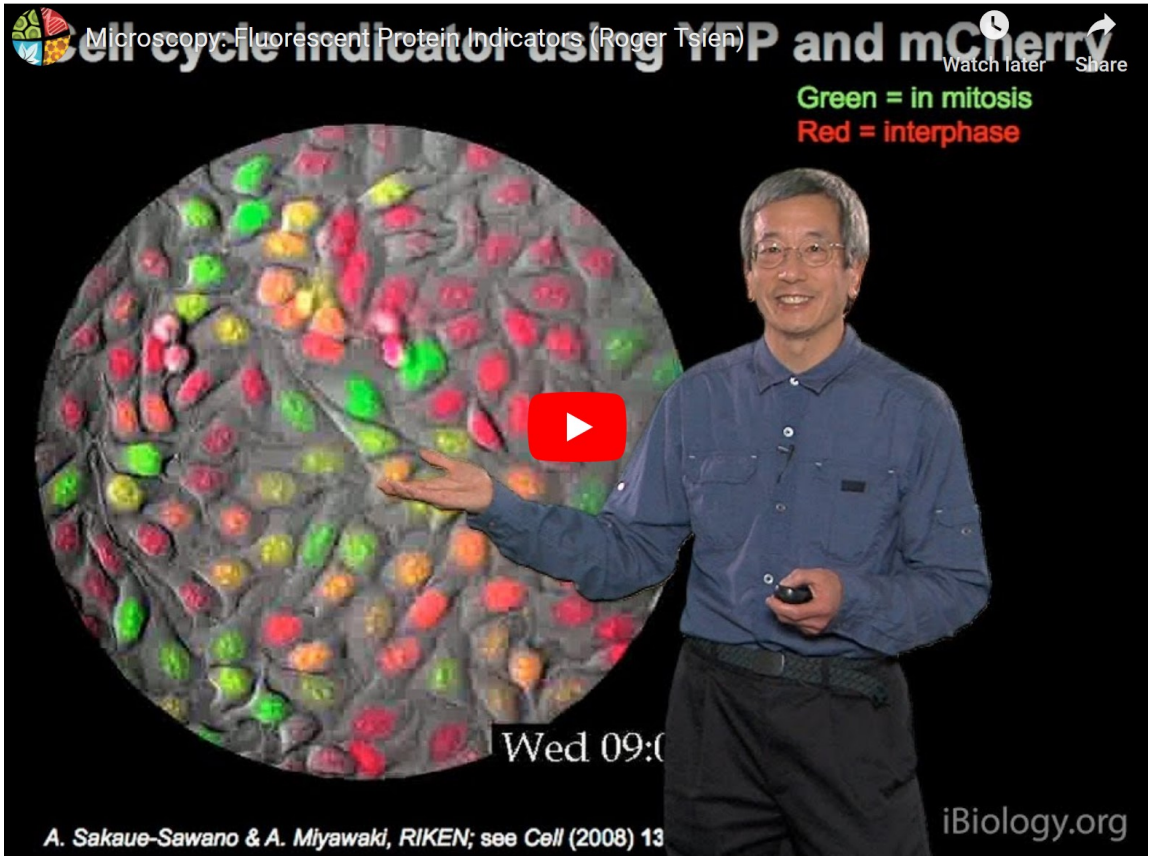
- **1 acting as Ca^{2+} buffer/chelator**
- **1 acting as fluorophore**
- **Ca^{2+} binding to the chelator moiety must affect some property of the fluorophore**



There are 2 major types of fluorescent Ca^{2+} indicators:

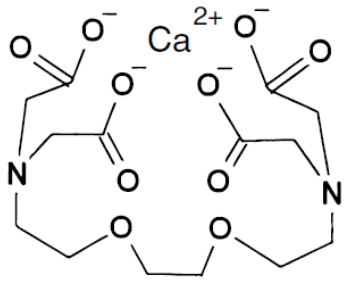
- **Small molecule indicators (SMIs)**, synthetic organic dyes developed since the beginning of the 1980s, mainly by Roger Tsien

- **Genetically-encoded Ca^{2+} indicators (GECIs)**



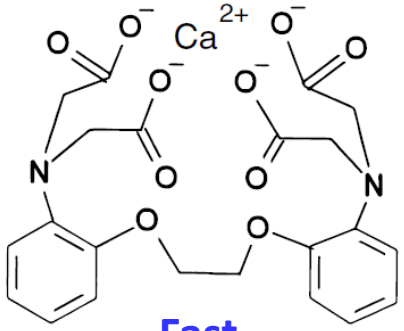
Small molecule indicators (SMIs) are based on:

A EGTA



Slow

B BAPTA



Fast

EGTA and BATA, the 2 major exogenous Ca^{2+} buffers

1. **They bind 1 Ca^{2+} → important for quantitative estimation of [Ca^{2+}]**
2. **with affinities comparable to endogenous Ca^{2+} binding proteins → they can 'read' physiological levels of [Ca^{2+}]**

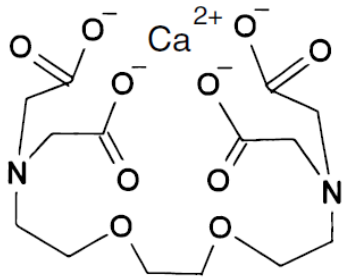
Table 1 | **Physicochemical properties of exogenous and endogenous Ca^{2+} buffers**

Chelator/Ca^{2+}-binding protein	Ca^{2+}-binding rate (k_{on})	Ca^{2+}-unbinding rate (k_{off})	Affinity (K_D)	Refs
BAPTA*	$4 \times 10^8\ M^{-1}\ s^{-1}$	$88\ s^{-1}$ ‡	220 nM	22,33,139
EGTA*	$1 \times 10^7\ M^{-1}\ s^{-1}$	$0.7\ s^{-1}$ ‡	70 nM	22,77
Calbindin	$7.5 \times 10^7\ M^{-1}\ s^{-1}$	$29.5\ s^{-1}$	293 nM‡	77,79
Calretinin§	$1.8 \times 10^6\ M^{-1}s^{-1}$ (T)	$1.29\ s^{-1}$ (T)	717 nM‡	78
	$3.1 \times 10^8\ M^{-1}\ s^{-1}$ (R)	$1.73\ s^{-1}$ (R)	5.6 nM‡	
Calmodulin N-lobe§	$7.7 \times 10^8\ M^{-1}\ s^{-1}$ (T)	$1.6 \times 10^5\ s^{-1}$ (T)	208 µM‡	79
	$3.2 \times 10^{10}\ M^{-1}\ s^{-1}$ (R)	$2.2 \times 10^4\ s^{-1}$ (R)	688 nM‡	
Calmodulin C-lobe§	$8.4 \times 10^7\ M^{-1}\ s^{-1}$ (T)	$2.6 \times 10^3\ s^{-1}$ (T)	31 µM‡	79
	$2.5 \times 10^7\ M^{-1}\ s^{-1}$ (R)	$6.5\ s^{-1}$ (R)	260 nM‡	

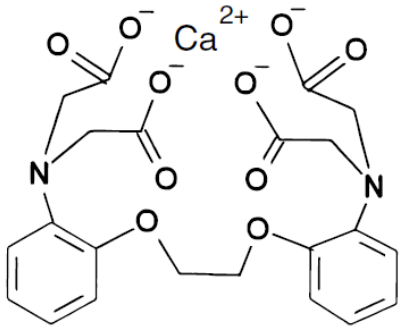
*For the exogenous chelators, the Ca^{2+}-binding rate (on rate) is ~40 times higher for BAPTA than for EGTA. By contrast, the affinity values are comparable; in fact the affinity is threefold lower for BAPTA than for EGTA. ‡ This value was calculated using $K_D = k_{off} / k_{on}$. §For the Ca^{2+}-binding proteins calretinin and calmodulin, Ca^{2+} binding is highly cooperative. Therefore, rates are given separately for tense (T) and relaxed (R) conformations of the protein.

Small molecule indicators (SMIs) are based on:

A EGTA

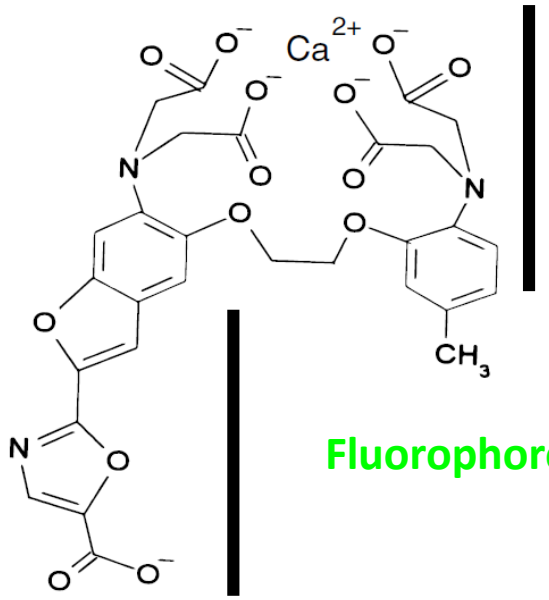


B BAPTA



EGTA and BATA are the 2 major exogenous Ca^{2+} buffers

C Fura-2



Ca^{2+}-chelating moiety

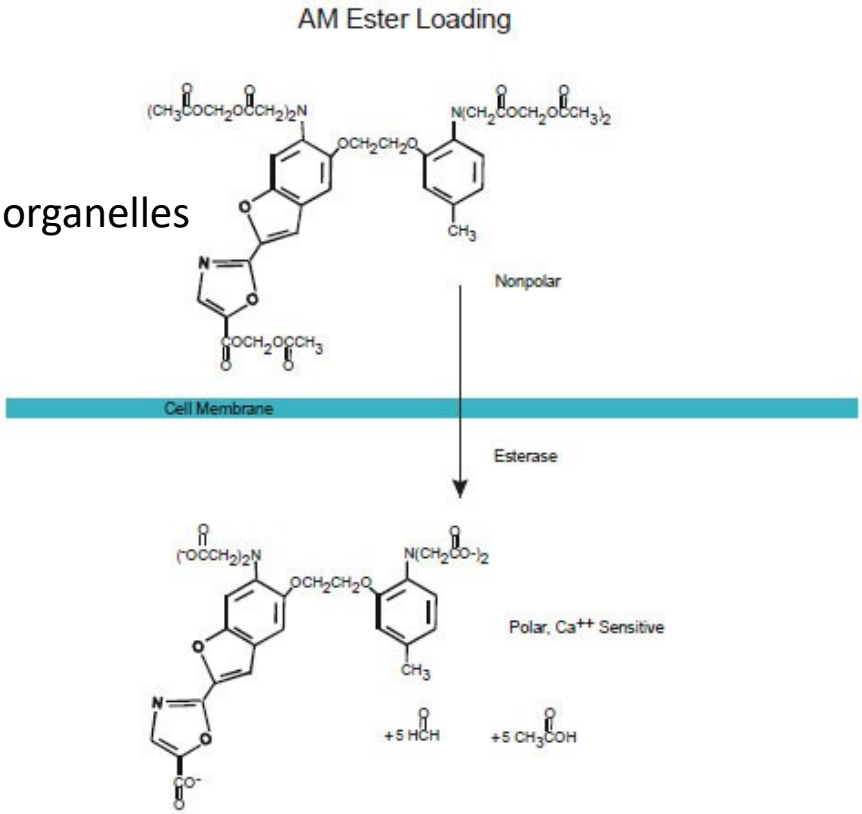
Fluorophore

Fura-2: an example of SMI based on BAPTA

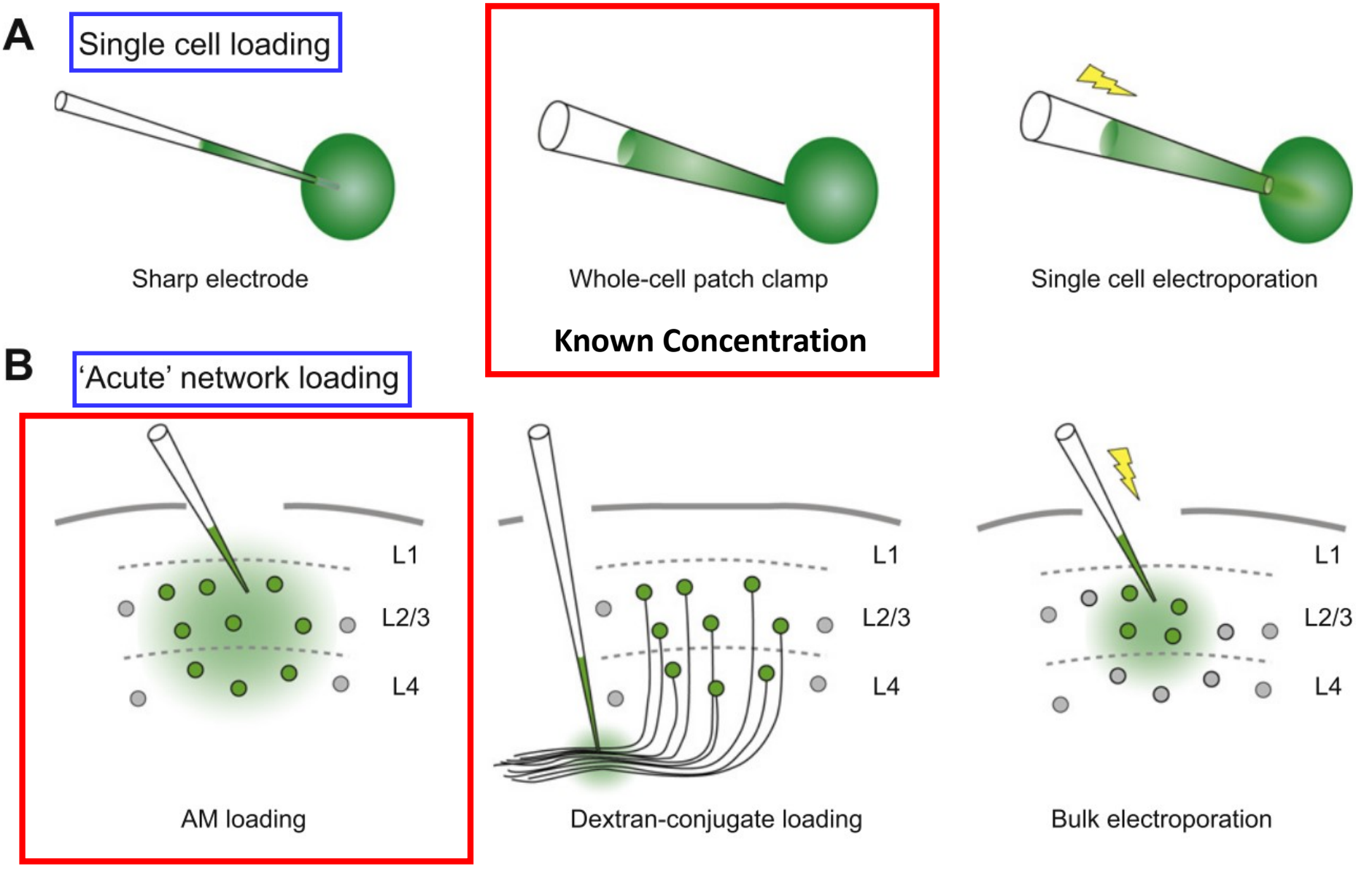
Small molecule indicators (SMIs)

- **SMIs are water soluble and diffuse readily through the cytosol**
  - ❑ Pros: you can know the concentration → important for quantitative estimation of $[Ca^{2+}]$
  - ❑ Cons: difficult to load, one cell at the time
- **Membrane permeable variants (acetoxymethyl ester (AM)-conjugated) have been developed**
  - ❑ Pros: easy to load
  - ❑ Cons: unknown concentration

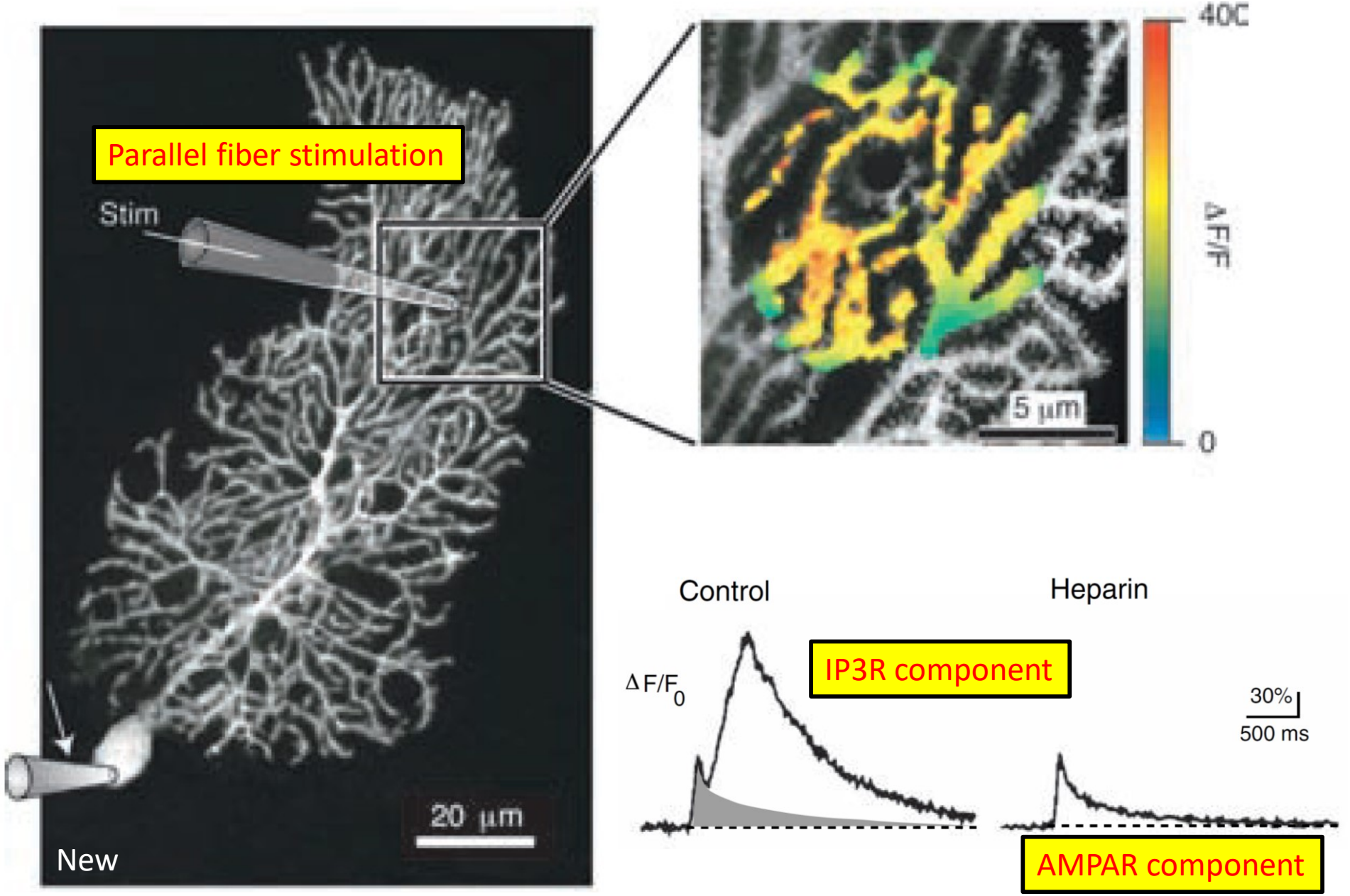
    They tend to accumulate in intracellular organelles



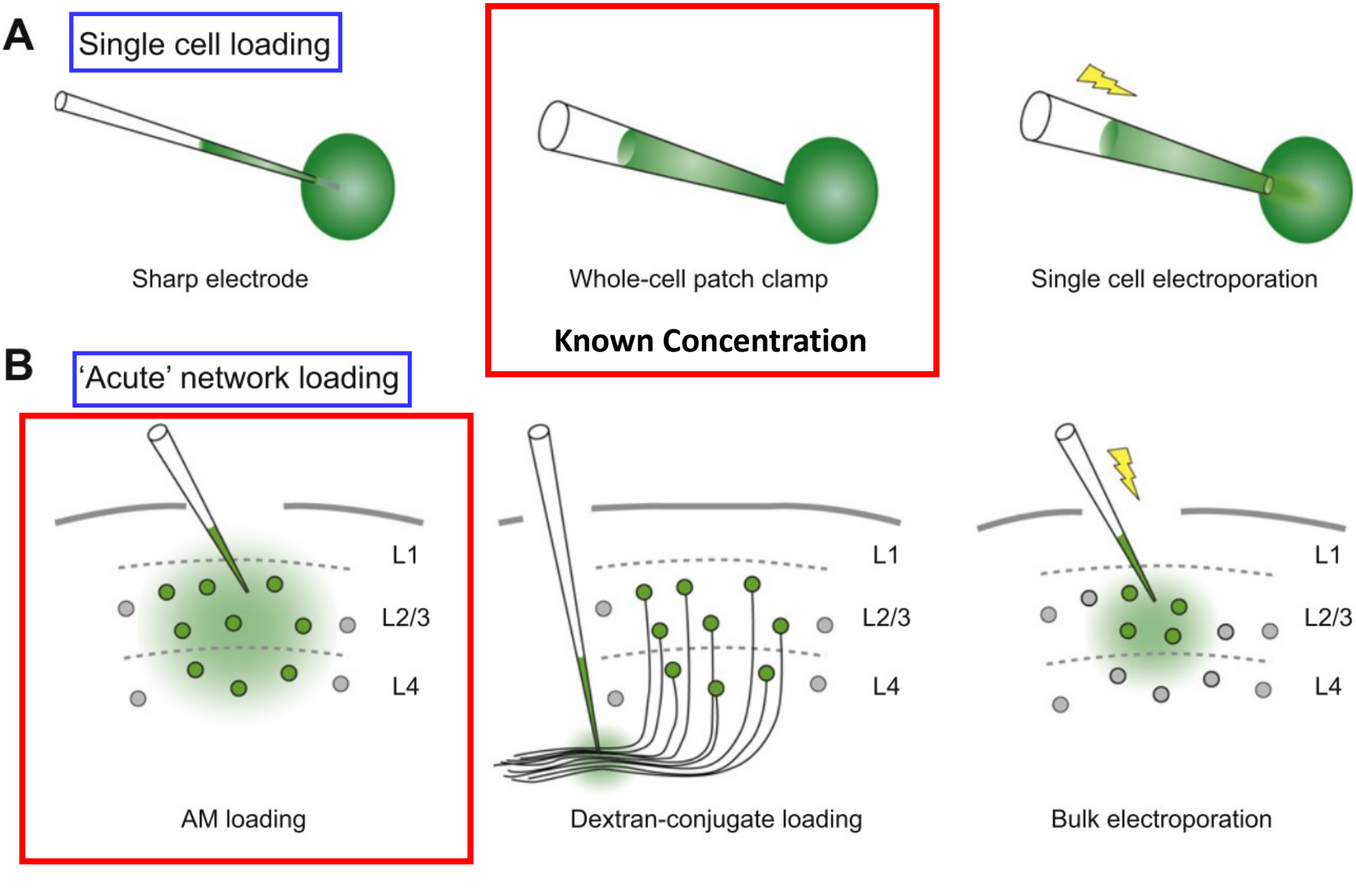
SMI loading


A
Single cell loading
Sharp electrode
Whole-cell patch clamp
Known Concentration
Single cell electroporation
B
'Acute' network loading
L1
L2/3
L4
AM loading
L1
L2/3
L4
Dextran-conjugate loading
L1
L2/3
L4
Bulk electroporation

mGluRs


Parallel fiber stimulation
Stim
20 µm
New
40
ΔF/F
0
5 µm
Control
Heparin
ΔF/F0
IP3R component
30%
500 ms
AMPAR component

SMI loading

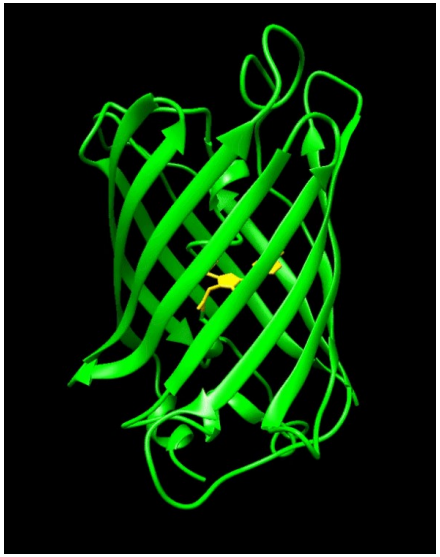

A
Single cell loading
Sharp electrode
Whole-cell patch clamp
Known Concentration
Single cell electroporation
B
'Acute' network loading
L1
L2/3
L4
AM loading
L1
L2/3
L4
Dextran-conjugate loading
L1
L2/3
L4
Bulk electroporation

Genetically-encoded Ca^{2+}-indicators

Most of them are based on EGFP

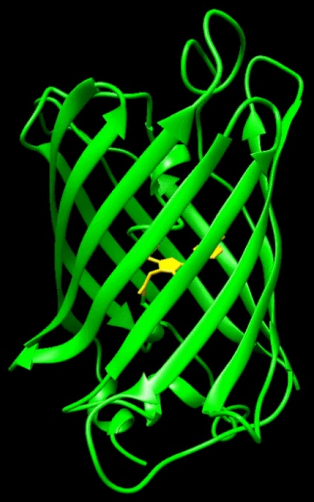
We can distinguish 2 major classes:

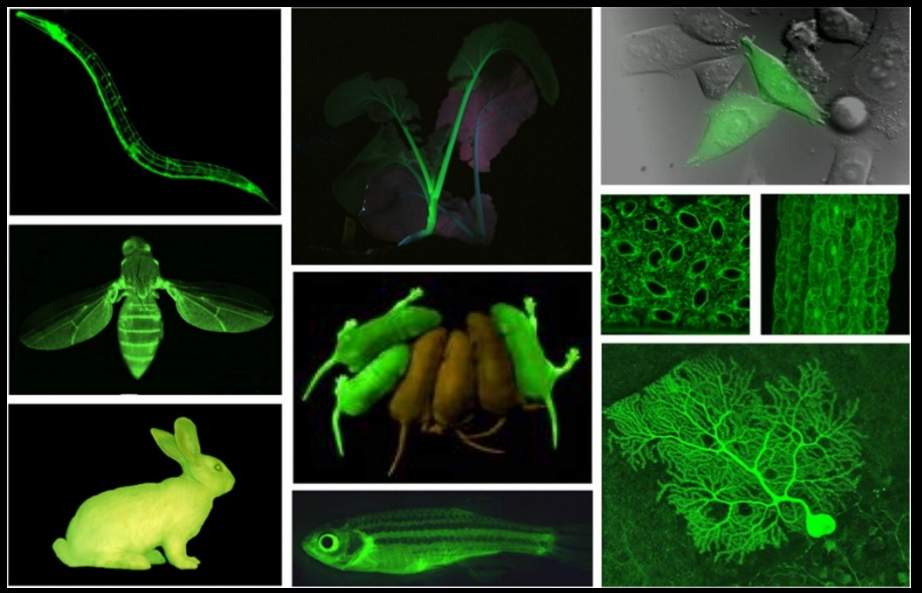
- **Fluorescence resonance energy transfer (FRET)-based GECIs (e.g. Cameleon family)**
- **Single-protein indicators (e.g. GCaMP family)**



An example: GFP (green fluorescent protein)

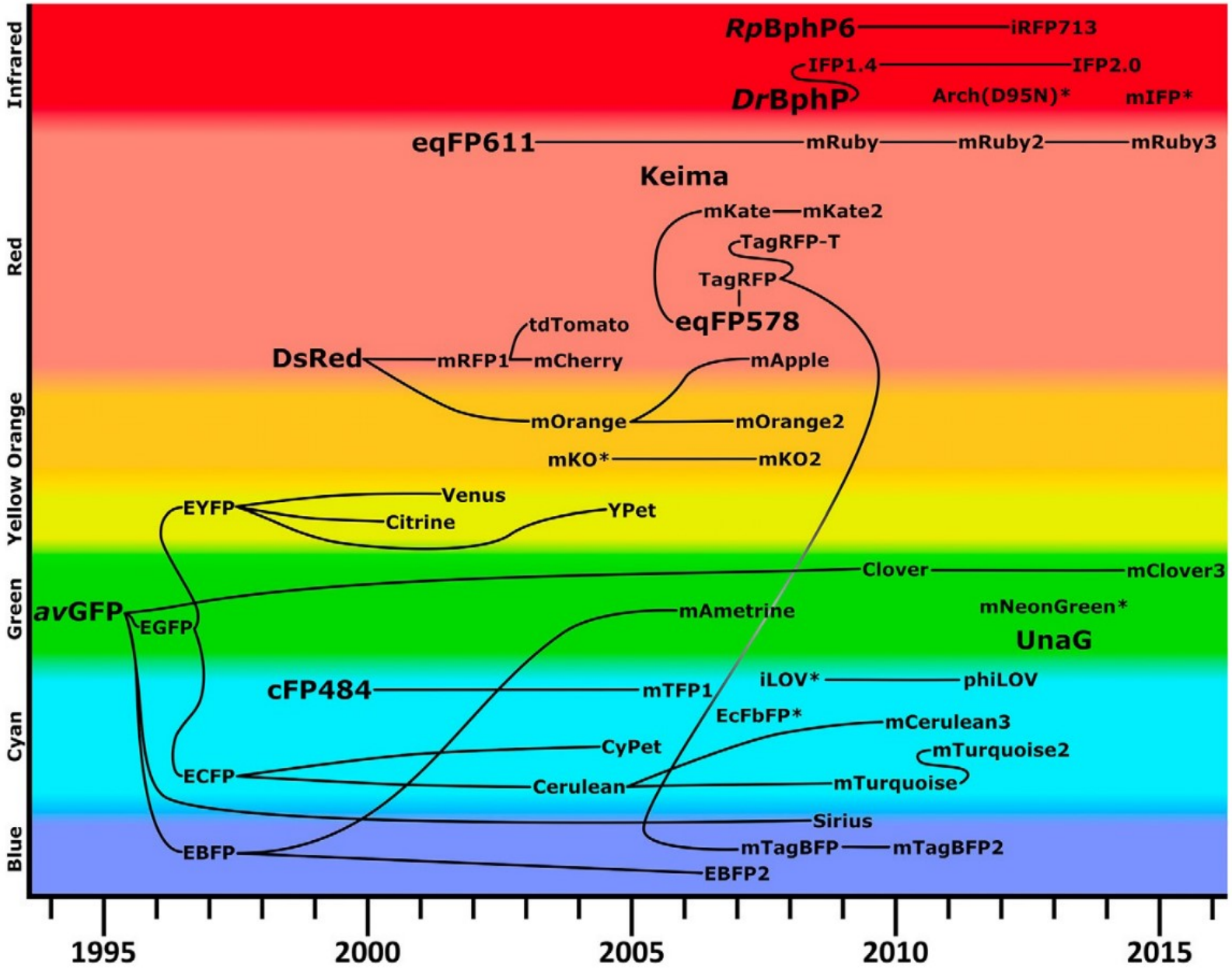
Aequorea victoria



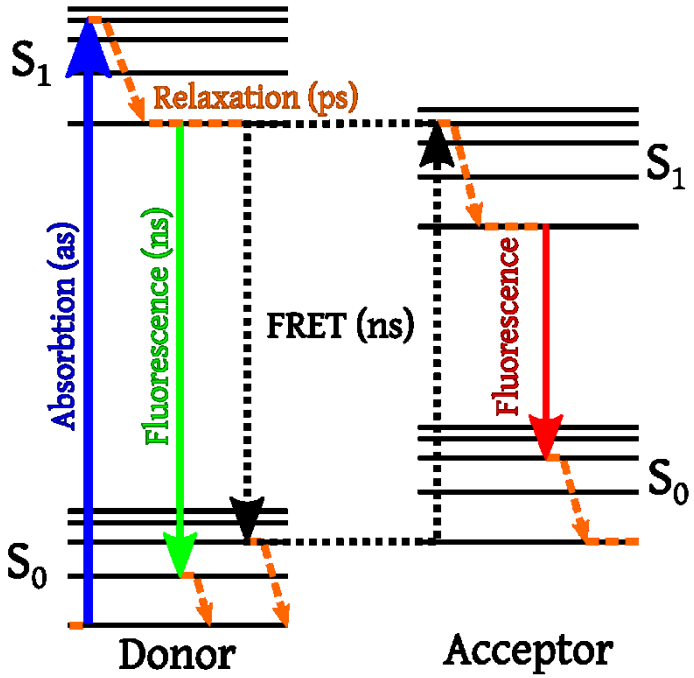


Nobel price 2008 for chemistry: Martin Chalfie , Osamiu Shimomura and Roger Tsien,

Fluorescent proteins

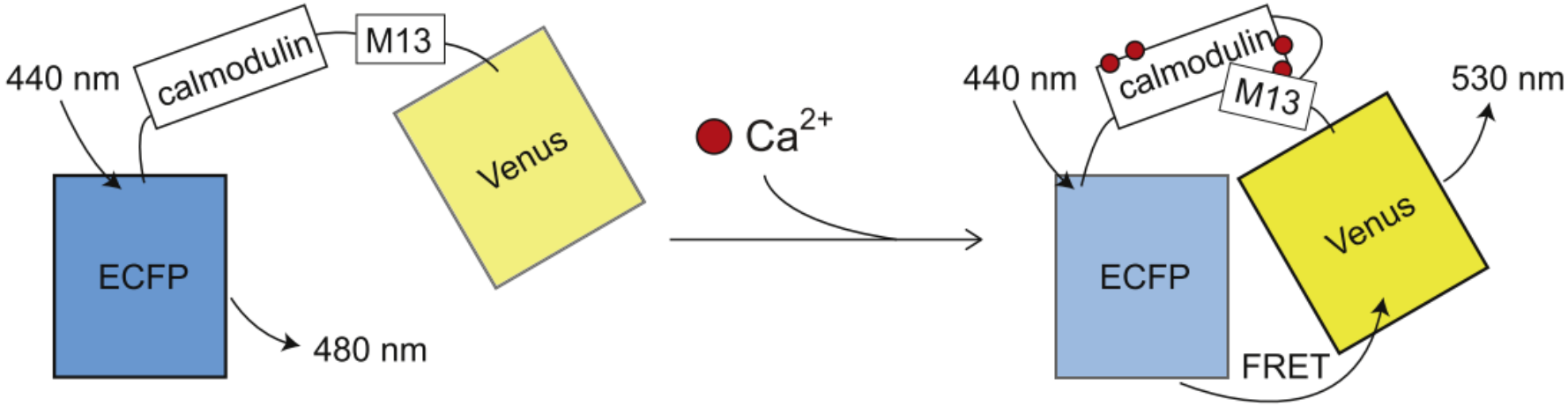


Fluorescence resonance energy transfer (FRET)-based GECIs



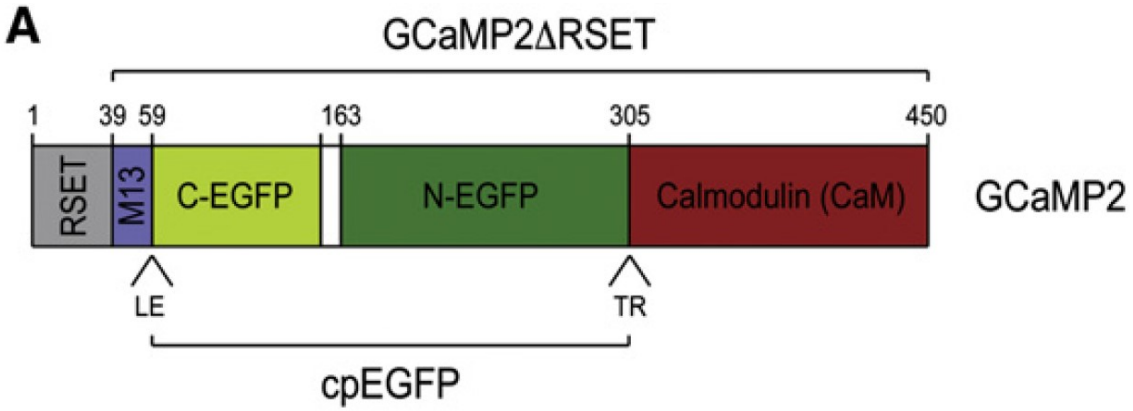
- The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making FRET extremely sensitive to small changes in distance
- Pros: ratiometric (we will come back to this)
- FRET has broad applications beyond Ca^{2+} imaging for example to detect protein-protein interactions, enzymatic reaction, protein cleavage, mechanical forces…

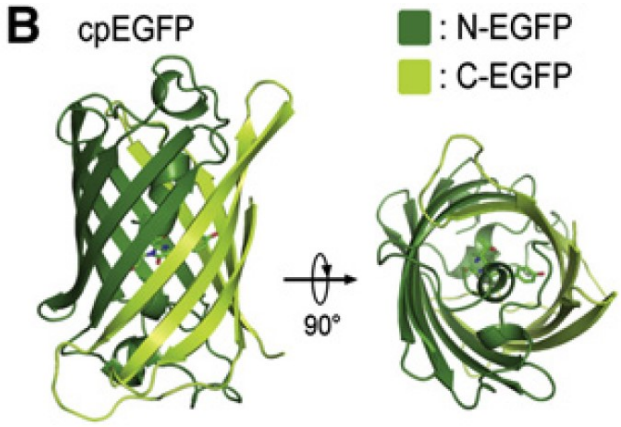
C FRET-based GECI

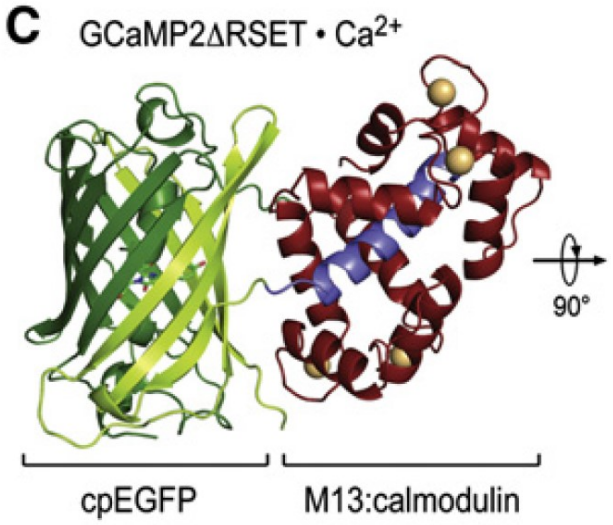


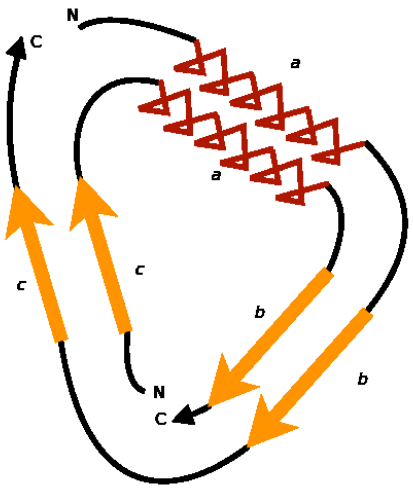
GCaMP family

Circularly permuted EGFP



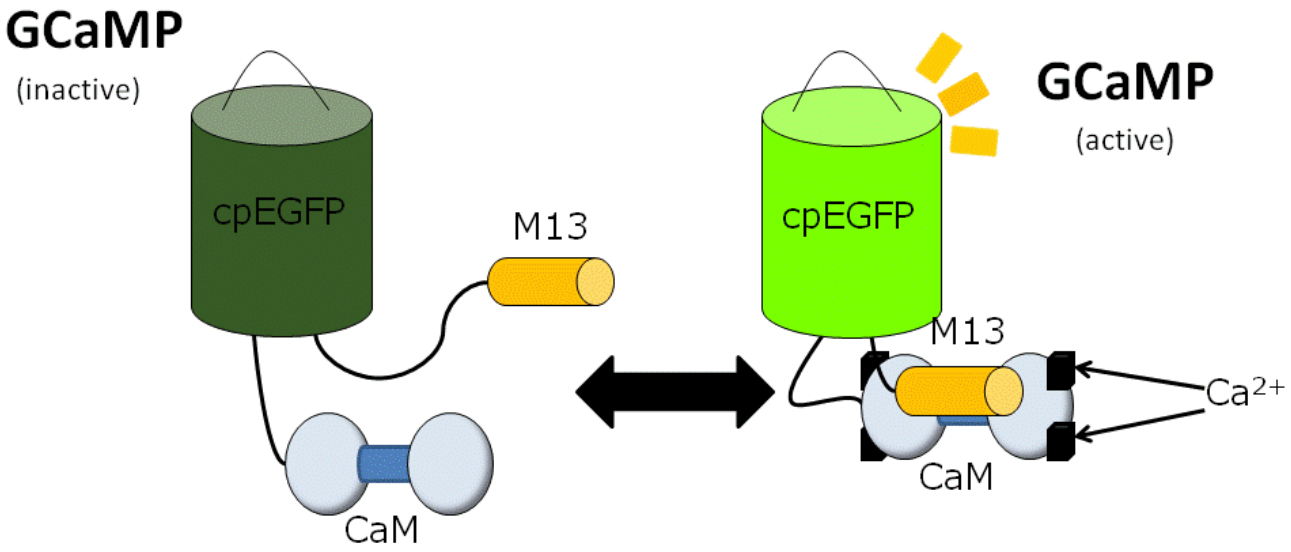


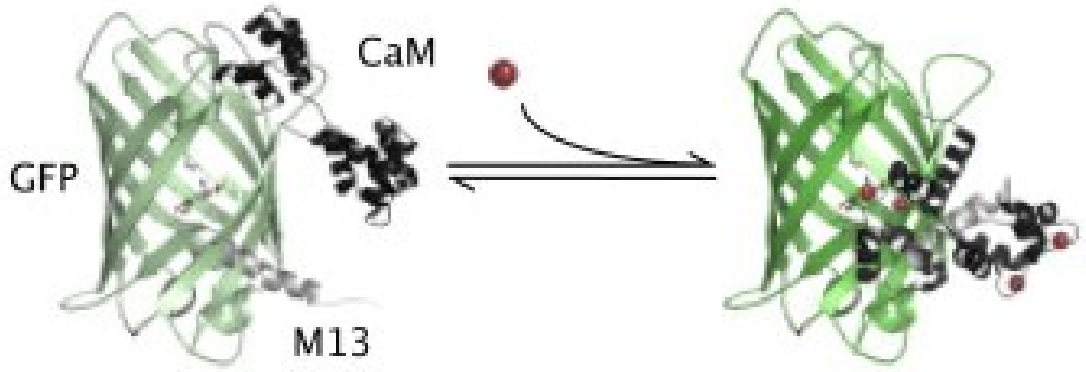




A **circular permutation** is a relationship between proteins whereby the proteins have a changed order of amino acids in their peptide sequence. The result is a protein structure with different connectivity, but overall similar 3D shape.

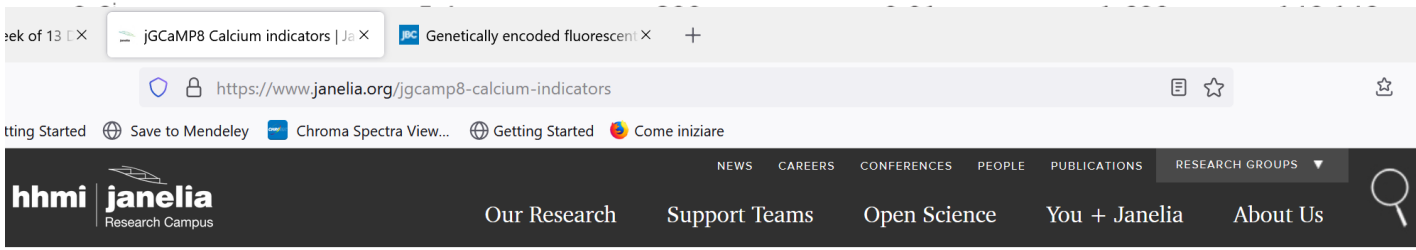
GCaMP family





Genetically-encoded Ca^{2+}-indicators

GECI	Maximum $\Delta F/F$ *in vitro*[a]	Ca^{2+}-free brightness (mM^{-1} cm^{-1})[b]	Ca^{2+}-saturated brightness (mM^{-1} cm^{-1})[b]	K_d *in vitro* (nM)[c]	$\Delta F/F$ per AP in tissue[d]	Half-decay rate in tissue (ms)[e]	Refs.
YC3.60	−0.66 (ECFP)	8.8[f]	3.1	780	−0.01	410	137,138
	+0.77 (cpVenus)	2.4[f]	11		+0.02		
YC3.60 3GS	−0.66 (ECFP)	8.8[g]	3.1	140	−0.01	470	139,140
	+0.77 (cpVenus)	2.4[g]	11		+0.01		
D3cpV	−0.46 (ECFP)	7.3[h]	3.6	530	−0.03	9,500	141,142
	+1.1 (cpVenus)	4.8[h]	10		+0.02		
TN-XXL	−0.5 (ECFP)						
	+1.0 (cpCitrine)						
Twitch-2B	−0.77 (mCerulean3)						
	+0.87 (cpVenus)						
GCaMP3	+12						
GCaMP5k	+9.4						
GCaMP6f	+52						
GCaMP6s	+63						
R-CaMP2	+4.8						
jRGECO1a	+11						
jRCaMP1b	+6.2						



jGCaMP8 Calcium indicators

Ultra-sensitive protein calcium sensors with fast kinetics

The Looger Lab and the GENIE Project Team at HHMI Janelia have developed a new suite of jGCaMP8 calcium indicators, built on the GCaMP scaffold. The jGCaMP8 sensors have fast kinetics without compromising sensitivity, setting a new standard for *in vivo* imaging. Sensors that have been extensively tested in mammalian neurons *in vivo* and *in vitro* are:

- **jGCaMP8f** (fast): 4x faster rise time, 2.5x faster decay time than jGCaMP7f
- **jGCaMP8m** (medium): almost 4x faster rise time and 3.5x more sensitive than jGCaMP7f
- **jGCaMP8s** (sensitive): 2x more sensitive than jGCaMP7s, >2x faster than jGCaMP7f (at 1 AP)

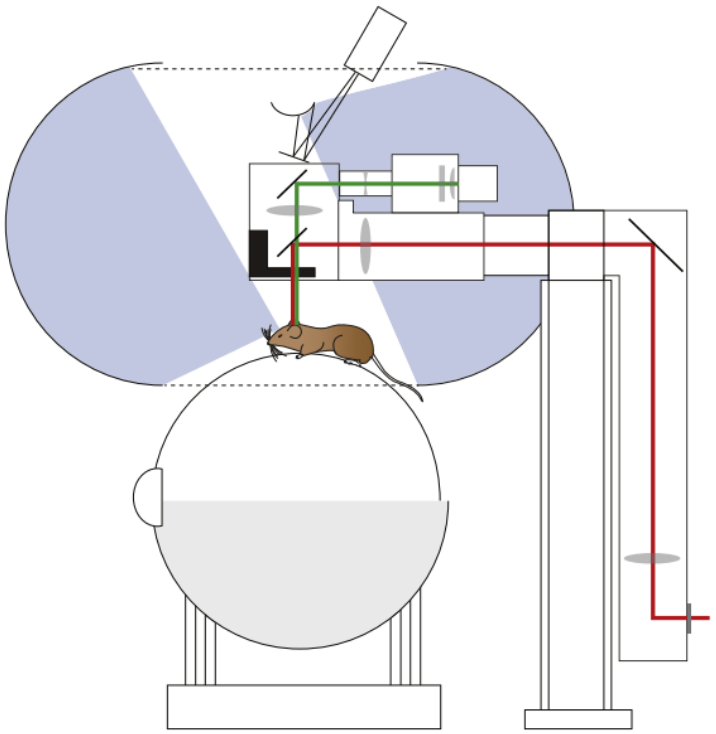
The plasmids are available on Addgene!

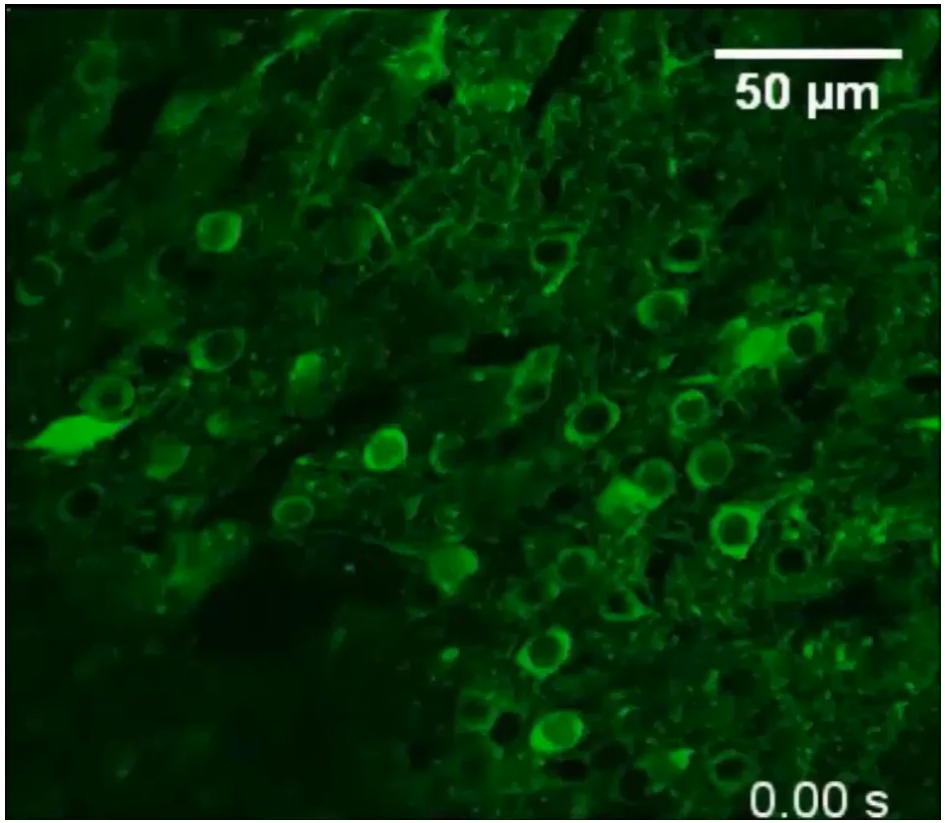
Update (6/23/2021):

jGCaMP8-expressing flies are now available from The Bloomington Drosophila Stock Center. Search for "jGCaMP8" to see all available strains. See results of testing in Drosophila below.

Pros and Cons of GECIs

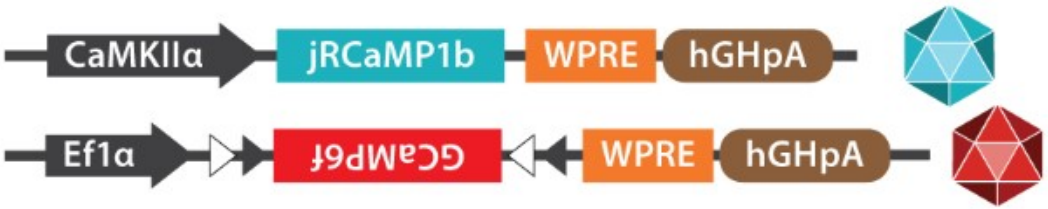
1) Long-term (days, weeks, months) expression and imaging *in vivo*

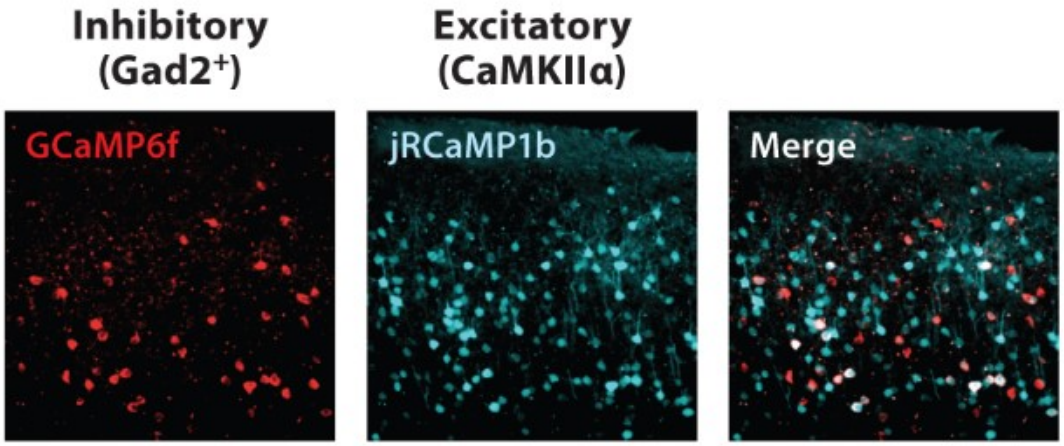


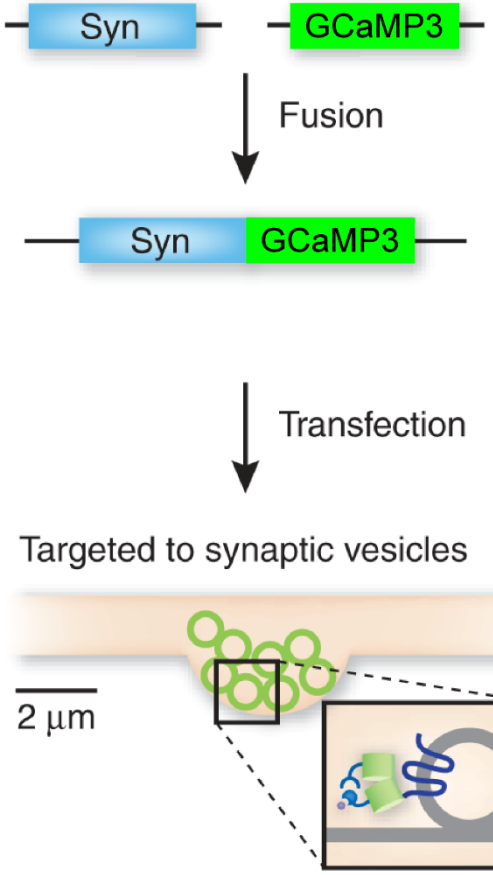


Pros and Cons of GECIs

2) Targeting to (i) specific subtypes of neurons or (ii) subcellular locations



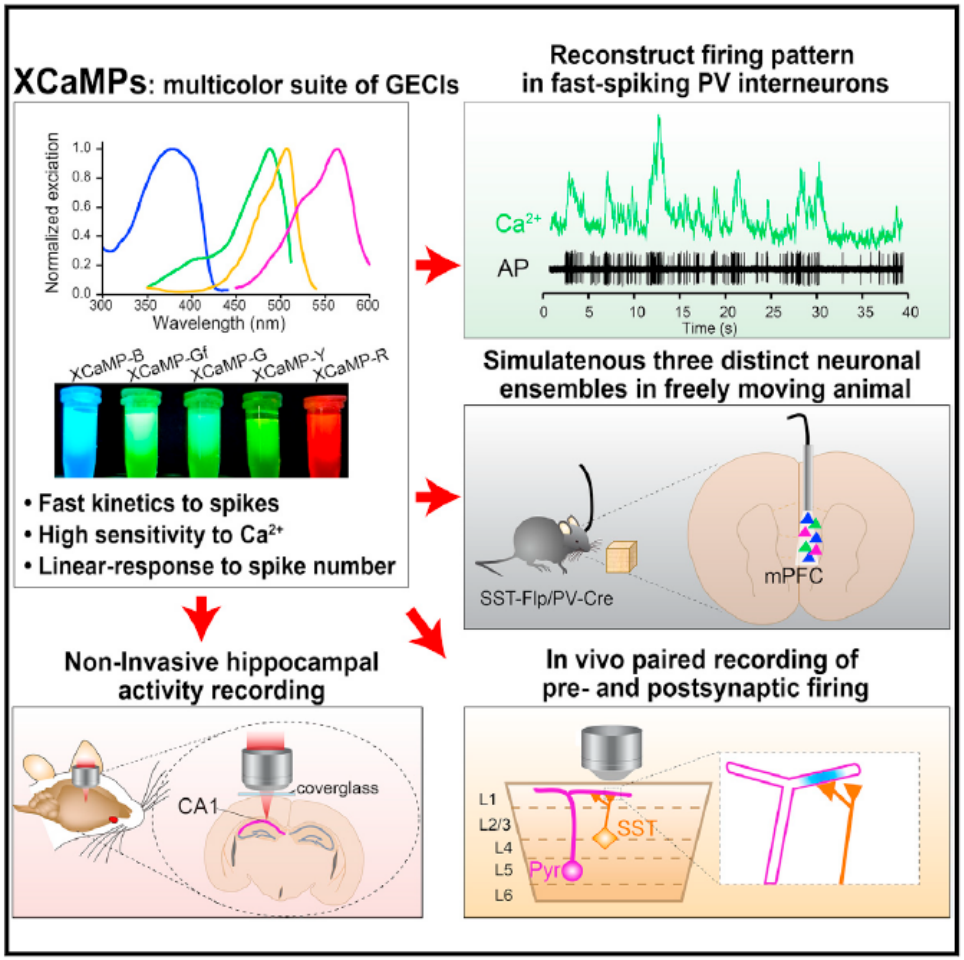




Cell

Rational Engineering of XCaMPs, a Multicolor GECI Suite for *In Vivo* Imaging of Complex Brain Circuit Dynamics

Graphical Abstract



Authors

Masatoshi Inoue, Atsuya Takeuchi, Satoshi Manita, ..., Karl Deisseroth, Kazuo Kitamura, Haruhiko Bito

Correspondence

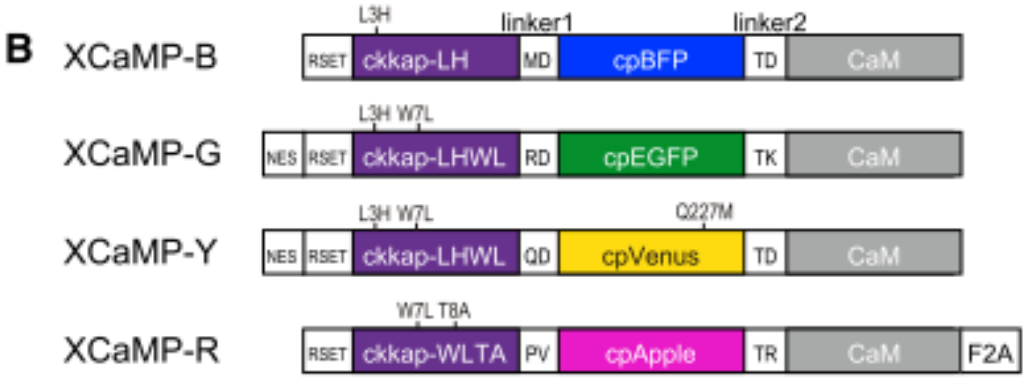
hbito@m.u-tokyo.ac.jp

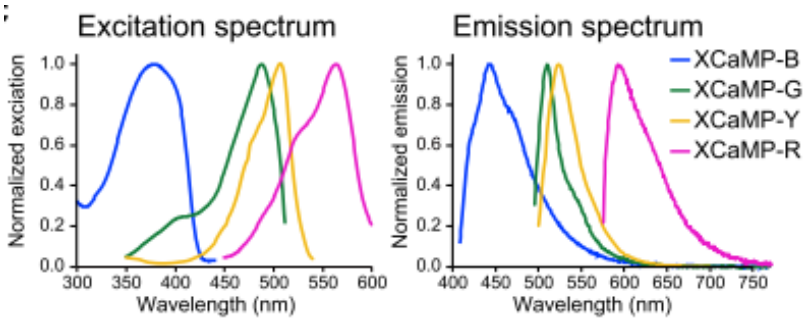
In Brief

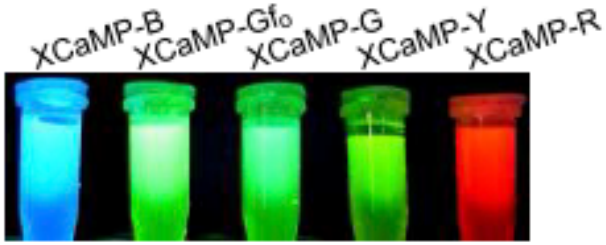
Quadricolor suite of genetically encoded calcium indicators for multiplex recording in the brain.

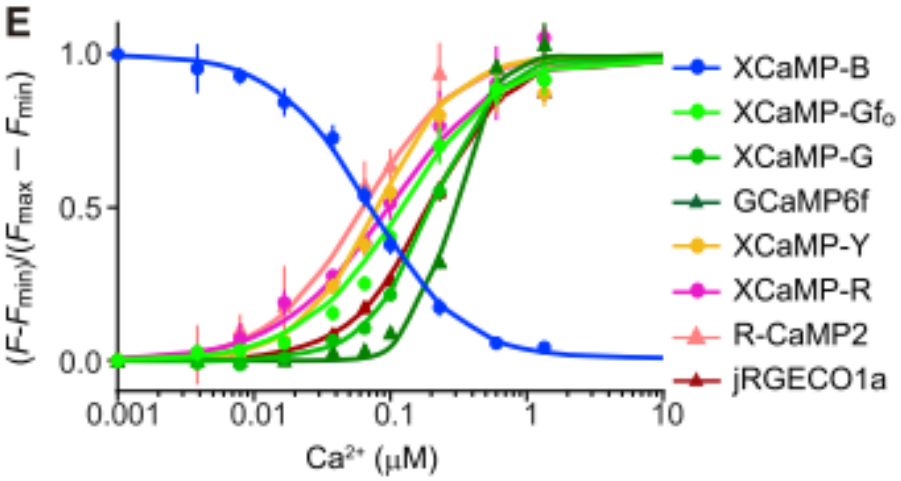
https://doi.org/10.1016/j.cell.2019.04.007

Multicolor XCamPs

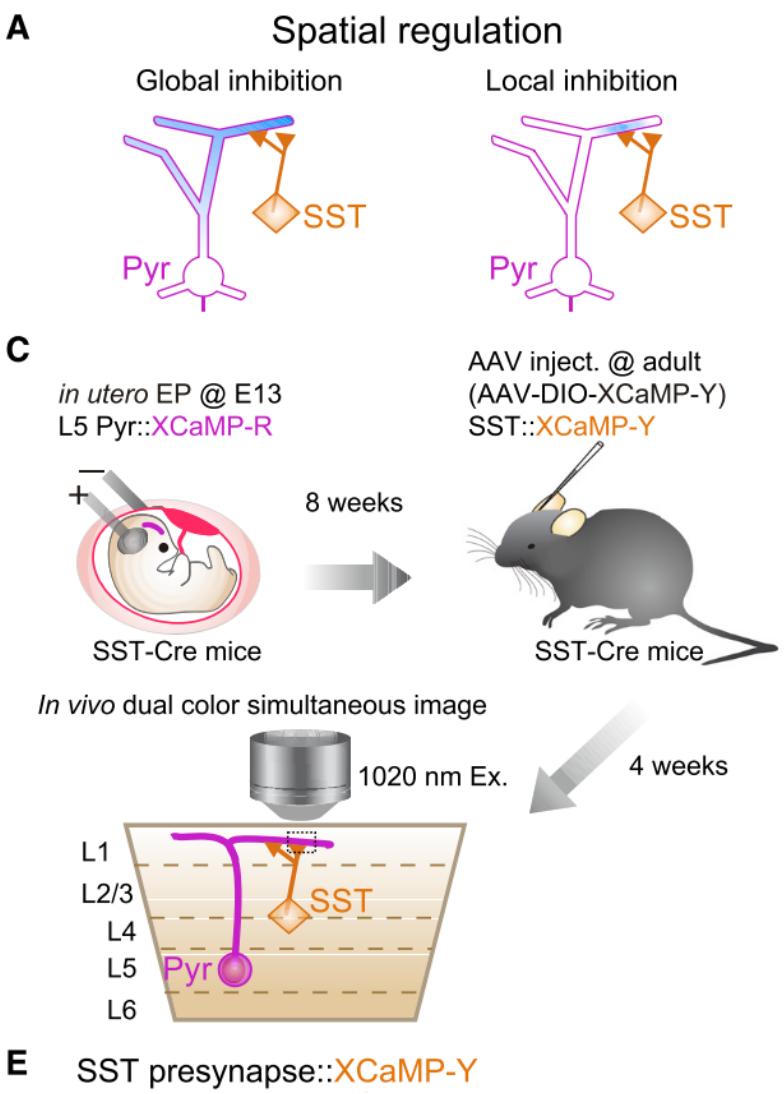


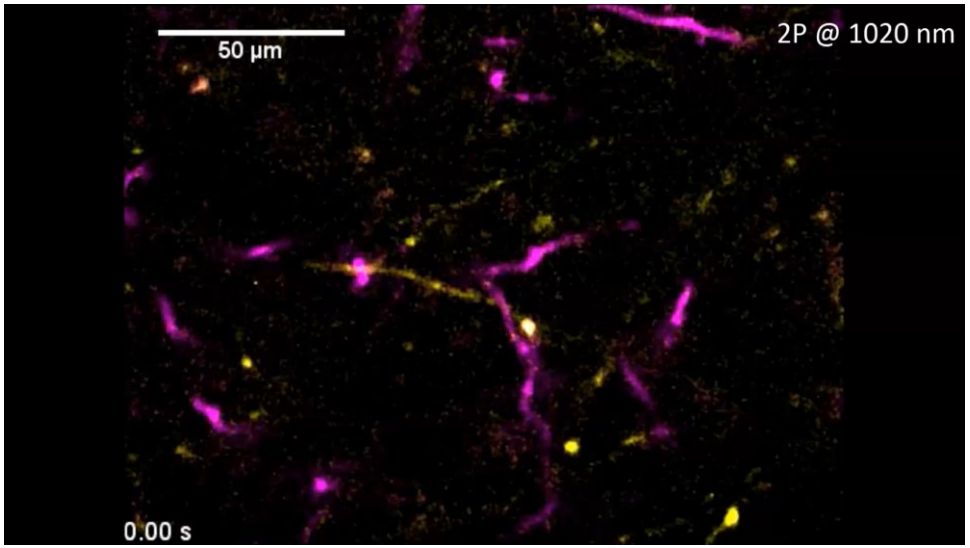


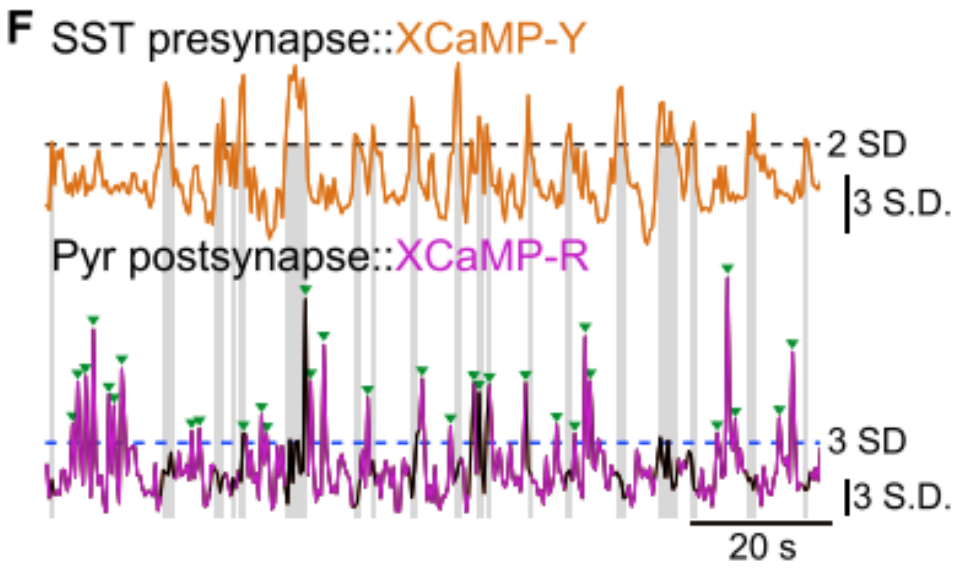




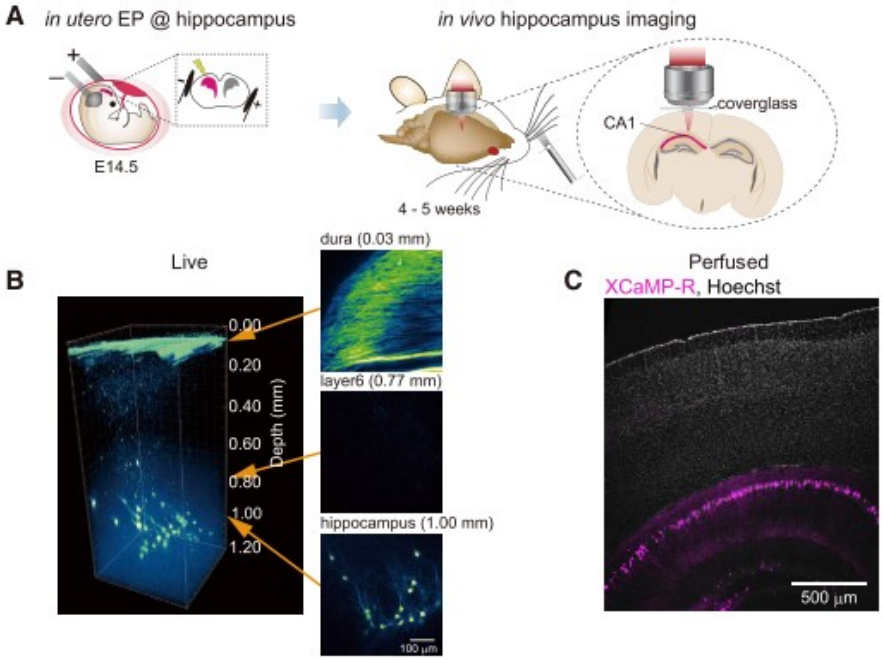
Dual color simultaneous imaging of axons and dendrites in the barrel cortex







Can we image deep brain structures non-invasively?



Fluorescence imaging with longer wavelength has the advantage of minimizing both the scattering of excitation light and the absorption of emission when compared to green probes

Pros and Cons of GECIs

1) Indicator concentration not known

2) Because (often) based on calmodulin (4 cooperative Ca^{2+} binding sites) → Ca^{2+} binding is cooperative

Difficult to relate ΔF to Δ[Ca^{2+}]

Conclusions

1. Ca^{2+} indicators are fluorescent Ca^{2+} indicators

2. Small molecule indicators (SMIs)
   a. Based on EGTA/BAPTA
   b. Soluble or membrane permeable
   c. Pros: They bind 1 Ca^{2+}
      For the soluble ones, we know the concentration

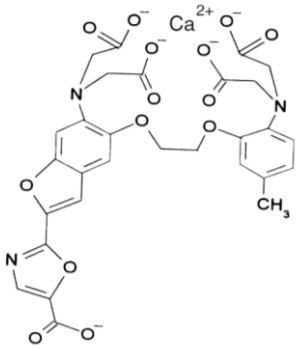
   } Quantitative

3. Genetically-encoded Ca^{2+} indicators (GECIs)
   a. Based on EGFP or other fluorescent proteins
   b. The most commonly used are those based on circularly permuted fluorescent proteins (e.g. GCaMPs)
   c. Pros: Long-term *in vivo* experiments
      Cell-type specific expression (e.g. excitatory vs. inhibitory neurons)
      Specific subcellular locations (e.g. presynaptic bouton)

Ca^{2+} imaging

- Review Ca^{2+} signaling
- Ca^{2+} indicators
- **Ca^{2+} binding**
  - **Dissociation constant**
  - **Calcium binding ratio**
  - **Cooperative binding**
- **Ca^{2+} diffusion**
- **Ca^{2+}-dependent fluorescence properties**
- **Simplified models of Ca^{2+} dynamics**
- **Imaging devises**
- **What can we do with it**

Independent Ca^{2+} binding



The indicator binds 1 Ca^{2+}

(Alternatively it binds multiple Ca^{2+} but it does so <u>independently</u>, <u>non-cooperatively</u>)

$$\mathrm{Ca}^{2+} + \mathrm{B} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \mathrm{CaB}$$

Temporal dynamics described

K_{on} association rate ($mM^{-1}\ s^{-1}$)

K_{off} dissociation rate (s^{-1})

$$\frac{\partial\,[\mathrm{Ca}]}{\partial t} = -k_{on}\,[\mathrm{Ca}]\,[\mathrm{B}] + k_{off}\,[\mathrm{CaB}]$$

$$\frac{\partial\,[\mathrm{B}]}{\partial t} = -k_{on}\,[\mathrm{Ca}]\,[\mathrm{B}] + k_{off}\,[\mathrm{CaB}]$$

$$\frac{\partial\,[\mathrm{CaB}]}{\partial\,t} = -\frac{\partial\,[\mathrm{B}]}{\partial t} = k_{on}\,[\mathrm{Ca}]\,[\mathrm{B}] - k_{off}\,[\mathrm{CaB}]$$

Independent Ca^{2+} binding

$$Ca^{2+} + B \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} CaB$$

At steady state, once equilibrium has been reached:

$$0 = -k_{on}\,[Ca]\,[B] + k_{off}\,[CaB]$$

Law of mass action

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[Ca]\,[B]}{[CaB]} = \frac{[Ca]\,([B]_T - [CaB])}{[CaB]}$$

$$[B]_T = [B] + [CaB]$$

$$S = \frac{[CaB]}{[B]_T} = \frac{[Ca]}{[Ca] + K_d}$$

Independent Ca^{2+} binding

$$Ca^{2+} + B \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} CaB$$

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[Ca]\,[B]}{[CaB]}$$

$$S = \frac{[CaB]}{[B]_T} = \frac{[Ca]}{[Ca] + K_d}$$

K_d = Dissociation constant. **When $[Ca^{2+}] = K_d$ 50% of the buffer is saturated.**

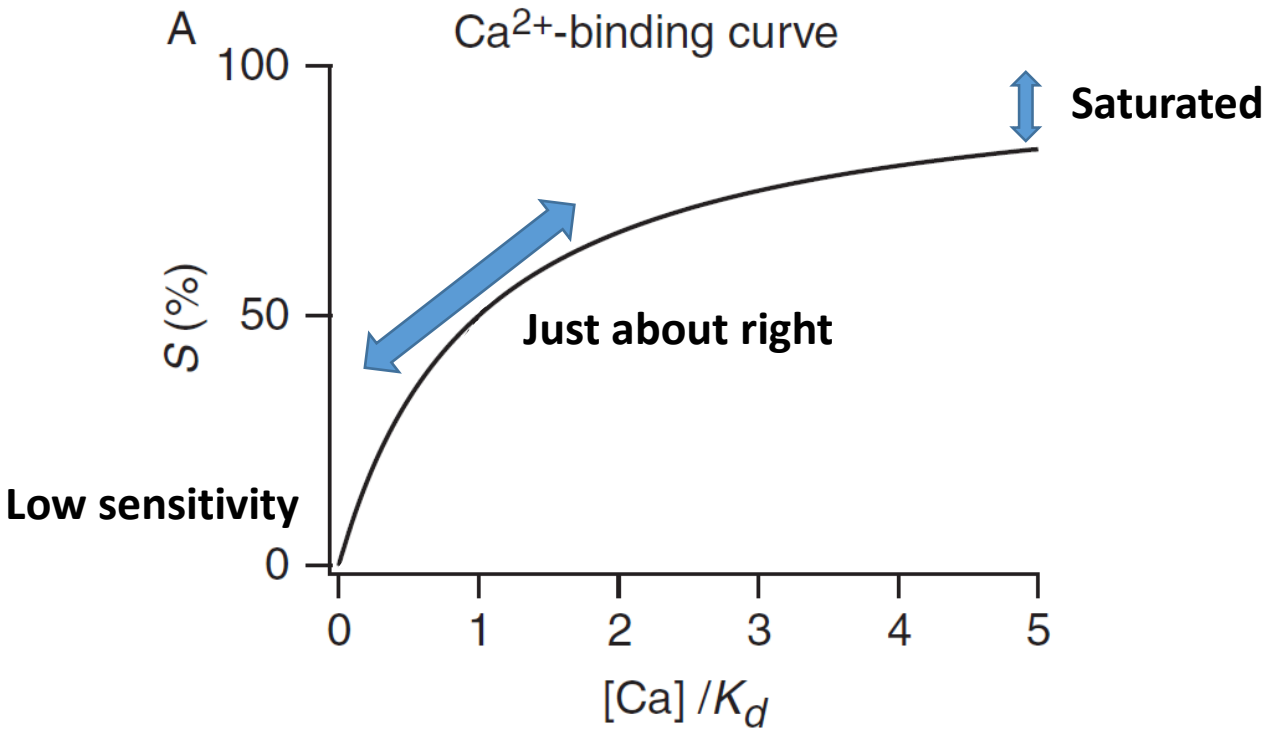
Attention: **Low K_d = high affinity** **High K_d = low affinity**

The affinity of the indicator should match the expected range of $[Ca^{2+}]$ 'seen' by the indicator

Affinity too low (K_d high) → not enough sensitivity **Affinity too high (K_d low) → saturation**

Independent Ca^{2+} binding

$$S = \frac{[\mathrm{CaB}]}{[\mathrm{B}]_T} = \frac{[\mathrm{Ca}]}{[\mathrm{Ca}] + K_d}$$



An example: Fluo family of Ca^{2+} indicators

https://www.thermofisher.com/it/en/home/references/molecular-p

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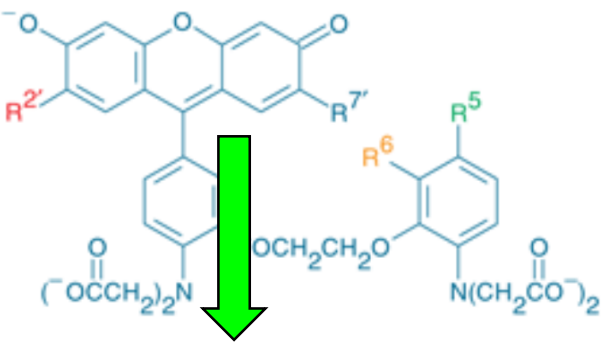
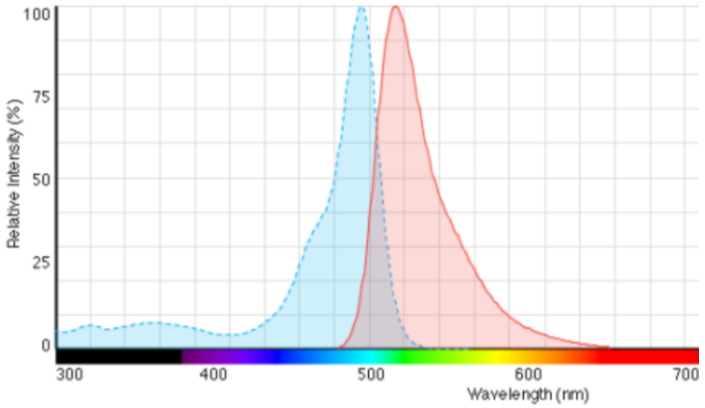
Technical Notes and Product Highlights



Indicator	$K_d(Ca^{2+})$	$R^{2'}$	$R^{7'}$	R^5	R^6
Fluo-3	0.39 µM	Cl	Cl	CH_3	H
Fluo-4	0.35 µM	F	F	CH_3	H
Fluo-5F	2.3 µM	F	F	F	H
Fluo-5N	90 µM	F	F	NO_2	H
Fluo-4FF	9.7 µM	F	F	F	F

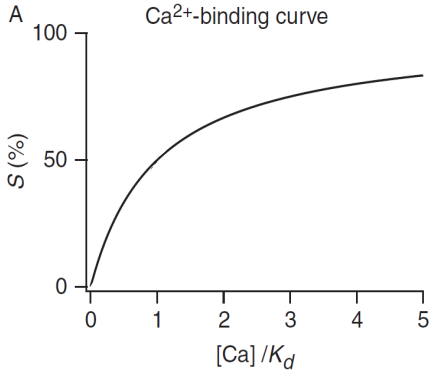
Figure 19.3.1 Fluo indicators.



Ca^{2+}-binding ratio

Buffering capacity = buffering strength = Ca^{2+}-binding efficiency = Ca^{2+}-binding ratio = κ_B

While K_d is an intrinsic property of the buffer, κ_B depends on intrinsic properties of B (K_d), on [B] and $[Ca^{2+}]$



$$Ca^{2+} + B \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} CaB$$

$$S = \frac{[CaB]}{[B]_T} = \frac{[Ca]}{[Ca] + K_d}$$

$$[CaB] = \frac{[B]_T\,[Ca]}{[Ca] + K_d}$$

Differentiate relative to $[Ca^{2+}]$

$$\kappa_B = \frac{\partial[CaB]}{\partial[Ca]} = \frac{[B]_T K_d}{([Ca] + K_d)^2}$$

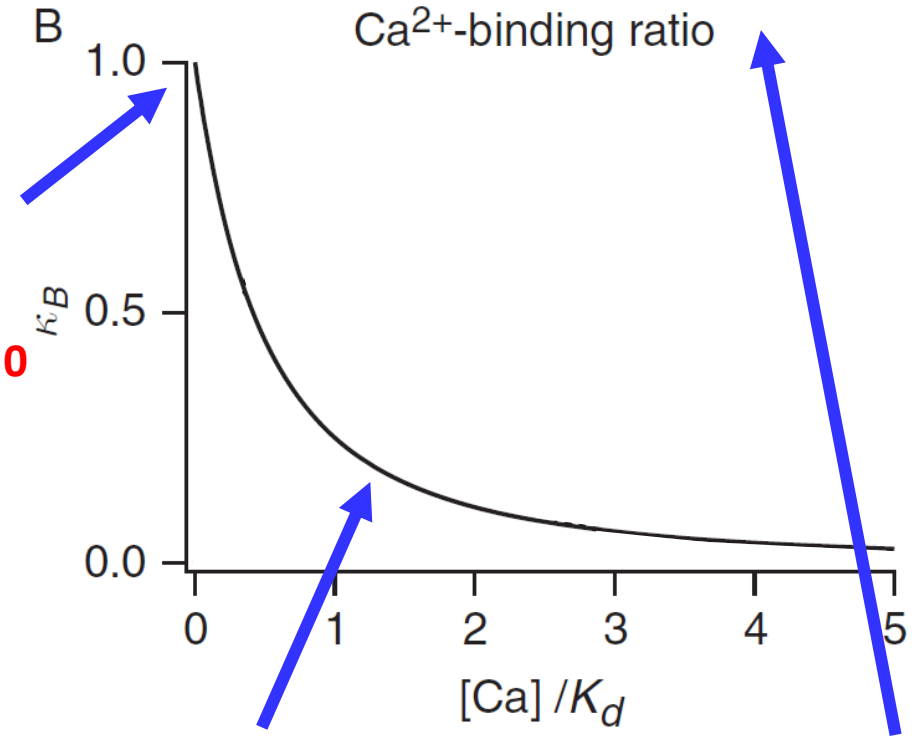
κ_B = how fast [CaB] changes relative to $\Delta[Ca^{2+}]$

= capacity of absorbing extra Ca^{2+}

= Ca^{2+} buffering capacity

Ca^{2+}-binding ratio

$$\kappa_B = \frac{\partial[\mathrm{CaB}]}{\partial[\mathrm{Ca}]} = \frac{[\mathrm{B}]_T K_d}{([\mathrm{Ca}] + K_d)^2}$$



$[Ca^{2+}] = 0 \rightarrow \kappa_B = \frac{[B]_T}{K_d}$

Buffering capacity is maximal at $[Ca^{2+}] = 0$

(it makes sense!)

κ_B decreases when $[Ca^{2+}]$ increases according to the 2 power of $[Ca^{2+}]$

Buffering capacity decreases quickly the more Ca^{2+} you give to B

(This also makes sense!)

Trends in Neurosciences

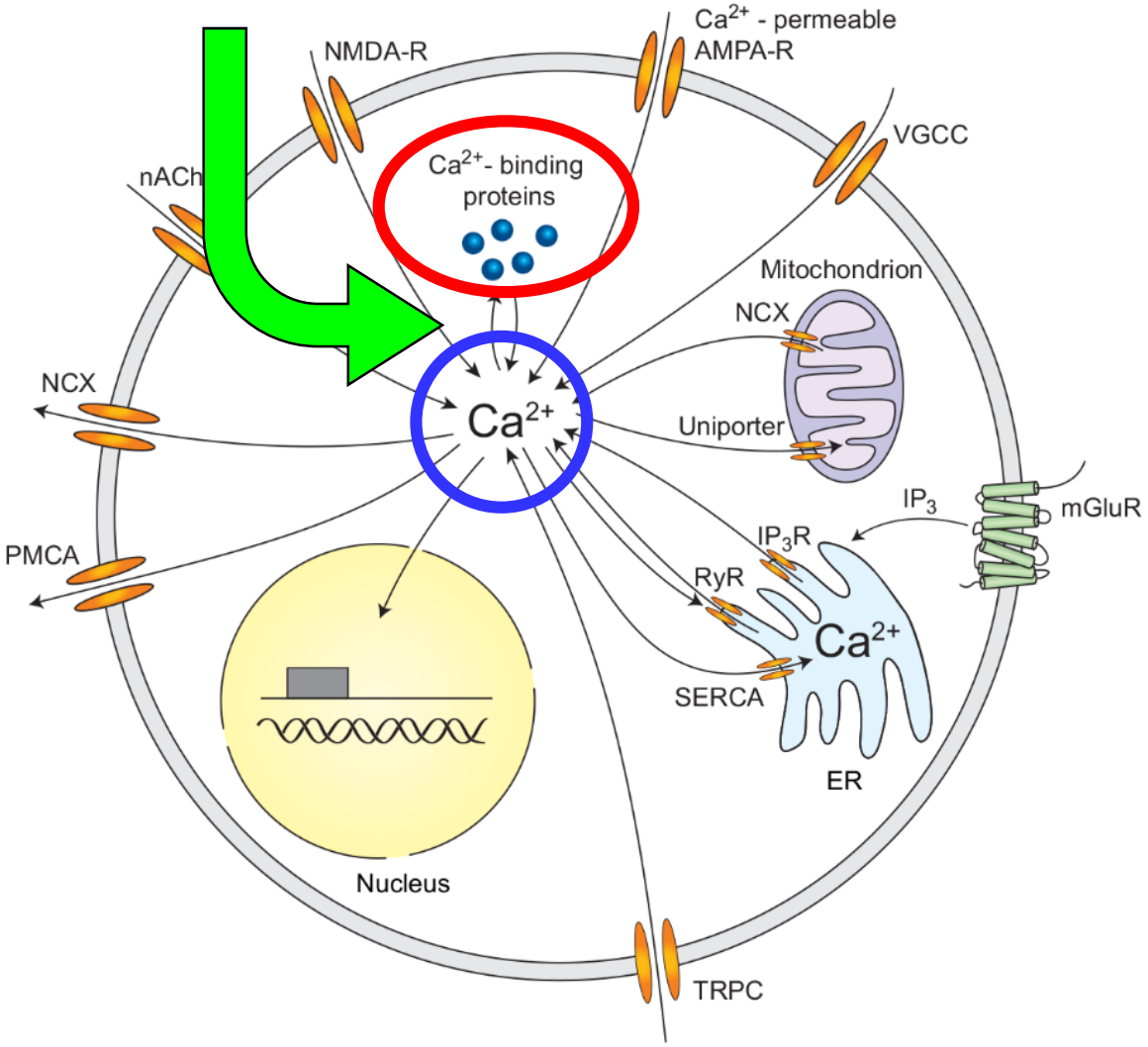
CellPress
REVIEWS

Opinion

An Inconvenient Truth: Calcium Sensors Are Calcium Buffers

Shane M. McMahon[1] and Meyer B. Jackson[1,*]

Exogenous vs. endogenous Ca^{2+}-binding ratio

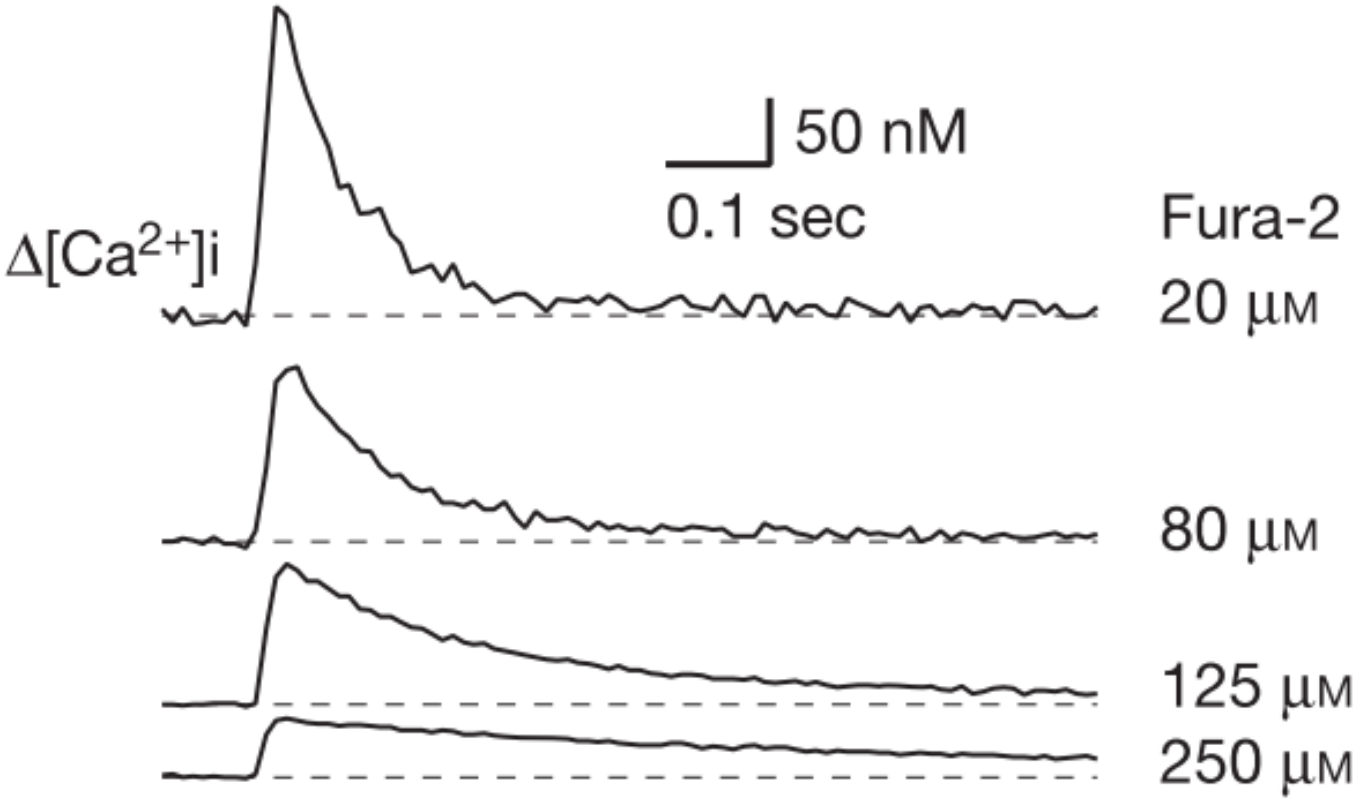


At any given moment, free $[Ca^{2+}]_i$ is determined by the balance between

- **Ca^{2+} influx**
- **Ca^{2+} efflux**
- **Ca^{2+} exchange with internal stores**
- **Ca^{2+} sequestration by endogenous buffers (Ca^{2+} -binding proteins)**

It is important to have a good estimation of κ_B of the added fluorescent indicator relative to the κ_B of the endogenous buffers, in order not to alter (too much) the free Ca^{2+} signal.

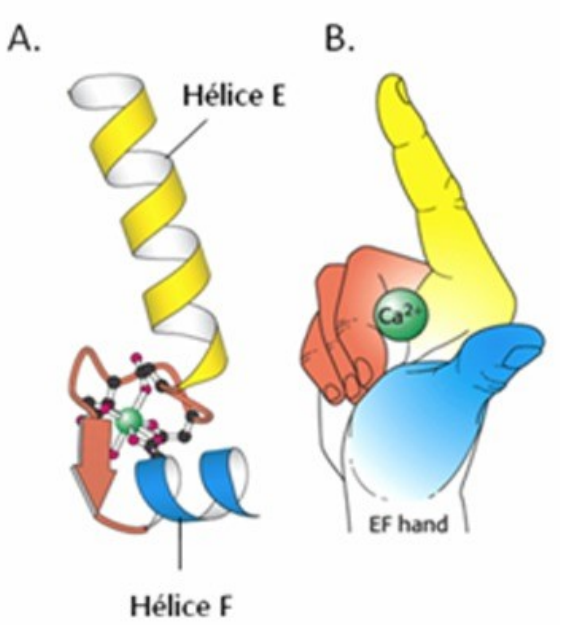
An added exogenous κ_B larger than the endogenous κ_B of the neuron deforms the very same signal we wish to measure

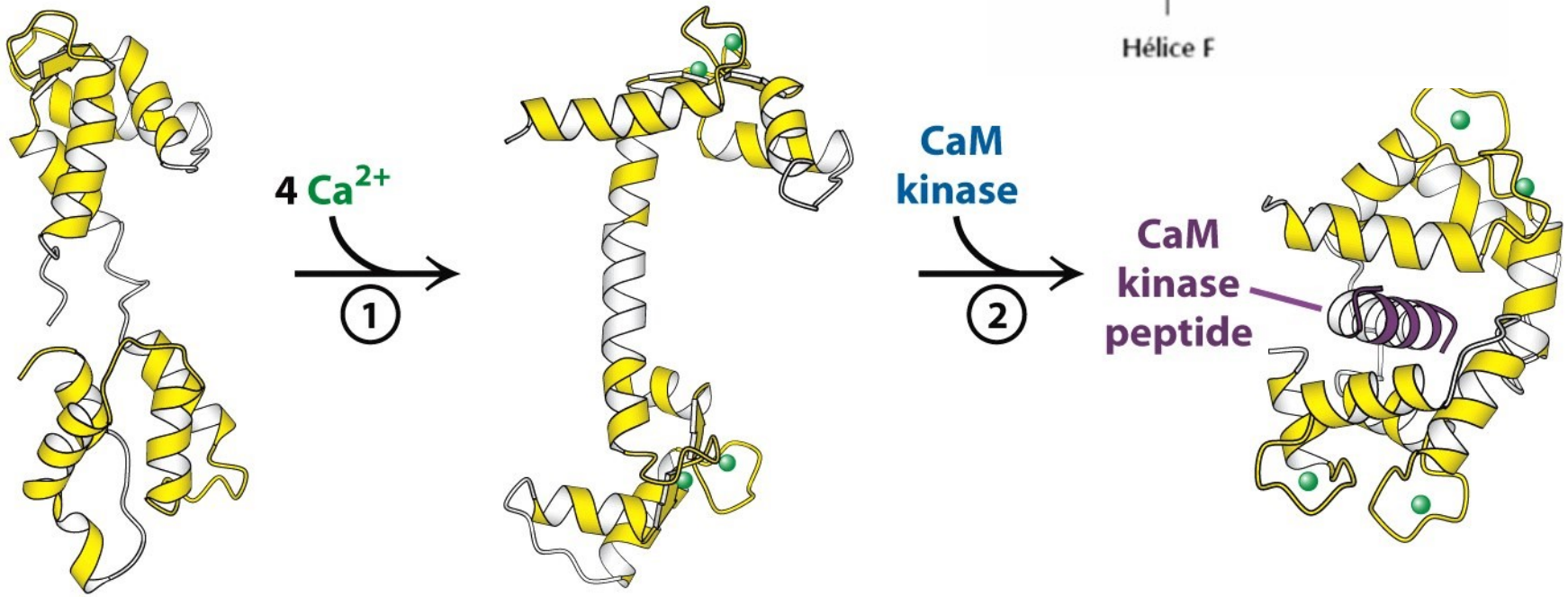


It is important to have a good estimation of κ_B of the added fluorescent indicator relative to the κ_B of the endogenous buffers, in order not to alter (too much) the free Ca^{2+} signal.

Many GECIs exhibit however cooperative Ca^{2+} binding

because many of them are based on Calmodulin





Cooperative Ca^{2+} binding

The buffer binds x Ca^{2+} non-independently, cooperatively (for calmodulin x = 4)

$$x\,Ca^{2+} + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} Ca^{x}B$$

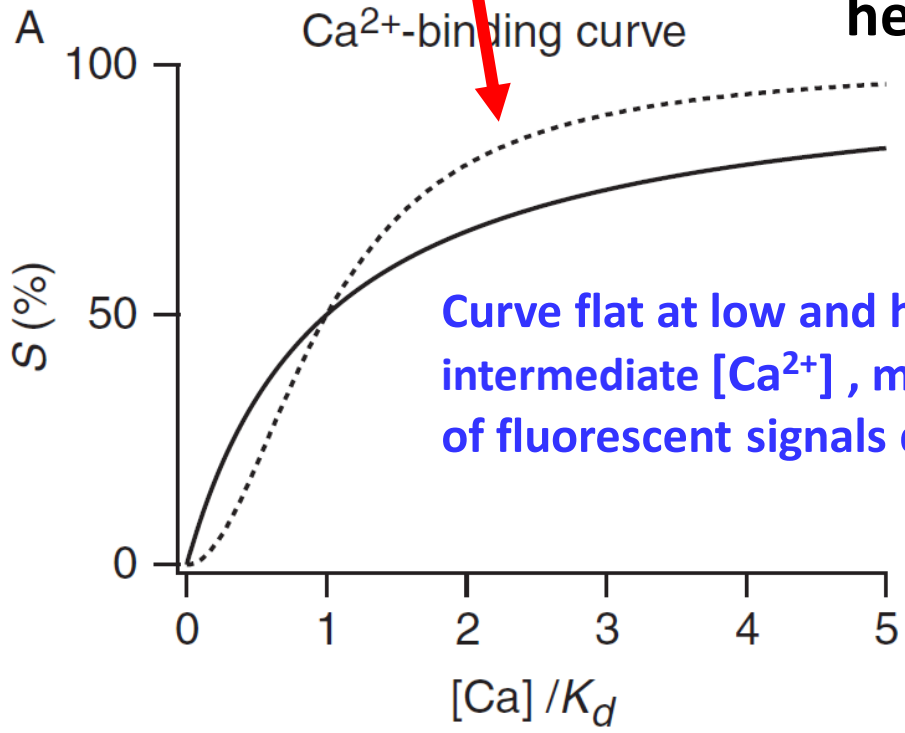
$$K_A = \frac{[Ca]^n[B]}{[Ca^nB]}$$

n = Hill coefficient (it is not the number of Ca^{2+} binding sites, but an empirical parameter describing cooperativity; generally ≤ binding sites)

K_A = apparent dissociation constant = K_d^n

Cooperative Ca^{2+} binding

$$S = \frac{[Ca^n B]}{[B]_T} = \frac{[Ca]^n}{[Ca]^n + K_A} = \frac{[Ca]^n}{[Ca]^n + K_d^n}$$



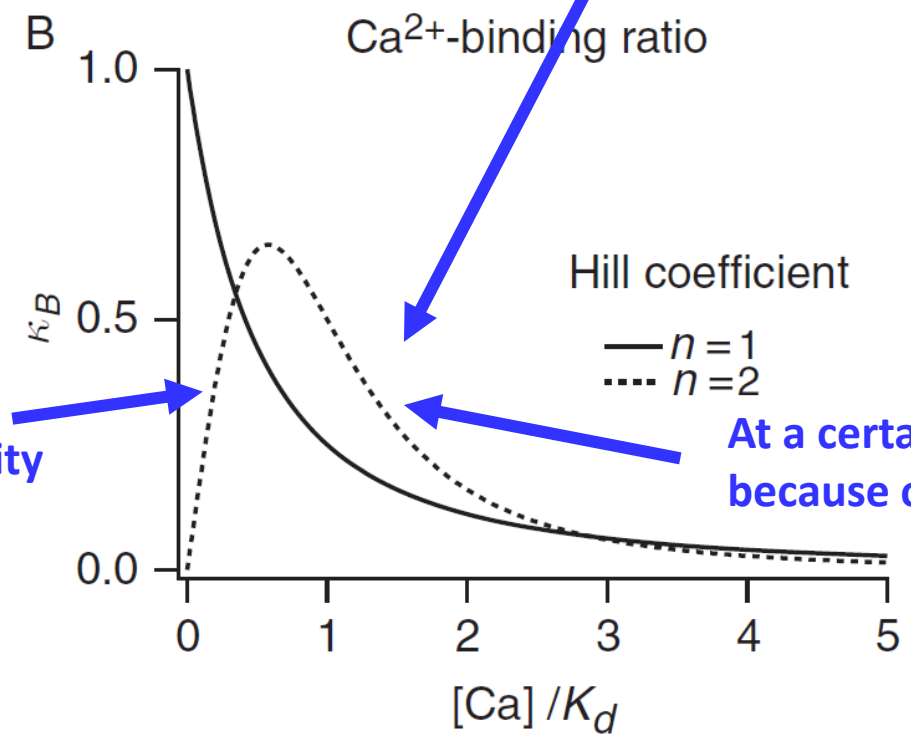
here n = 2

Curve flat at low and high $[Ca^{2+}]$, and steep at intermediate $[Ca^{2+}]$, making interpretation of fluorescent signals difficult

(the $[Ca^{2+}]$ at half-maximal occupancy is K_d , not $K_A = K_d^n$)

Cooperative Ca^{2+} binding

$$\kappa_B = \frac{\partial \left[\mathrm{Ca}^n \mathrm{B}\right]}{\partial \left[\mathrm{Ca}\right]} = [\mathrm{B}]_T \frac{n\,[\mathrm{Ca}]^{n-1}\,K_d^n}{([\mathrm{Ca}]^n + K_d^n)^2}$$

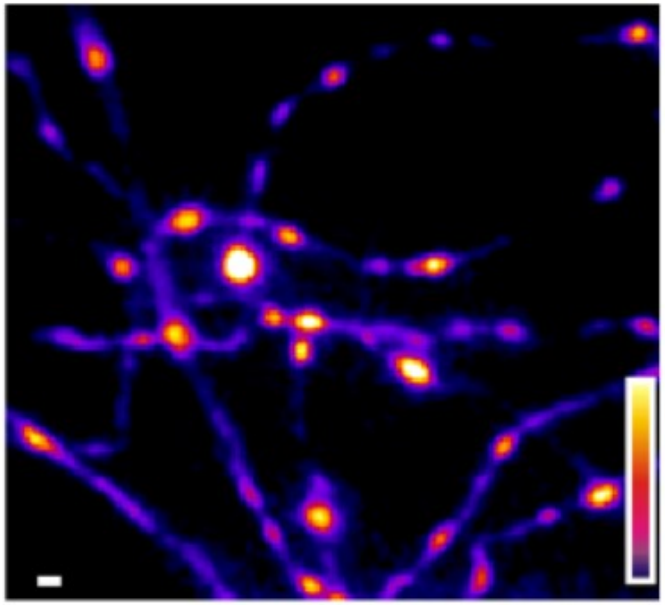


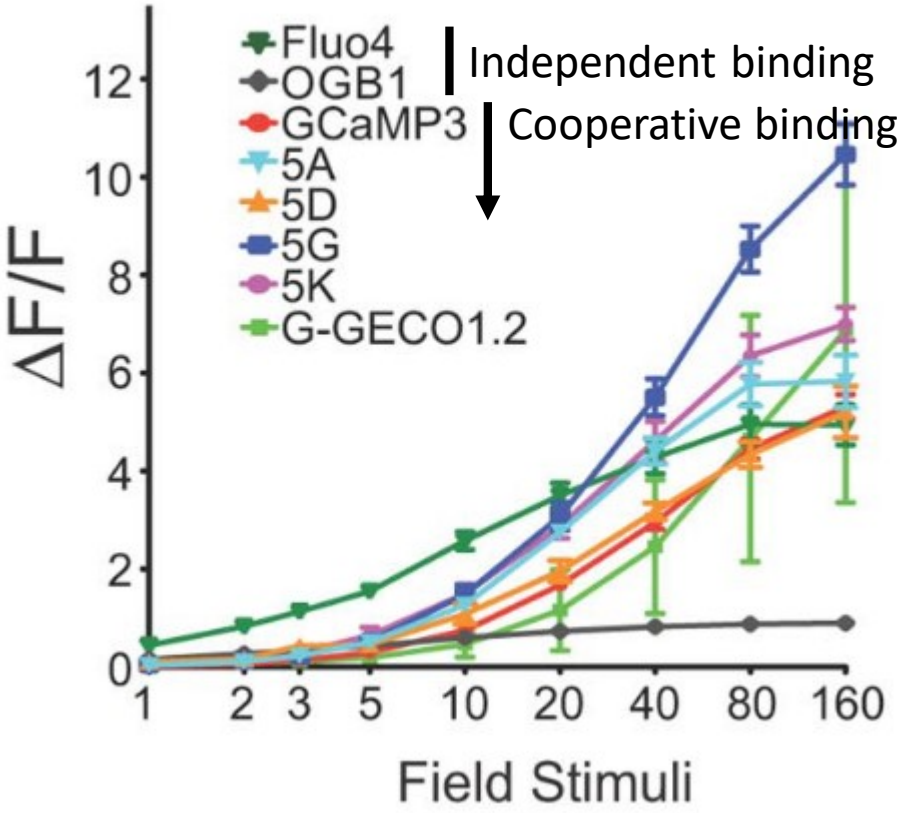
κ_B initially increases because of cooperativity binding

At a certain point, κ_B decreases because of saturation

Relationship non-monotonic, making interpretation of fluorescent signals extremely difficult

Cooperative Ca^{2+} binding





Cooperative binding makes it difficult to correlate fluorescence to [Ca^{2+}]

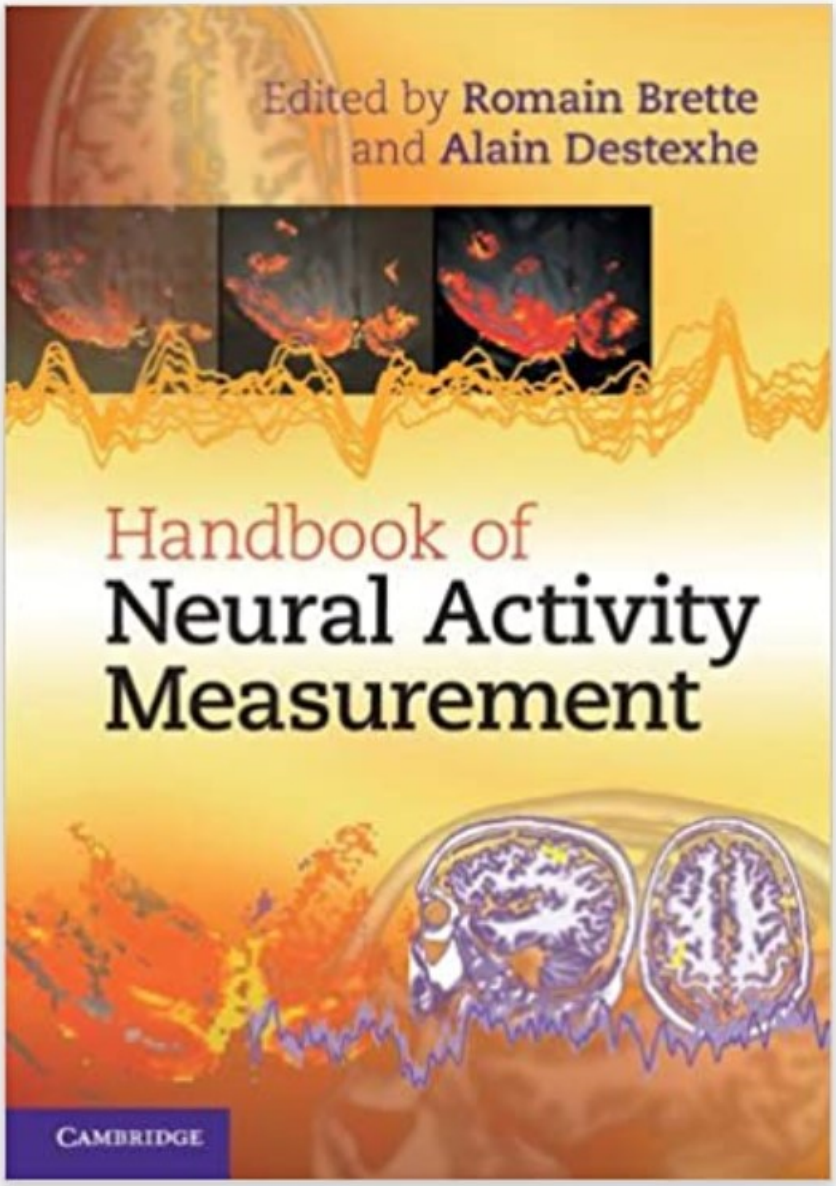
Conclusion

1. K_d = Dissociation constant
   a. It depends on the intrinsic properties of B
   b. It needs to match the $[Ca^{2+}]$ we want to detect

2. κ_B = Ca^{2+}-binding ratio
   a. It depends on
      - [B]
      - intrinsic properties of B (K_d)
      - $[Ca^{2+}]$
   b. κ_B of exogenous indicators needs not to overwhelm κ_B of endogenous buffers

3. Cooperativity binding makes quantitative Ca^{2+} imaging arduous

Further readings



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Neuron
Primer

Imaging Calcium in Neurons

Christine Grienberger[1] and Arthur Konnerth[1,*]
[1]Institute of Neuroscience, Technical University Munich, Biedersteinerstr. 29, 80802 Munich, Germany
*Correspondence: arthur.konnerth@lrz.tum.de
DOI 10.1016/j.neuron.2012.02.011

Calcium ions generate versatile intracellular signals that control key functions in all types of neurons. Imaging calcium in neurons is particularly important because calcium signals exert their highly specific functions in well-defined cellular subcompartments. In this Primer, we briefly review the general mechanisms of neuronal calcium signaling. We then introduce the calcium imaging devices, including confocal and two-photon microscopy as well as miniaturized devices that are used in freely moving animals. We provide an overview of the classical chemical fluorescent calcium indicators and of the protein-based genetically encoded calcium indicators. Using application examples, we introduce new developments in the field, such as calcium imaging in awake, behaving animals and the use of calcium imaging for mapping single spine sensory inputs in cortical neurons in vivo. We conclude by providing an outlook on the prospects of calcium imaging for the analysis of neuronal signaling and plasticity in various animal models.

Diffusion

- Review Ca^{2+} signaling
- Ca^{2+} indicators
- Ca^{2+} binding
- **Ca^{2+} diffusion**
- **Ca^{2+}-dependent fluorescence properties**
- **Simplified models of Ca^{2+} dynamics**
- **Imaging devises**
- **Applications**

Objective

To understand how the timescale of diffusion relates to length scales

Thermal energy

For every degree of freedom (= for every way that a particle can move, either forward and backward, left and right, up and down, or rotations), **a particle has a kinetic energy proportional to the temperature.**

The proportionality constant is the **Boltzmann constant (k) = 1.38 x 10^{-23} J/K**

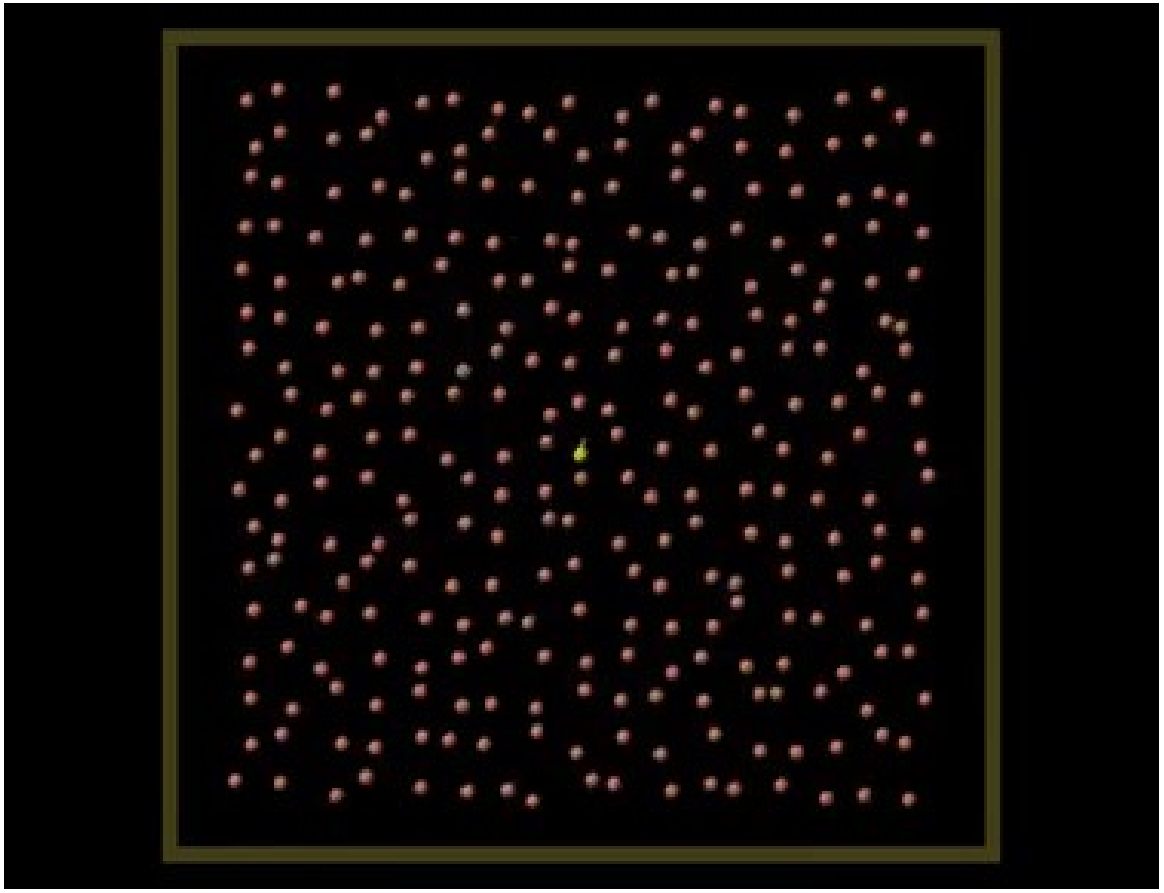
Kinetic energy: $\left\langle \frac{1}{2} m v_x^2 \right\rangle = \frac{1}{2} KT$

The mass of a calcium ion is 6.66 x 10^{-26} Kg; Room temperature = 300 K

$\left\langle v_x^2 \right\rangle$ = 6 x 10^4 m^2/s^2 → $\overline{v_x}$ = 250 m/s (900 km/hr)

Collisions produce a random walk

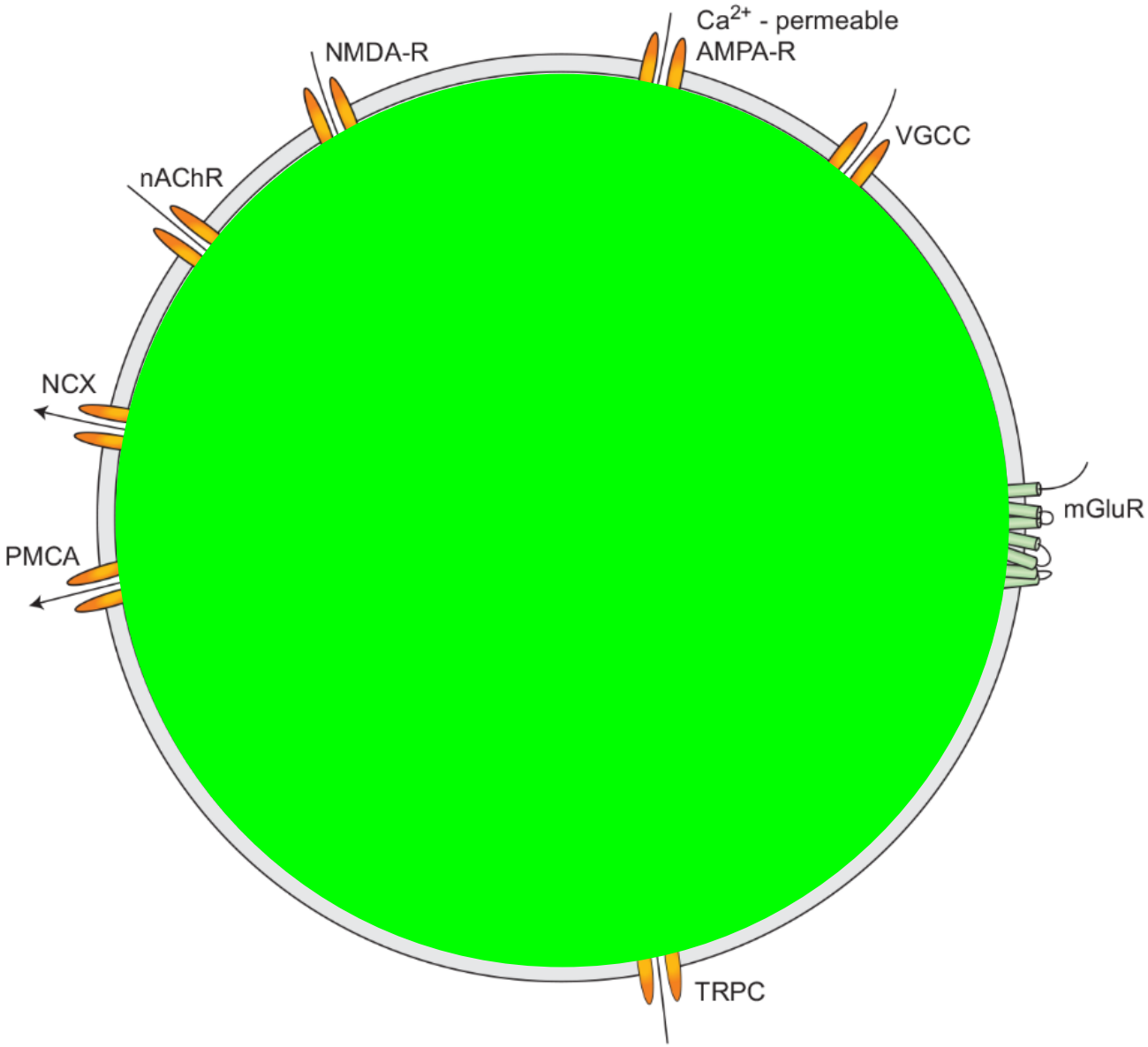
A particle in solution constantly (10^{13} times per second) bumps into H_2O and other molecules, which constantly changes its direction.



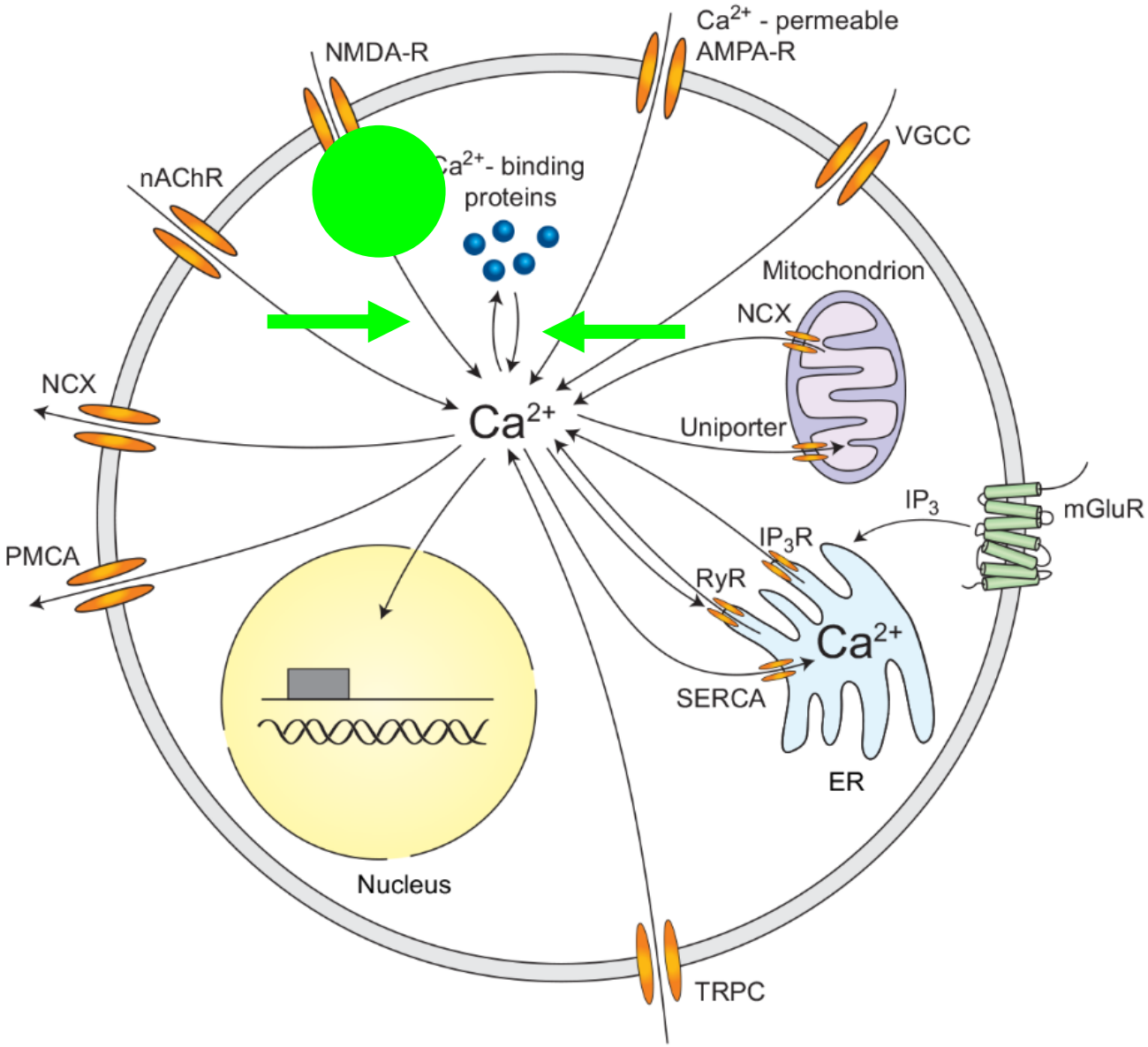
The so called **brownian motion**

(Robert Brown, botanist)

Ca^{2+} diffusion



Ca^{2+} diffusion



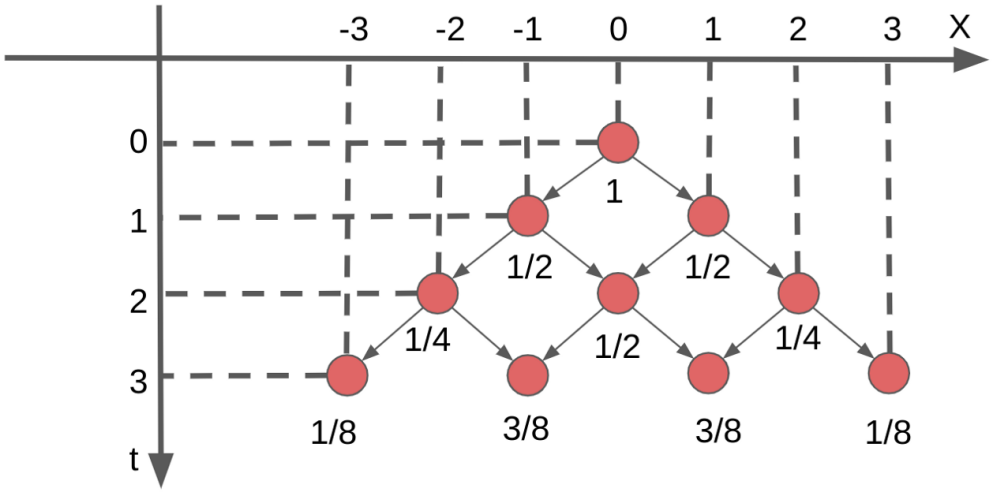
Relationship between spatial and temporal scales

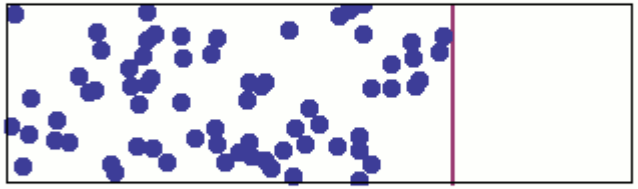
Diffusion is fast at short length scales and becomes slower and slower at long length scales

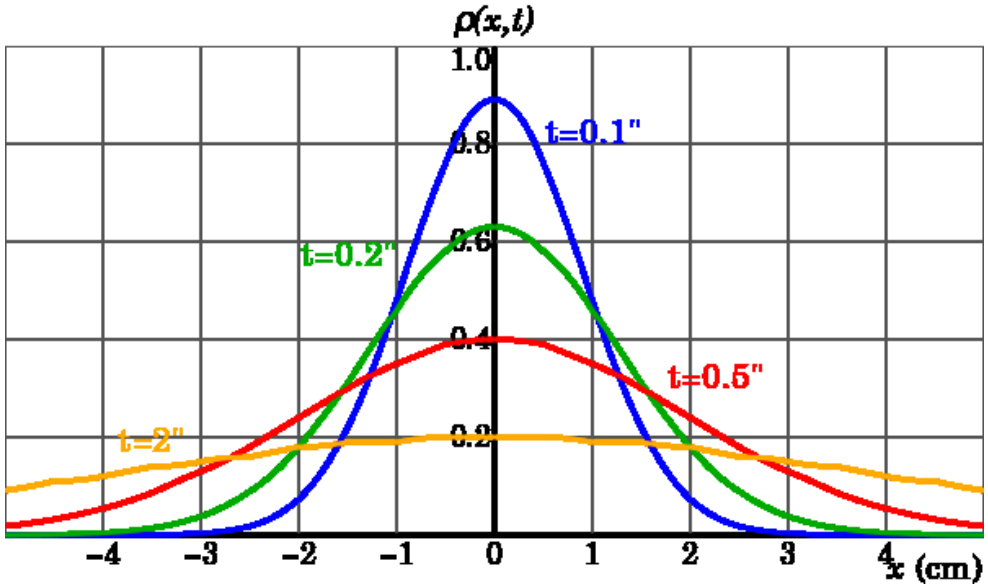
- To diffuse across a **synapse** (**1 µm**), it takes Ca^{2+} **8 ms (not 4 ns)**
- To diffuse across a **soma** (**10 µm**), it takes Ca^{2+} **800 ms (not 40 ns)**
- To diffuse down a **dendrite** (**1 mm**), it takes Ca^{2+} **???**
- To diffuse down an **axon** (**1 m**), it takes Ca^{2+} **????**

Diffusion in 1-D

You can get an intuition why this is the case by analyzing how ions diffuse in 1-D, for example along an axon



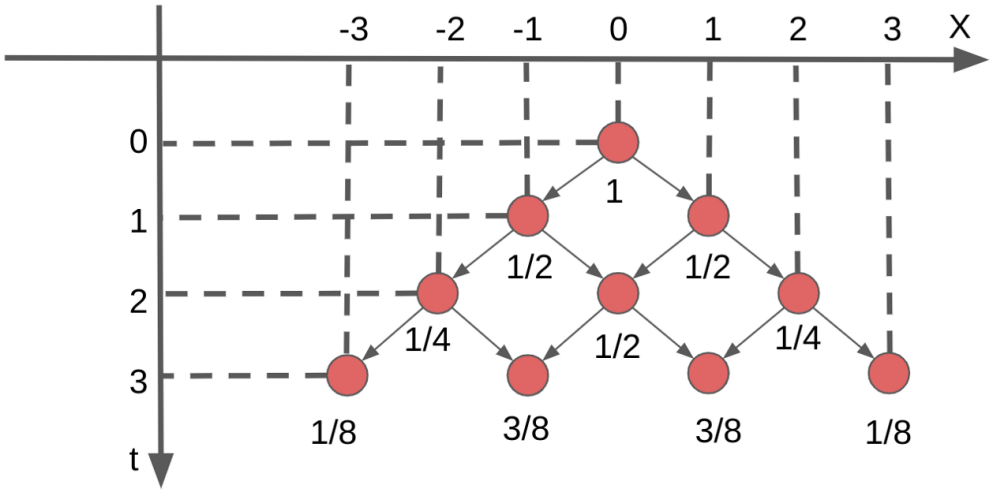


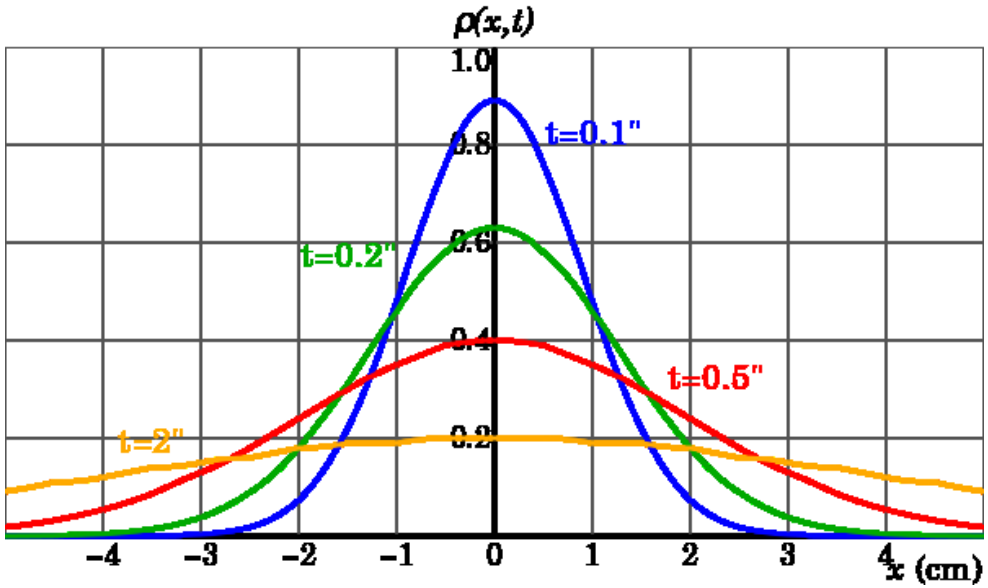


Diffusion in 1-D

What is the average position of all the particles?

- **Each particle is moving left (p=0.5) or right (p=0.5) by a distance δ at a velocity $\overline{v_x}$ for some time τ before a collision.**
- **After each collision $\overline{v_x}$ is randomly reset to the left or to the right.**
- **Each particle is independent**
- **On average, particles stay clustered around the initial position = the peak of the Gaussian stays fixed at the center (μ = 0)**





What is diffusion?

On average, how far do particles travel/get from where they started?

This is simply how wide the Gaussian distribution is

$$\rho(x,t) = \frac{1}{\sqrt{4\pi Dt}} e^{-\frac{x^2}{4Dt}}$$

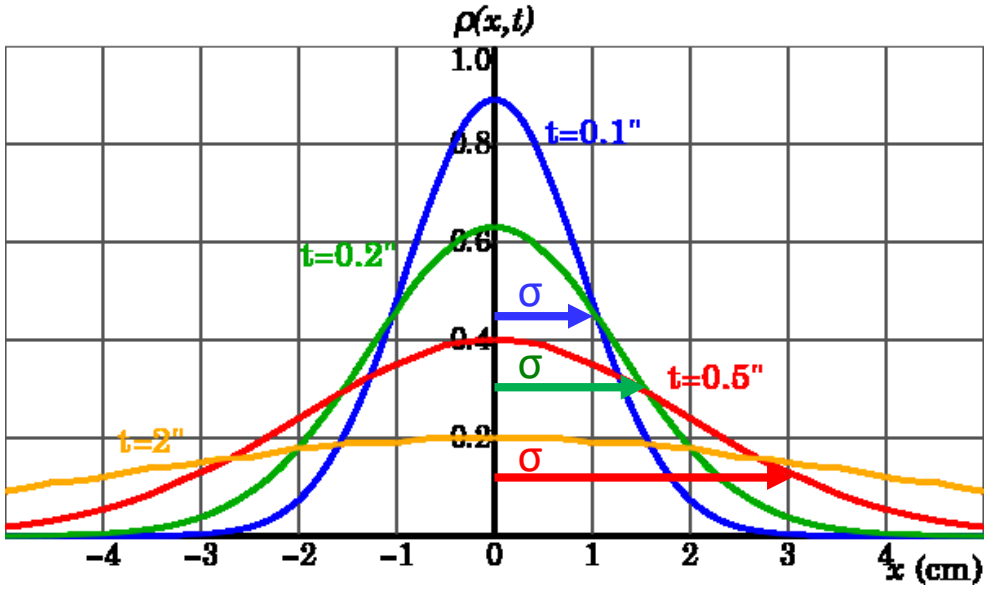
With μ = 0

$2\sigma^2 = 4Dt$

$\sigma^2 = 2Dt$

$\sigma = \sqrt{2Dt}$

(D = diffusion coefficient)



Mean displacement = r = standard deviation = σ = $\sqrt{Var} = \sqrt{2Dt}$

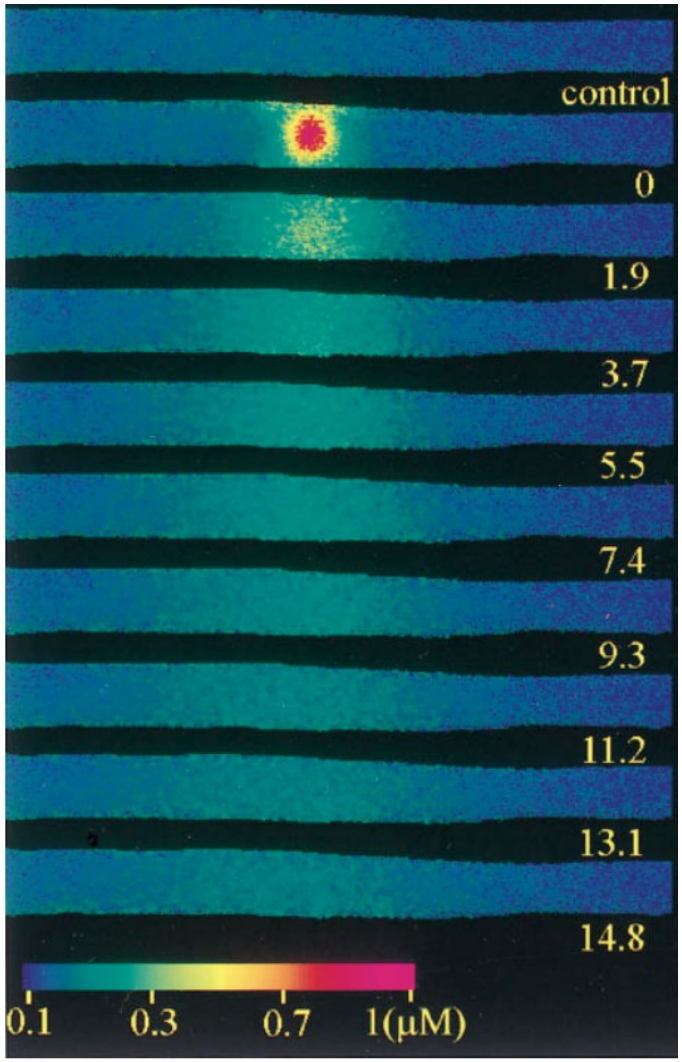
with D = $\frac{\delta^2}{2\tau}$ (D is the diffusion coefficient)

(I have posted an easy to understand derivation of this important result)

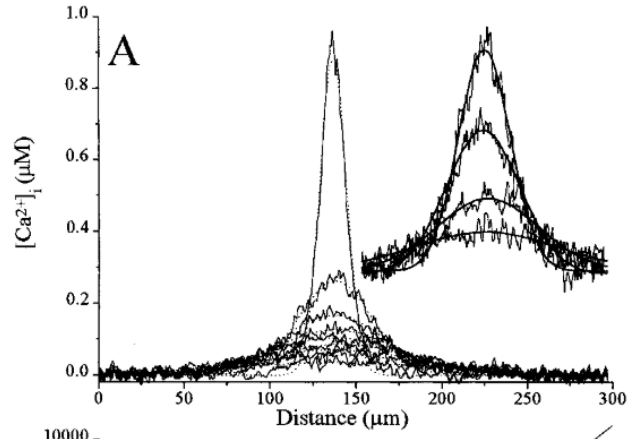
$$f(x \mid \mu, \sigma^2) = \frac{1}{\sqrt{2\pi\sigma^2}} e^{-\frac{(x-\mu)^2}{2\sigma^2}}$$

Neuron, Vol. 18, 473–481, March, 1997, Copyright ©1997 by Cell Press

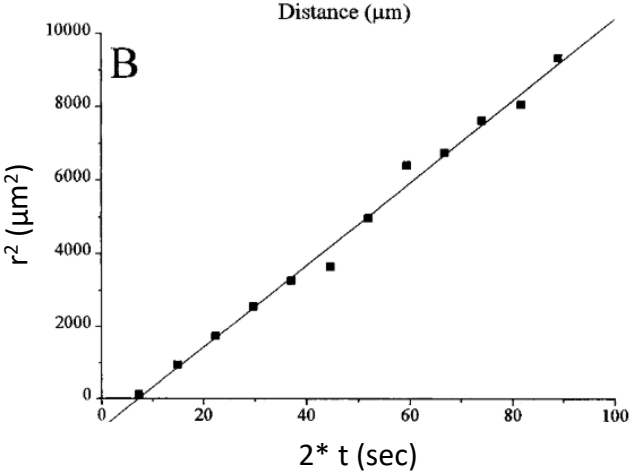
Low Mobility of the Ca^{2+} Buffers in Axons of Cultured Aplysia Neurons



- **Neuron loaded with the indicator Fura-2**
- **Ca^{2+} (100 μM) pressure injected in the center of the axon at time 0**



$$r^2 = \sigma^2 = D_{Ca}2t$$



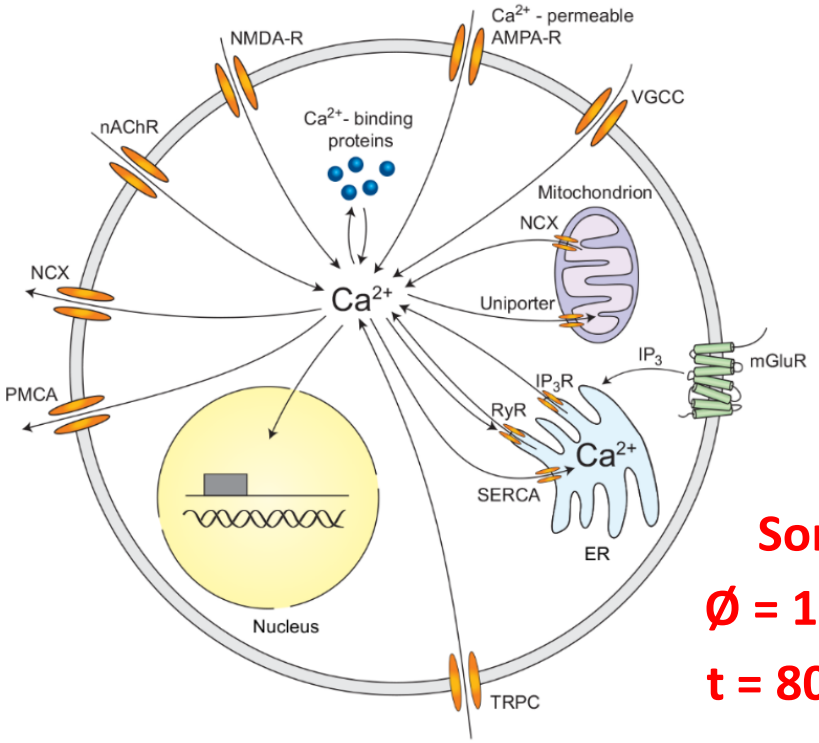
$$D_{Ca} \approx 20\ \mu m^2 s^{-1}$$

How long will it take for Ca^{2+} to be uniformly distributed across a subcellular compartment?

In one dimension: mean displacement = r = σ = $\sqrt{2Dt}$ → t = $\frac{r^2}{2D}$

In two dimension: mean displacement = r = σ = $\sqrt{4Dt}$ → t = $\frac{r^2}{4D}$

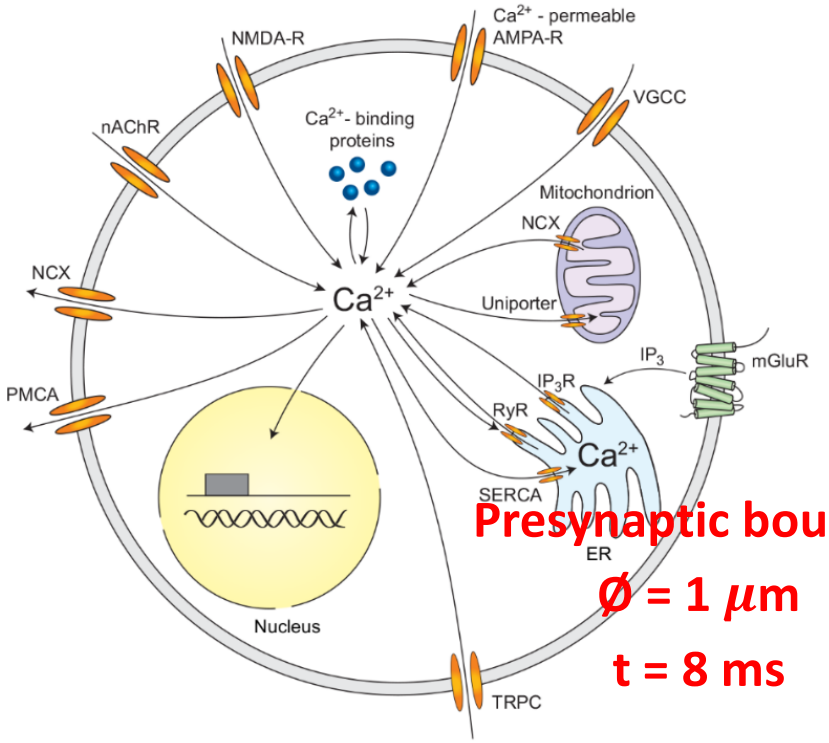
In three dimension: mean displacement = r = σ = $\sqrt{6Dt}$ → t = $\frac{r^2}{6D}$



Soma

Ø = 10 μm

t = 800 ms



Presynaptic boutons

Ø = 1 μm

t = 8 ms

5) **Some axons in your brain can be 1 m long. Assuming a diffusional constant for Ca^{2+} (D_{Ca}) of 20 $\mu m^2 s^{-1}$, how long would it take for Ca^{2+} to diffuse from the soma to the terminal bouton if there were only free diffusion in 1 D?**

a) **80 min**

b) **80 hours**

c) **80 days**

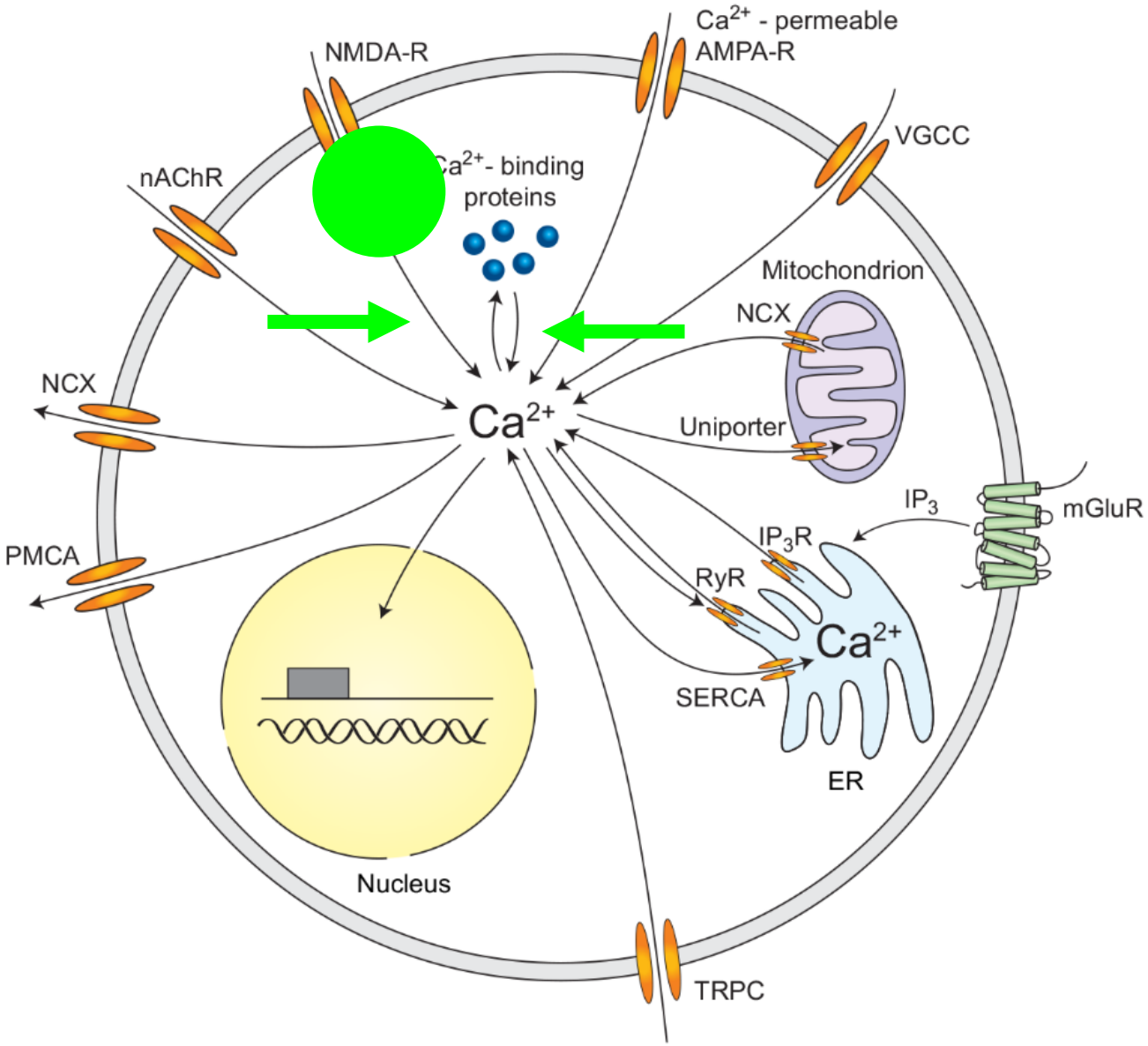
d) **8 months**

e) **8 years**

f) **80 years**

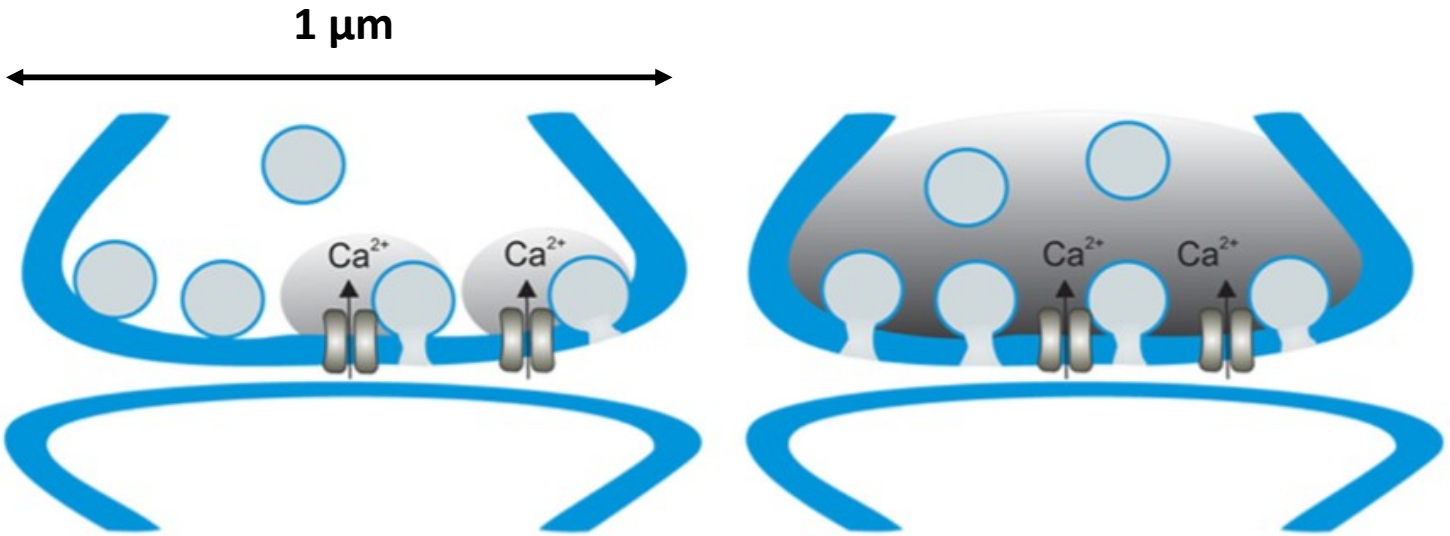
g) **800 years**

Lower limit of (Ca^{2+}) imaging (1)

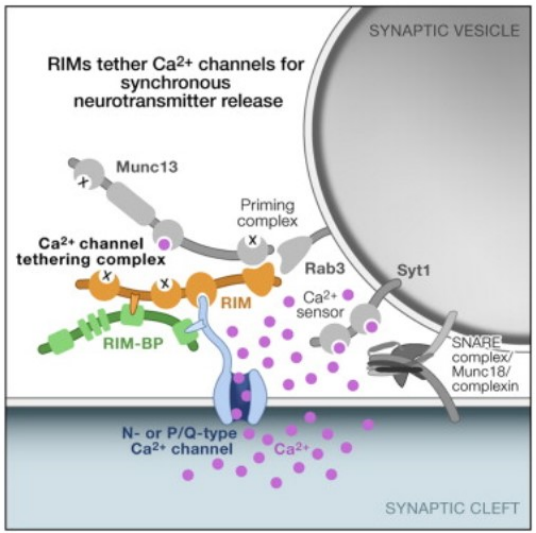


1) We can only image the average 'residual' $[Ca^{2+}]$ that remains after initial concentration gradients have equilibrated by diffusion

Lower limit of (Ca^{2+}) imaging (1)



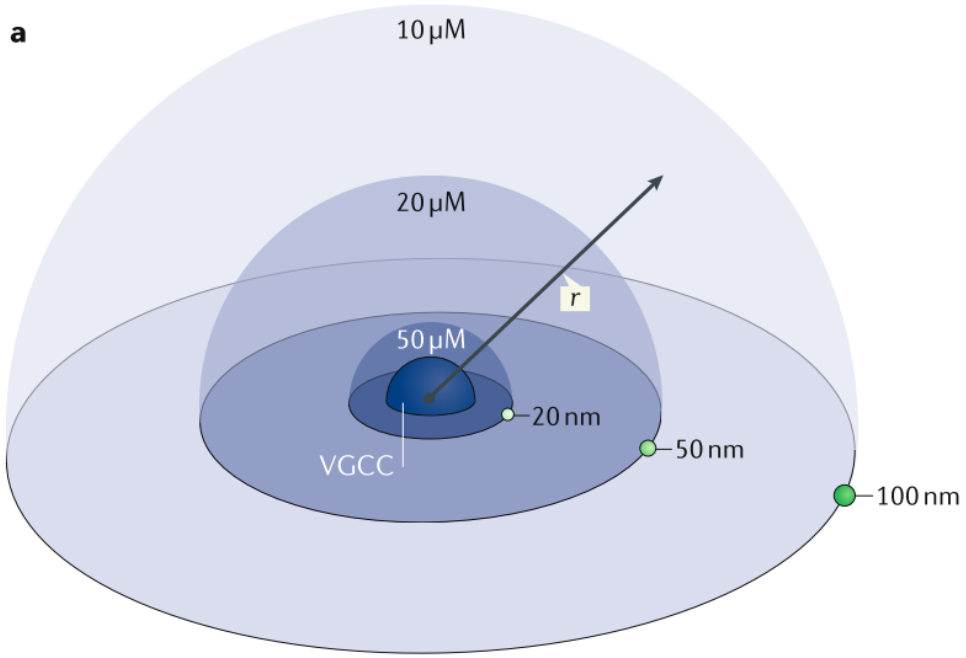
Ca^{2+} important for synaptic transmission



Average residual Ca^{2+} we can 'see' with Ca^{2+} imaging after <u>diffusion</u> and <u>equilibration</u> with <u>endogenous</u> and <u>exogenous</u> (= Ca^{2+} indicators) <u>buffers</u>

Lower limit of (Ca^{2+}) imaging (1)

Many Ca^{2+}-dependent events (e.g. synaptic transmission) depends on local nanodomains (few hundreds nanometers) of high Ca^{2+} (100 µM) that develop and collapse within tens to hundreds of microseconds following the opening of Ca^{2+} permeable channels



10 - 100 µM Ca^{2+} are required for synaptic transmission

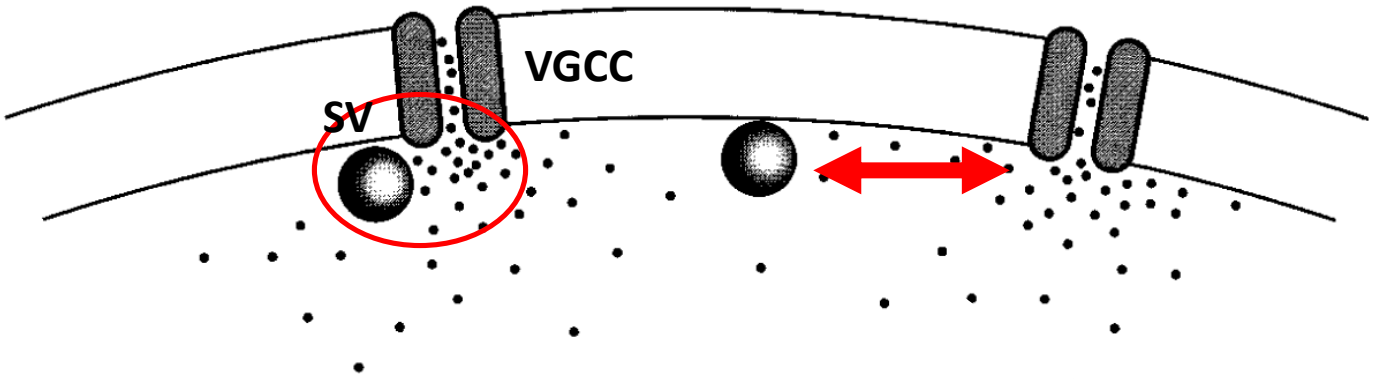
Residual Ca^{2+} in the presynaptic bouton, as measured with Ca^{2+} imaging, is ≈1 µM

Lower limit of (Ca^{2+}) imaging (2)

Due to the diffraction limit (≈ 200 nm)

(a) (b) (c)

Comparing slow (EGTA) and fast (BAPTA) Ca^{2+} buffers to estimate the distance between intracellular targets and Ca^{2+} channels



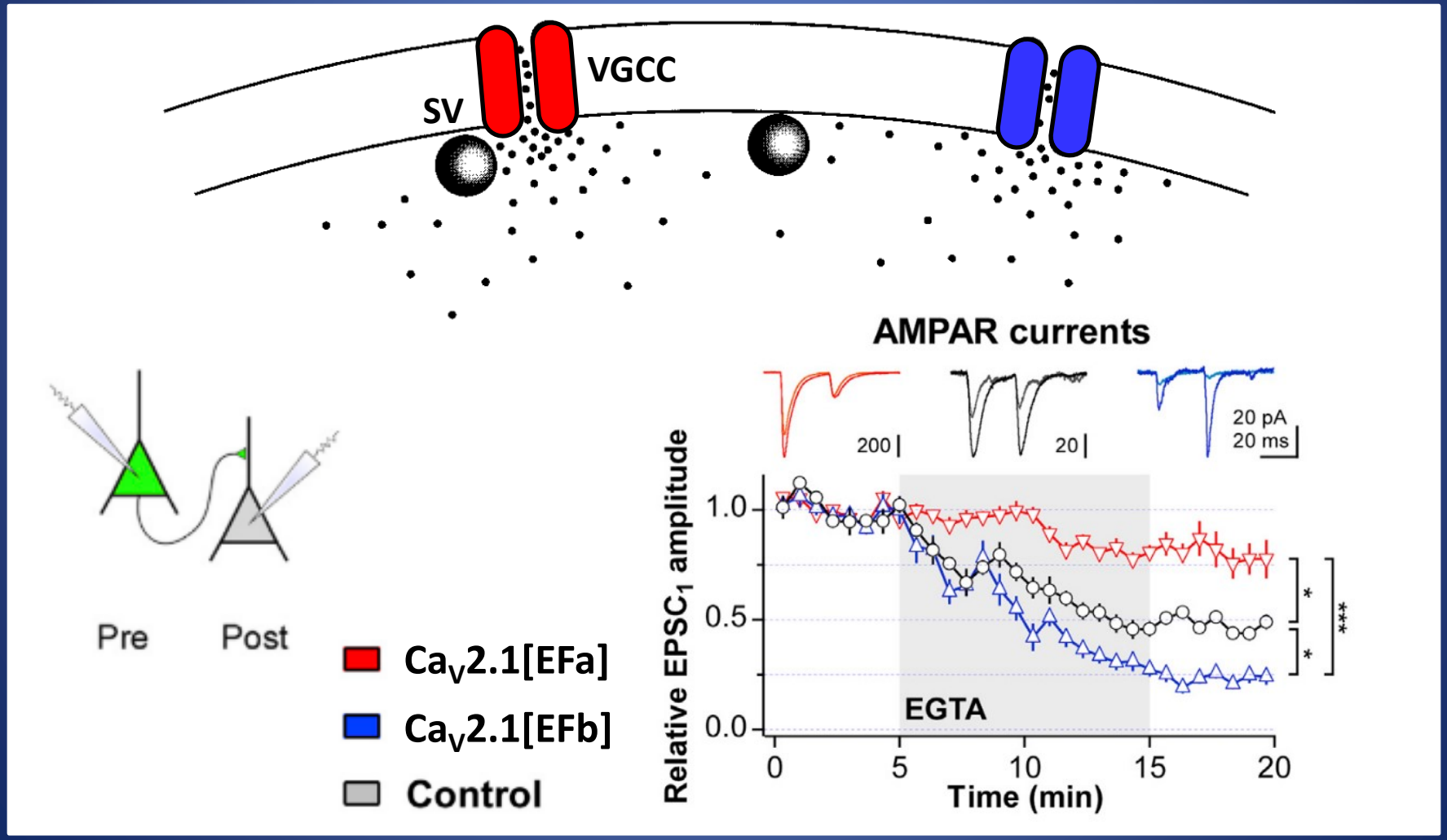
At 10-20 nm distance:

- high $[Ca^{2+}]$, high P_r
- **Sensitive to BAPTA**
- **Insensitive to EGTA**

At 100-200 nm distance:

- low $[Ca^{2+}]$, low P_r
- **Sensitive to BAPTA**
- **Sensitive to EGTA**

Example: sensitivity of different $Ca_V2.1$ splice variants to EGTA-AM



Thalhammer et al., 2017

Conclusion

1. A particle has a kinetic energy proportional to the temperature ($\overline{v_x}$ = 250 m/s at RT for Ca^{2+})

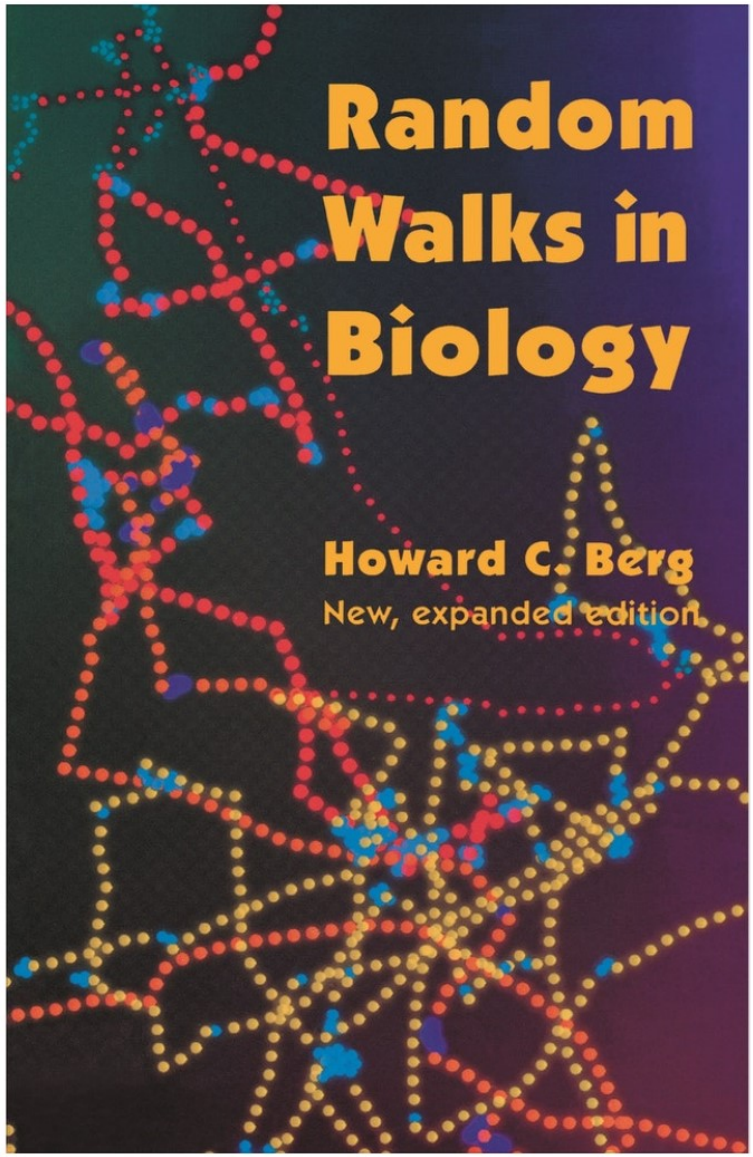
2. A particle in solution constantly (10^{13} times per second) bumps into other particles, which changes its direction

3. As a result, diffusion is fast at short length scales and slower at long length scales or

4. $t_{diffusion} = \frac{r^2}{2D}$ (in one dimension)

5. We can only image the average 'residual' [Ca^{2+}] that remains after initial concentration gradients have equilibrated by diffusion

Further readings



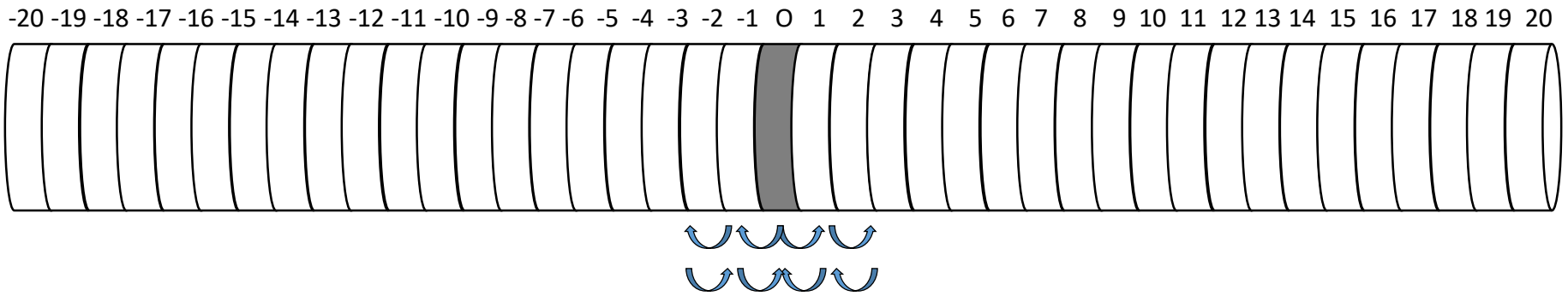
REVIEWS

The control of release probability at nerve terminals

Jeremy S. Dittman and Timothy A. Ryan**

Abstract | Exocytosis is a fundamental membrane fusion process by which the soluble or membrane-associated cargoes of a secretory vesicle are delivered to the extracellular milieu or the cell surface. While essential for all organs, the brain relies on a specialized form of exocytosis to mediate information flow throughout its vast circuitry. Neurotransmitter-laden synaptic vesicles fuse with the plasma membrane on cue with astonishing speed in a probabilistic process that is both tightly regulated and capable of a fascinating array of plasticities. Here, we examine progress in the molecular understanding of synaptic vesicle fusion and its control.

Supplementary material



- We have N particles at position x = 0 at time t = 0

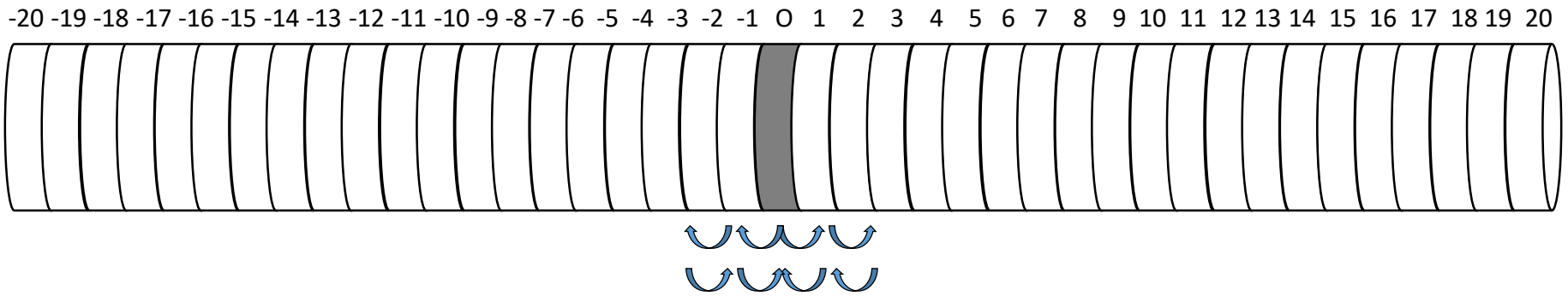
- At every time step τ, each particle moves independently from the others:
  - Half of the particles step right by a distance $\delta = + \overline{v_x}\ \tau$
  - Half of the particles step left by a distance $-\delta = - \overline{v_x}\ \tau$

- $x_i(n)$ = position of the i^{th} particle on time-step n, with $n = \frac{t}{\tau}$

- Thus, we can write the position of each particle at time-step n as a function of the position at previous time-step:

$$x_i(n) = x_i(n - 1) \pm \delta$$

Supplementary material



What is the average position of all the particles?

$$\langle x_i(\mathrm{n})\rangle = \frac{1}{N}\sum_i x_i(\mathrm{n})$$

$$\langle x_i(\mathrm{n})\rangle = \frac{1}{N}\sum_i \left[x_i(\mathrm{n}-1) \pm \delta\right]$$

$$\langle x_i(\mathrm{n})\rangle = \frac{1}{N}\sum_i \left[x_i(\mathrm{n}-1)\right] + \frac{1}{N}\sum_i \left(\pm \delta\right)$$

0

$$\langle x_i(\mathrm{n})\rangle = \langle x_i(\mathrm{n}-1)\rangle \qquad \langle x_i(\mathrm{n})\rangle = \mu = 0$$

Supplementary material

On average, how far do particles travel in absolute terms?

$\langle |x_i(n)| \rangle$ →

$$x_i(n) = x_i(n-1) \pm \delta$$

$$x_i^2(n) = (x_i(n-1) \pm \delta)^2$$

$$x_i^2(n) = x_i^2(n-1) \pm 2\,\delta\, x_i(n-1) + \delta^2$$

Supplementary material

At each step the variance grows linearly by δ^2

$$\sigma^2 = \langle x_i^2(n) \rangle = \langle x_i^2(n-1) \rangle + \delta^2$$

$$\langle x_i^2(0) \rangle = 0\,, \quad \langle x_i^2(1) \rangle = \delta^2\,, \quad \langle x_i^2(2) \rangle = 2\delta^2\,, \quad \boxed{\langle x_i^2(n) \rangle = n\delta^2}$$

We can change time steps between collisions (n) to continuous time, with $n = \frac{t}{\tau}$

$$\sigma^2 = \langle x_i^2(t) \rangle = \frac{\delta^2}{\tau} t$$

$$\sigma^2 = \langle x_i^2(t) \rangle = 2Dt, \quad \text{with } 2D = \frac{\delta^2}{\tau} \quad \text{(Diffusion coefficient; length}^2\text{/ time)}$$

$$\sigma = \sqrt{\langle x_i^2\,(n) \rangle} = \sqrt{2Dt}$$