Monitoring intracellular nanomolar [Ca^{2+}] using fluorescent lifetime imaging microscopy (FLIM)

Basic Principle of Fluorescent Lifetime Imaging Microscopy (FLIM)

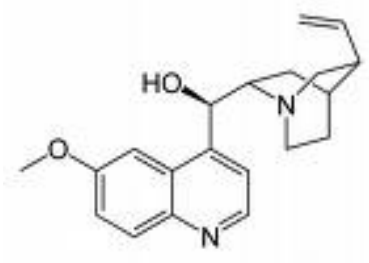
Calcium indicators that are suitable for FLIM

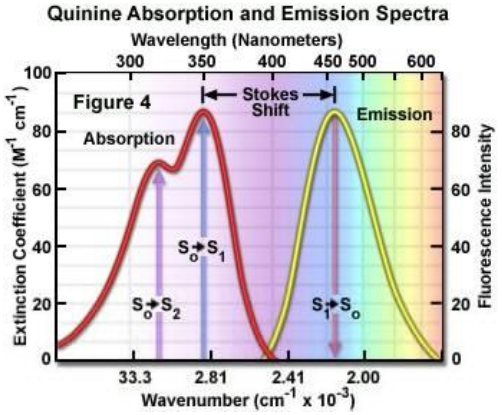
Calibration Procedure

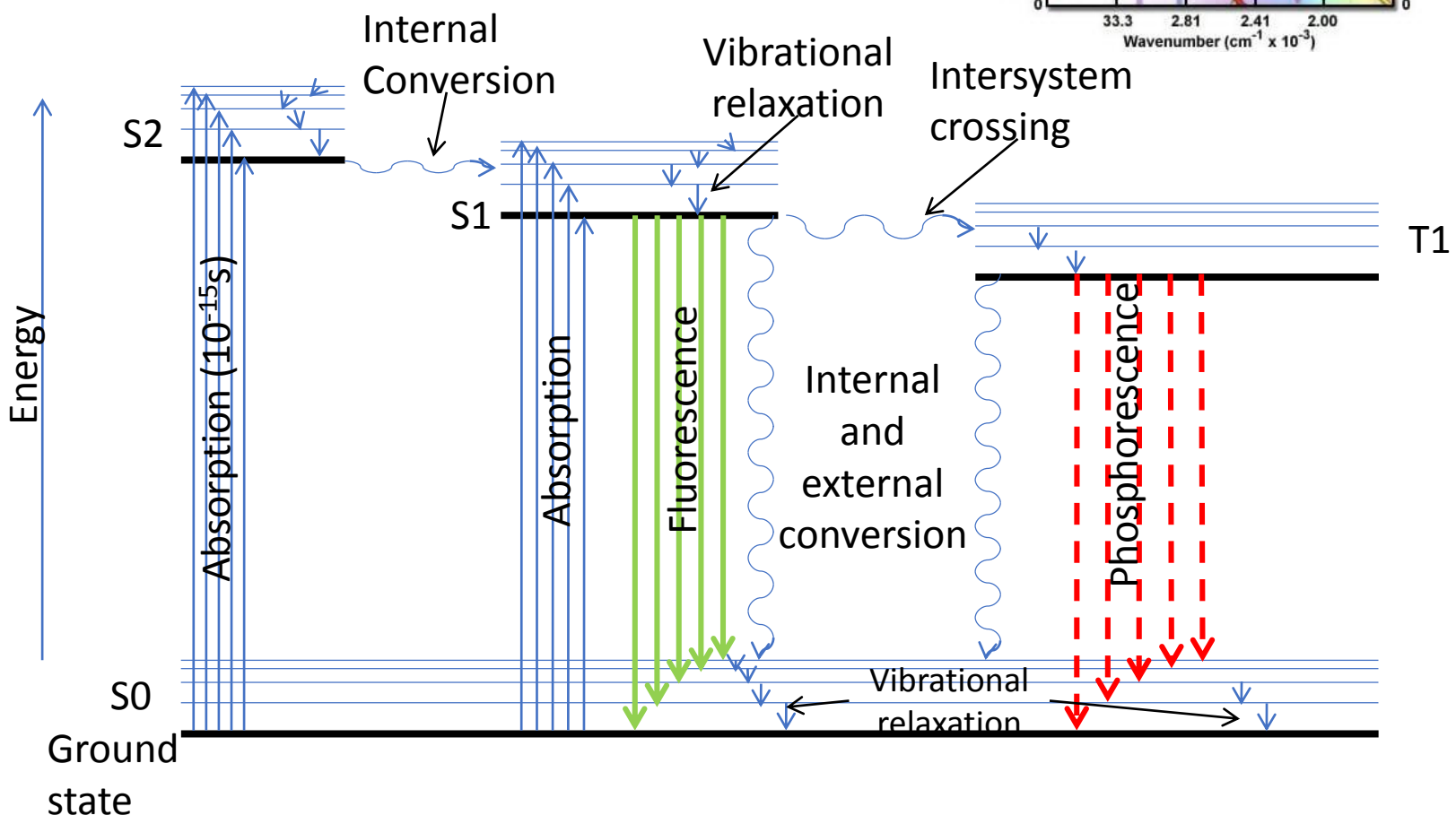
Measurement of nanomolar baseline [Ca^{2+}] in tissue samples

Utilisation of FLIM in improving measurement signals

Principle of Fluorescent Lifetime



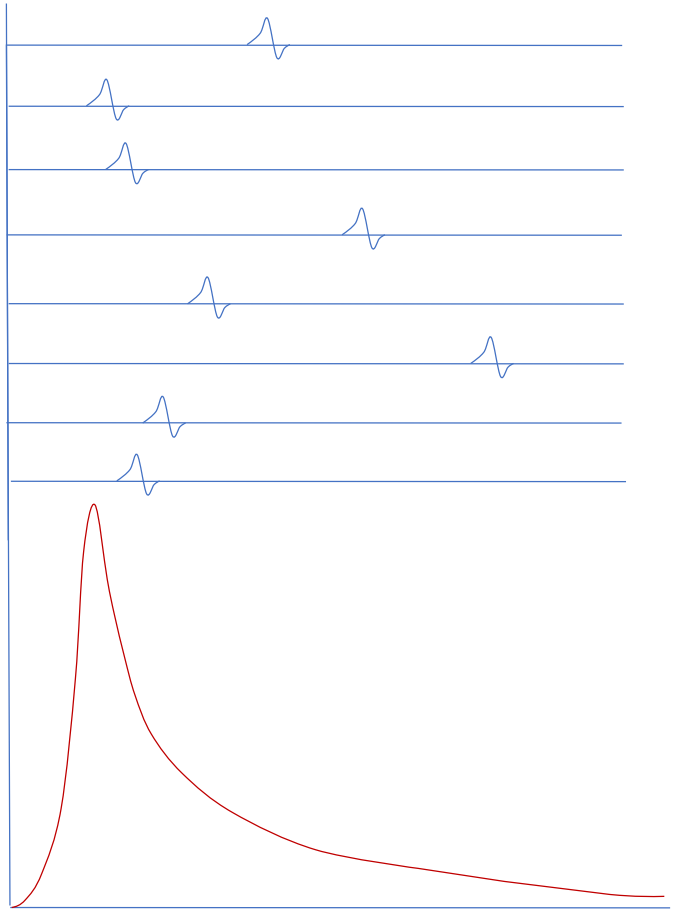


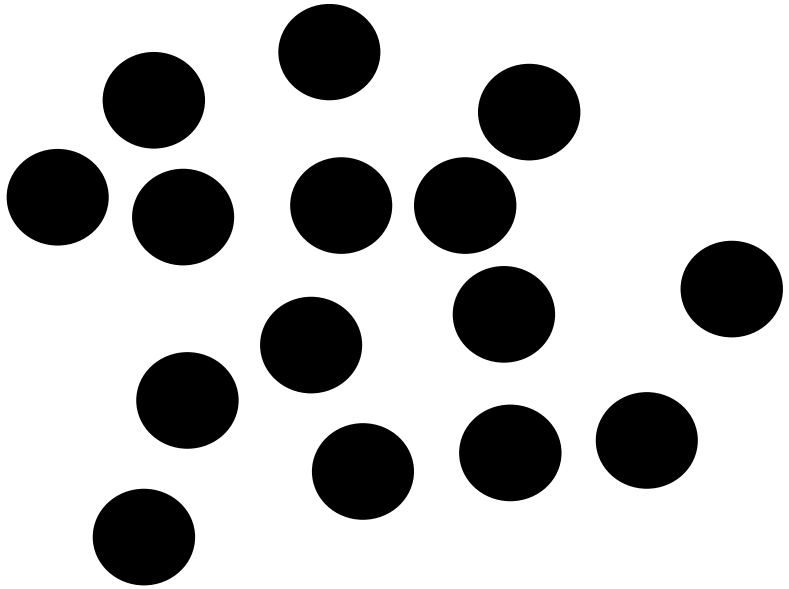


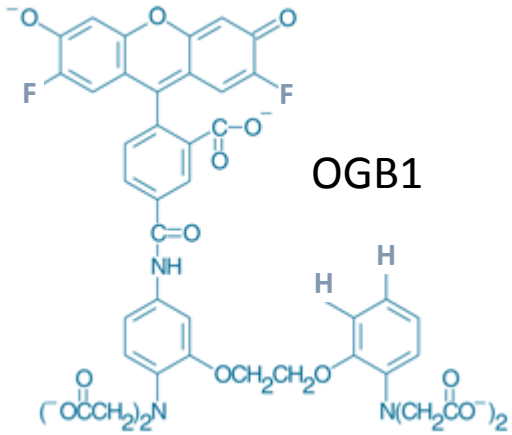
The fluorescence lifetime is a molecular property generally independent of variations in

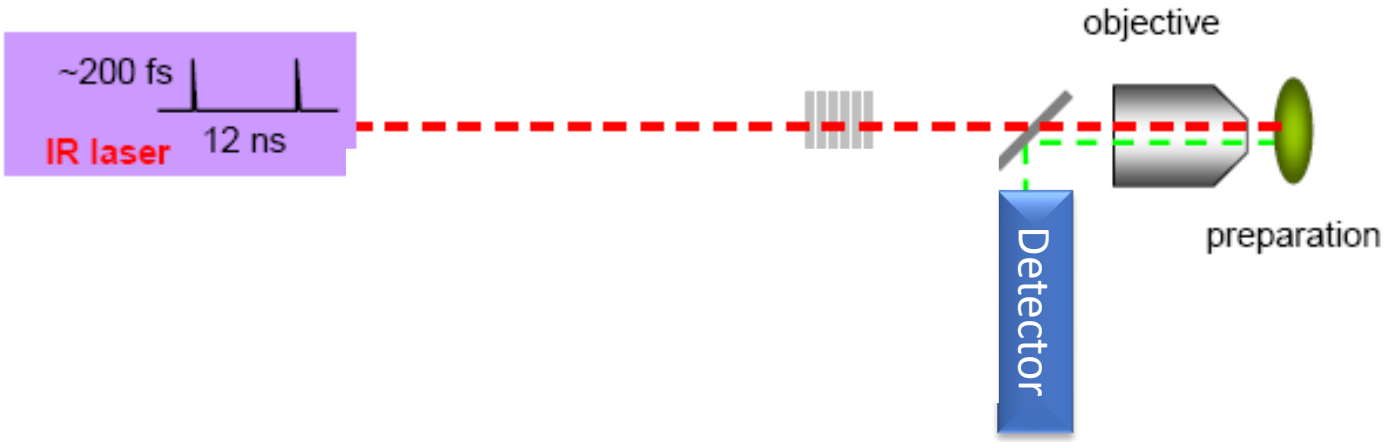
- **Fluorophore concentration**
- **Illumination intensity**
- **Short light path length difference**
- **Scattering**
- **Photo bleaching**

Principle of Fluorescent Lifetime









Principle of Fluorescent Lifetime - Pros and Cons

Advantage

- Useful for the study of complex formation and conformational changes

- The fluorescence lifetime is a molecular property generally independent of variations in fluorophore concentration, illumination intensity, light path length, scattering,

- FLIM can probe the fluorophore's local environment quantitatively and directly, *e.g.* viscosity, pH, ions concentration *etc.*

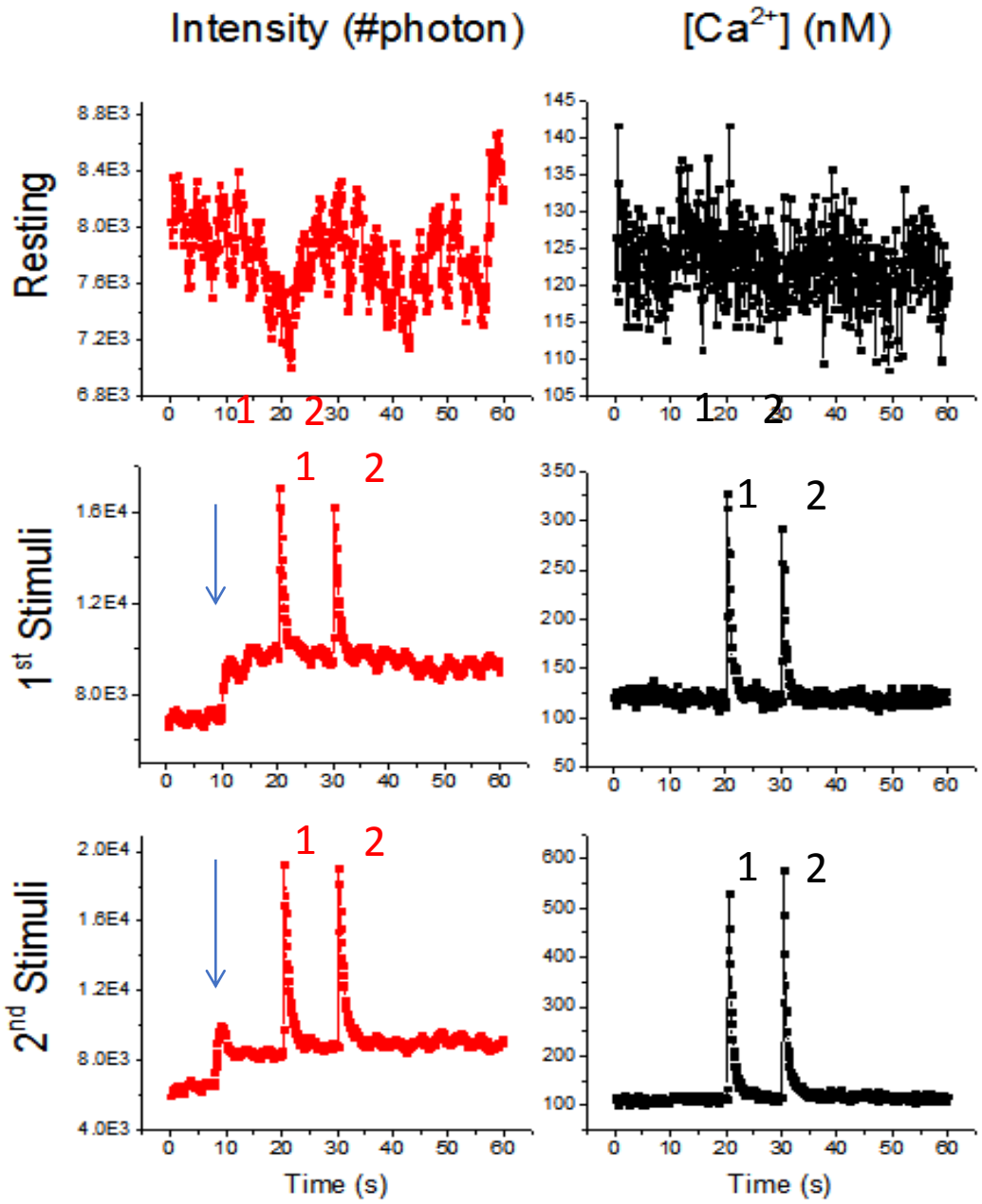
Disadvantage

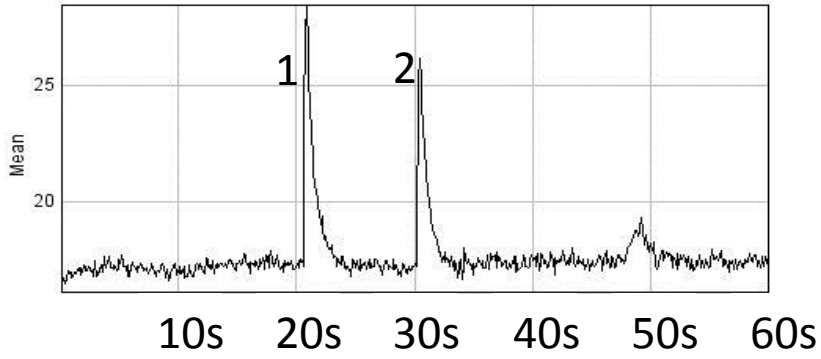
- Fluorescence decay is a complex process, spectroscopy expertise required for data interpretation, and in worst cases decay time cannot be resolved to give quantitative measures

- Many assumptions have been made about all related physical models, one has to be careful about what applies and what need modification

- Complex and expensive equipment required (~£40k)

Principle of Fluorescent Lifetime - Pros and Cons





FLIM signal is immune to z drift

Things often forgotten

Indicator	Kd in vitro (nM)	Kd in situ (nM)	Cell/Tissue Type
Calcium Green-1	190	930	HeLa cells
Fluo-3	390	2570	Frog skeletal muscel
OGB-1	170	430	HeLa cells
Fura-2	145	371	U373-MG astrocytoma cell
Fura-2	145	350	Rabbit gastric gland

- **Biological materials are fragile, prone to photodamage and phototoxicity**
- **Appropriate probes, indicators are buffers themselves**
- **Signal to noise issues, noises are always present**
- **Appropriate analytical tools, fitting is not always the best way**
- **2p and 1p don't excite the same state (S2,S1), that's why absorption spectra usually look similar but blue shifted**
- **Fluorescent tags that work well in one system don't necessarily translate into another system, so know the limitations and variations in different biological systems**

Basic Principle of Fluorescent Lifetime Imaging Microscopy

Calcium indicators that are suitable for FLIM

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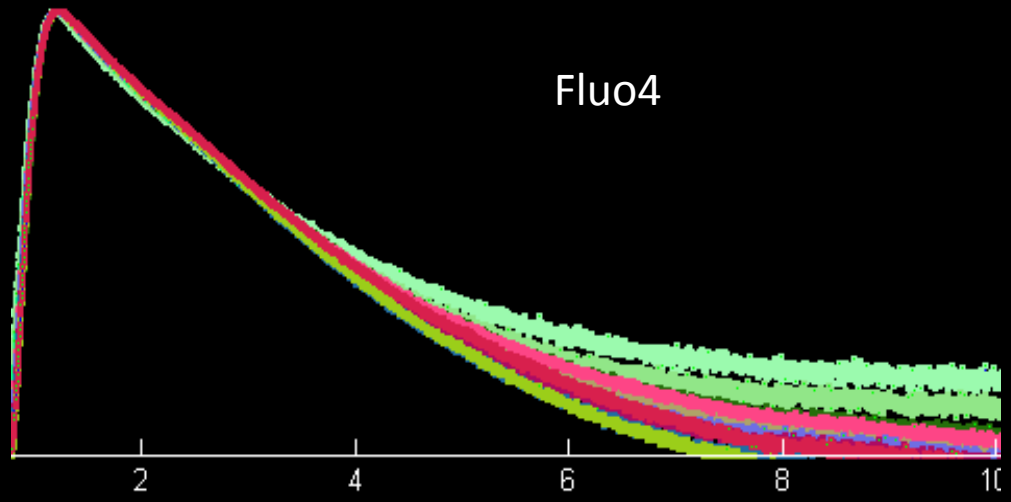
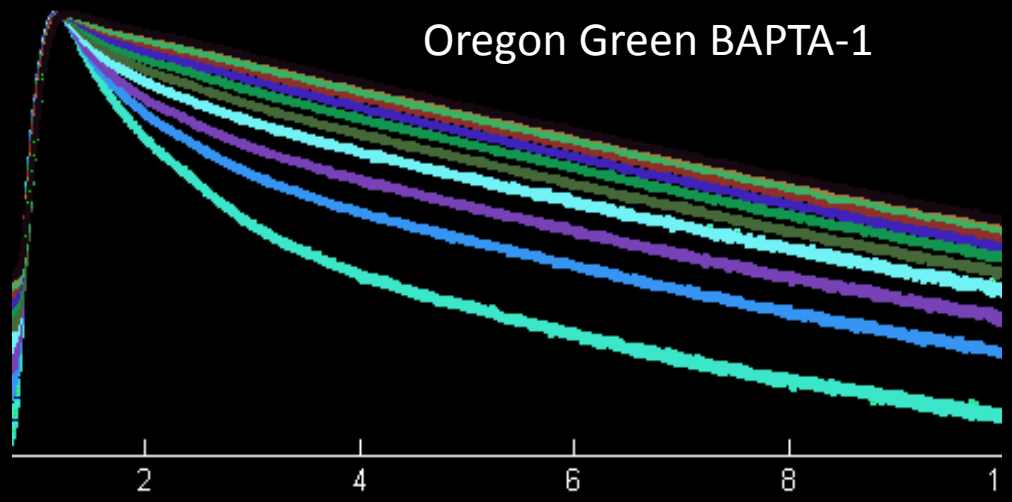
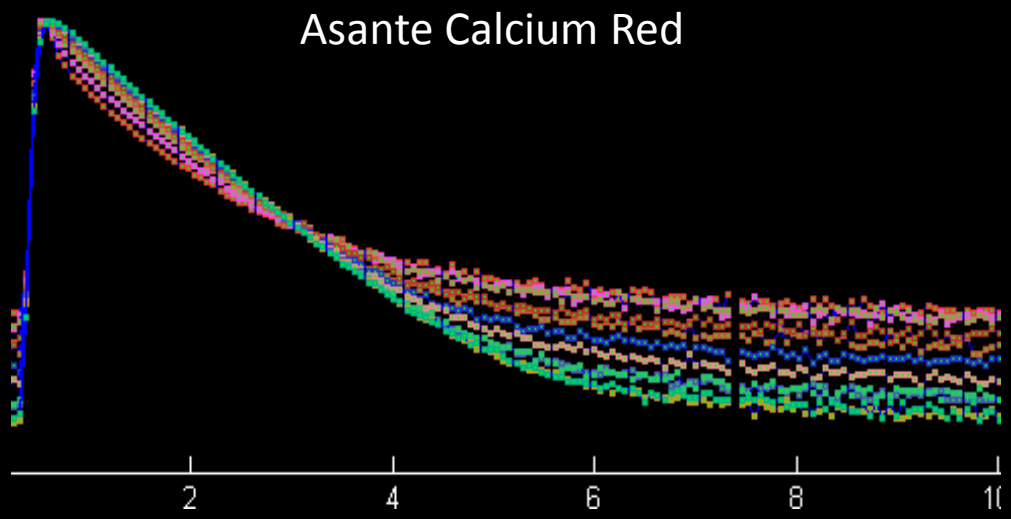
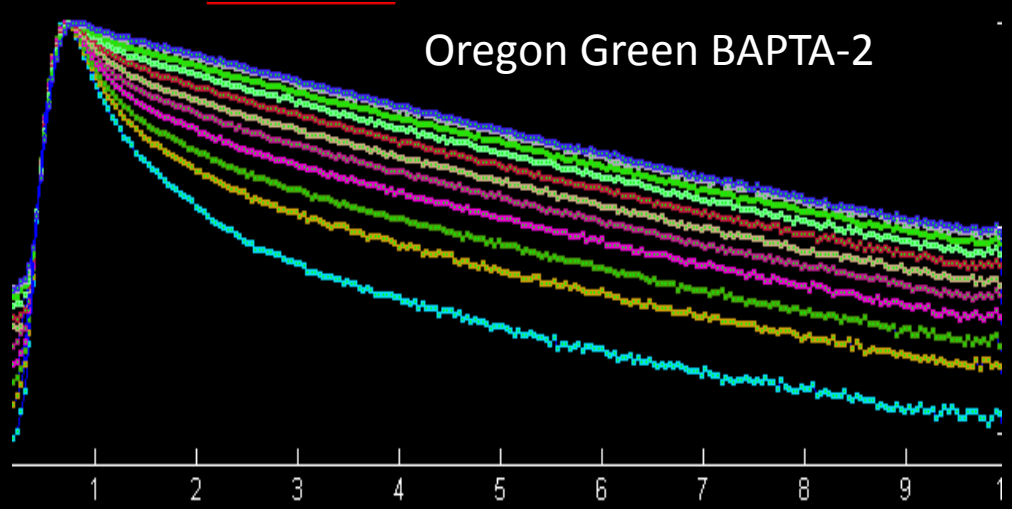
Utilisation of FLIM in improving measurement signals

Not all common calcium indicator can work under FLIM



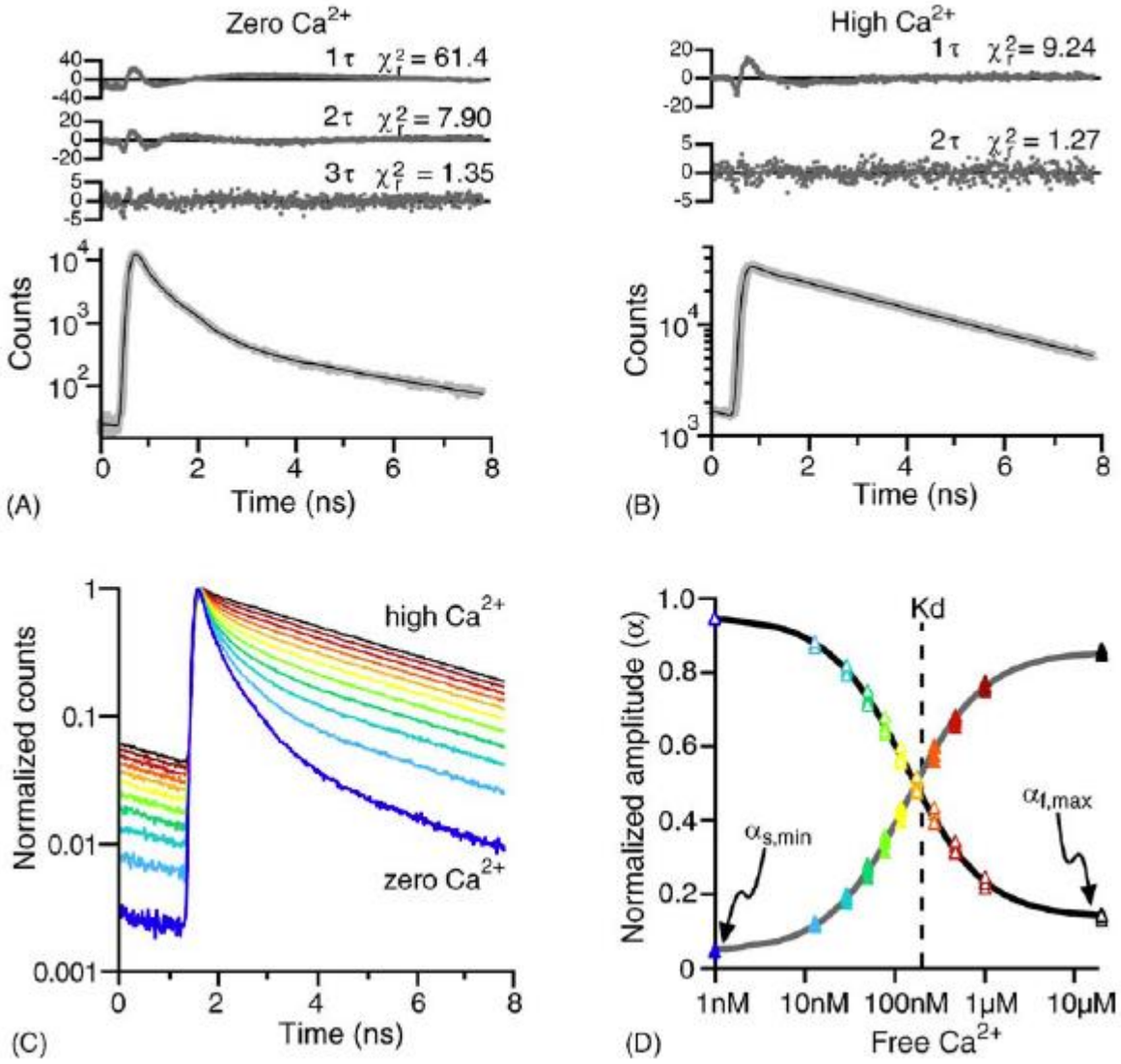




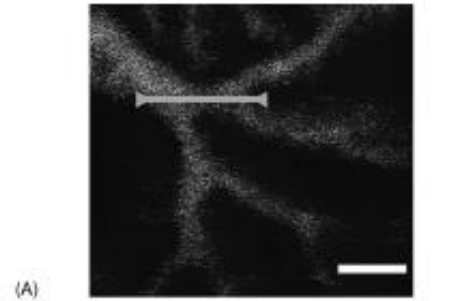


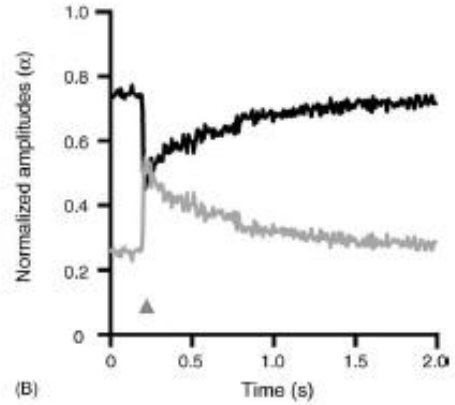
Oregon Green BAPTA -1 is a good candidate

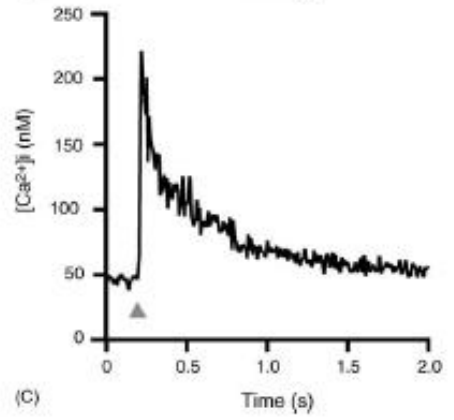
Curvette Calibration using intracellular solution



Fast transient measurement



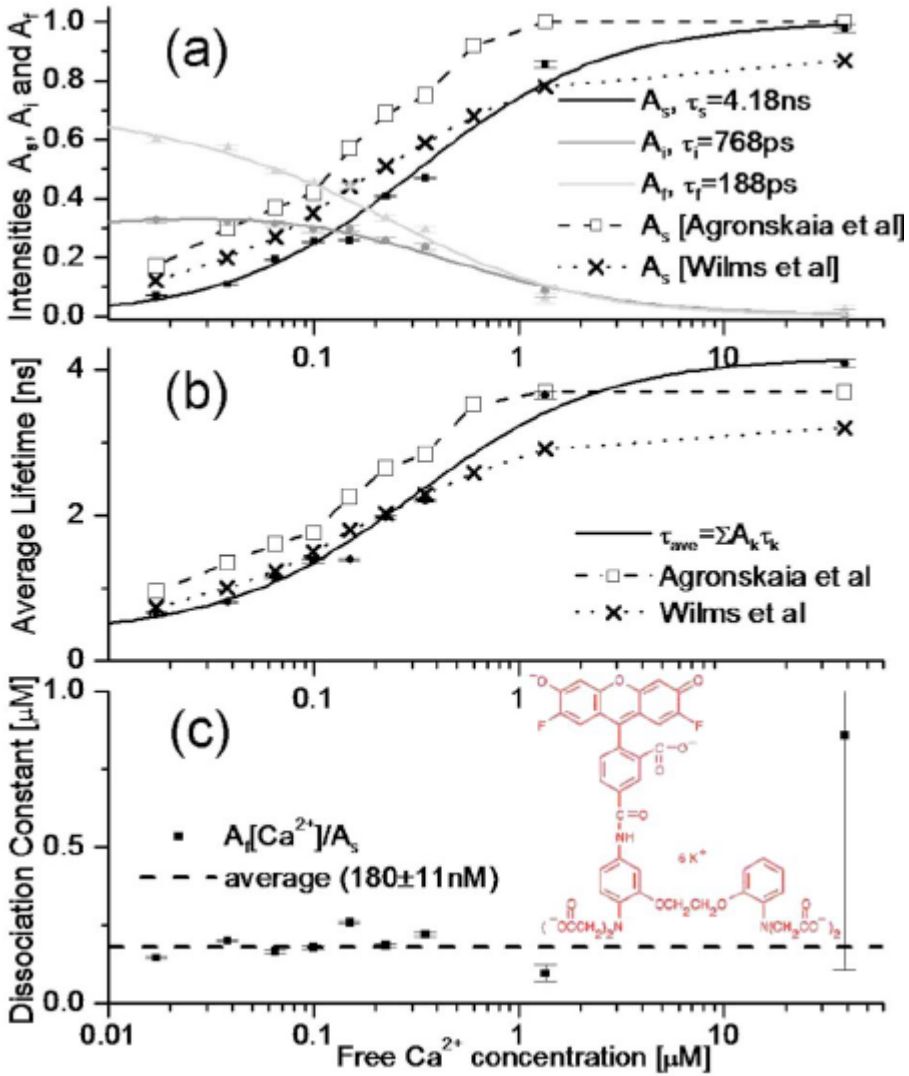




- **OGB family (OGB1, OGB2, OGB5N) all works well with different Kd**
- **Calcium Green also works but not as good**

Christian D. Wilms *et al*, Cell Calcium 2006

OGB1 decay time is triple exponential



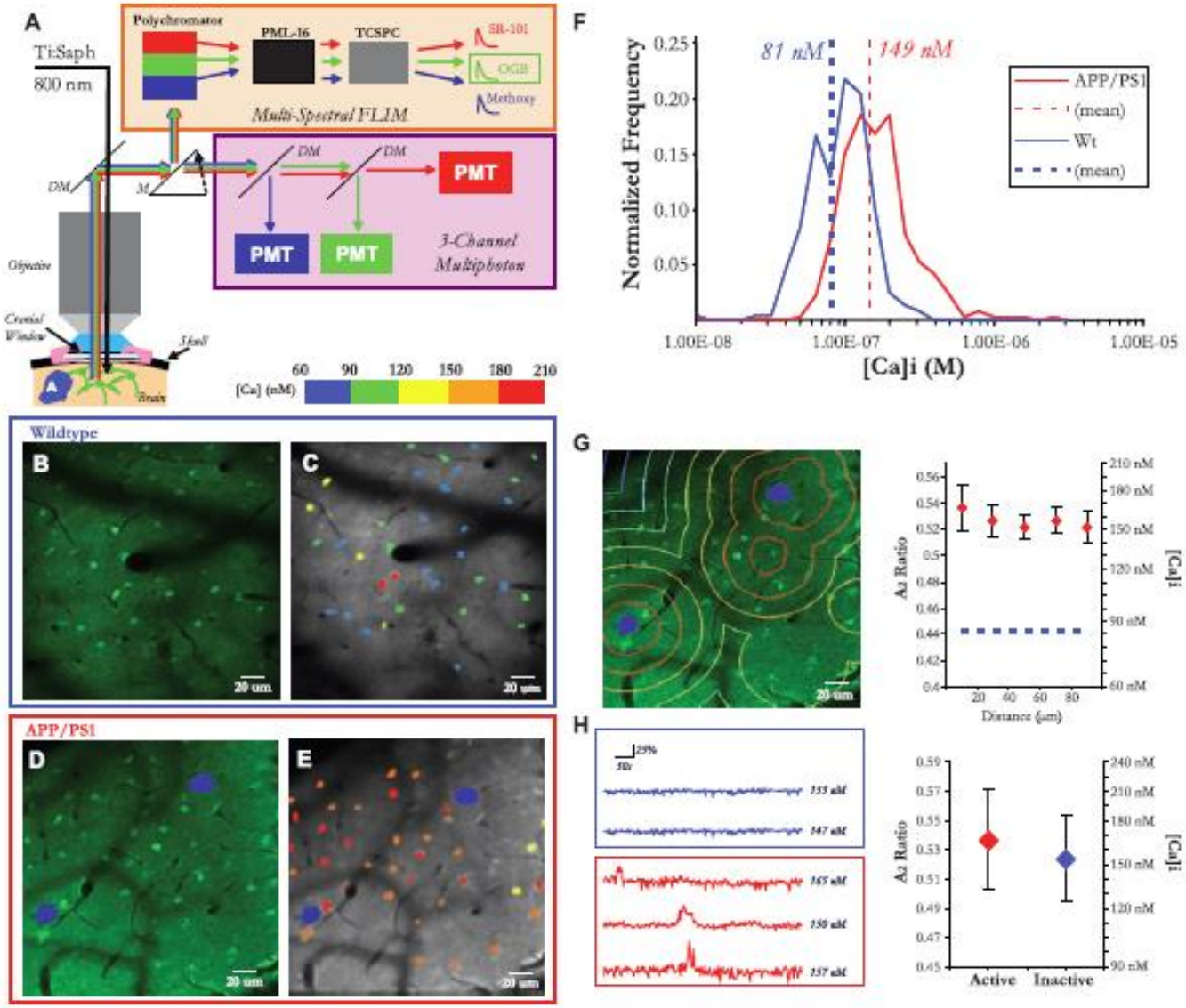
M. Gersbach *et al*, Optics Letters 2009

A better temporal resolution show that triple exponential fitting is needed for OGB-1 rather than double exponential fitting to give accurate measurements

Analysis can be complicated

OGB1 FLIM in vivo demonstrated

Synchronous Hyperactivity and Intercellular Calcium Waves in Astrocytes in Alzheimer Mice



OGB-1 (astrocyte+neuron)
SR-101 (astrocyte)

Measurement from astrocyte soma in somatosensory cortex

Kishore V. Kuchibhotla *et al,* Science 2009

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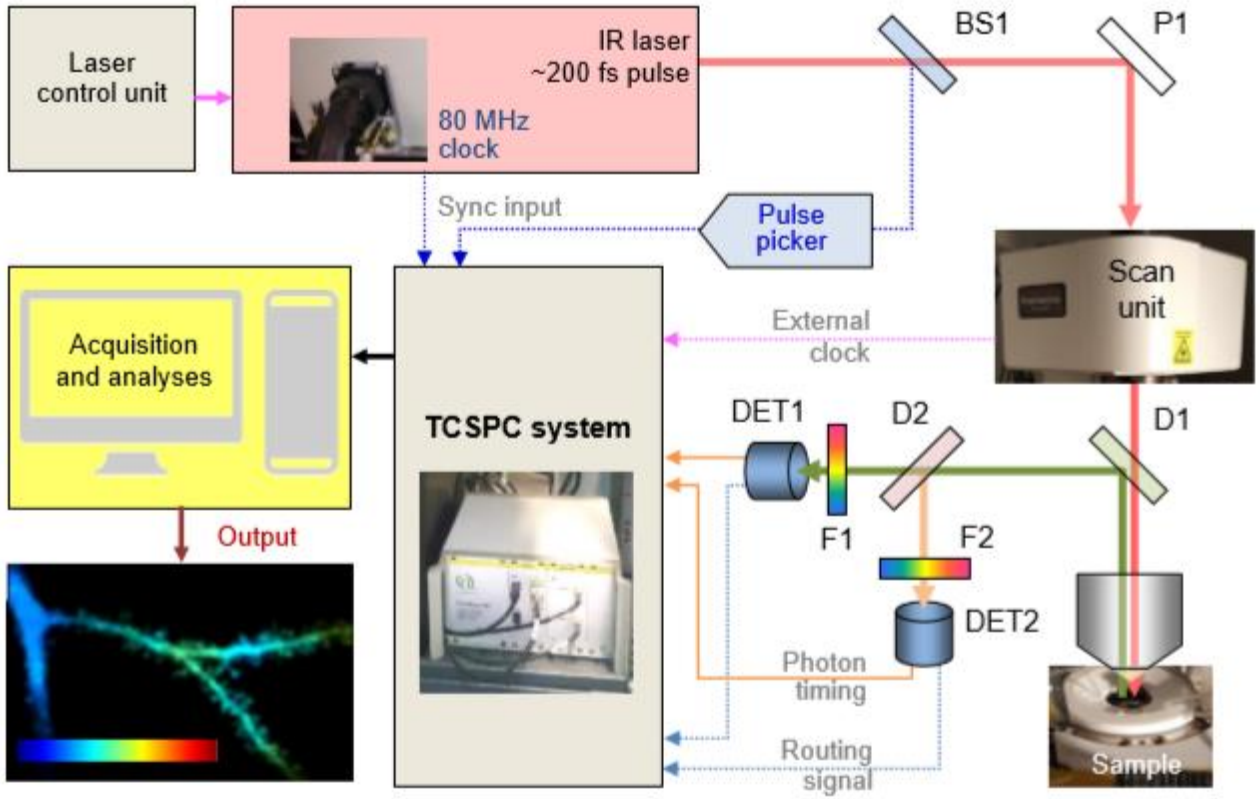
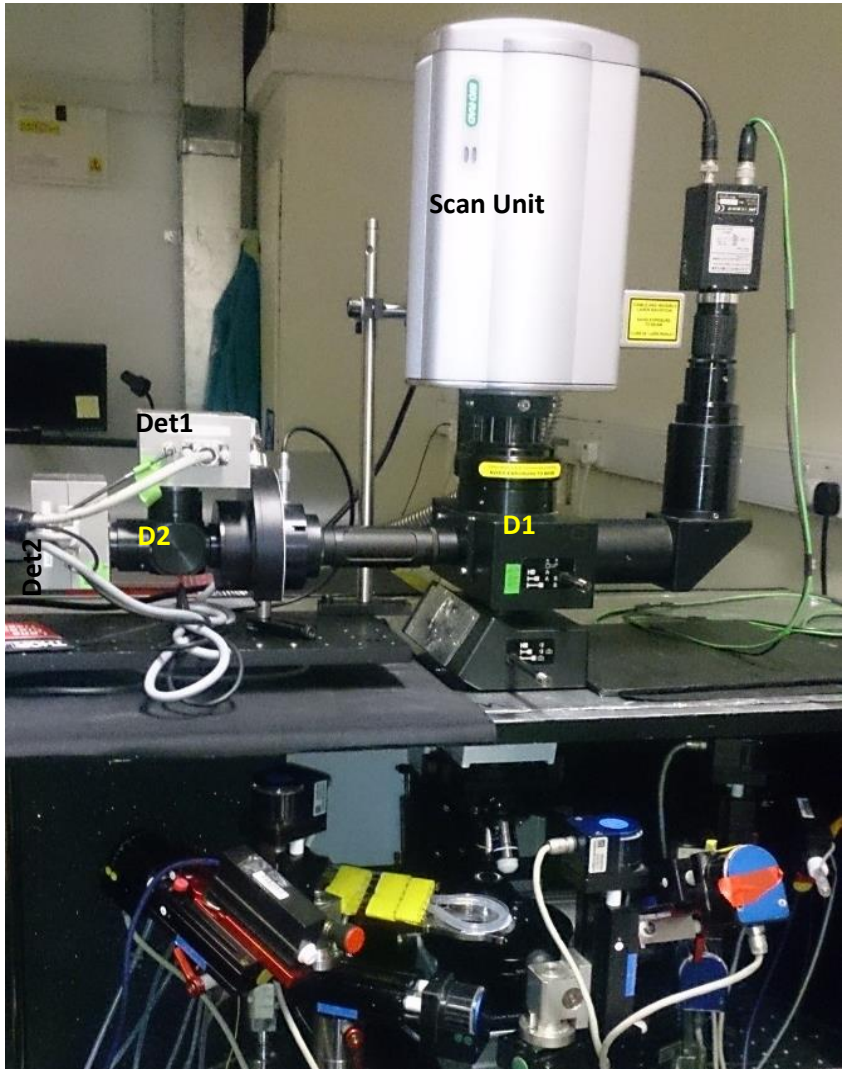
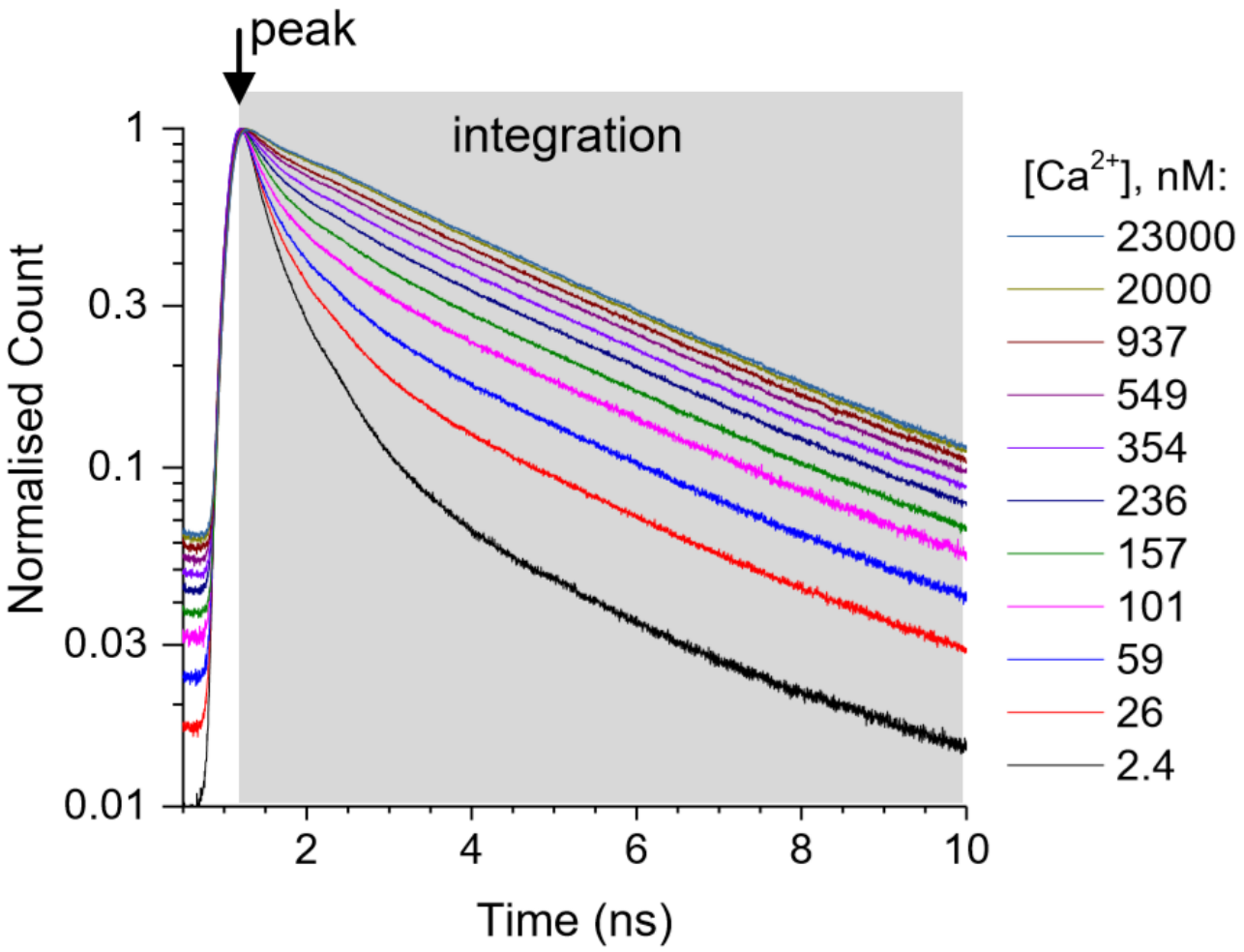
Utilisation of FLIM in improving measurement signals

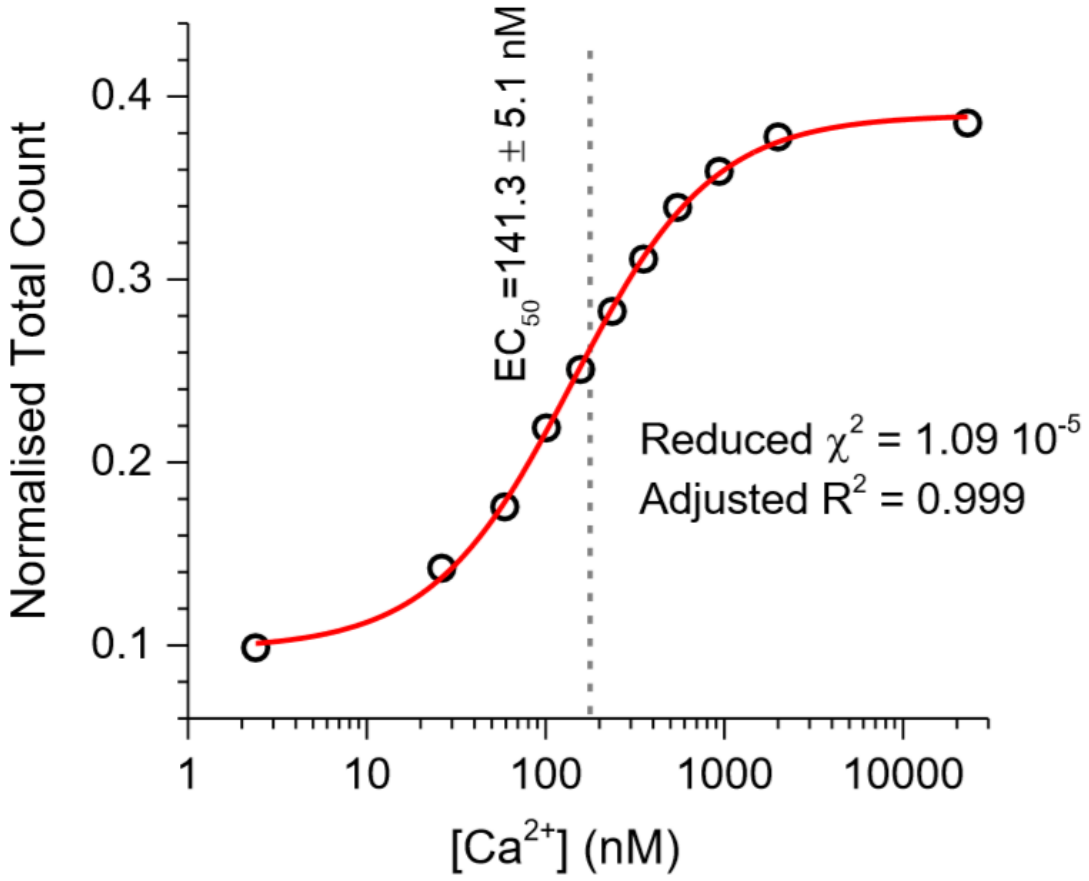
A FLIM setup


Laser control unit
IR laser ~200 fs pulse
80 MHz clock
BS1
P1
Sync input
Pulse picker
Scan unit
External clock
Acquisition and analyses
TCSPC system
DET1
D2
D1
F1
F2
DET2
Output
Photon timing
Routing signal
Sample

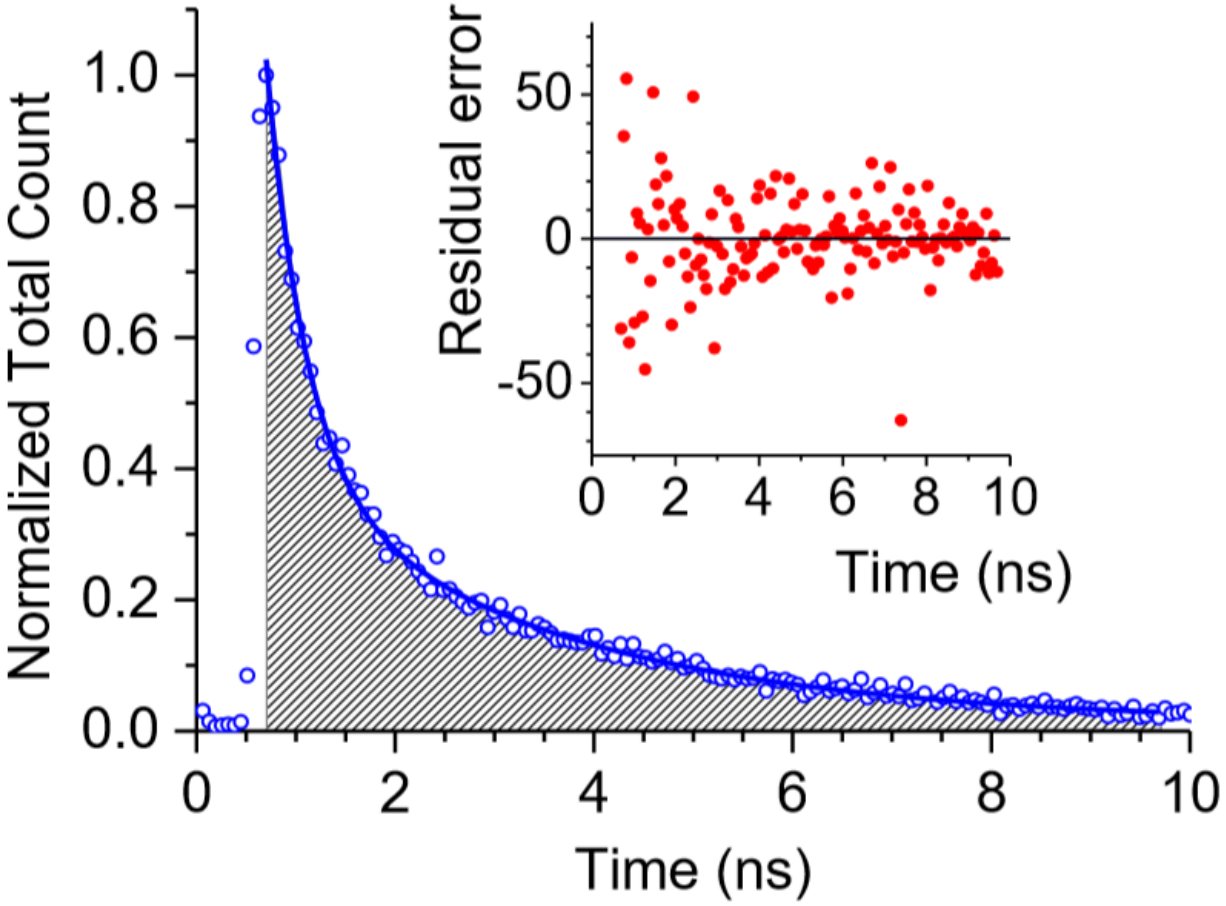

Scan Unit
Det1
D2
D1
Det2

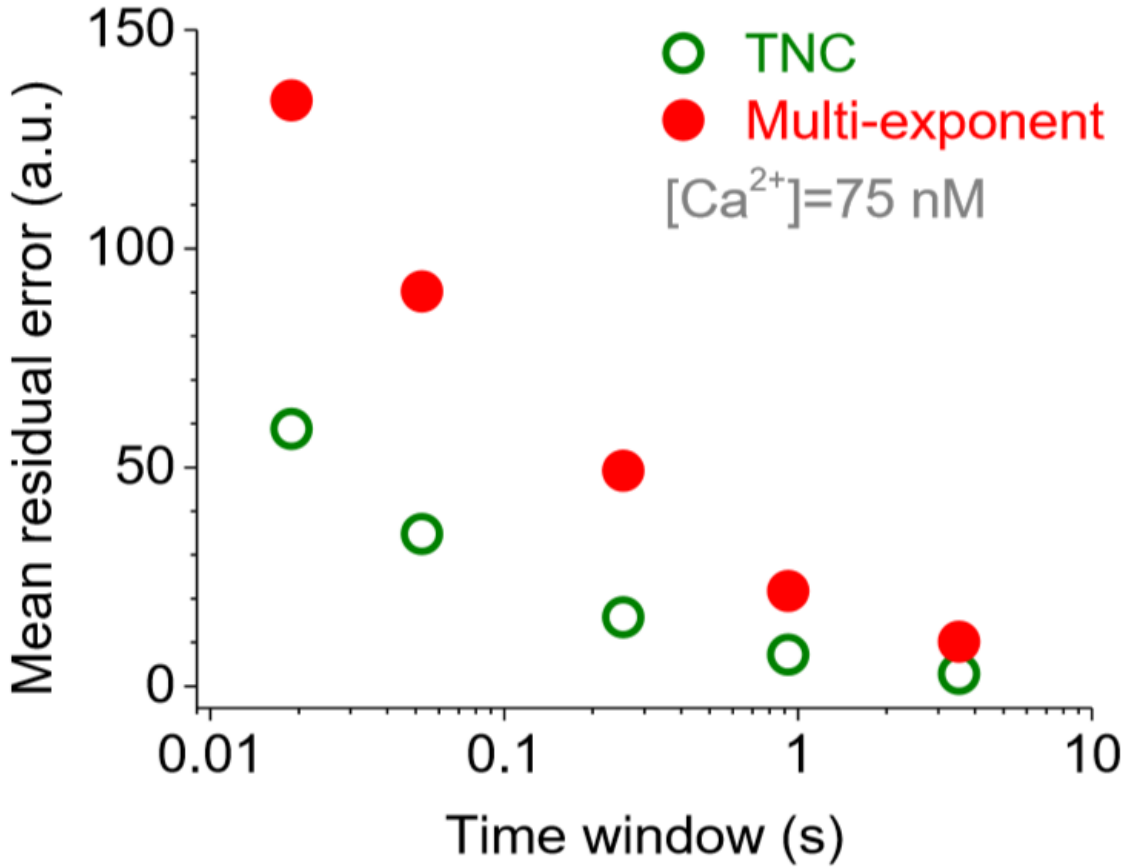
Use simple integration to avoid triple exponential fitting



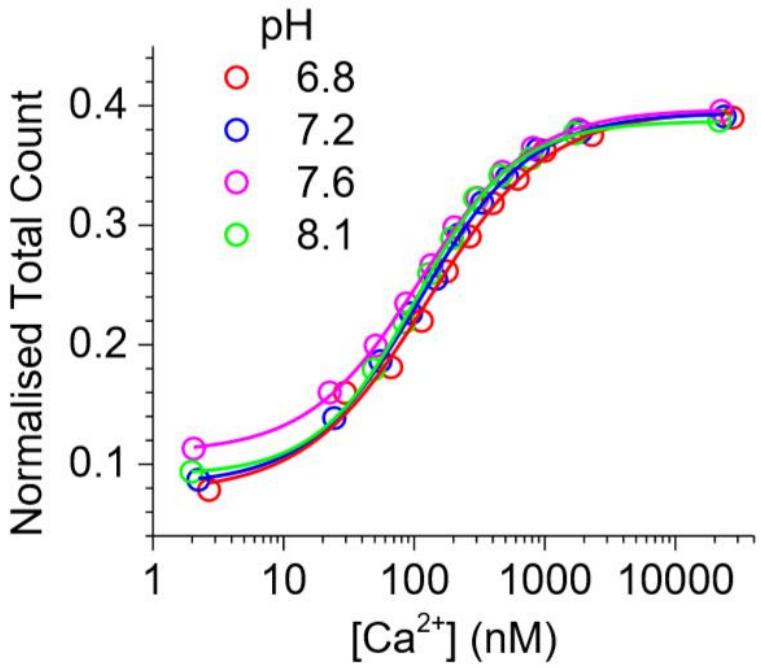


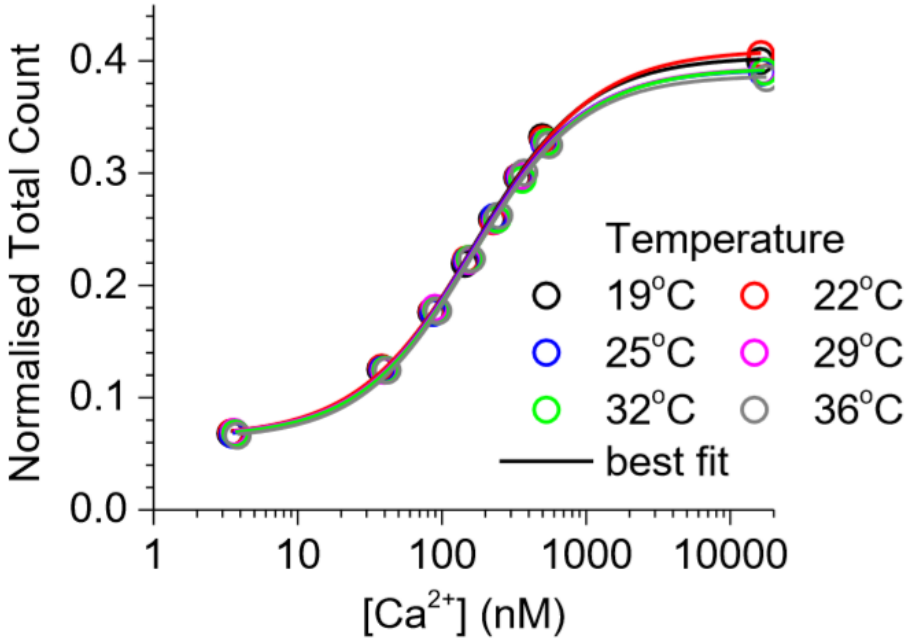
Comparison of NTC and multi-exponential fitting

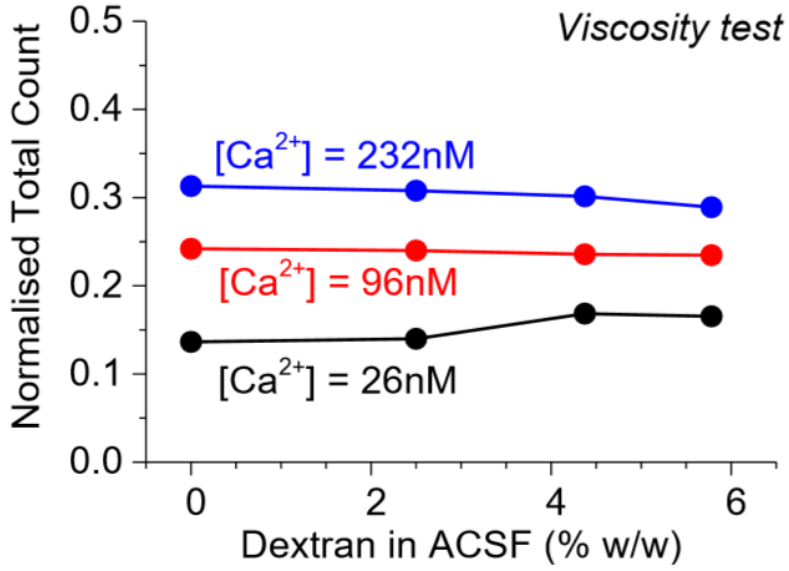


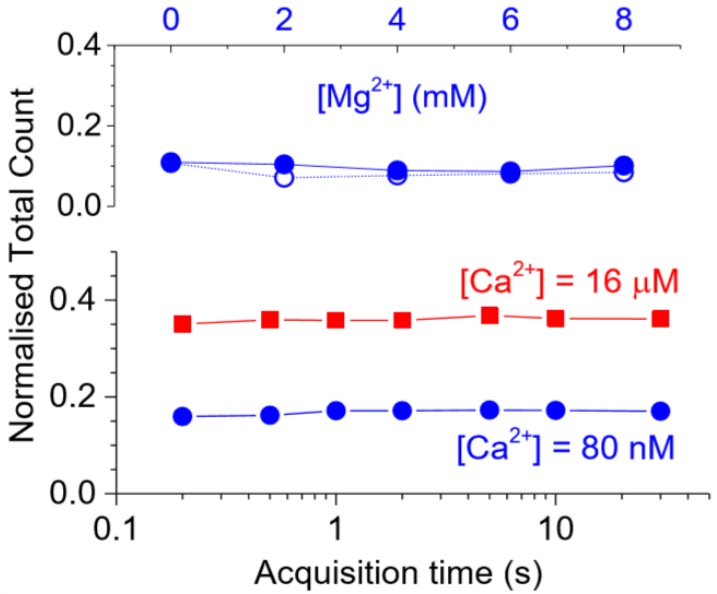


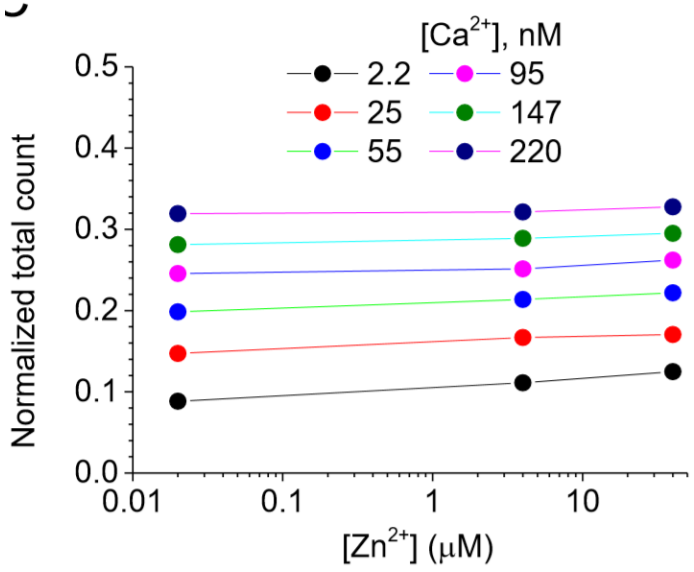
Testing OGB1 FLIM reliability to concomitant elements

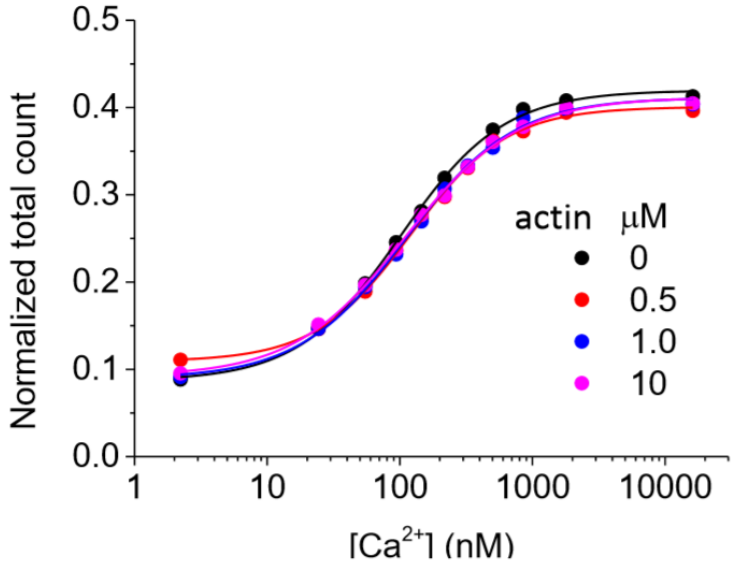




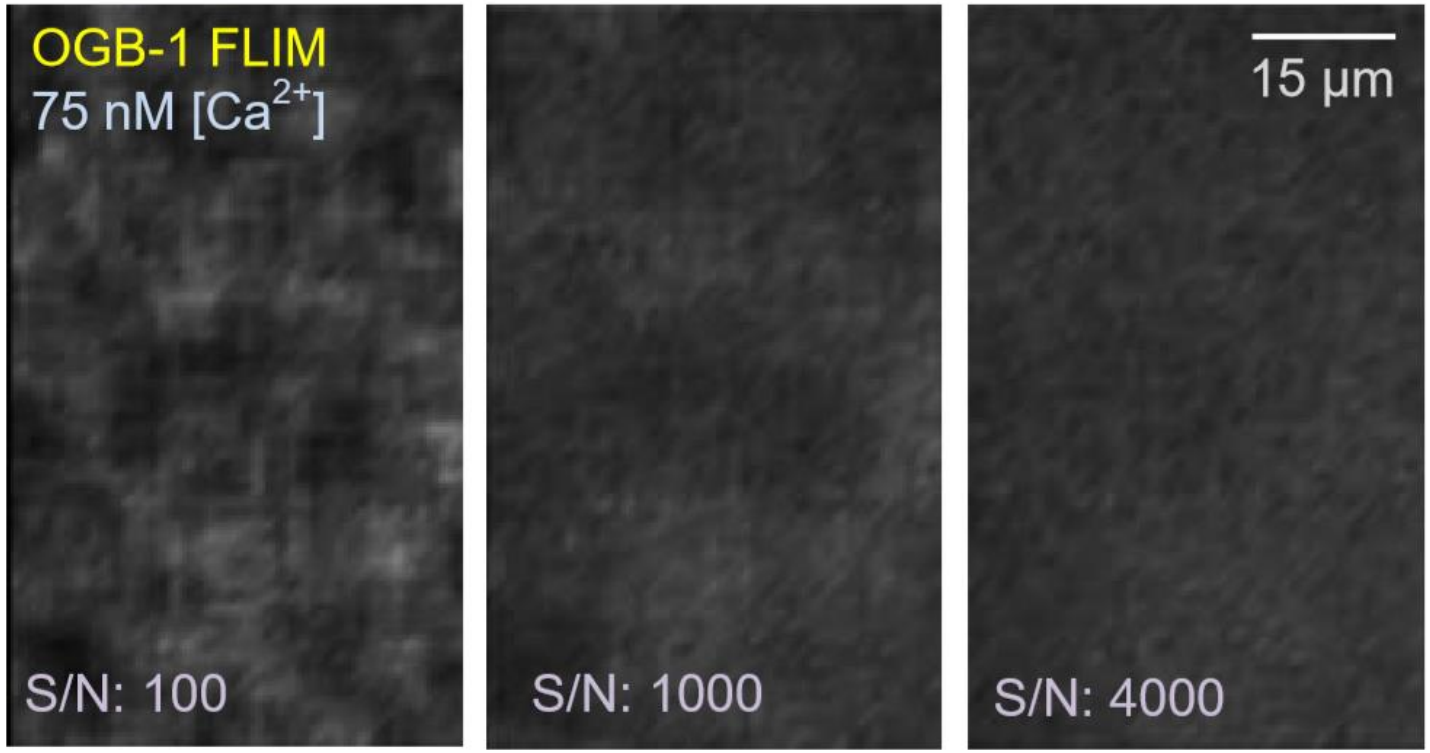


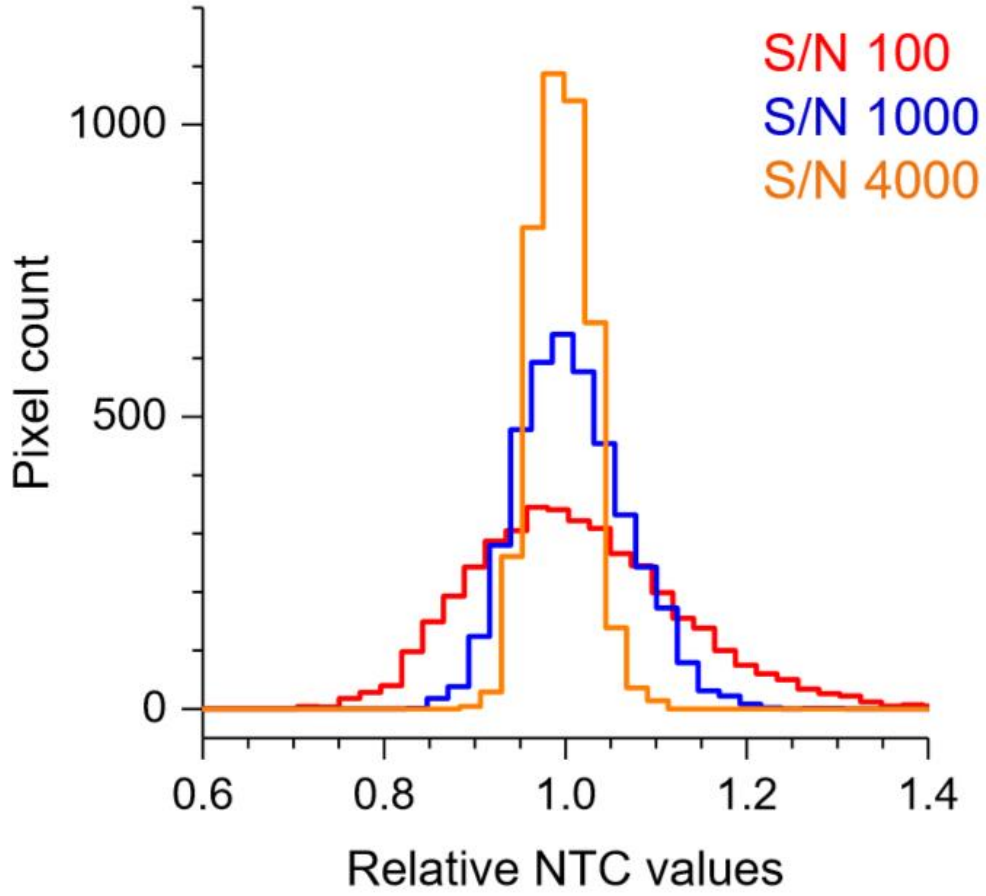






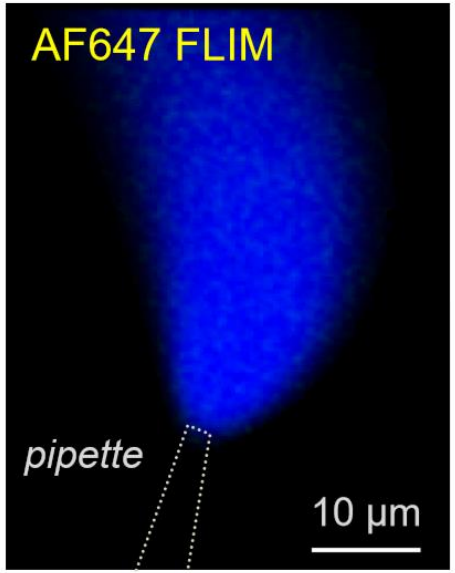
Noise! Always noise

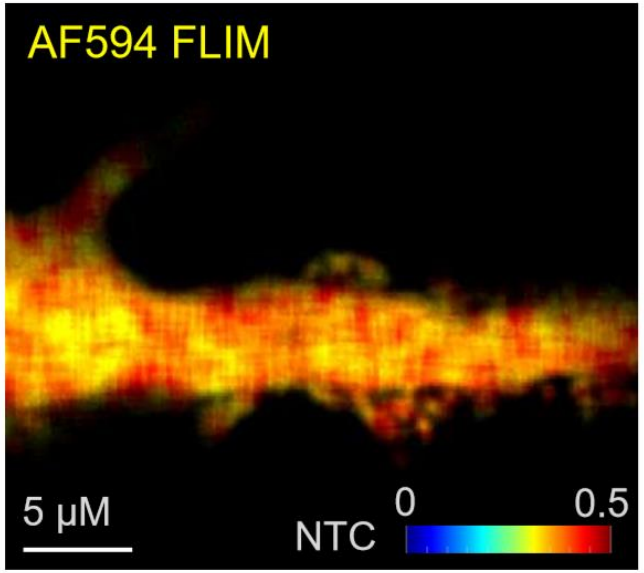


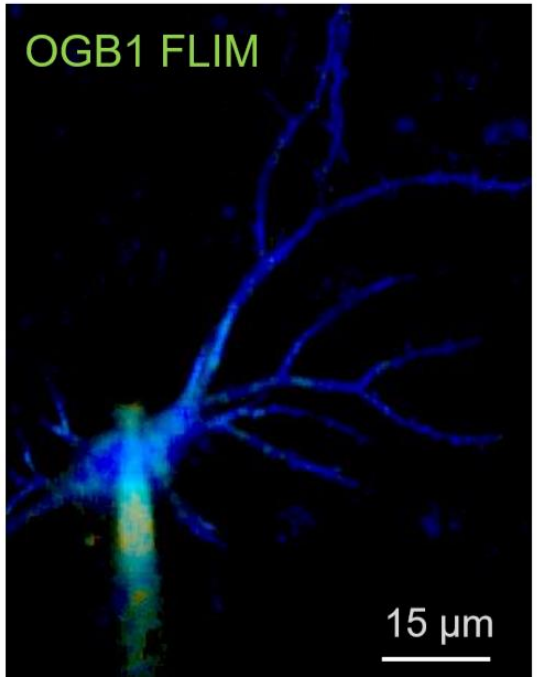


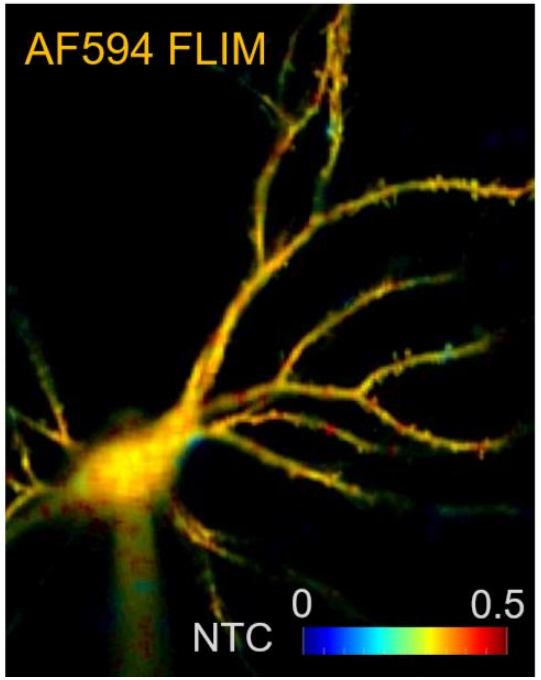
Bath medium of calibrated 75nM [Ca^{2+}]

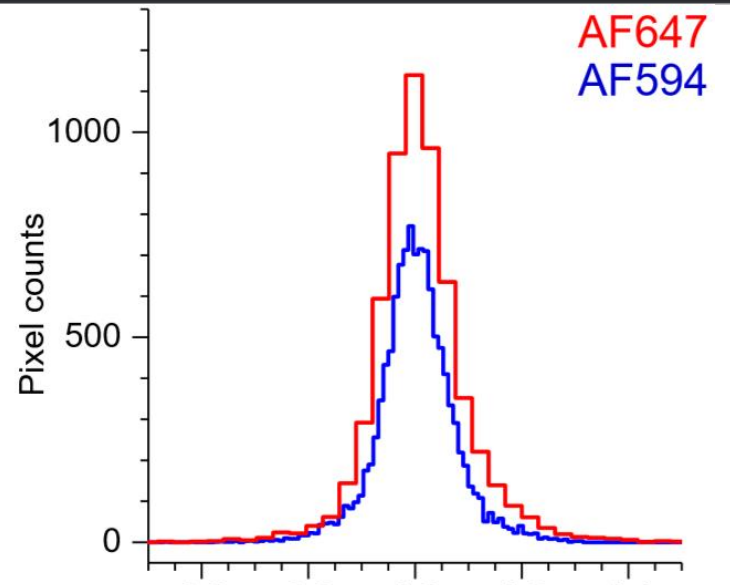
More noise in situ

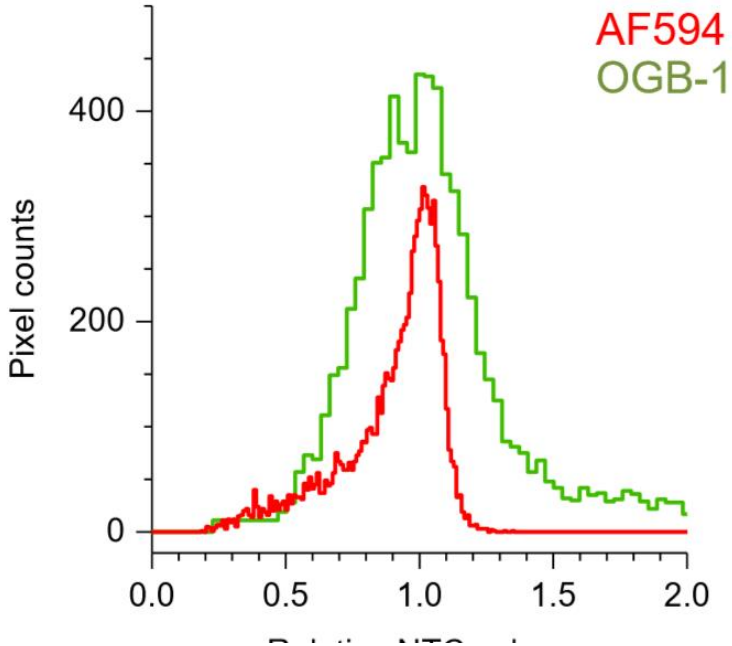




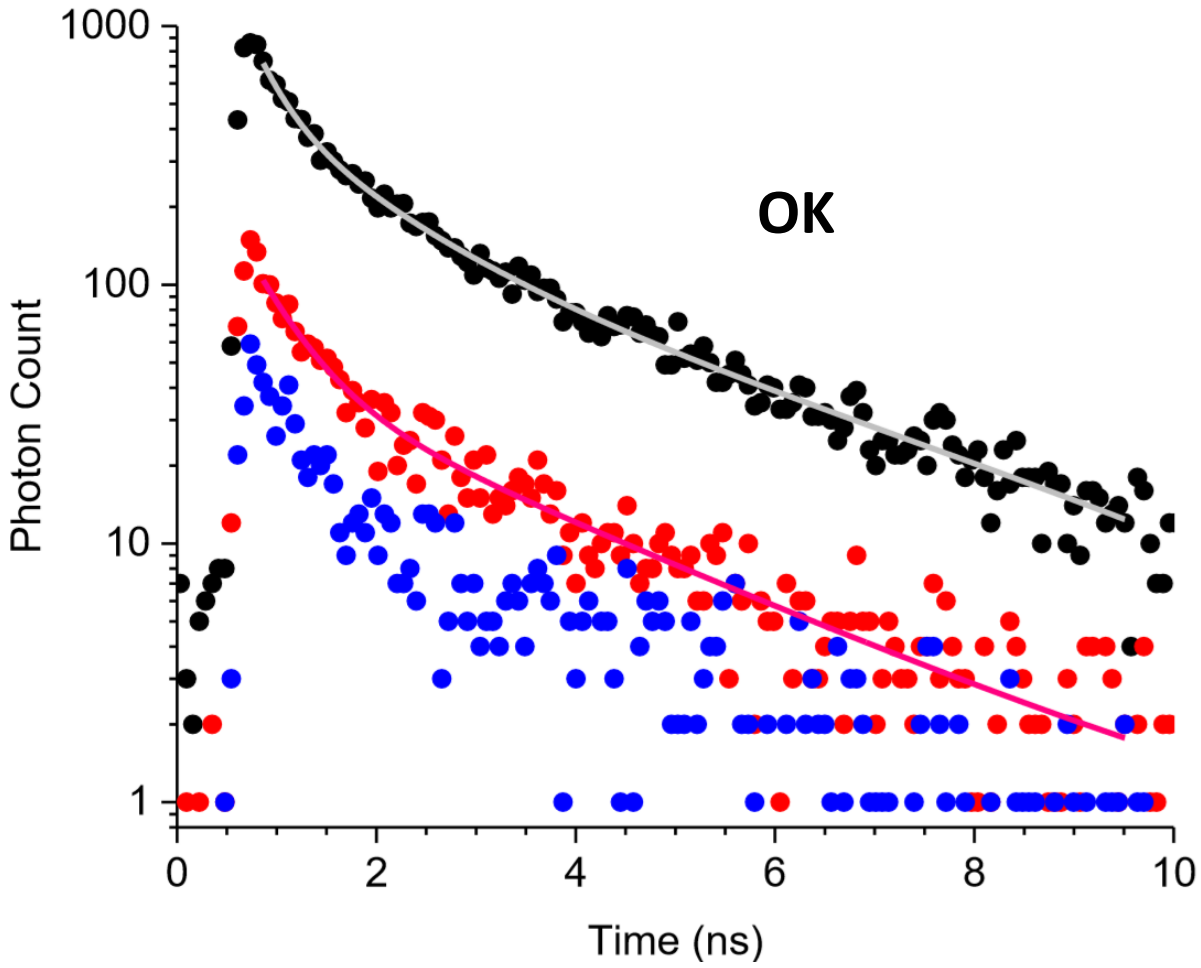








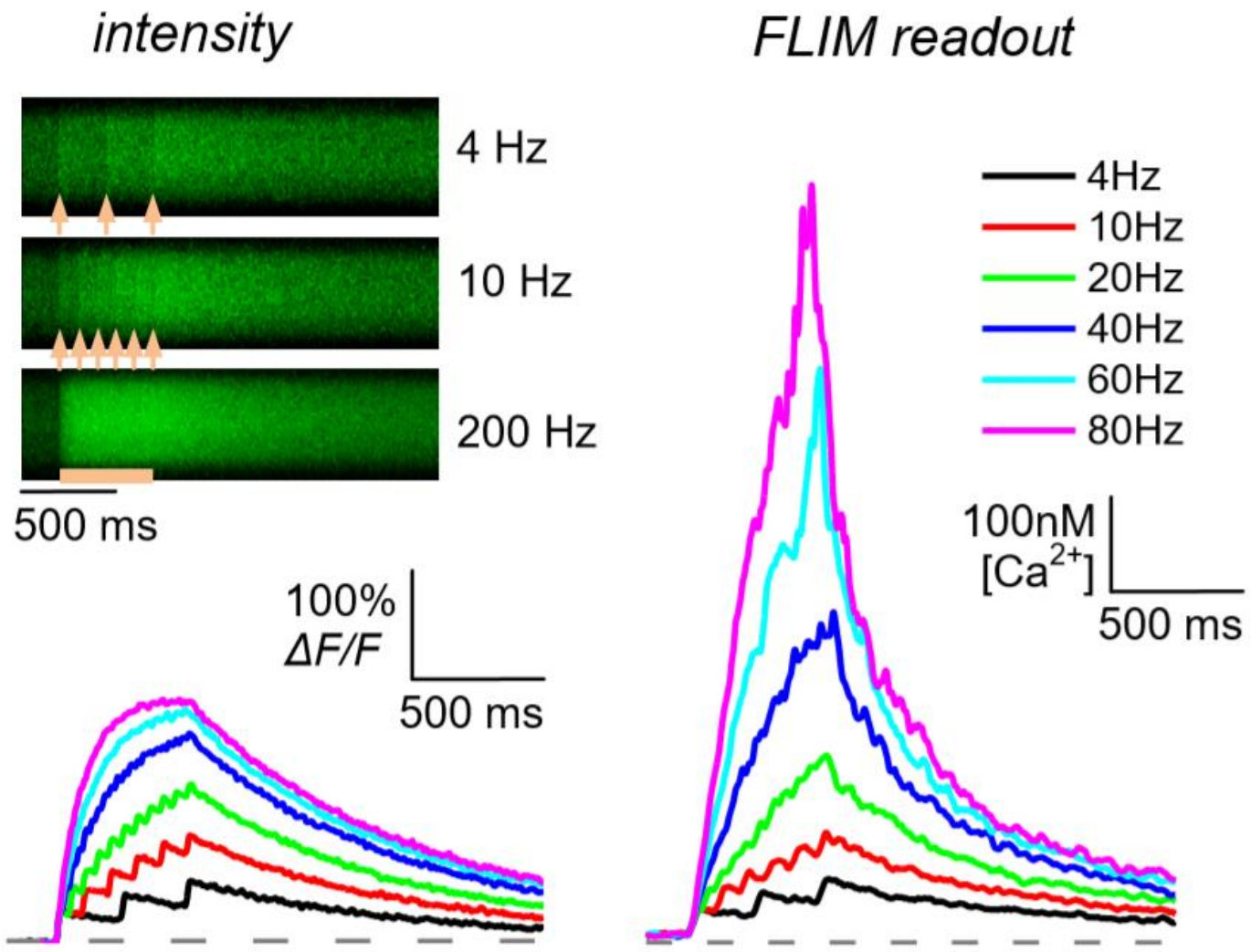
Acceptable noise for FLIM imaging using OGB1

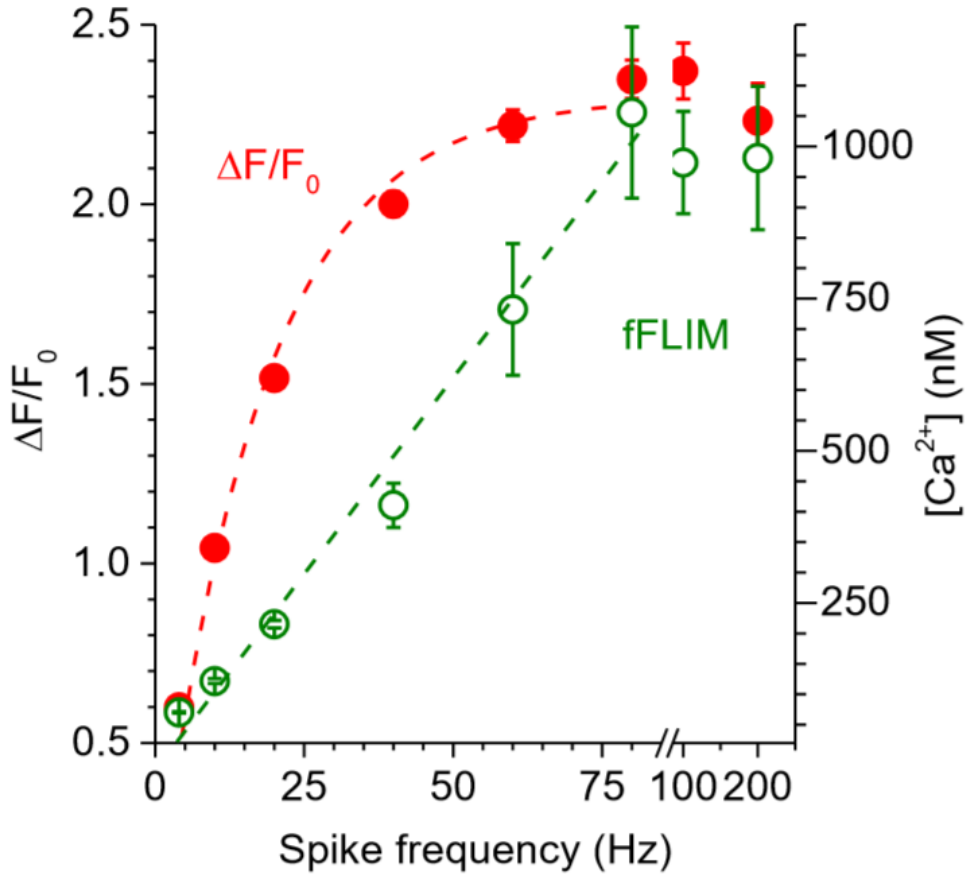


Just about

Throw away don't look back

OGB1 FLIM has good linear response





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Calcium in Cellular Signalling

Discovered in isolated rat hearts by Sidney Ringer [Ringer, S. (1883) J. Physiol. 4, 29–43] when making saline medium out of London tap water

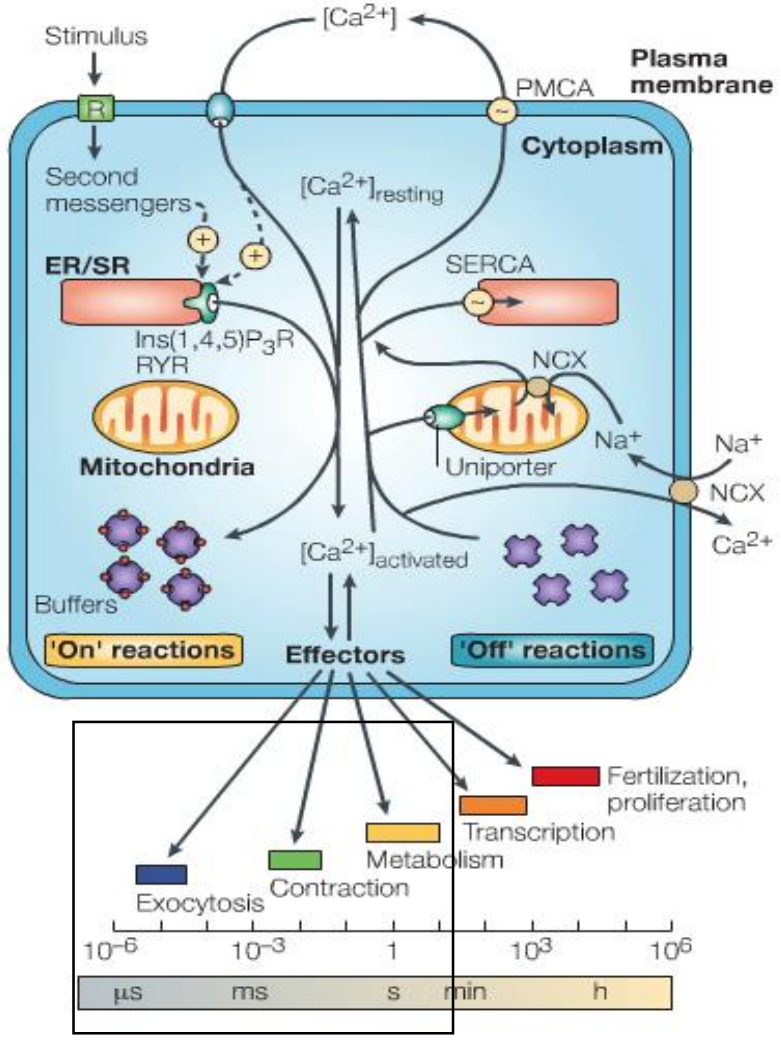
Ubiquitous in biological organisms.

Function-wise: smooth muscle contraction, ***neurotransmitter release***, gene expression, ***memory formation by LTP and LTD in hippocampal and cortical regions***, cell apoptosis, ***cell metabolism***, fertilisation

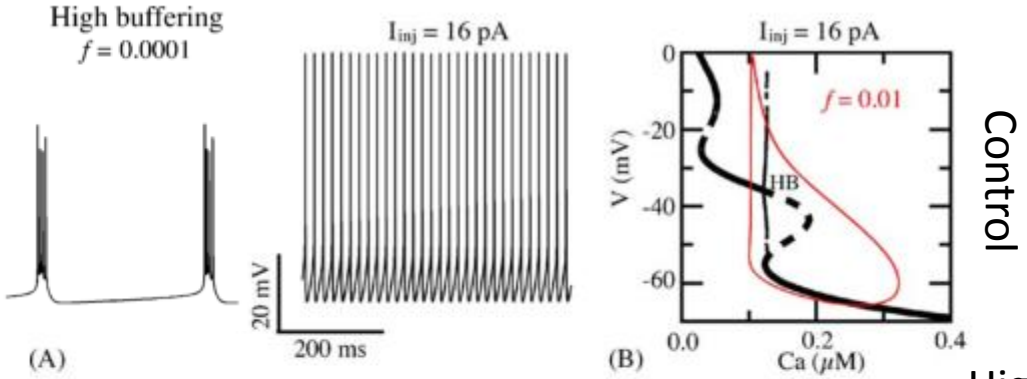
Location-wise: mitochondria, cytoplasm, endoplasmic reticulum of all cells

Potency

Acts as primary, secondary messengers and interacting with huge repertoire of molecules with generally quite high binding efficiency (Kd in the nM range)

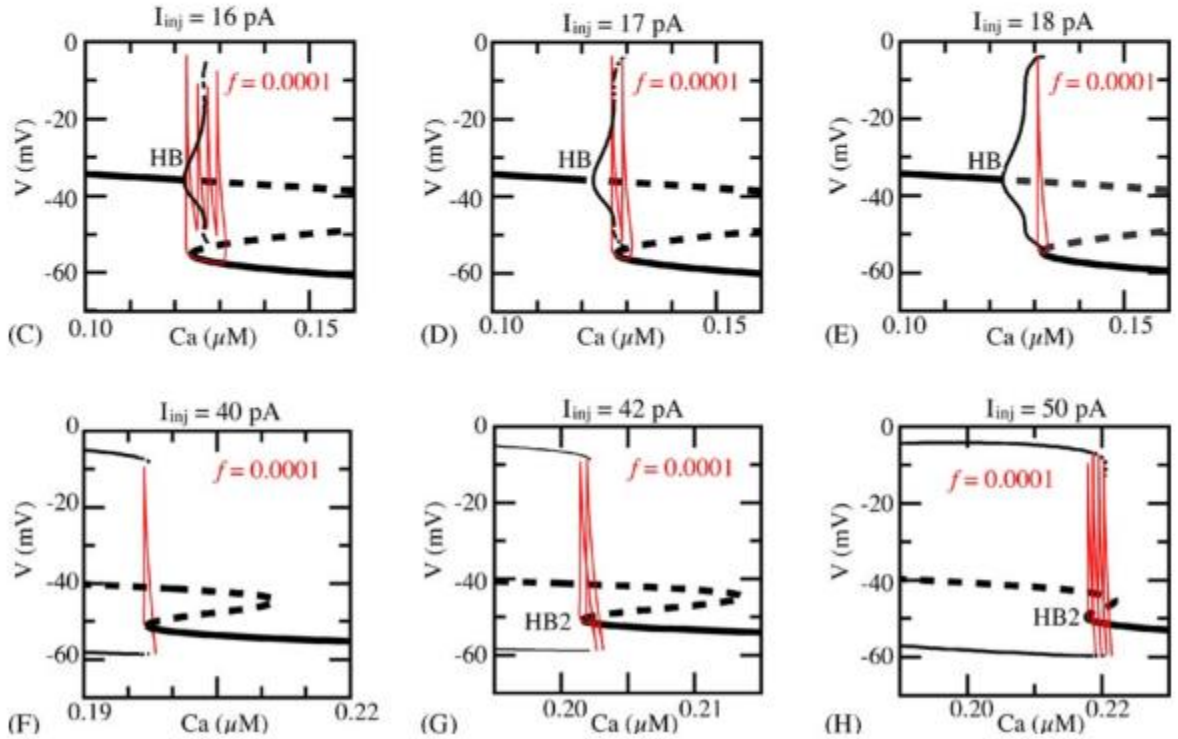


Basal $[Ca^{2+}]_i$ on cerebella granule cell firing properties



Control

High buffer

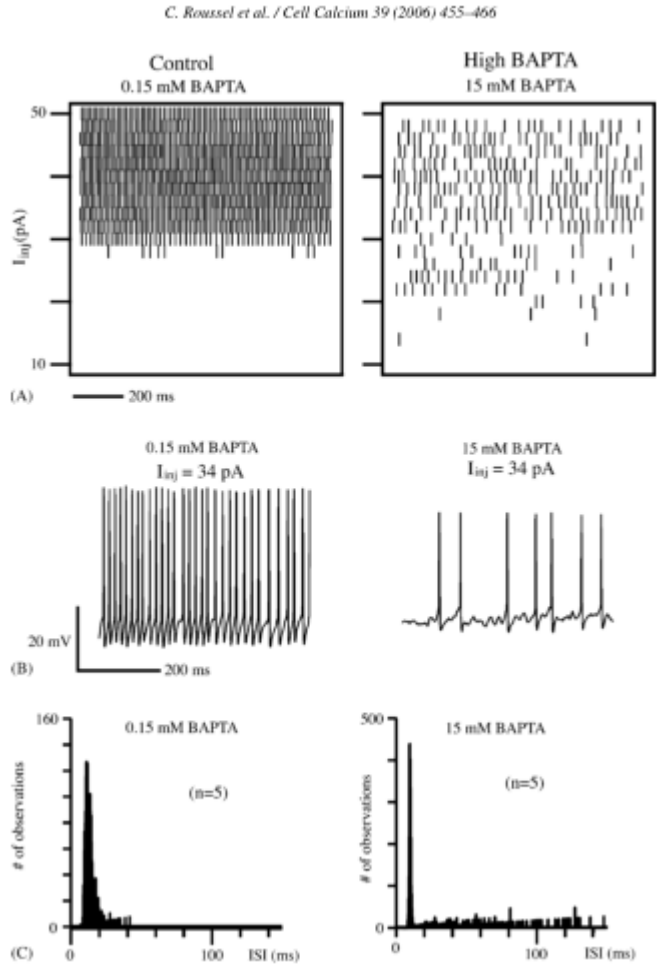


Solid line = stable steady state

Dash line = unstable steady state

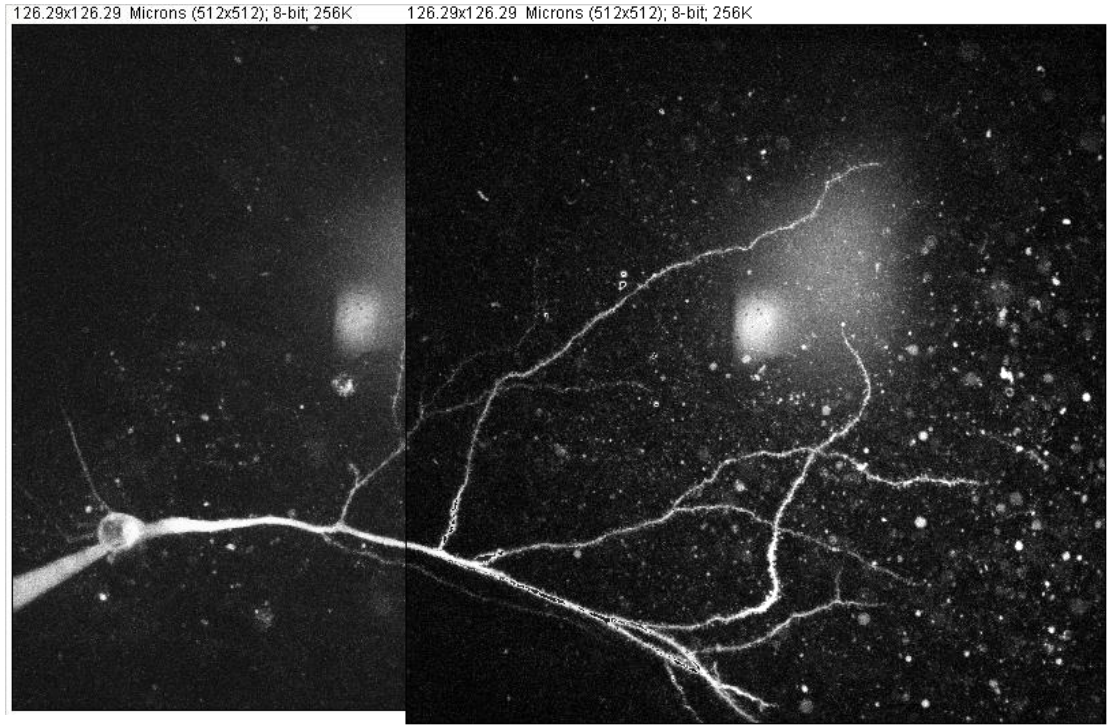
$$\frac{dCa}{dt} = f\left[-\frac{I_{Ca}}{2FV_d} - \beta_{Ca}Ca\right]$$

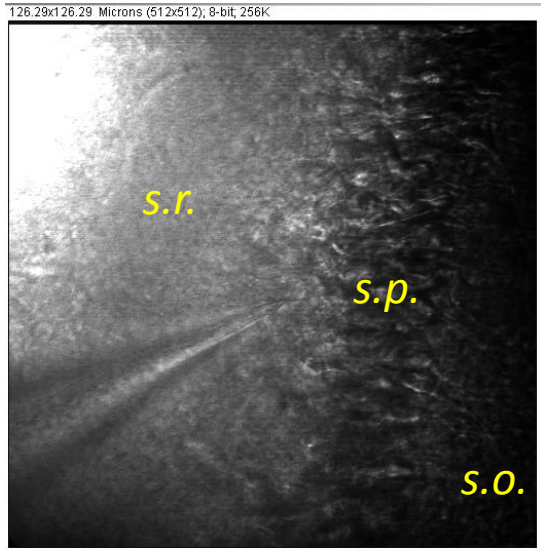
f = cytosolic buffering capacity



D. Gall (Cell Calcium 2006)

Patching and loading OGB1 & AF594 into neuron





P7-21 SD rats

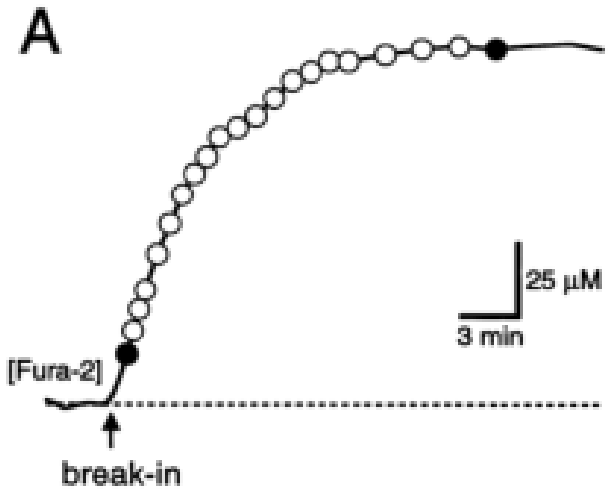
Hipppocampal slice, CA1 , *s.p.,* Pyramidal Cells, V_m=55-65mV

Temperature = 30-33C

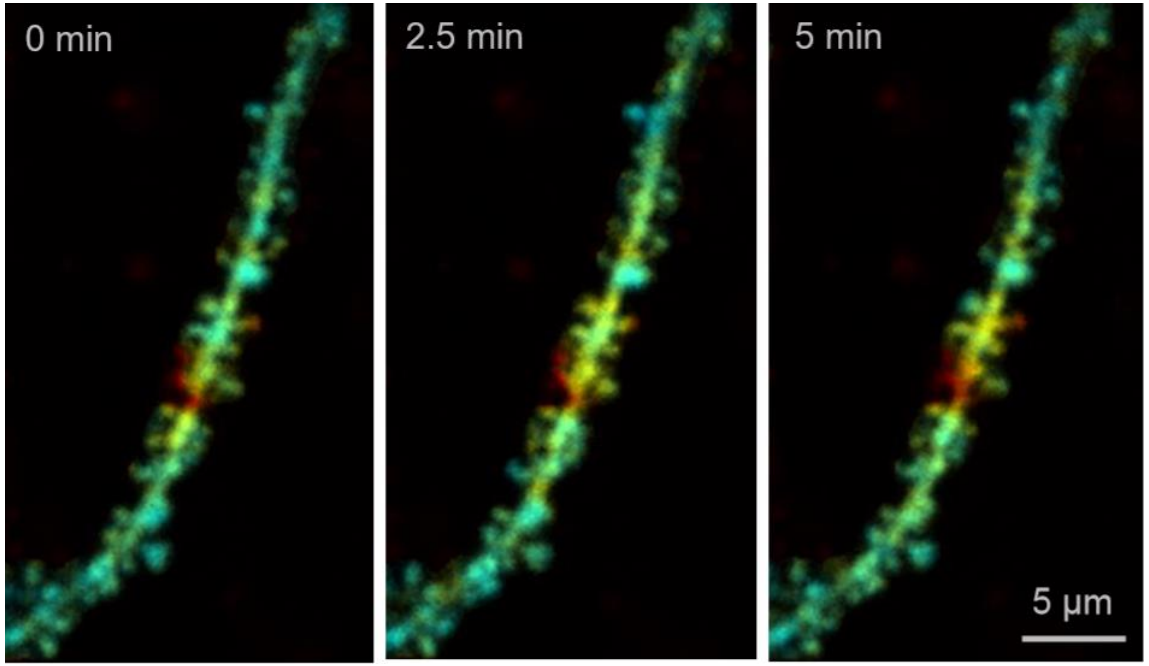
200µM OGB1

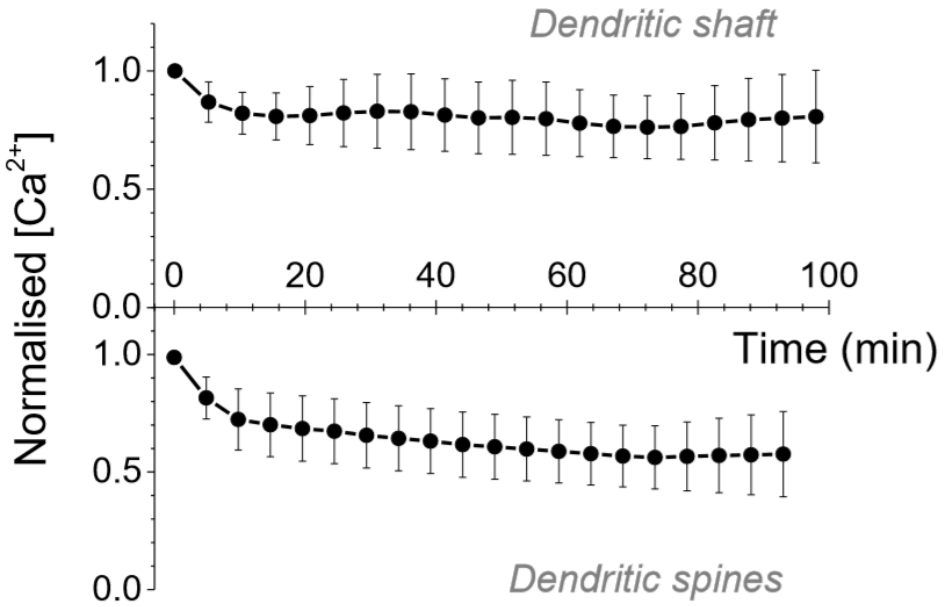
Patience is a virtue, Happy cell happy life

Fluorescent dye loading Bert Sakmann (Biophys J. 1996)

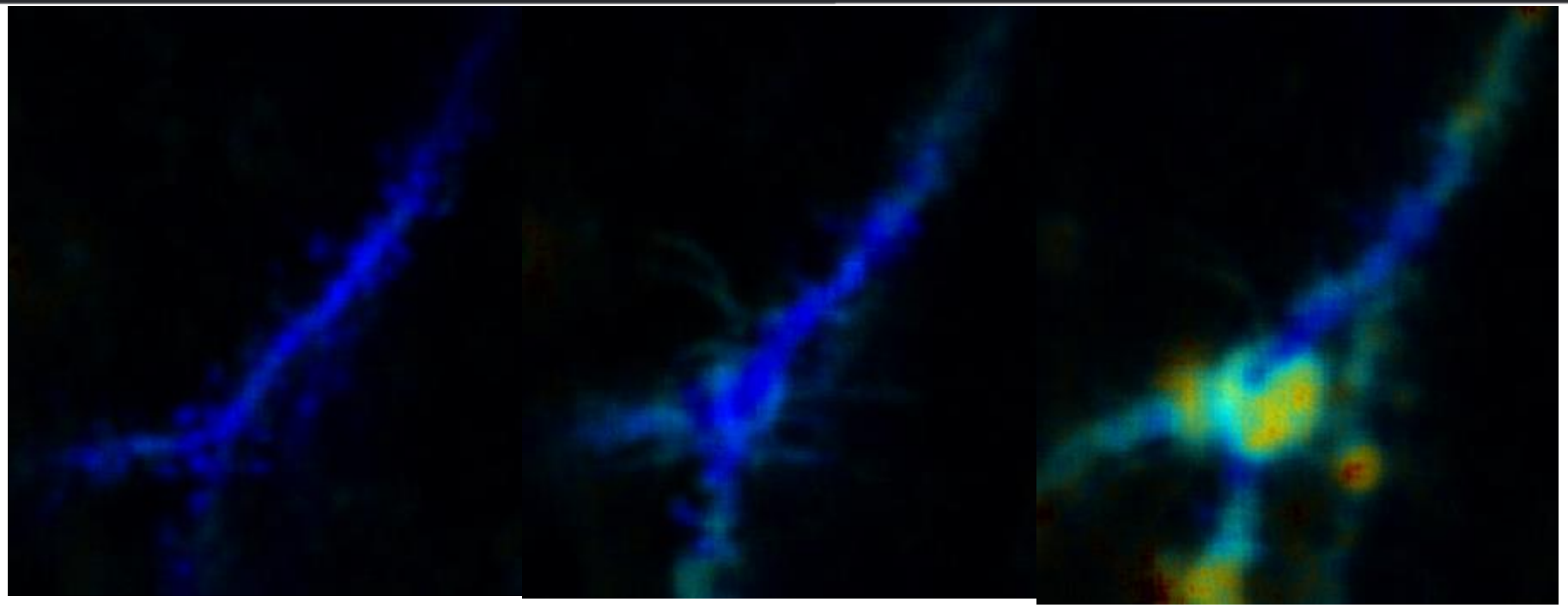


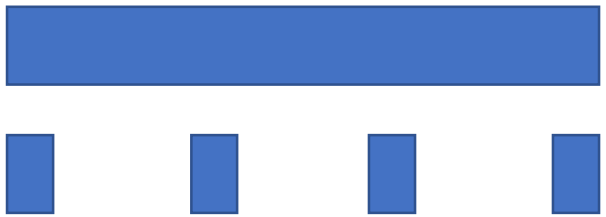
- **To minimise OGB1 buffering effect on somatic basal calcium levels, we measure it between 5-10min post break in.**
- **However for dendritic and spine measurement it is impossible to measure accurately until after 30min or so.**





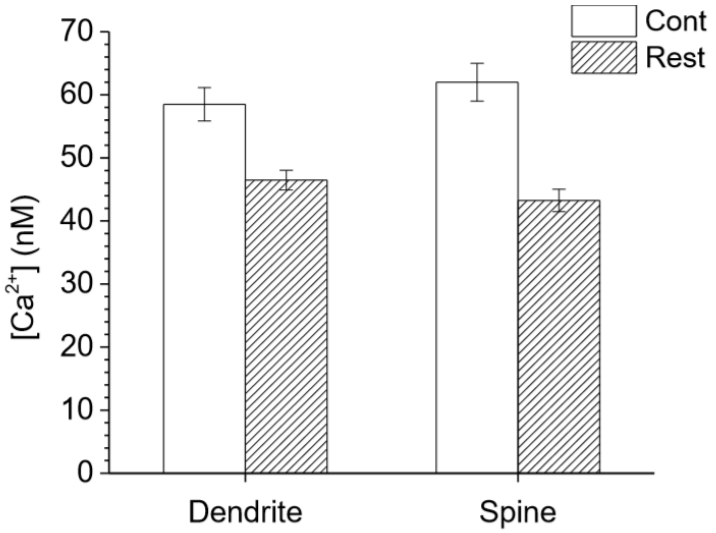
Cells are fragile, be gentle



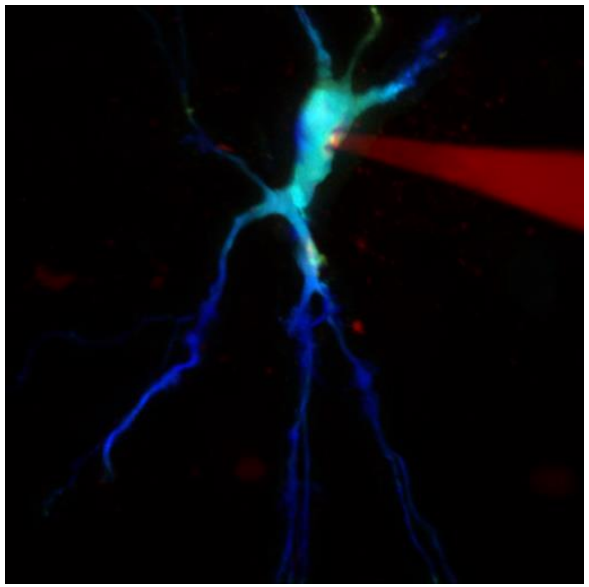


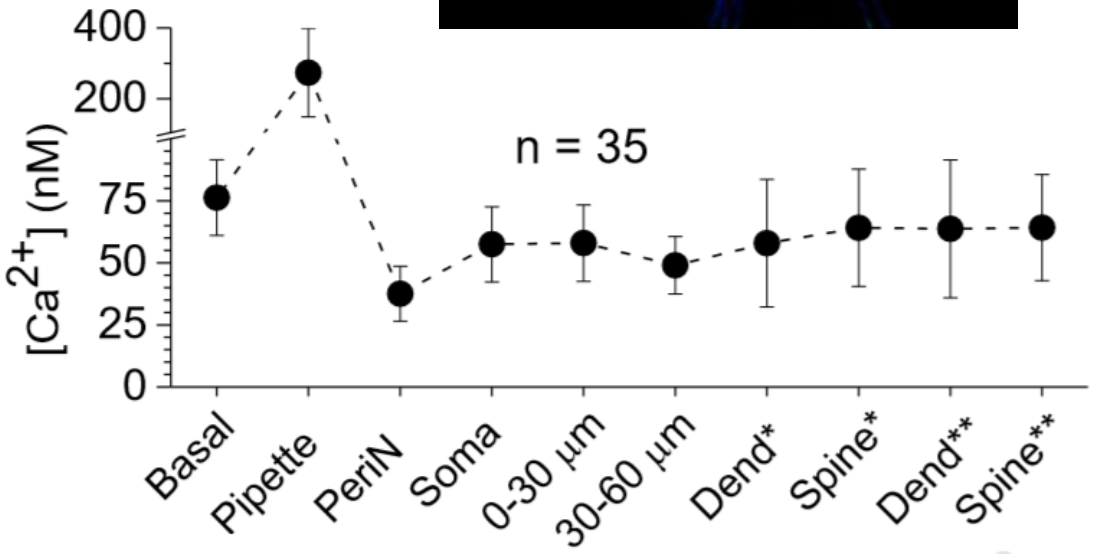
Continuous scan

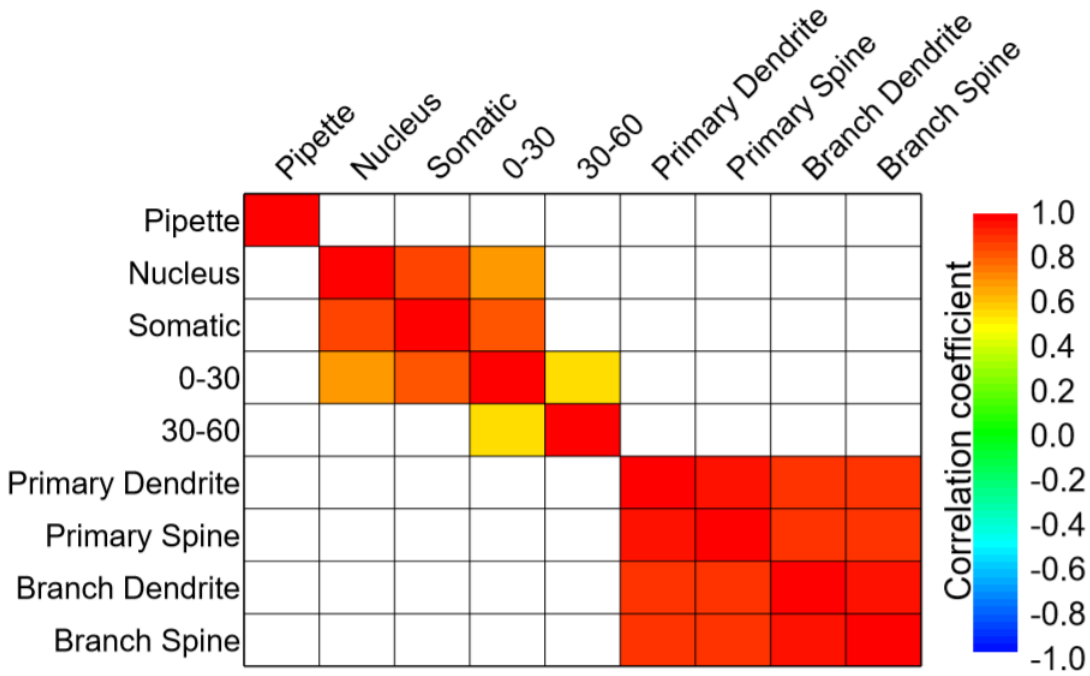
Resting scan



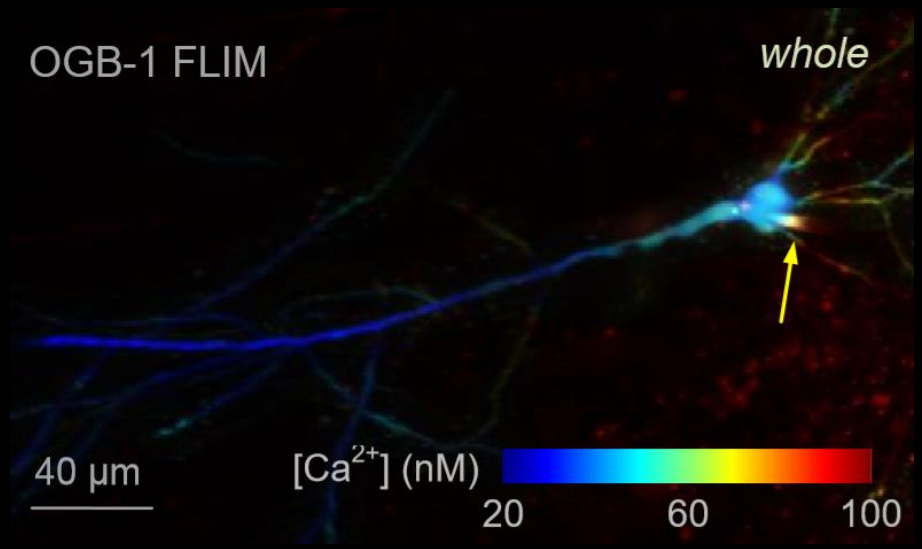
Neurons are good at maintain calcium levels

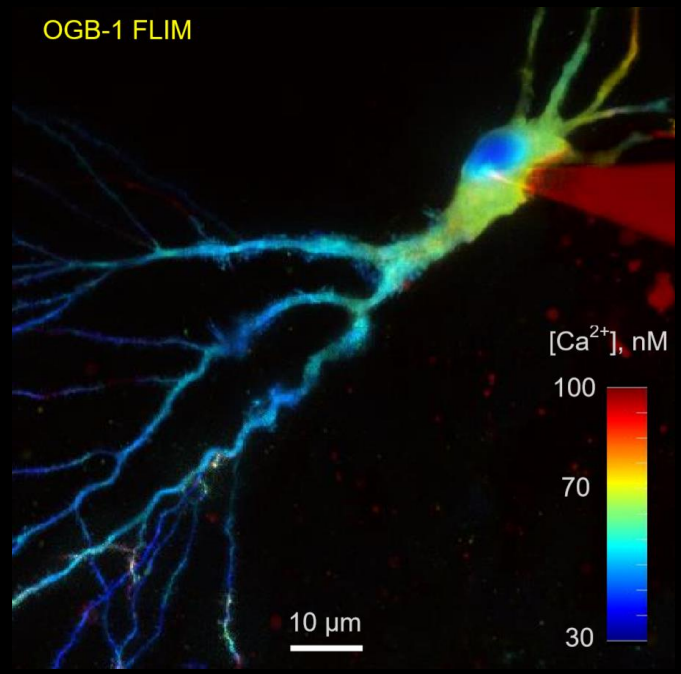


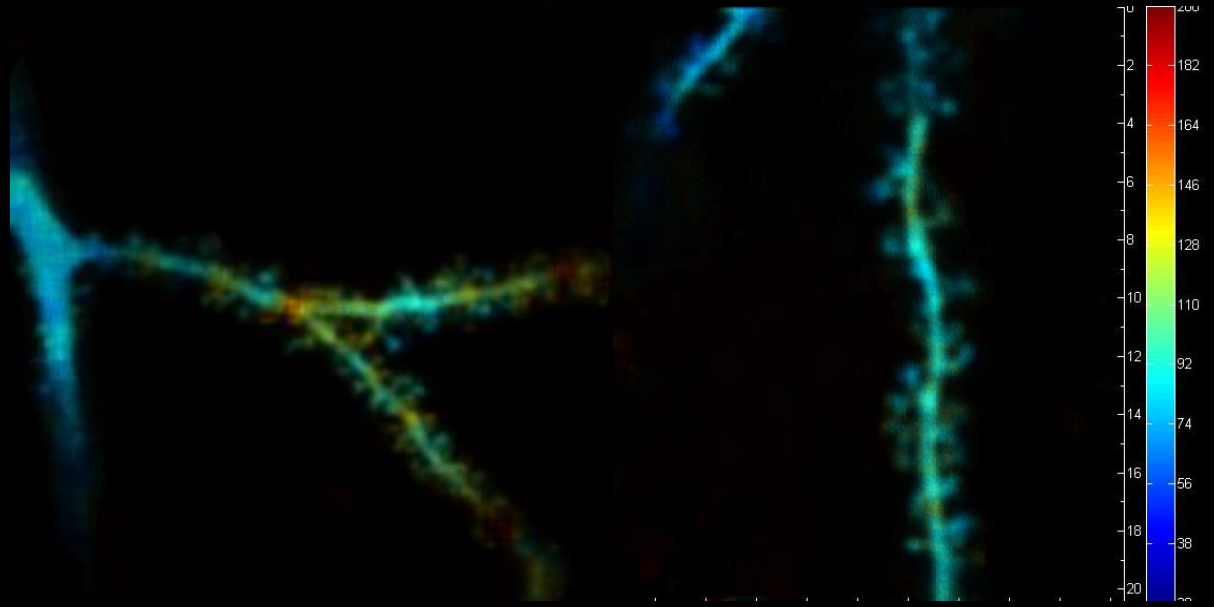


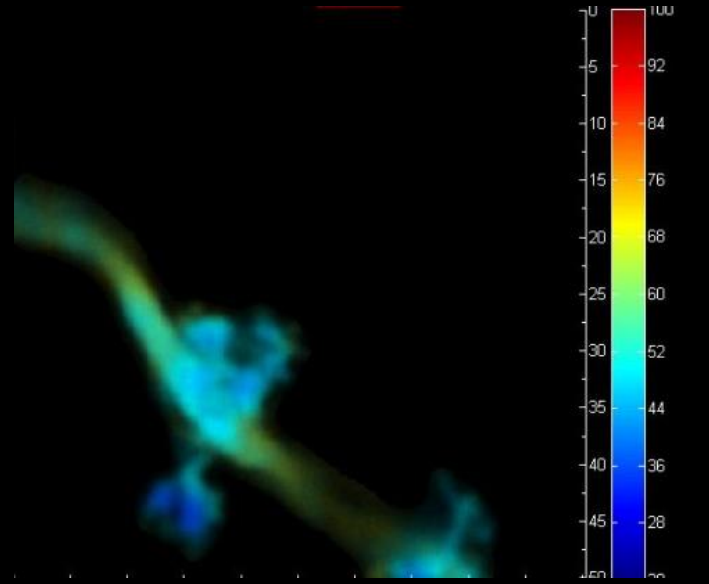


Basal $[Ca^{2+}]_i$ map in some hippocampal neurons (CA1 and CA3 PC)

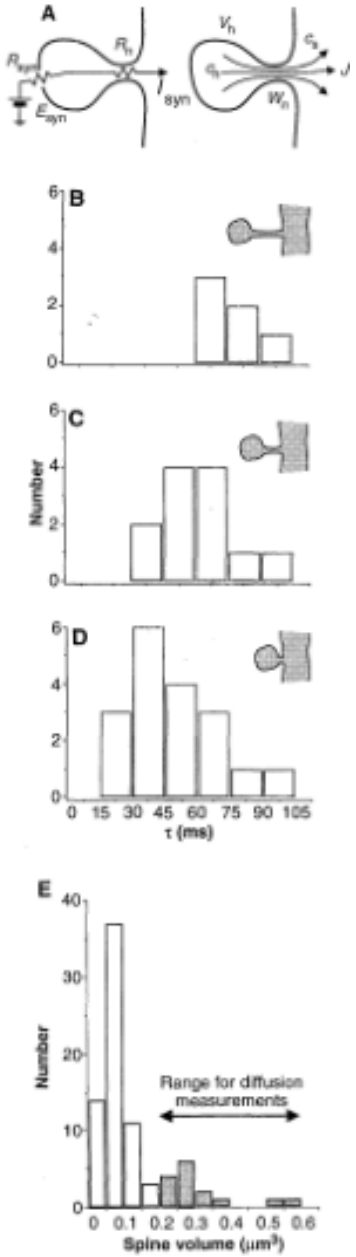


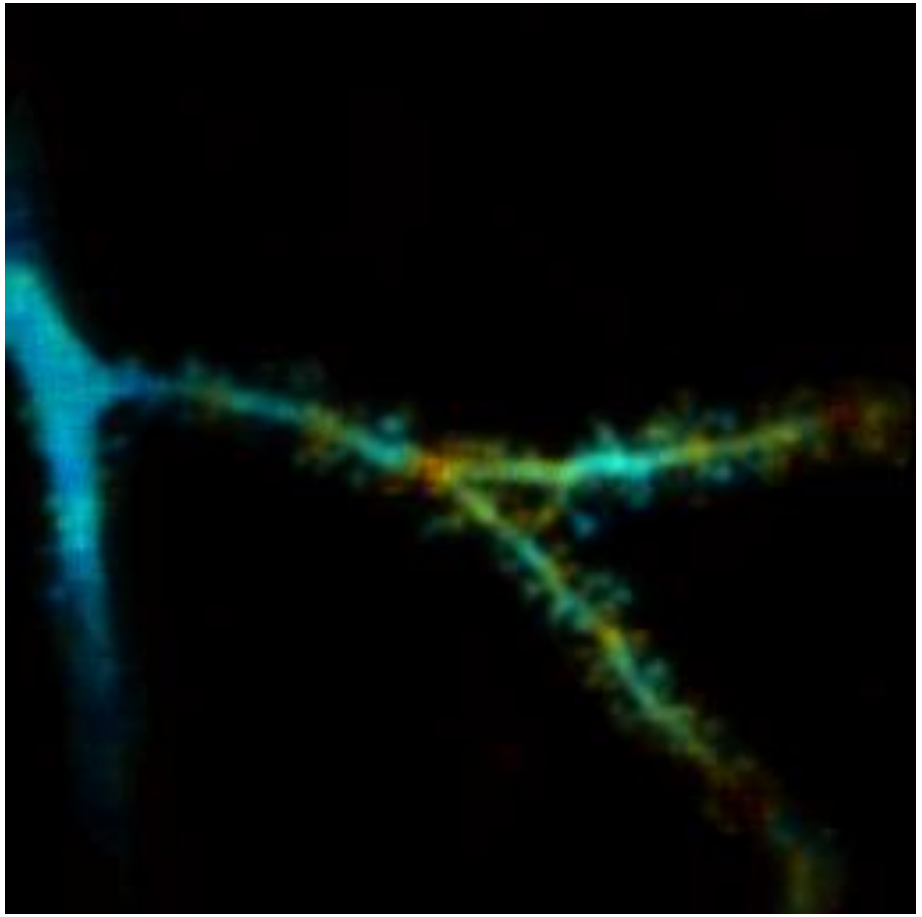


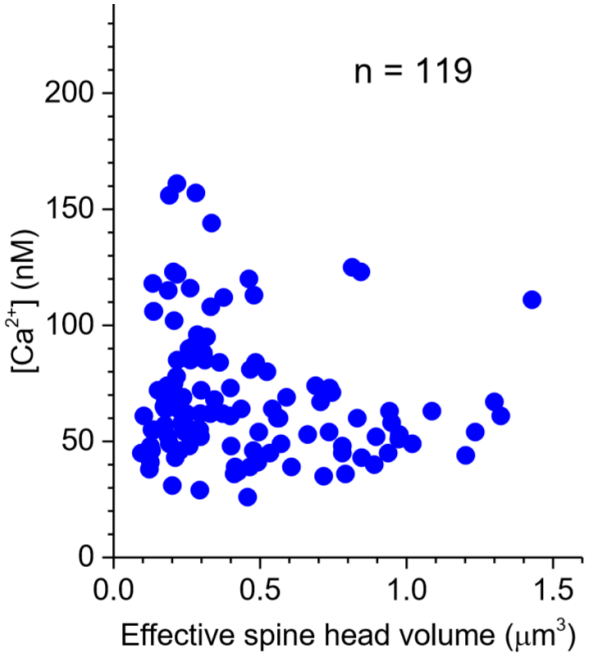


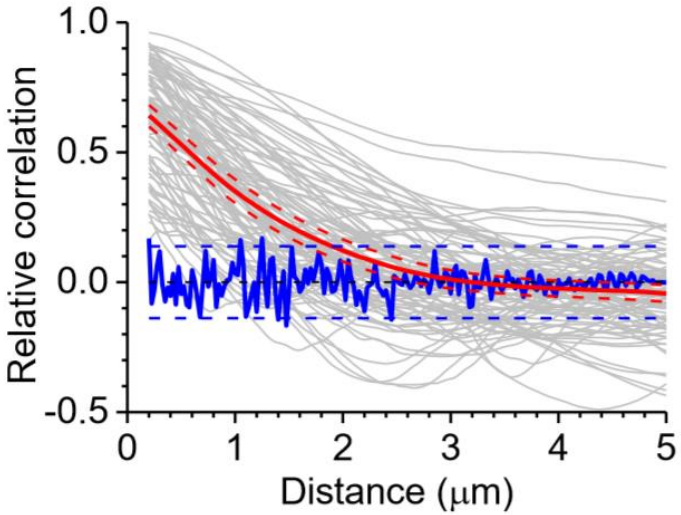


Compartmentalisation of calcium in neuron



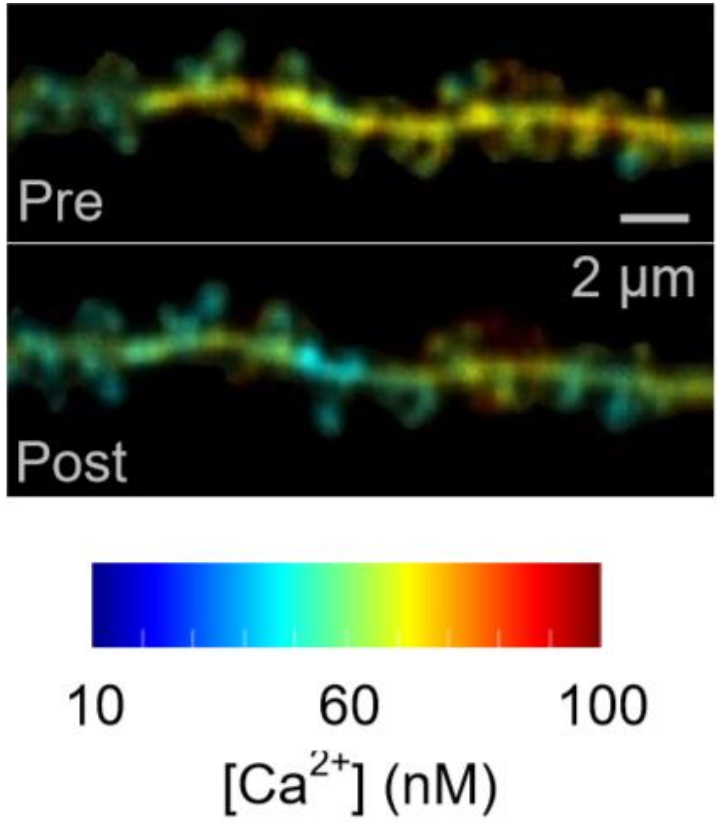


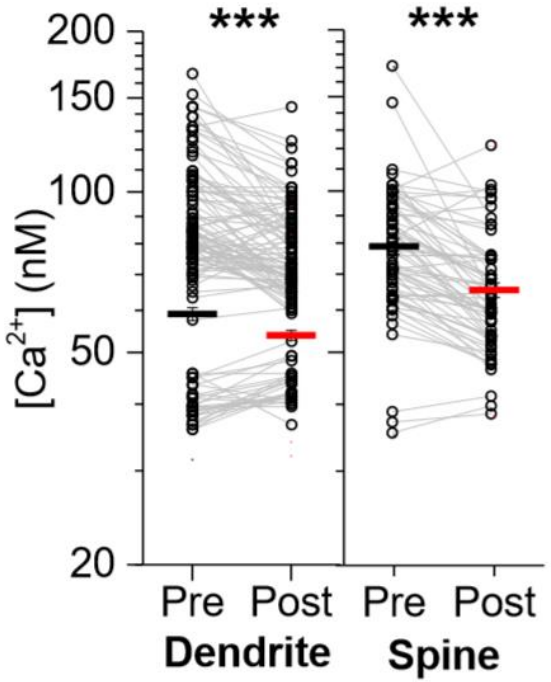




Svoboda, Tank, Denk (Science 1996)

Activity (bAP) induced resting calcium changes in neuron



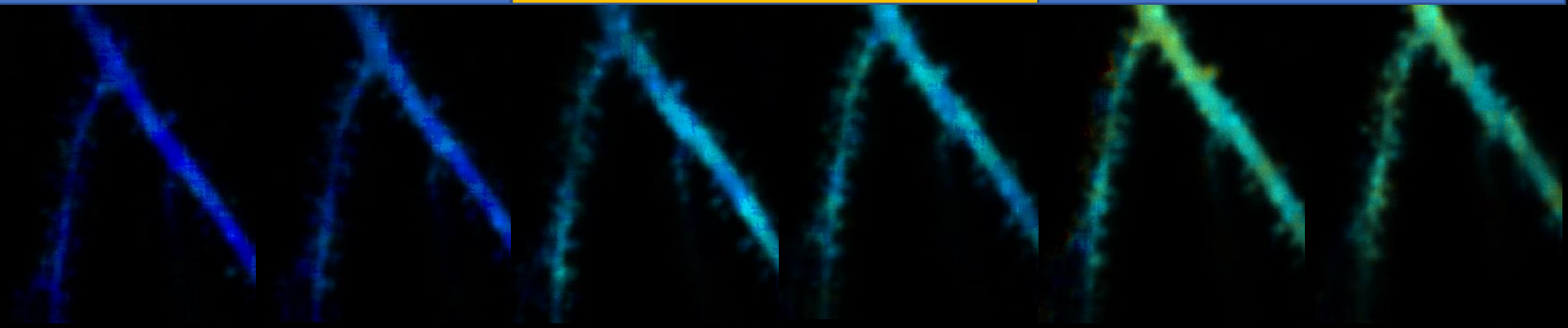


Low Mg^{2+} ACSF has varying results

H

H

Normal Mg^{2+} ACSF | Low Mg^{2+} wash in | Normal Mg^{2+} ACSF wash out

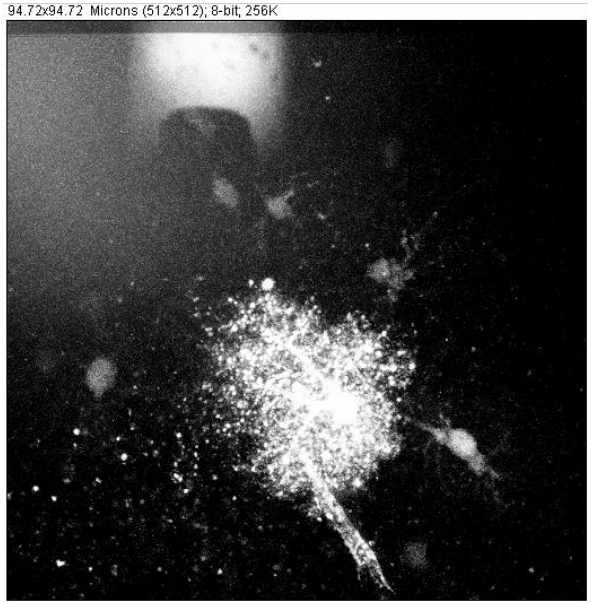


Normal Mg^{2+} ACSF | Low Mg^{2+} wash in | Normal Mg^{2+} ACSF

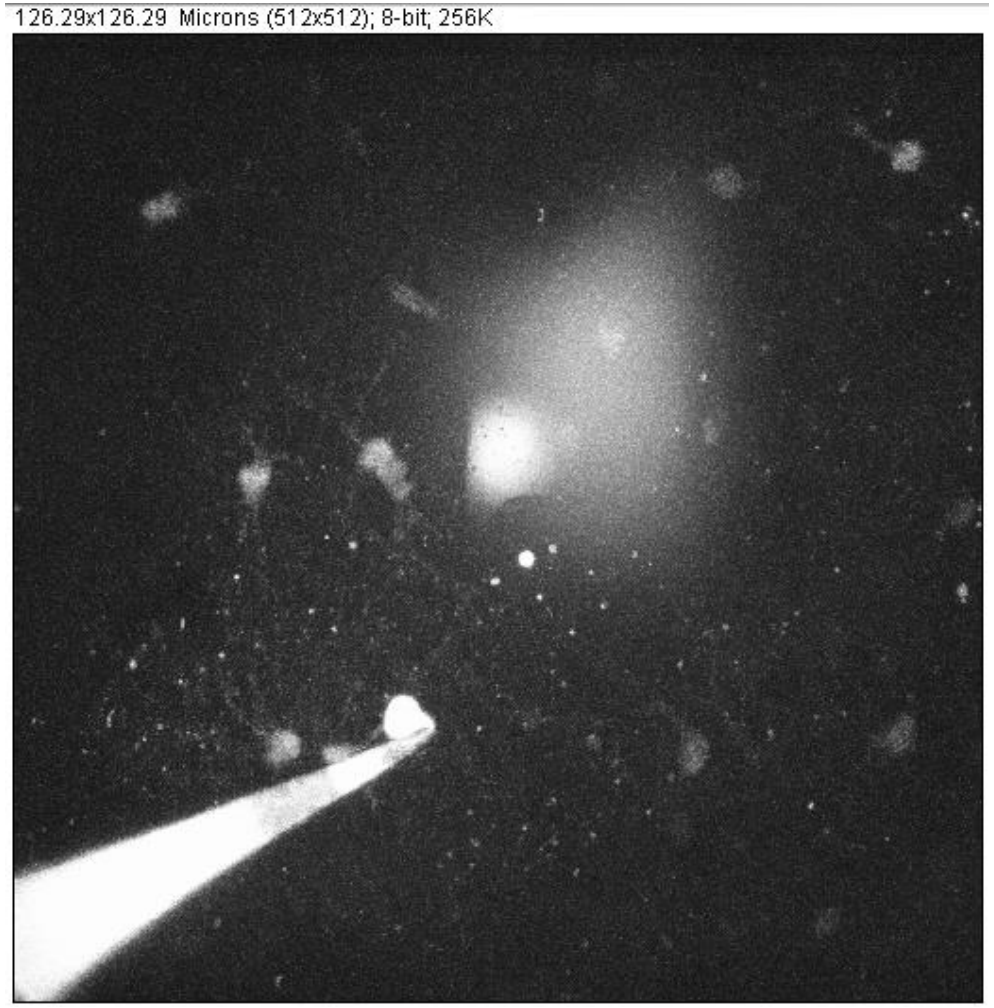
- **Patched neurons have resting calcium level of 40-70nM once reached equilibrium**
- **There is a calcium compartmentalisation in patched neuronal structures**
- **Patched neurons has large soma and sufficient machinery to maintain its calcium levels despite dialysed by the patch pipette**
- **Resting scan has to be adopted to avoid cells stress from prolonged laser scanning**

Patching and loading OGB1 & AF594 into astrocyte

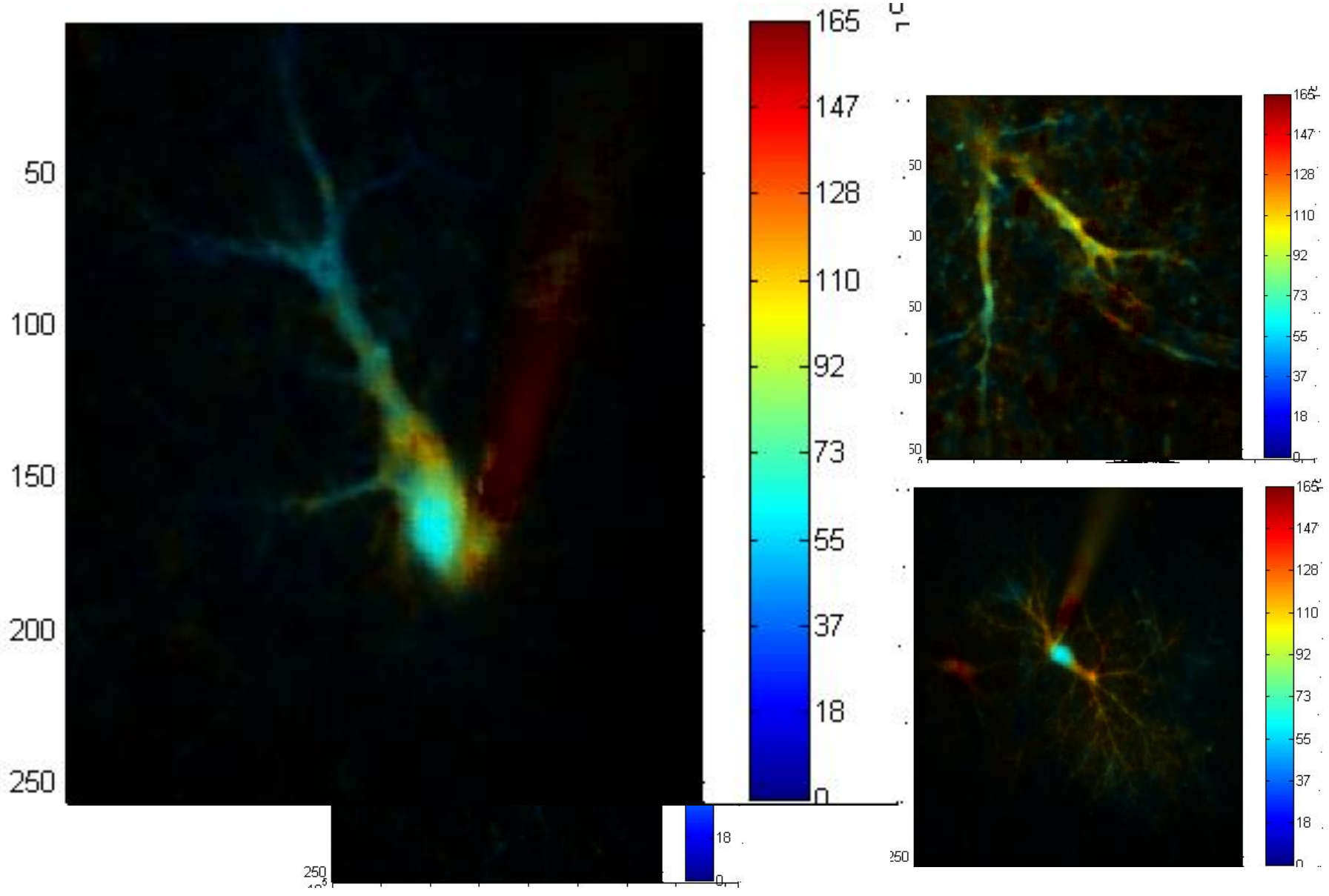
Patched Astrocyte



Patched Astrocyte has many gap junctioned cells (GJCs)

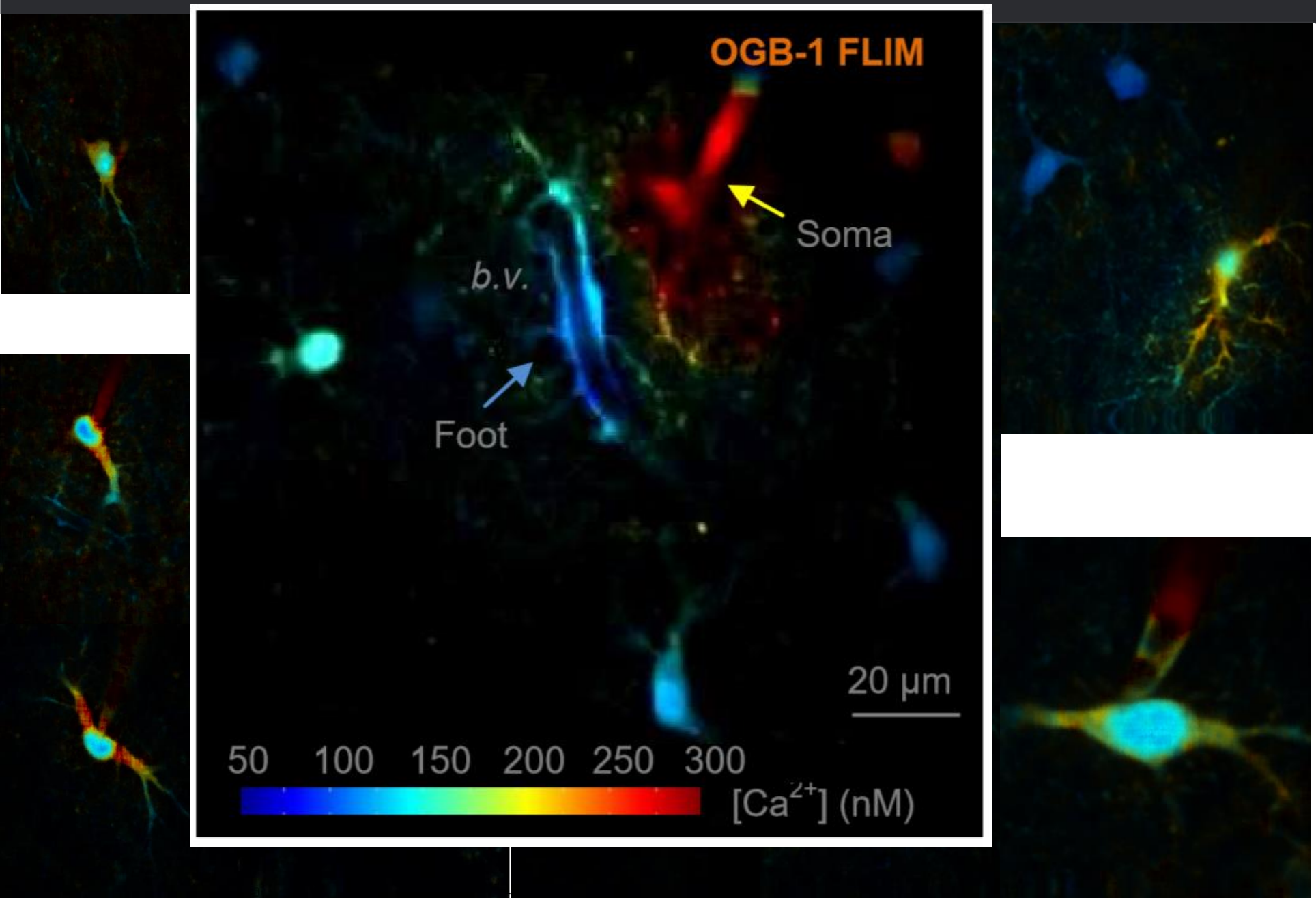


Basal $[Ca^{2+}]_i$ map in patched hippocampal astrocytes



Patched astrocytes cannot maintain calcium homeostasis

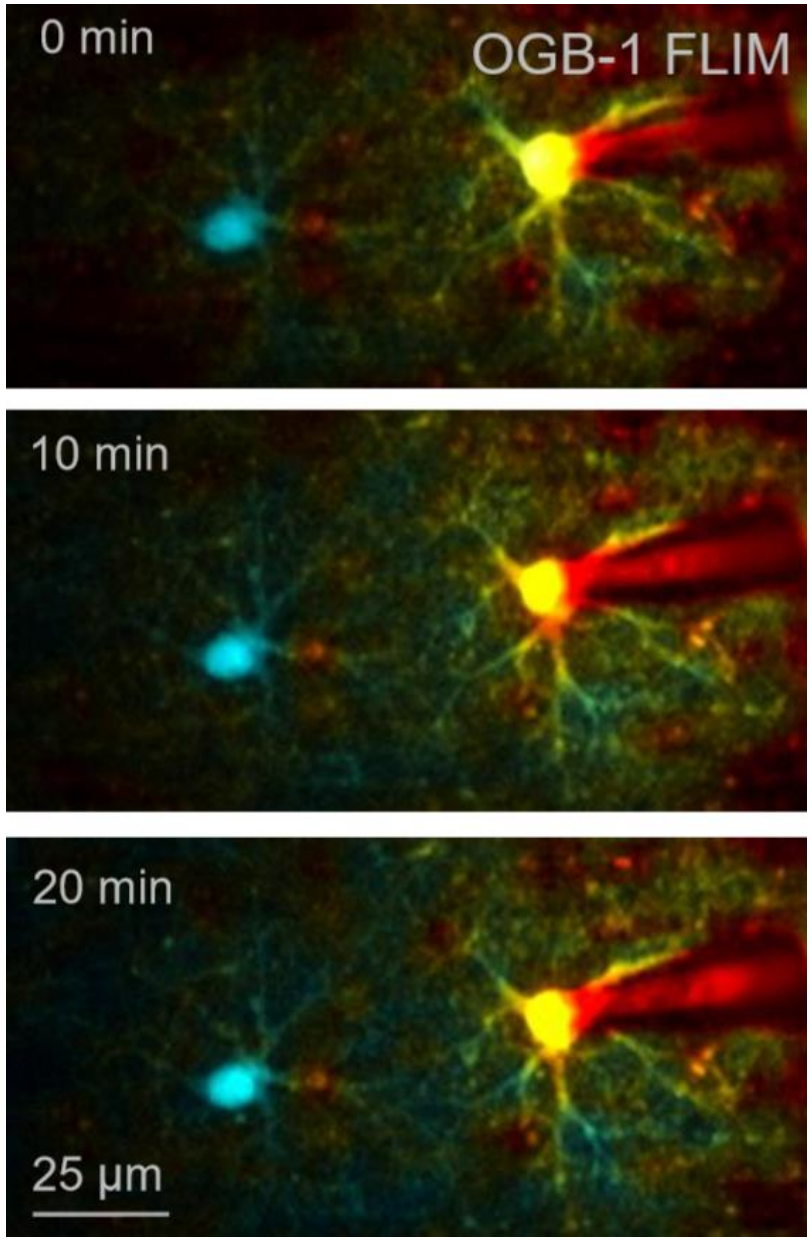
Basal $[Ca^{2+}]_i$ map in patched hippocampal astrocytes

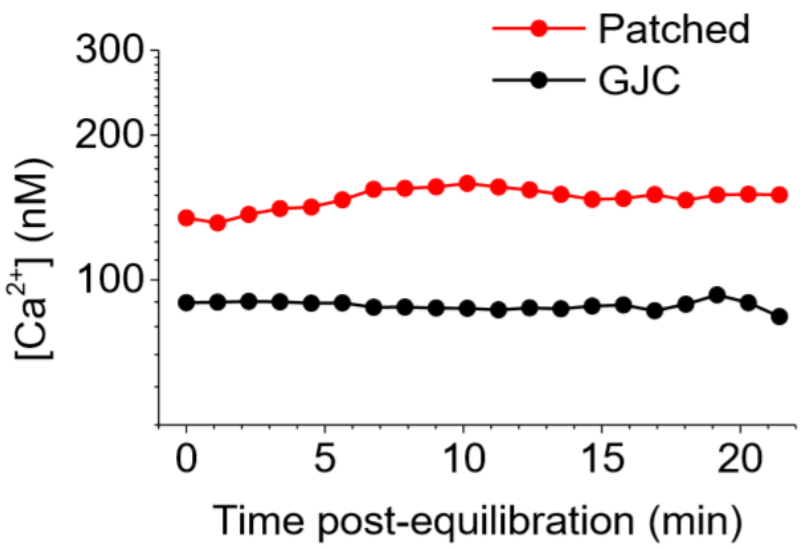


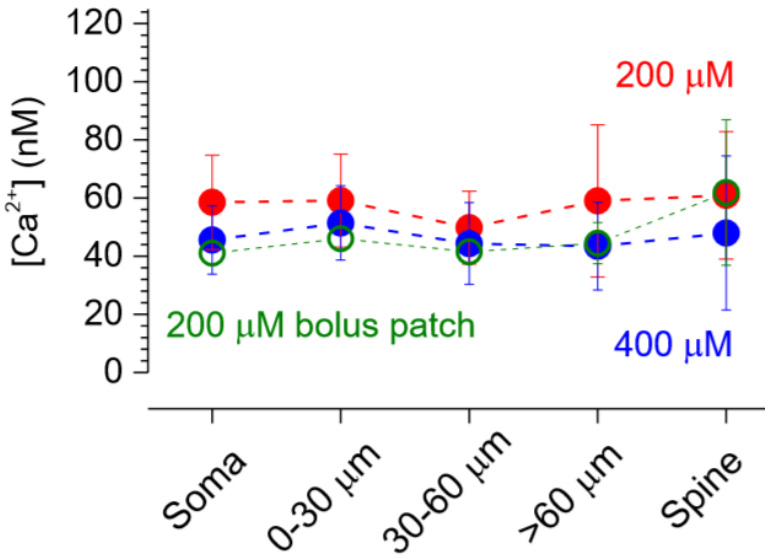
Patched astrocytes cannot maintain calcium homeostasis

But Gap Junctioned Cells are not affected

Patience is a virtue, Happy cell happy life



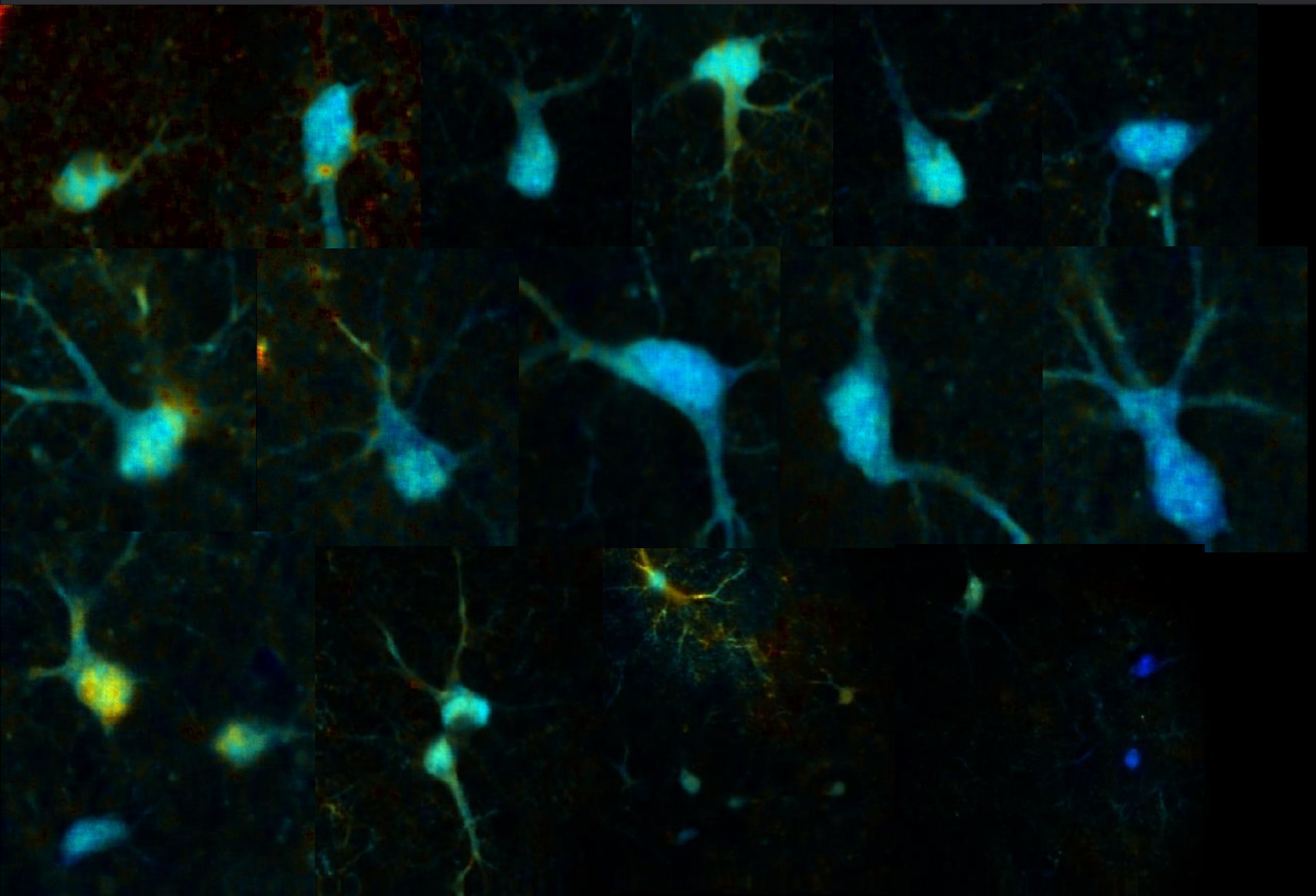




Cell calcium levels can reach stable state within reasonable time after breakin (~40min)

OGB1 has some buffering effect, therefore keep the dye concentration as low as possible

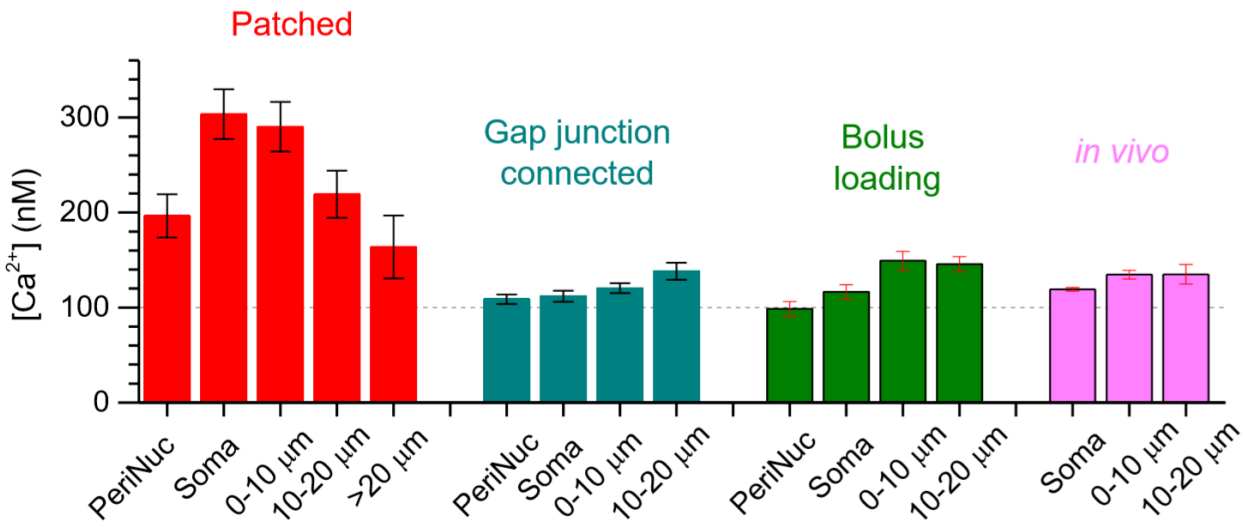
Basal $[Ca^{2+}]_i$ map in hippocampal gap junctioned astrocytes

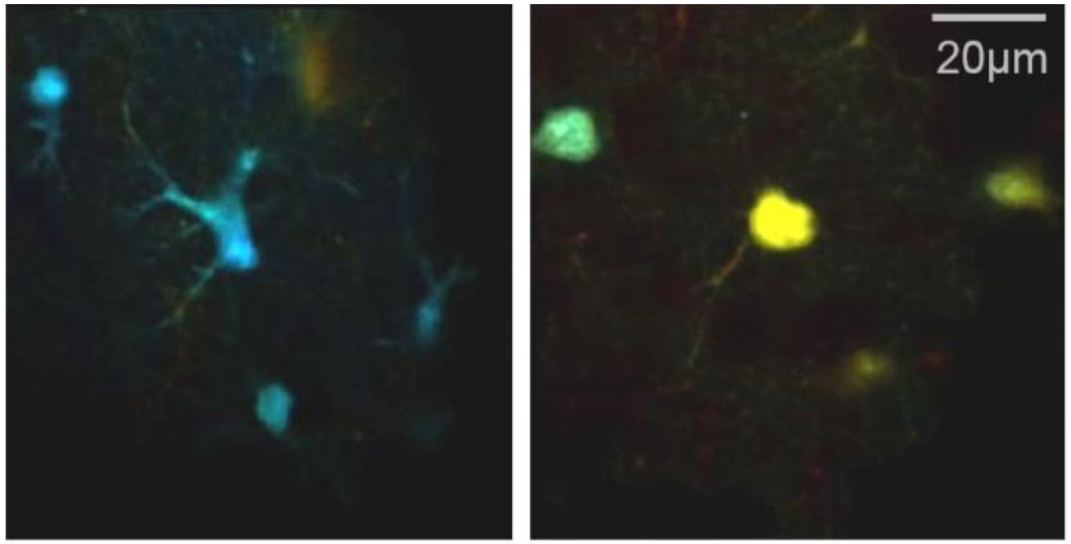


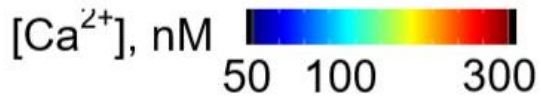
Gap junctioned astrocytes has level gradient within itself

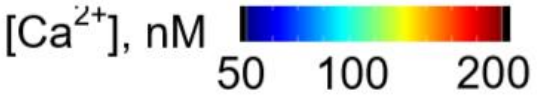
Variability between gjcs are significant

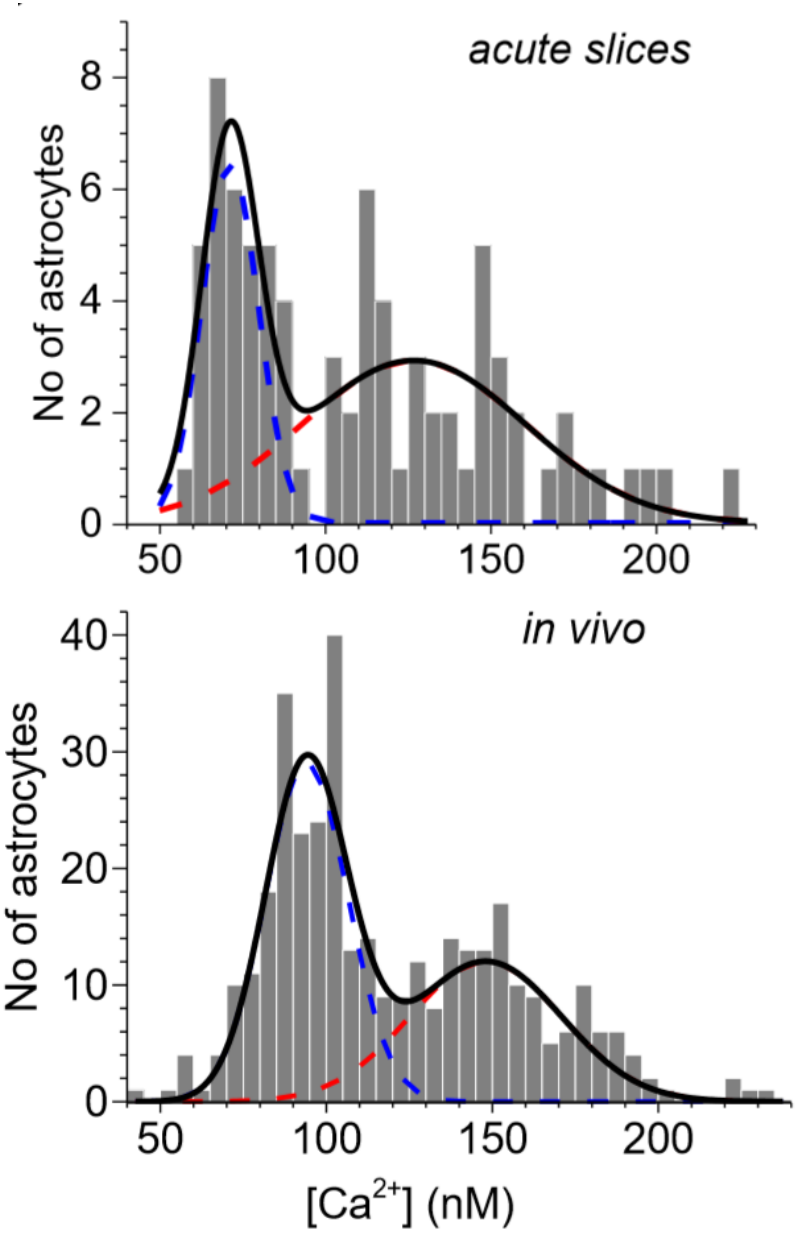
Astrocytes population groups



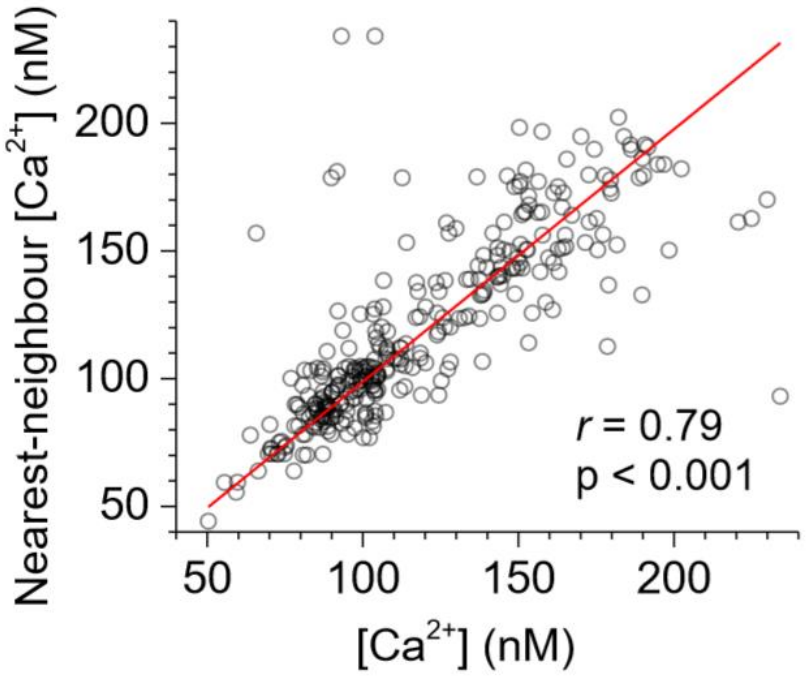






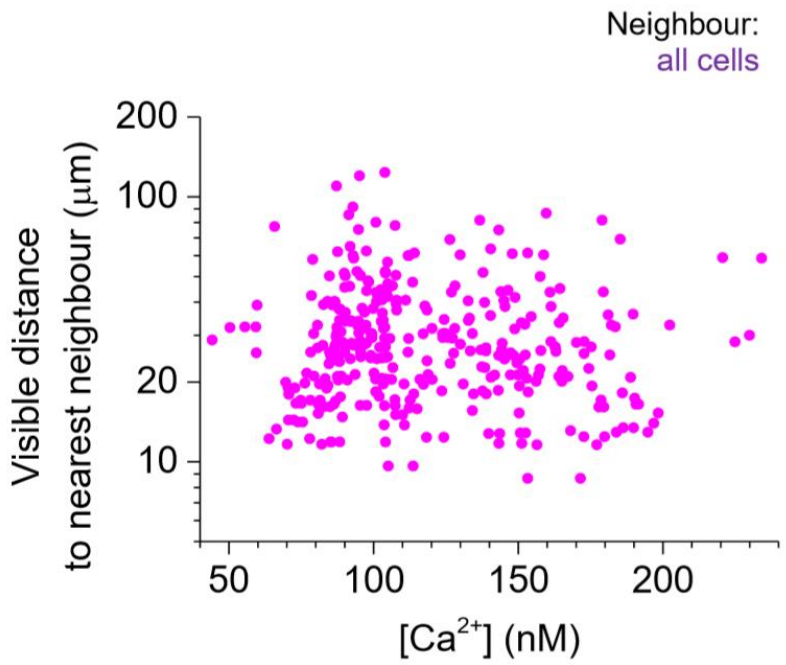


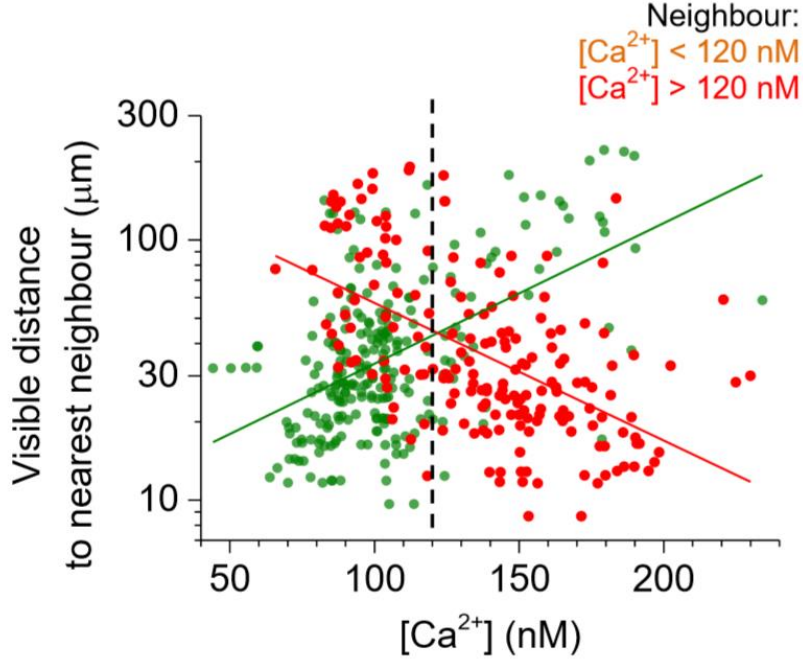
Astrocytes population groups similar levels stay together



Lower resting calcium group's nearest neighbour also has lower resting calcium

Higher resting calcium has neighbours further away



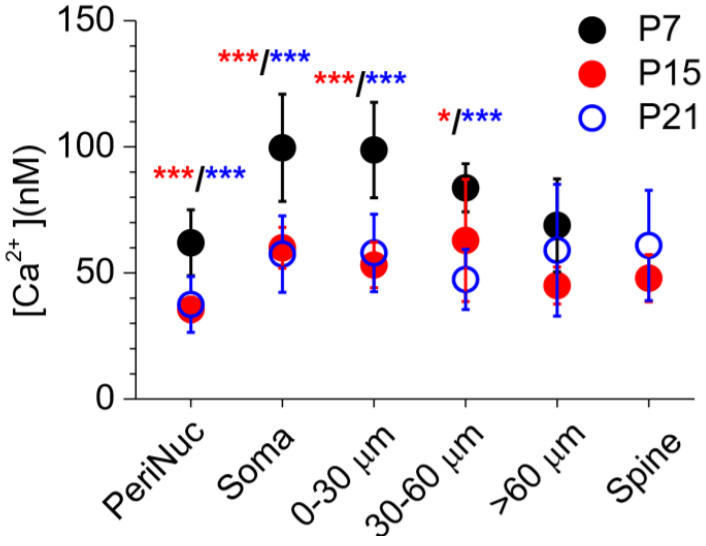


Basal $[Ca^{2+}]_i$ map in hippocampal astrocytes

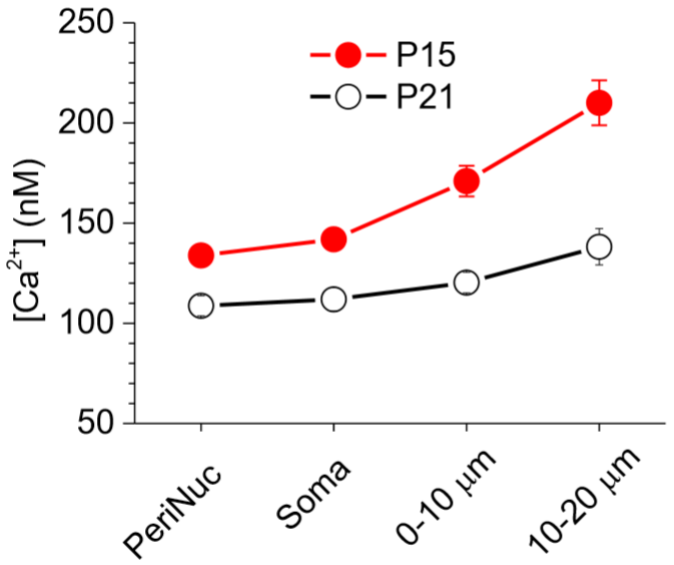
- Patched astrocytes have resting calcium level of 100-400nM depending on post breakin duration and cell health
- Gradient in patched astrocytes, soma can have lower apparent values because of nucleus
- Patched astrocyte has small soma dialysed by the patch pipette, therefore cannot faithfully report the resting level in astrocytes
- Gap junctioned astrocytes have resting levels between 50-150nM.
- There is no clear dependence on distance from the patched astrocytes

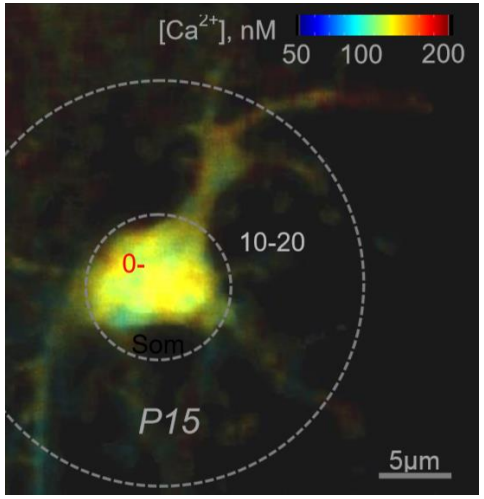
Animal maturation and different resting calcium levels

Neuronal



Astroglia

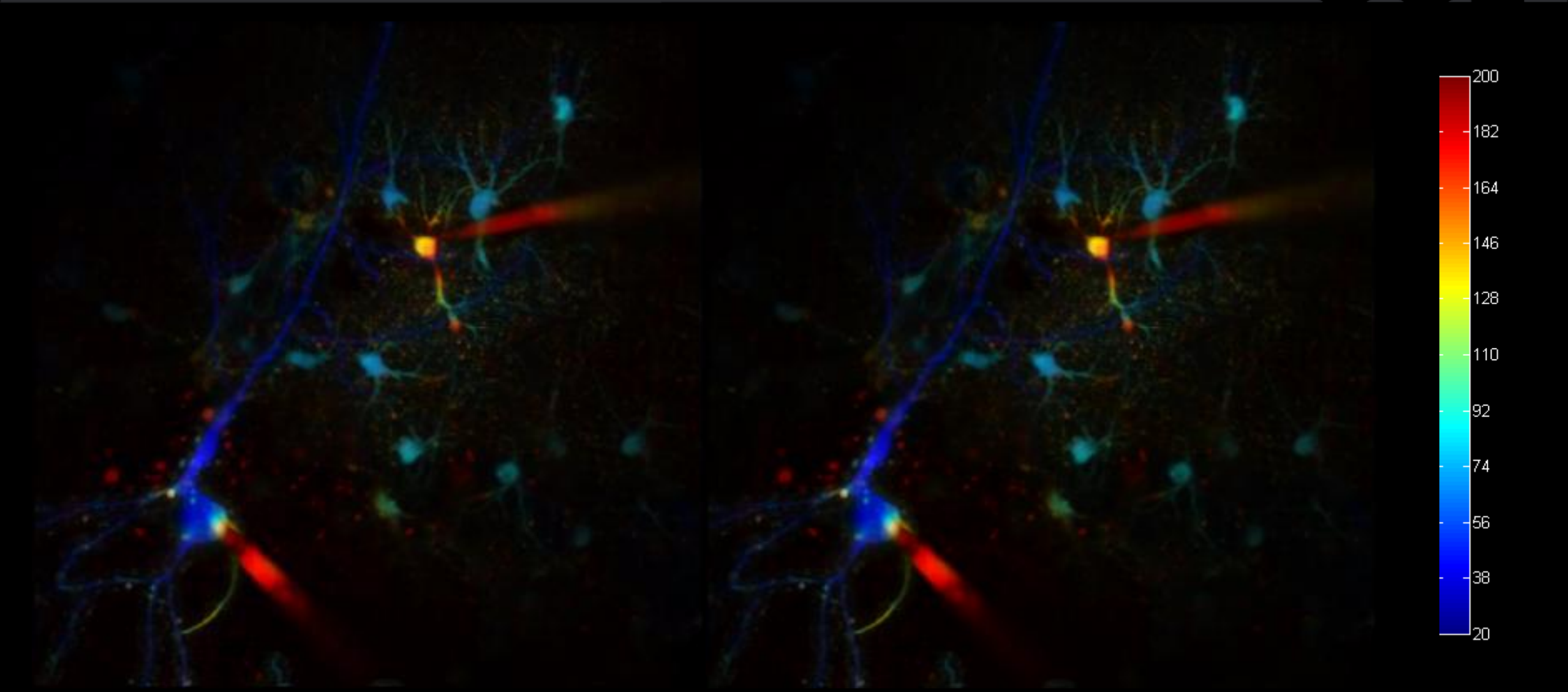




No P7 spine because immature neurons have very smooth dendritic tree

No P7 astrocyte because the cell has very immature morphology

Measurement of basal $[Ca^{2+}]_{free}$ in CA1 *s.p.* PCs and astrocytes



Thank you

University College London

UCL Institute of Neurology

wellcome

erc
European Research Council

Basic Principle of Fluorescent Lifetime Imaging Microscopy

Calcium indicators that are suitable for FLIM

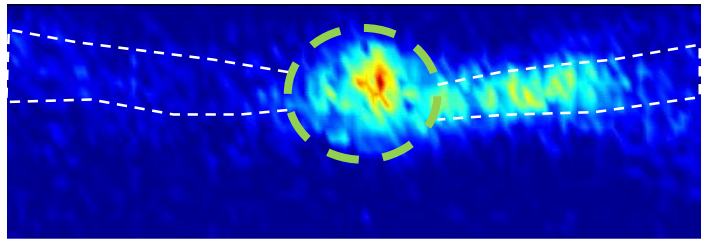
Calibration Procedure

Measurement of nanomolar baseline [Ca^{2+}] in tissue samples

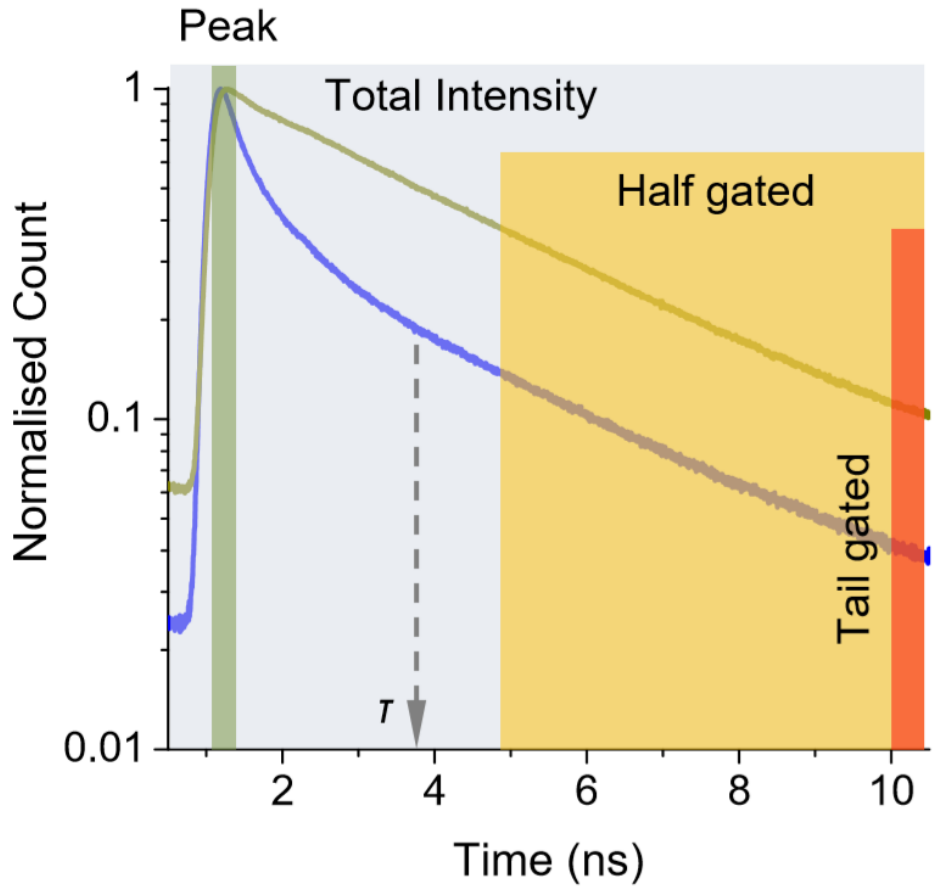
Utilisation of FLIM in improving measurement signals

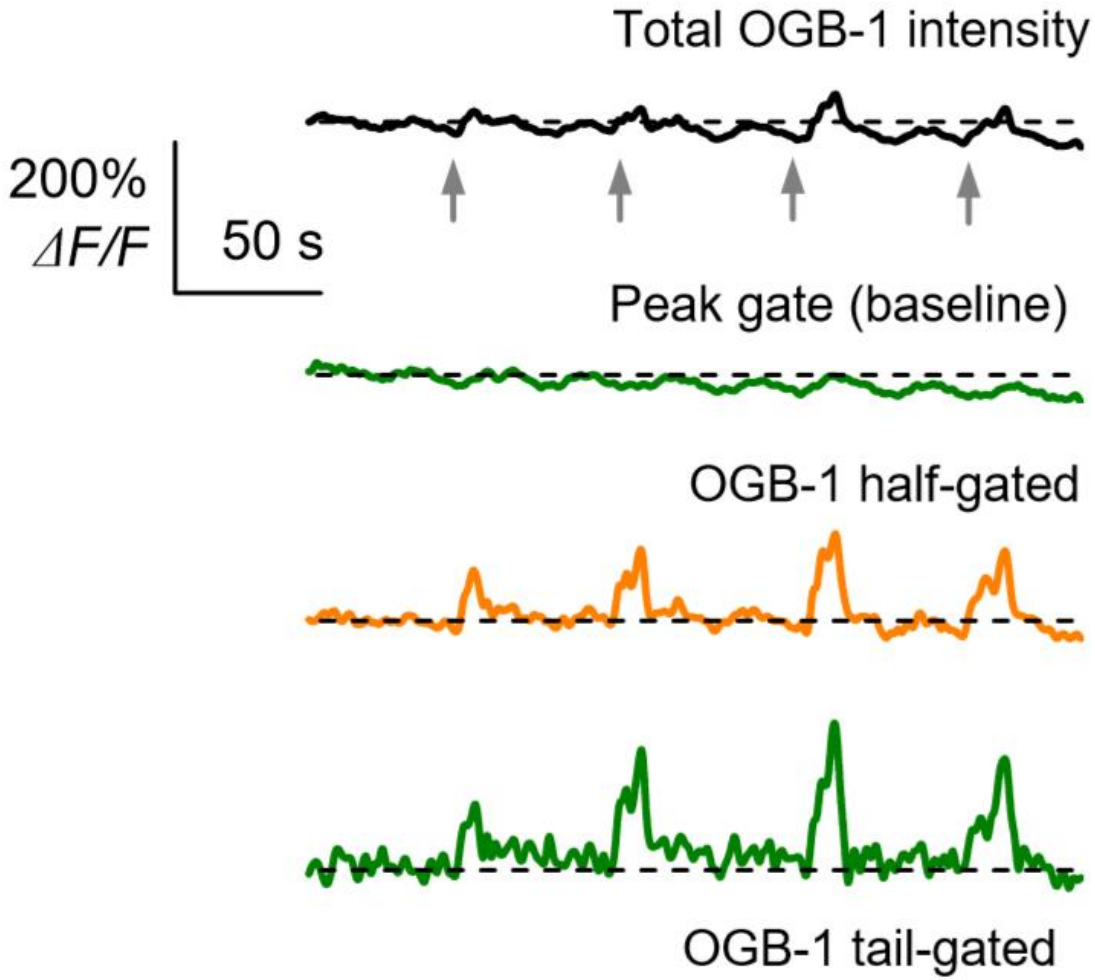
Gating improve functional signal

CA3 Pyramidal Cell Bouton

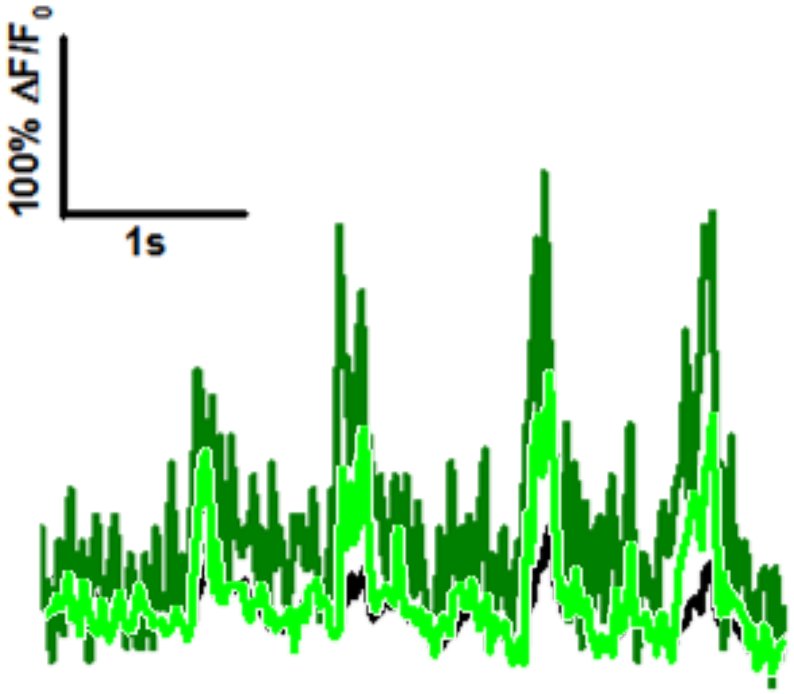


Examples of OGB1 FLIM decay trace for high and low [Ca^{2+}]

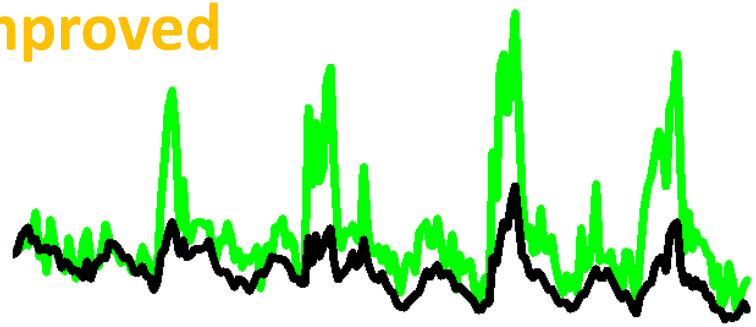




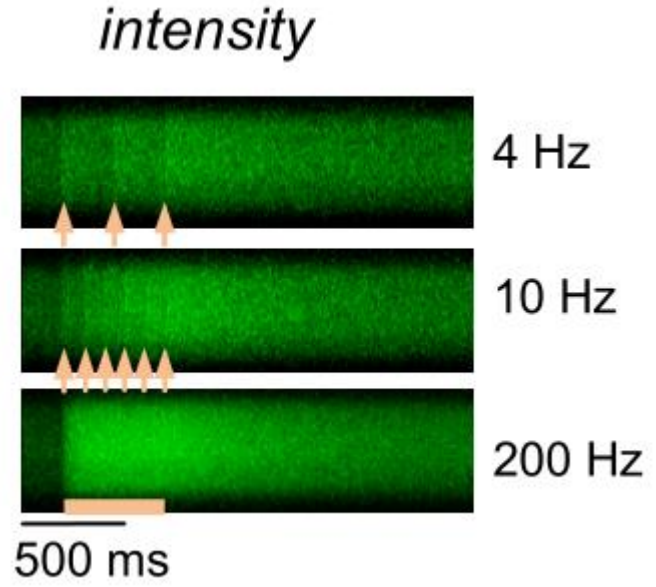
Gating improves $\Delta F/F_0$ signal by several fold

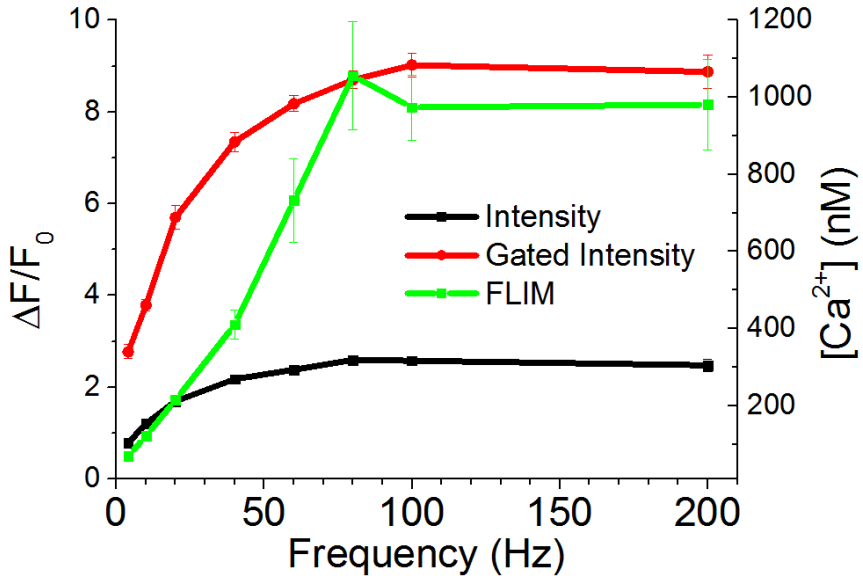


Improved



Original

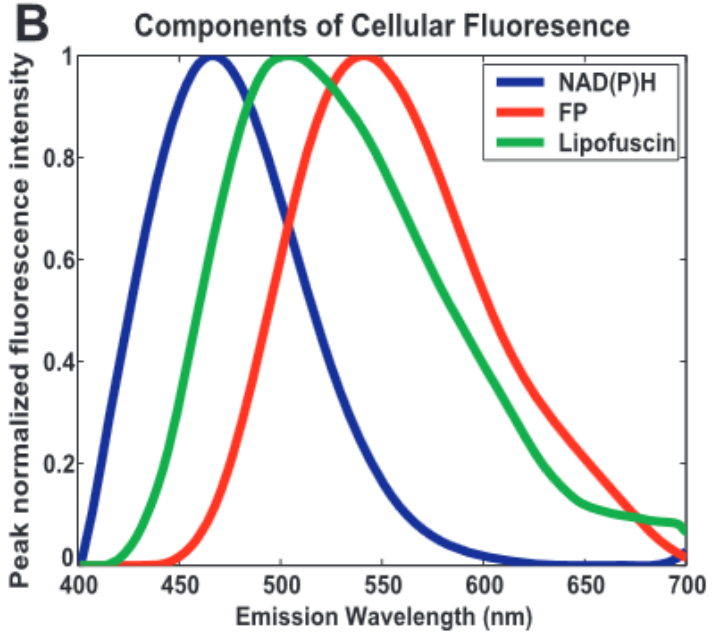




Autofluorescence in tissues

Flavoprotein - FAD
(flavin adenine dinucleotide)

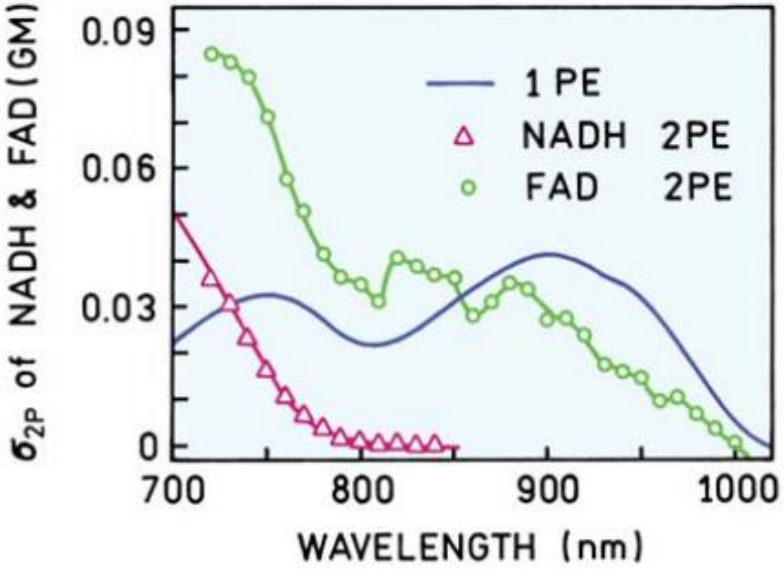
47 ps for dimer
200 ps for monomer
2.28 ns for free FAD
0.3-1ns protein bound



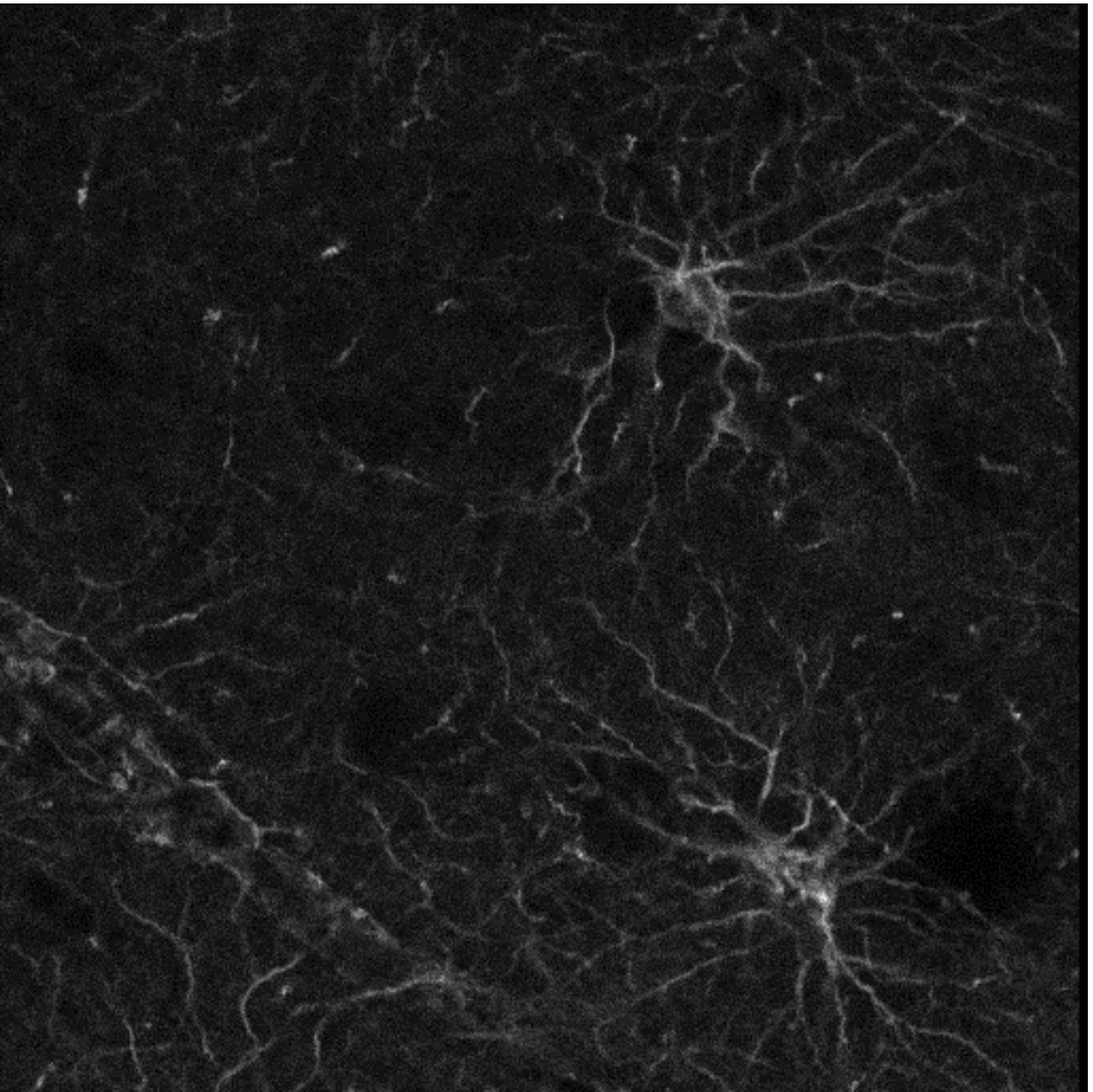
2PE @
755 nm
or
860 nm

NADH
(Nicotinamide adenine dinucleotide)

~0.4ns in H_2O
<1.2ns in protein bound form

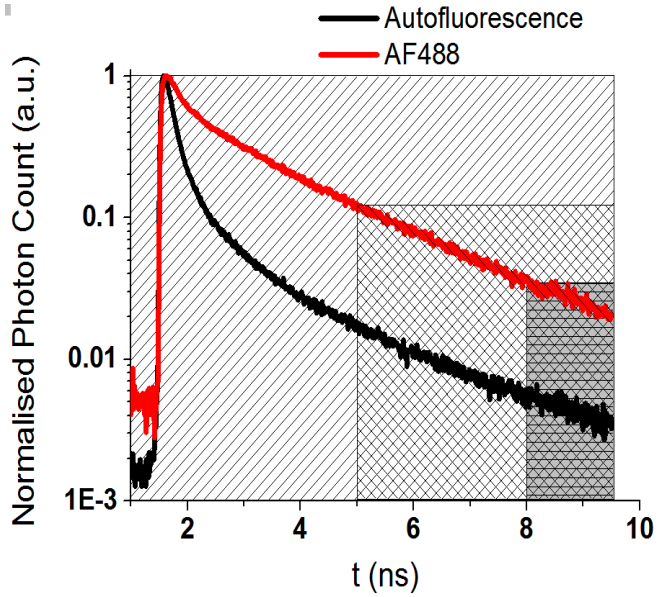


Gating can remove autofluorescence more efficiently



Example:

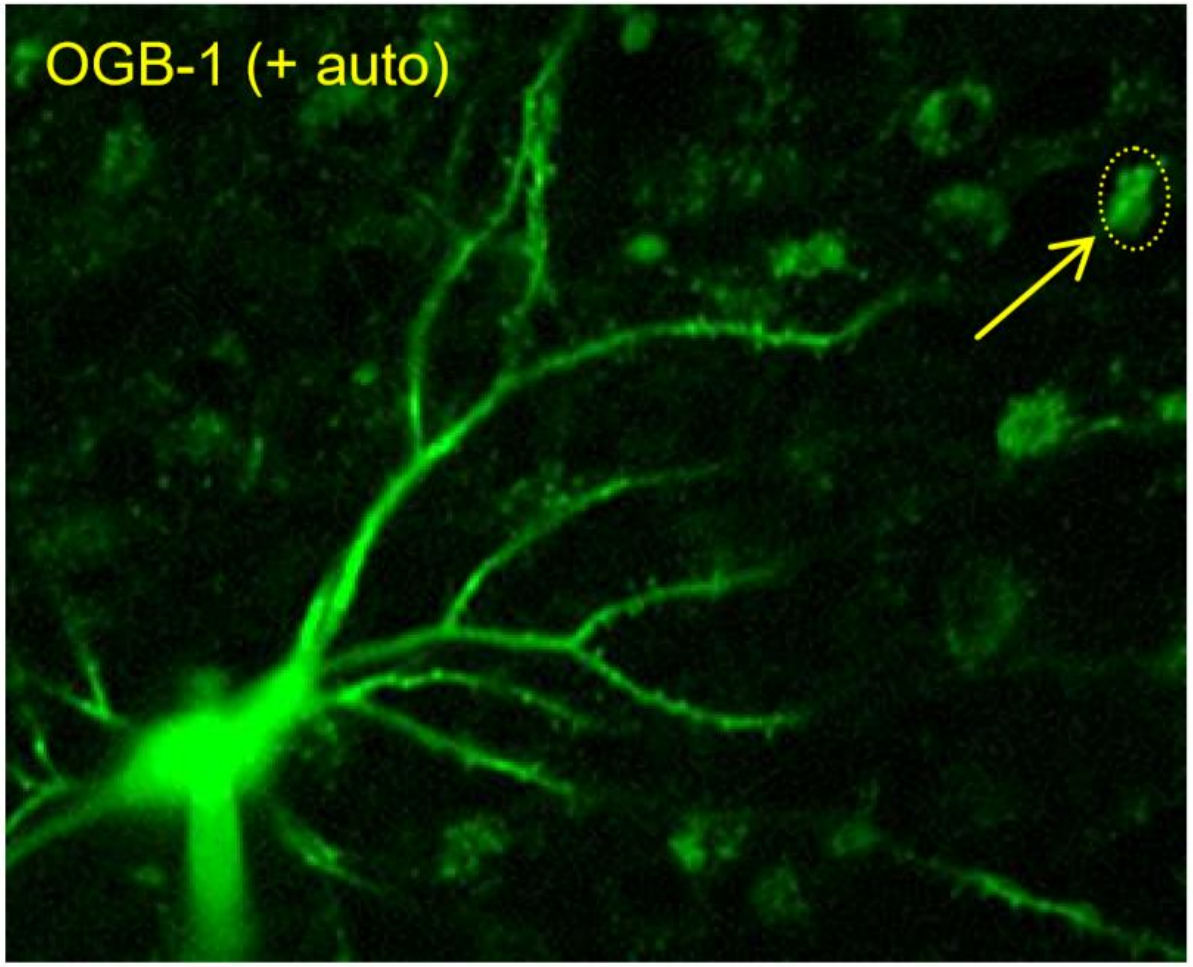
Human Temporal Lobe Slice GFAP stained with Alexa Fluor 488

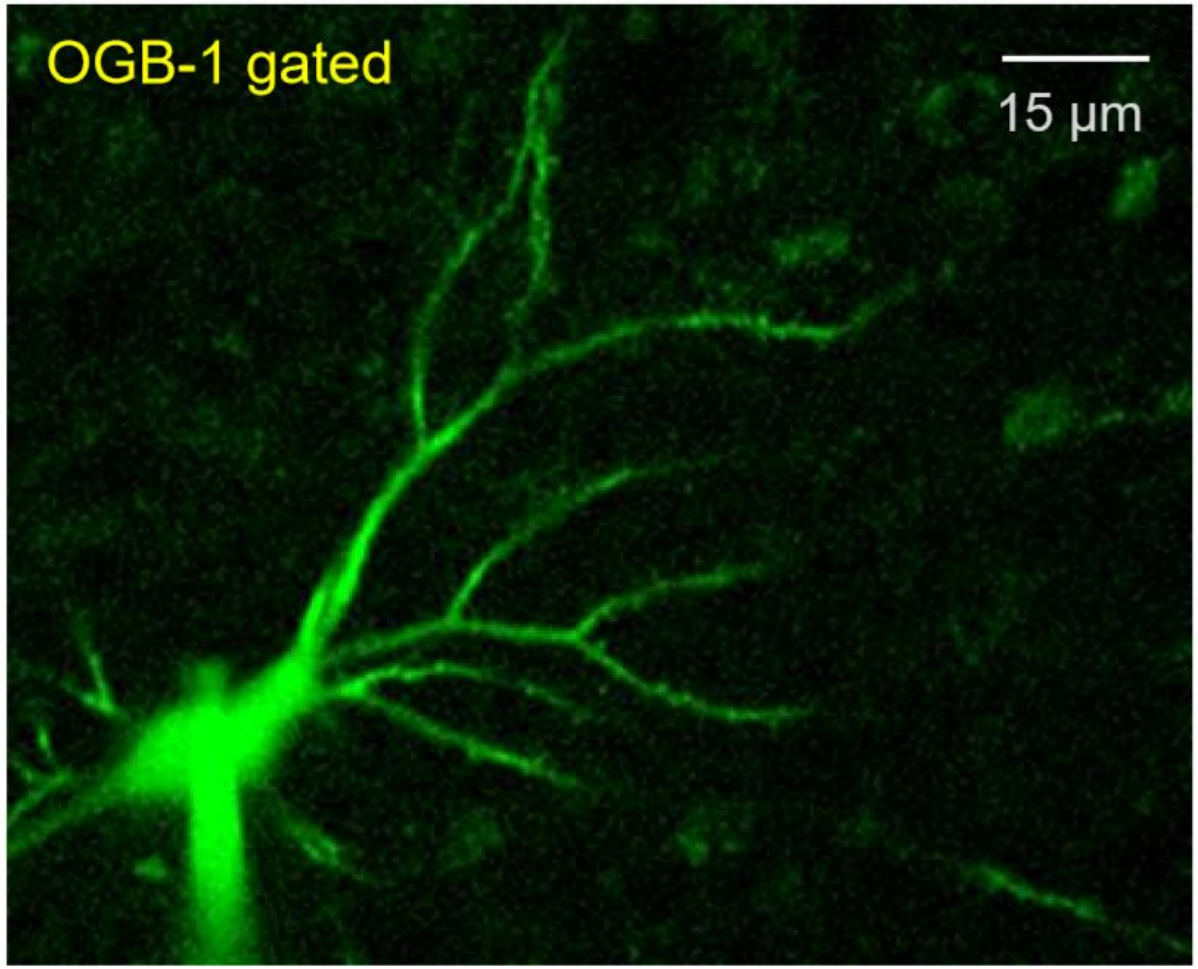


Gating can remove autofluorescence more efficiently

Example:

OGB1 in organotypic slices





Gated Intensity Technique using Fluorescence Life Time information can help

- **Improve traditional ΔF/F contrast by 3.5 fold using OGB1**
- **Intelligently remove autofluorescence from any images of fluorophores with lifetime >3ns**