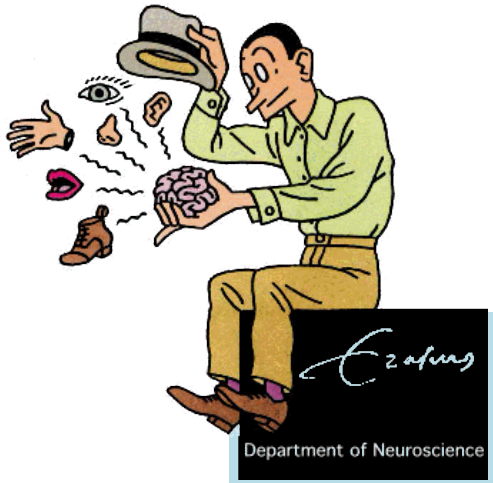
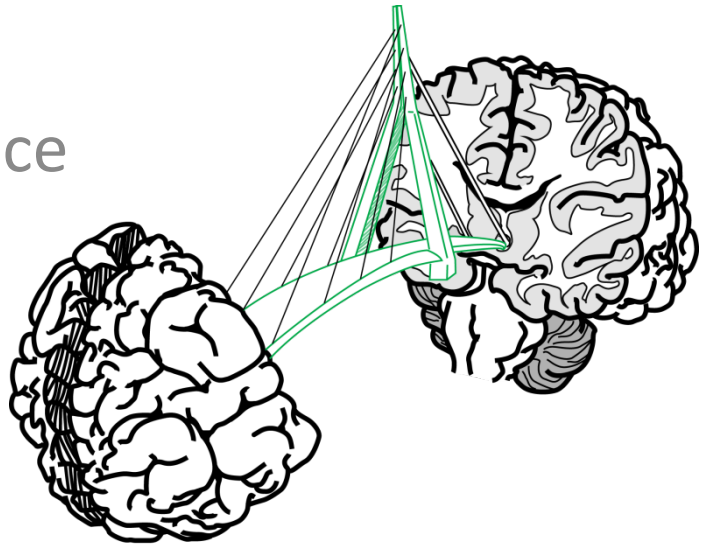


Multi-photon Microscopy

Aaron B. Wong

11th Nov, 2026

Dept. of Neuroscience

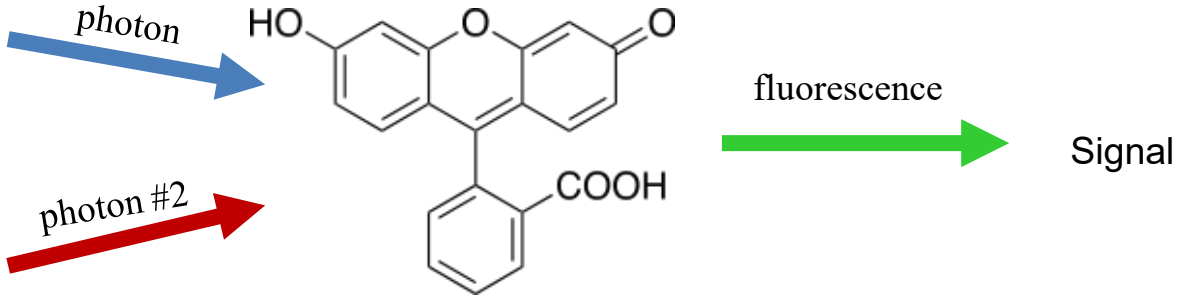


Multi-photon Microscopy

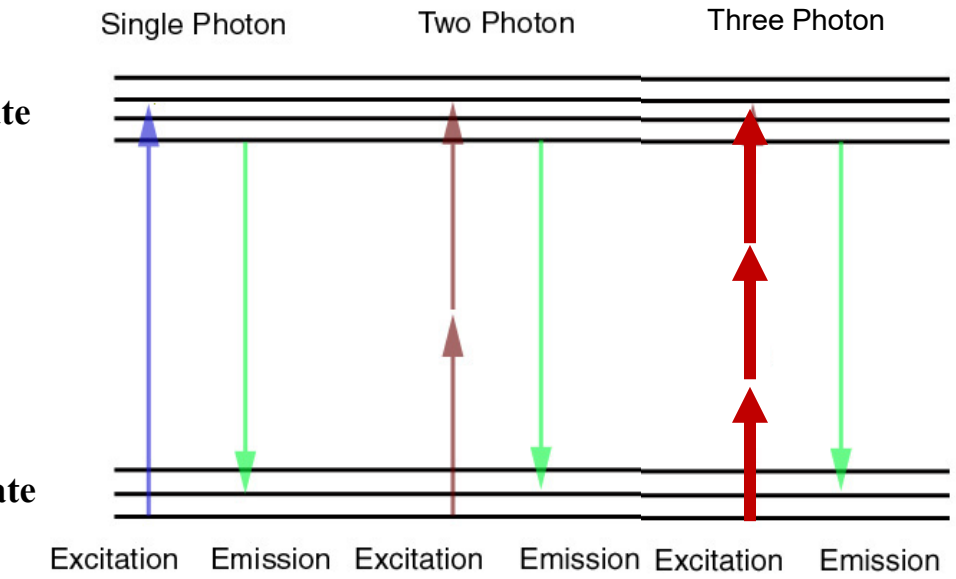
- What is multiphoton microscopy?
  - Multiphoton excitation
- Why and when would you use it?
  - Properties
  - Applications
- Practical considerations

Multiphoton excitation

- Absorption of **multiple** photons at the “same” time
  - Predicted by Maria Göppert-Mayer in 1931



fluorescein



Jablonski diagram

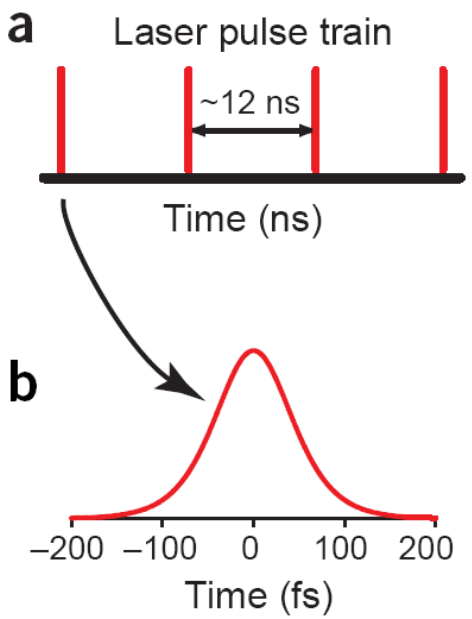
Multiphoton excitation

- Absorption of **multiple** photons at the “same” time
  - Predicted by Maria Göppert-Mayer in 1931
  - a rare event:
    - “In bright sunlight, a molecule of rhodamine B…
    - 1-photon process: once a **second**
    - 2-photon absorption: once **10 million years**;
    - no 3-photon absorption is expected throughout the entire age of the universe.”

Denk W, Svoboda K (1997) Photon Upmanship: Why Multiphoton Imaging Is More than a Gimmick. *Neuron* 18:351–357.

Multiphoton excitation

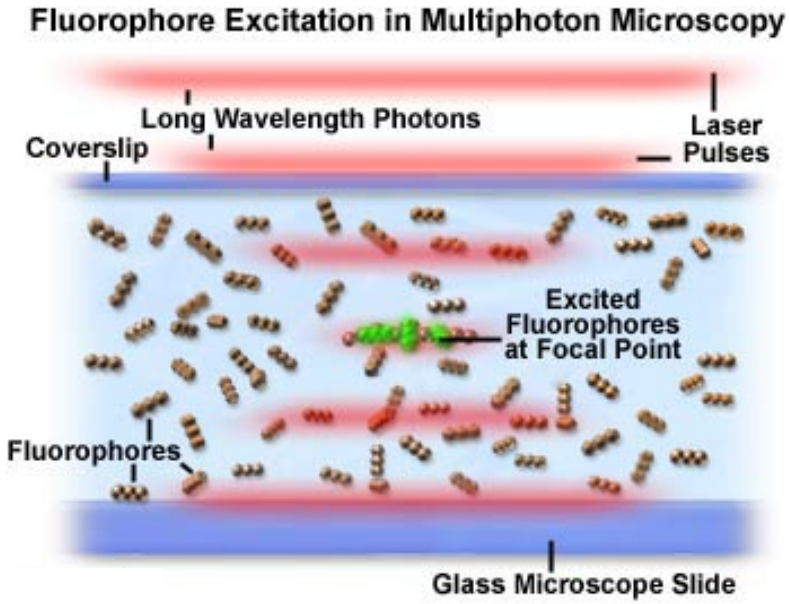
- Absorption of **multiple** photons at the “same” time
  - Predicted by Maria Göppert-Mayer in 1931
  - A high **photon flux** is necessary
    - Using pulse laser to achieve two-photon excitation



Pulse train from a laser (mode-locked Ti:S) at 80 MHz.

~100 fs

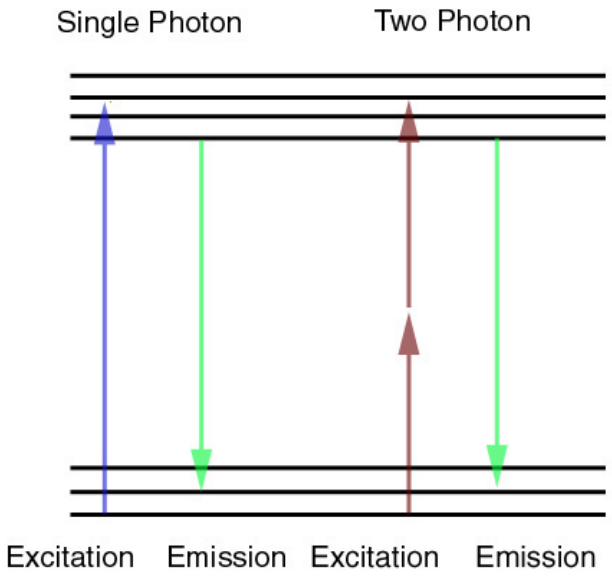
1/125000 of 12.5 ns



From:Zipfel et al, 2003

http://micro.magnet.fsu.edu

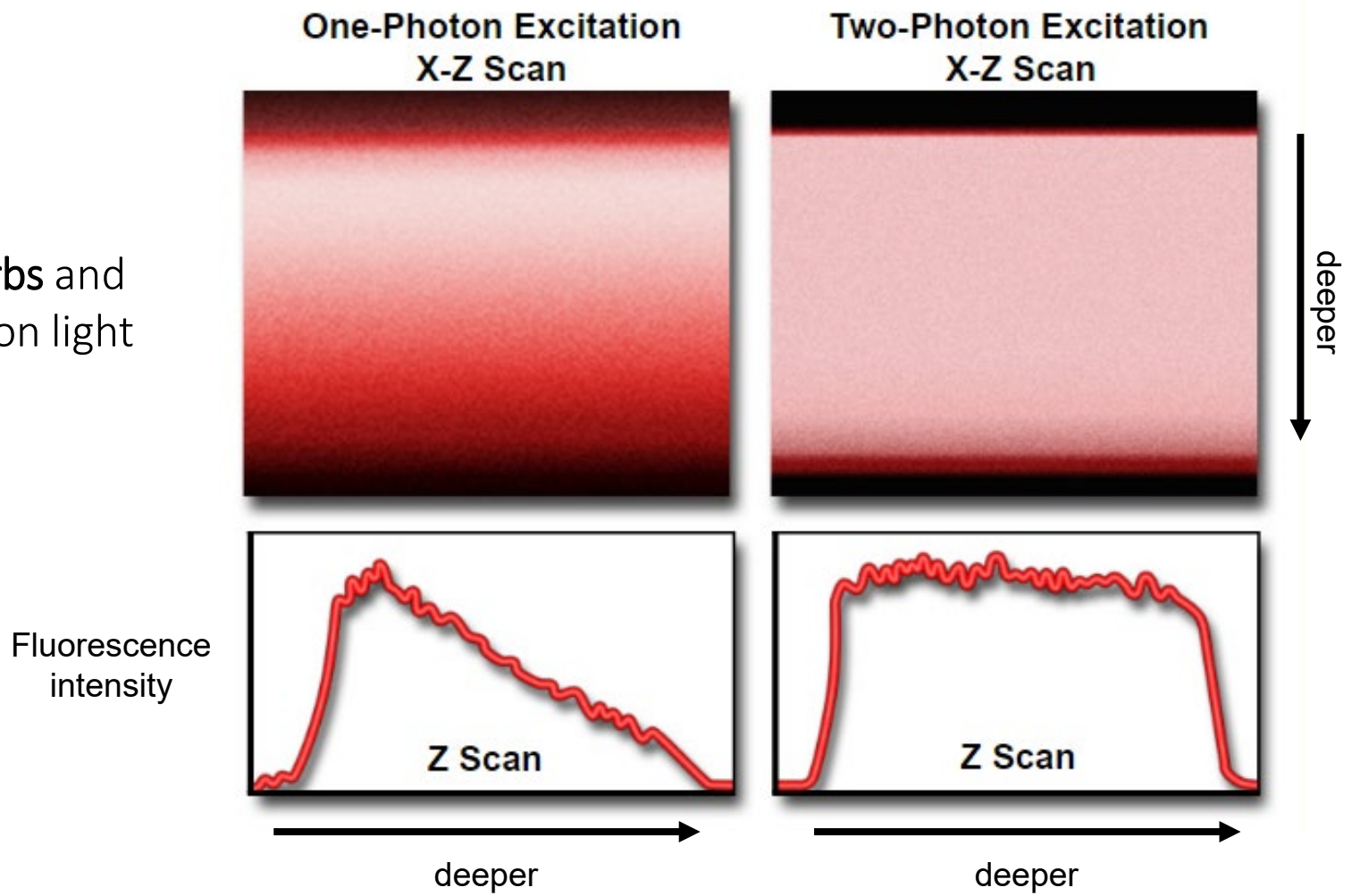
Why do we care? – Useful properties of two-photon excitation



- 1 high energy photon -> 2 lower energy photon
- UV-Visible (shorter λ) -> **infrared** (longer λ)

Infrared excitation allows deeper excitation

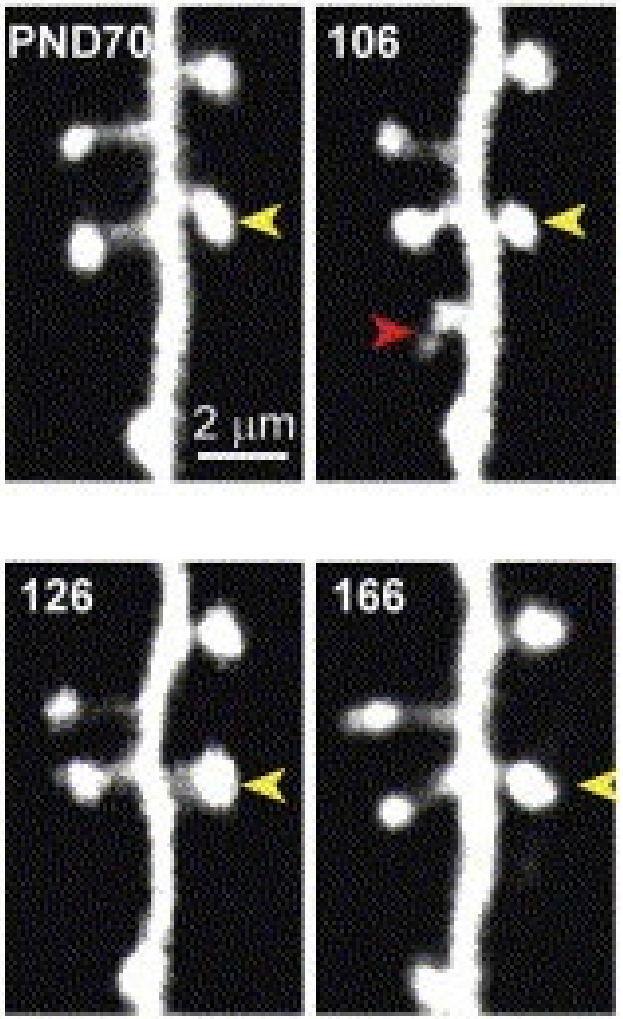
Specimen **absorbs** and **scatters** excitation light



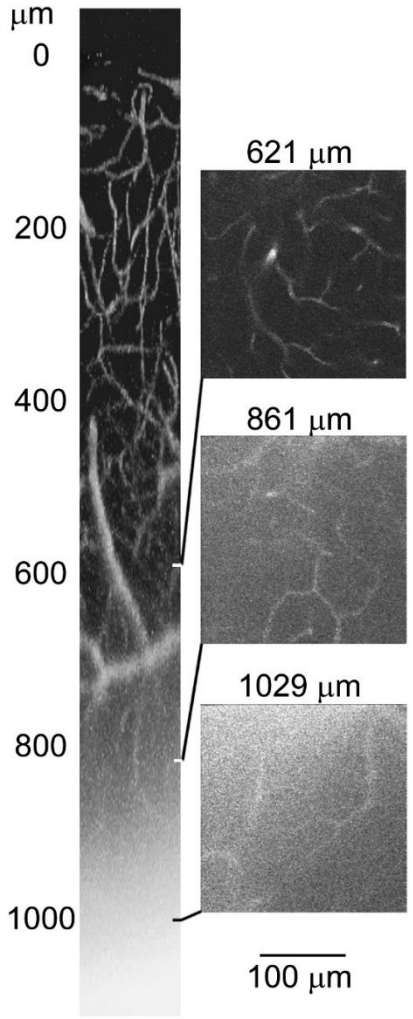
https://www.microscopyu.com/techniques/multi-photon/multiphoton-microscopy

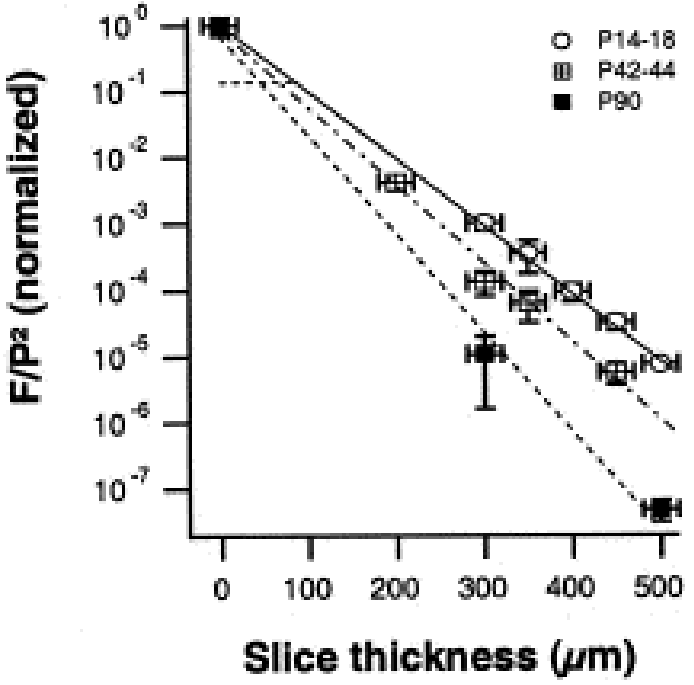
Imaging through thick, highly scattering tissue (*in vivo*)

Dendrites and spines in neurons expressing GFP



Cerebral Blood Vessels Imaged *In Vivo*



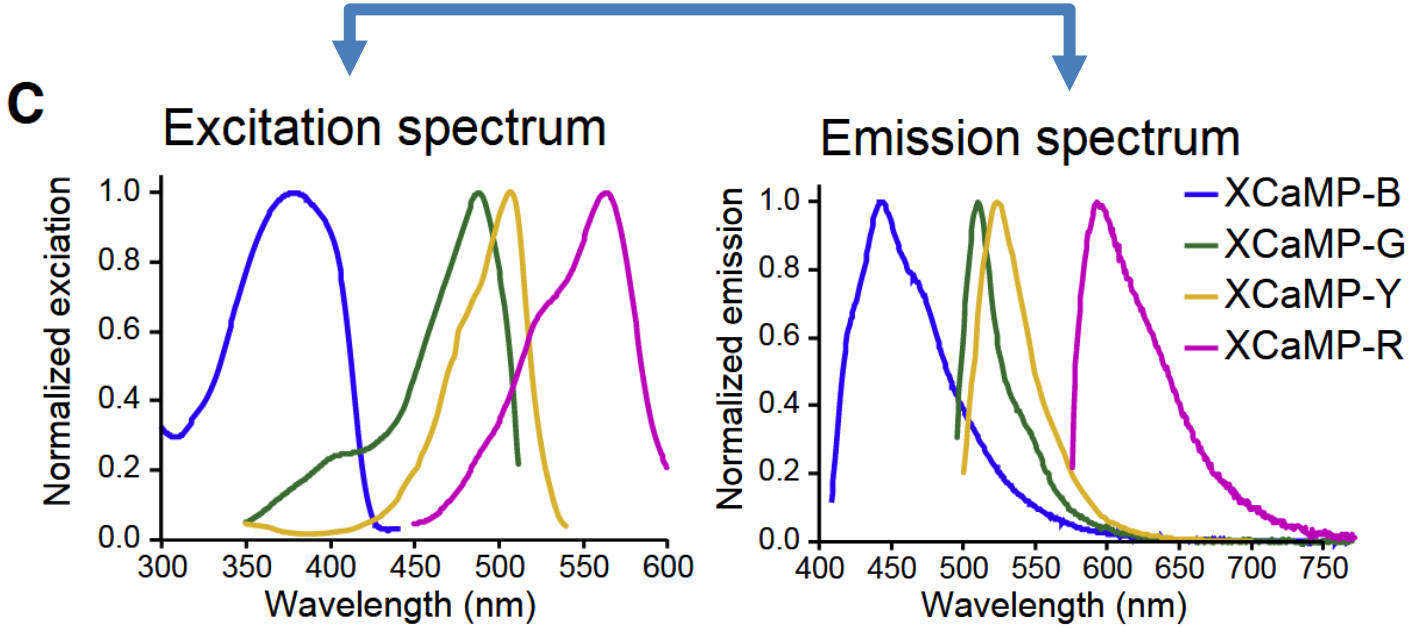


From: Oheim et al, 2001

Svoboda et al, 2006

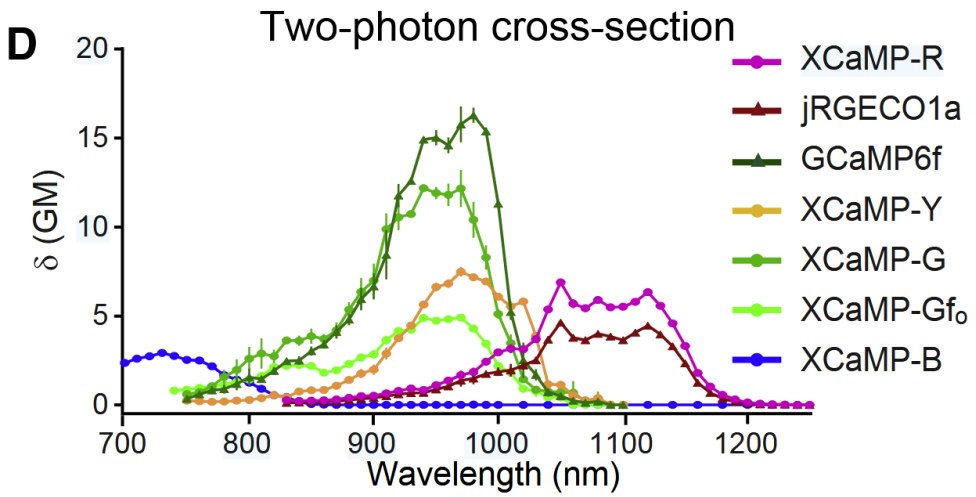
Easier separation of excitation from emission for multiple fluorophores

Overlap between emission and single photon excitation



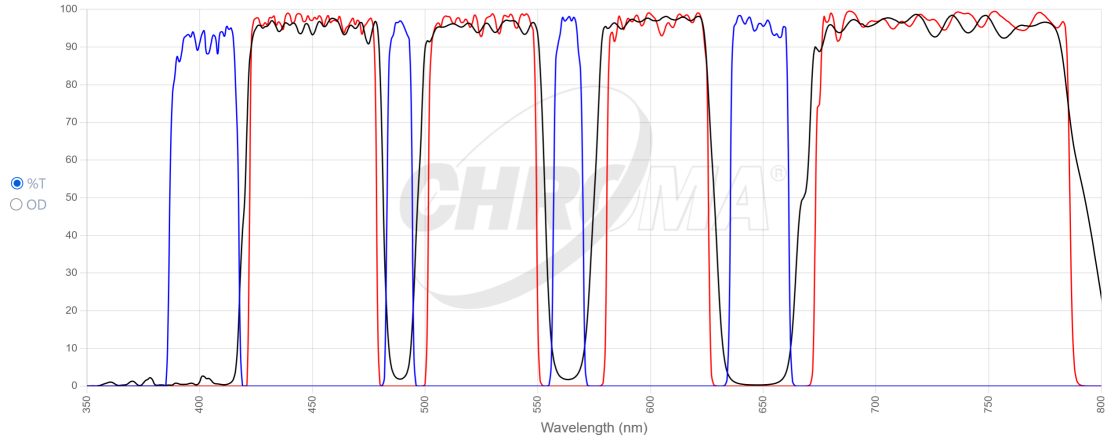
Infrared two photon excitation

- easy separation from emission with a dichroic mirror



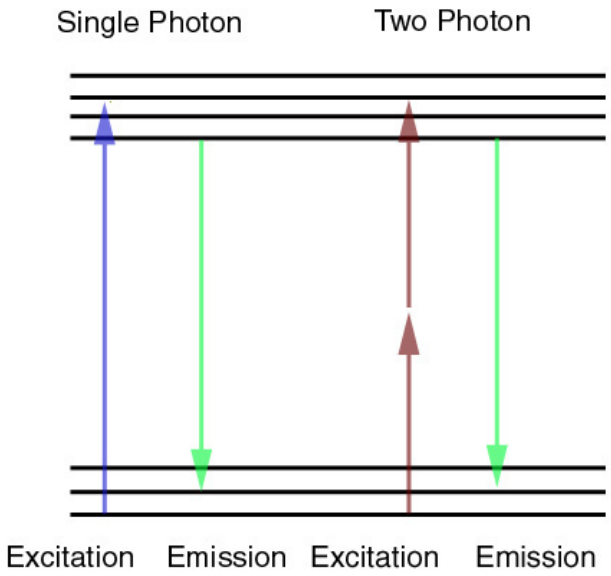
Inoue et al. 2019. '*Cell*

Complicated filter sets



ET - 405/488/561/647nm Laser Quad Band Set (from Chroma) chroma.com

Why do we care? – Useful properties of two-photon excitation

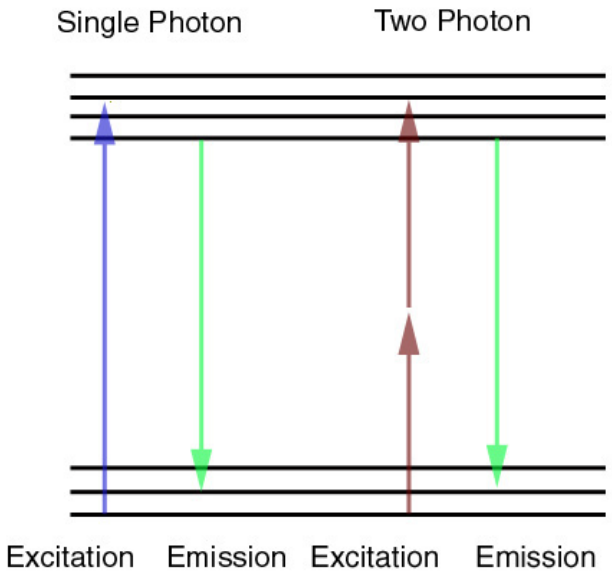


- Infrared excitation
  - Imaging thick, high-scatter samples (>100 µm) or *in vivo*
  - Easier separation of multiple excitation and emission
  - Avoid phototoxicity with UV excitation

Why do we care? – Useful properties of two-photon excitation

$$I_{flu}^{SPA} \propto \sigma \cdot I_{exc}$$

σ: SPA cross-section



$$I_{flu}^{TPA} \propto \delta \cdot (I_{exc})^2$$

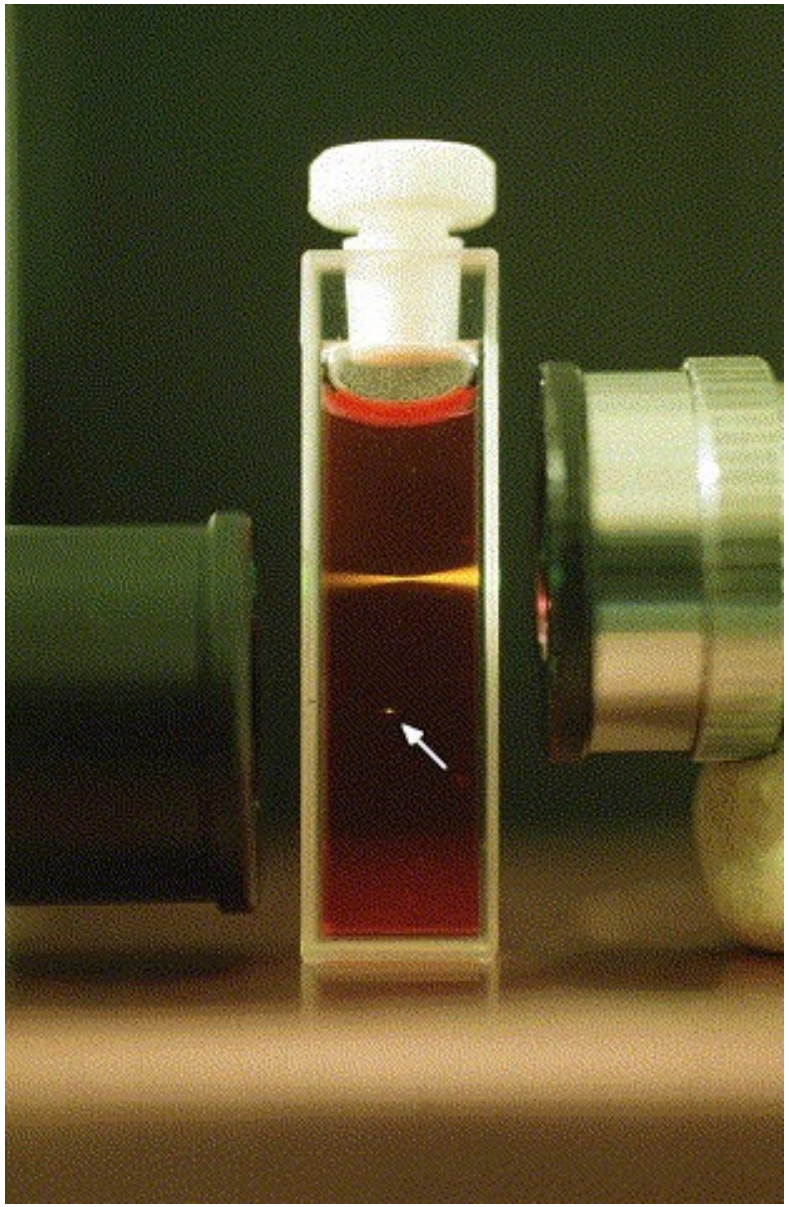
δ: TPA cross-section

- Infrared excitation
- Non-linear excitation
  - Fluorescence intensity scales with the **square** of the illumination intensity

Nonlinear absorption limits excitation to focal plane

Two-photon → (infrared)

Only fluorophores very close to the focal point are excited

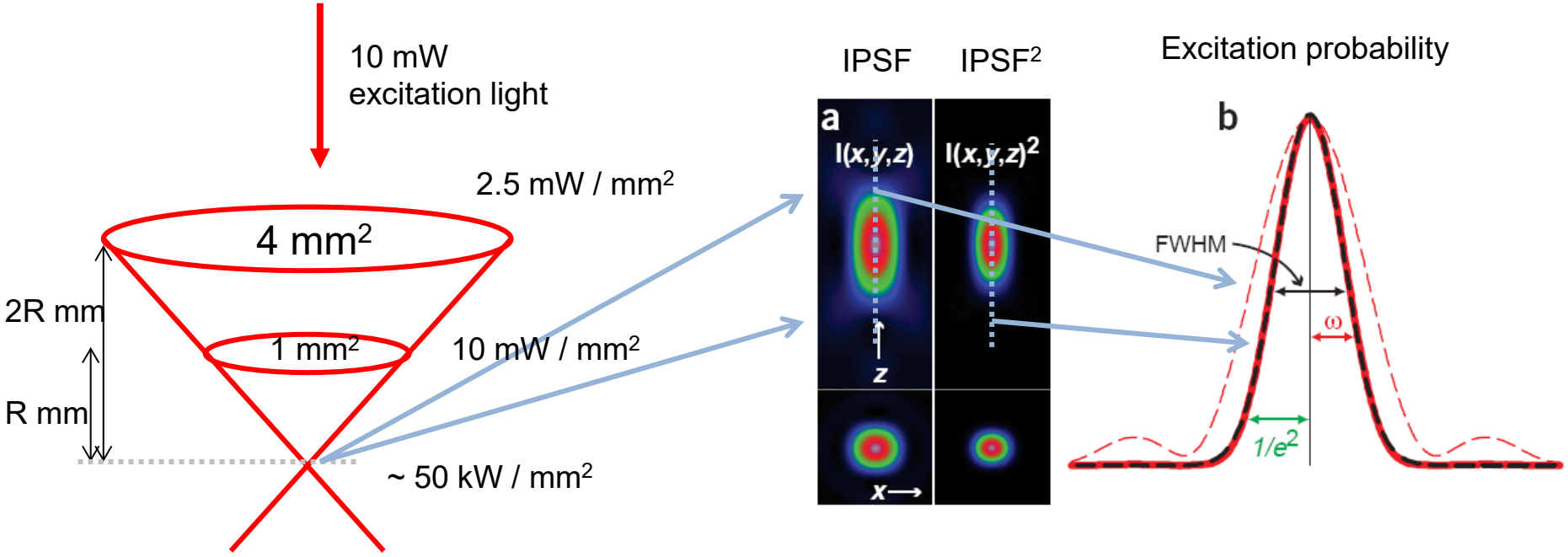


← Single-photon (green)

Brad Amos, Science Photo Library London

Nonlinear absorption limits excitation to focal plane

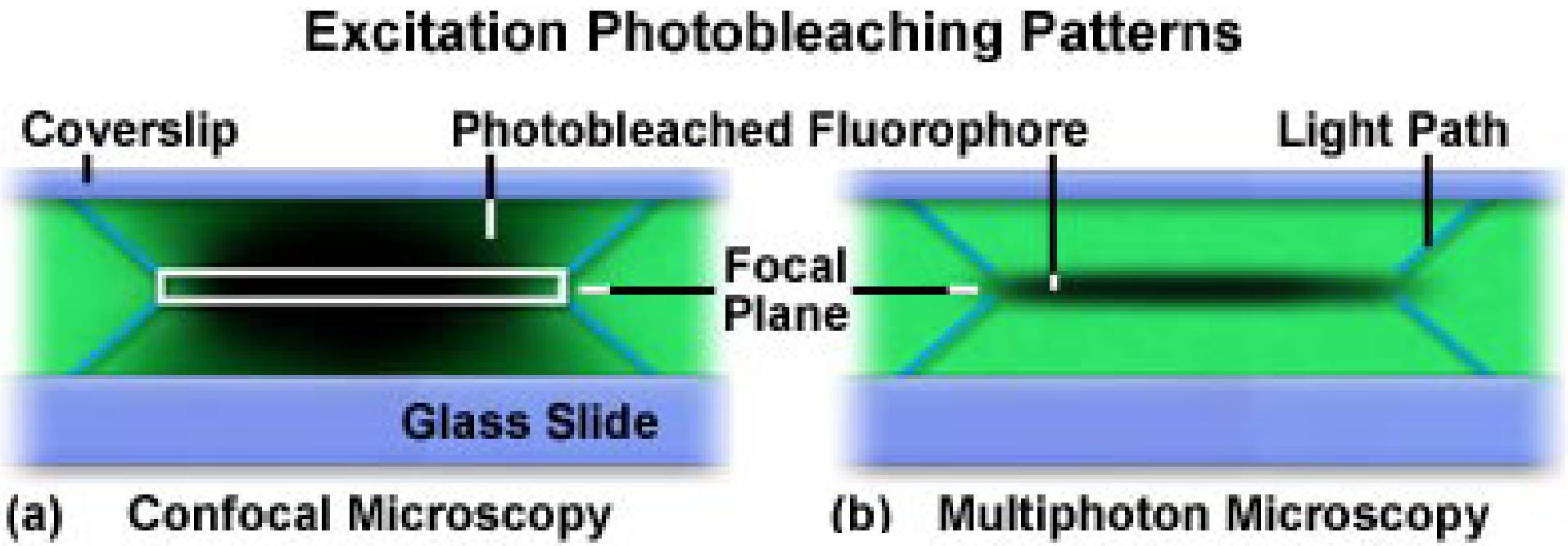
Since illumination scales with square of the distance from focus, and excitation, again, scales with square of illumination intensity, Fluorescence (2P) scales with the **fourth** power of distance from the focus



IPSF: illumination point spread function

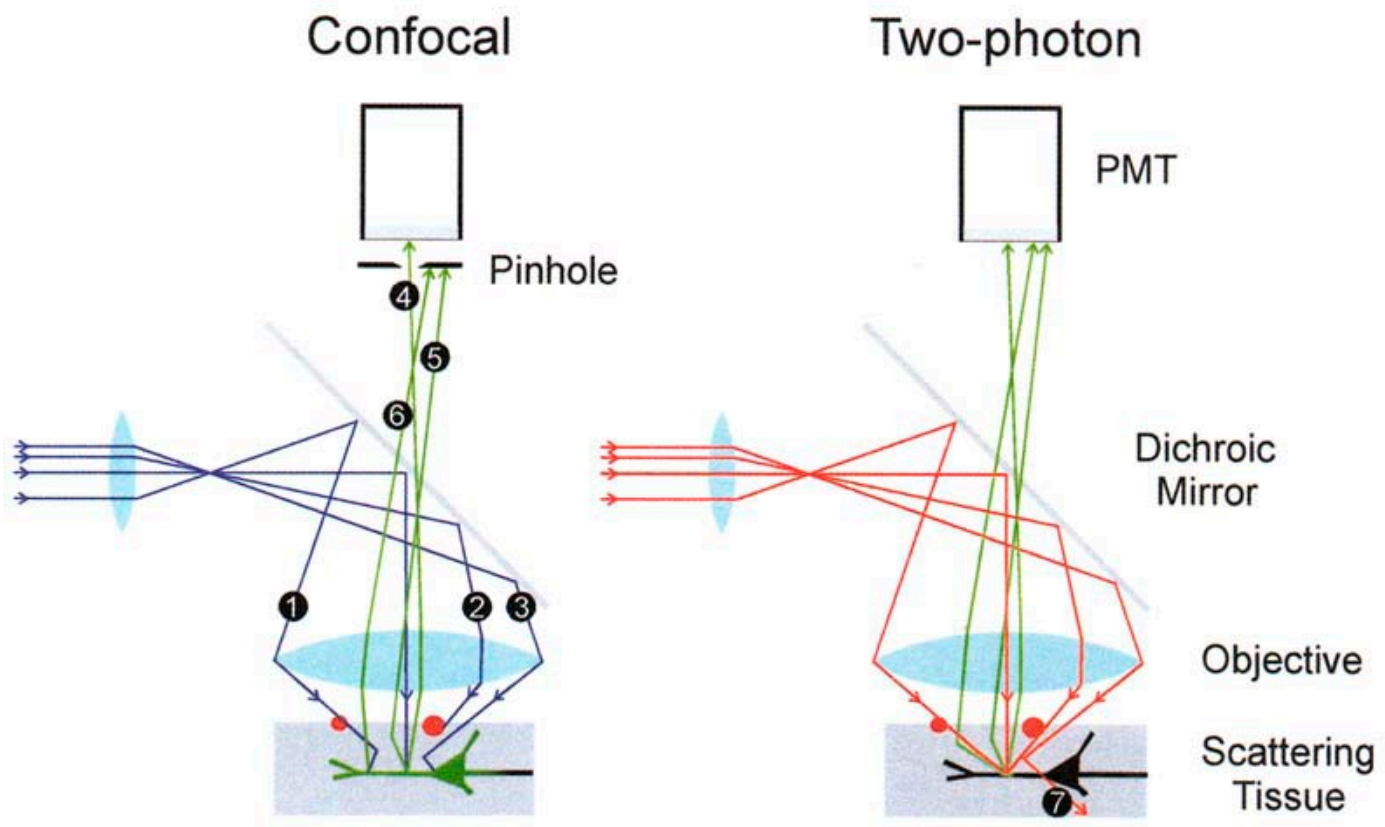
From: Zipfel et al, 2003

Restricted excitation, photobleaching and phototoxicity



http://micro.magnet.fsu.edu

Pinhole not required in two-photon microscopy

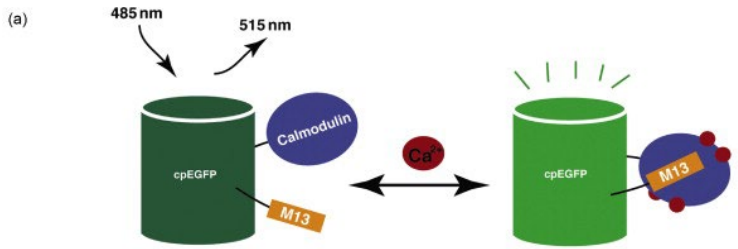


SOME APPLICATIONS

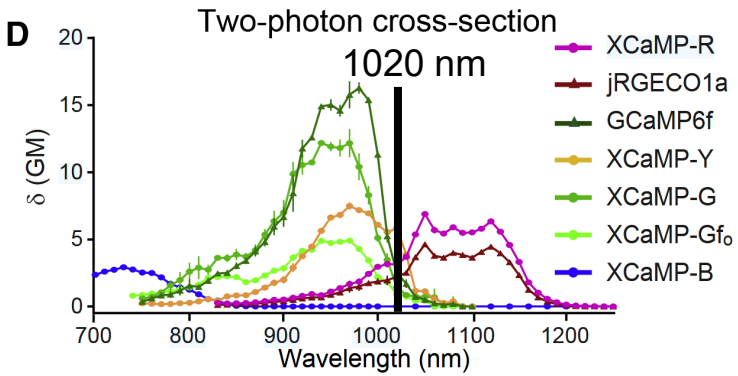
Calcium, glutamate, voltage, etc.

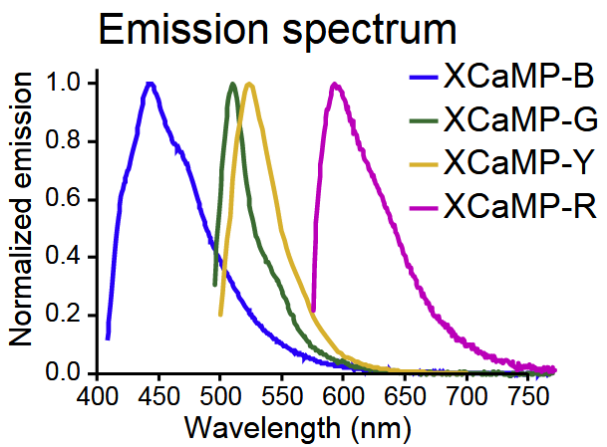
IN VIVO FUNCTIONAL IMAGING

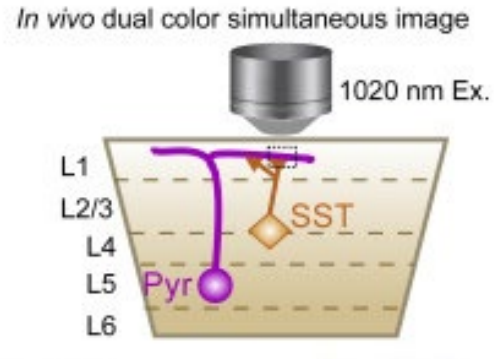
Dual-color *in vivo* Ca^{2+} imaging with XCaMPs

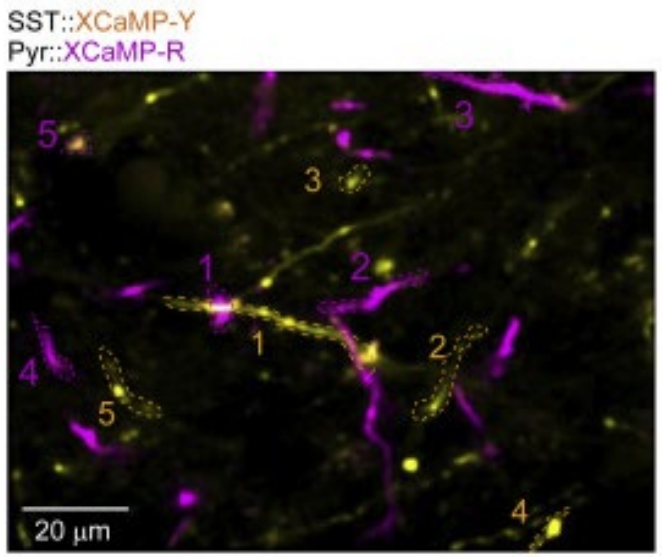


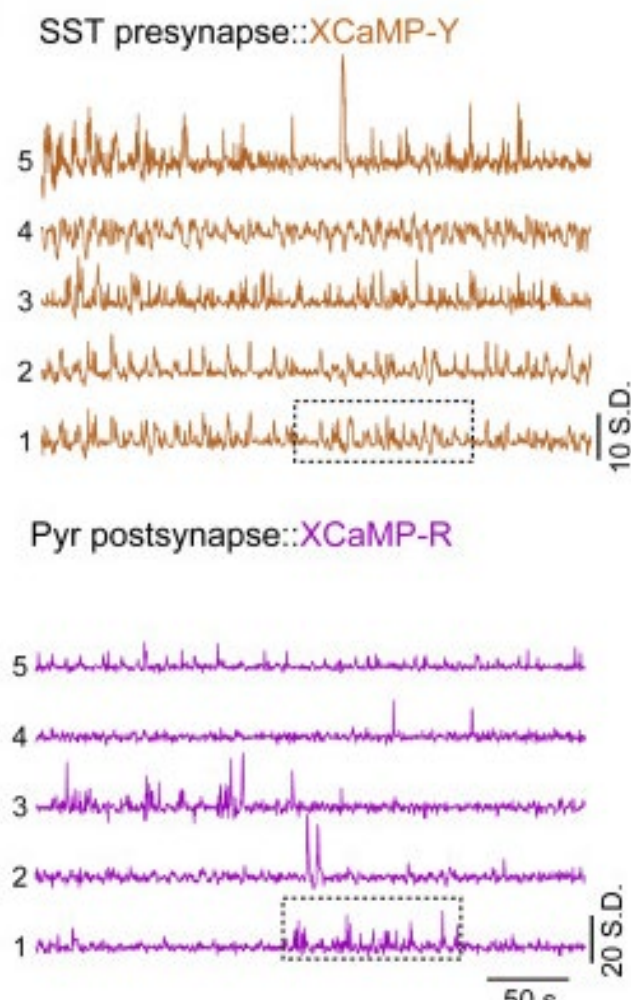
Tian et al, 2012





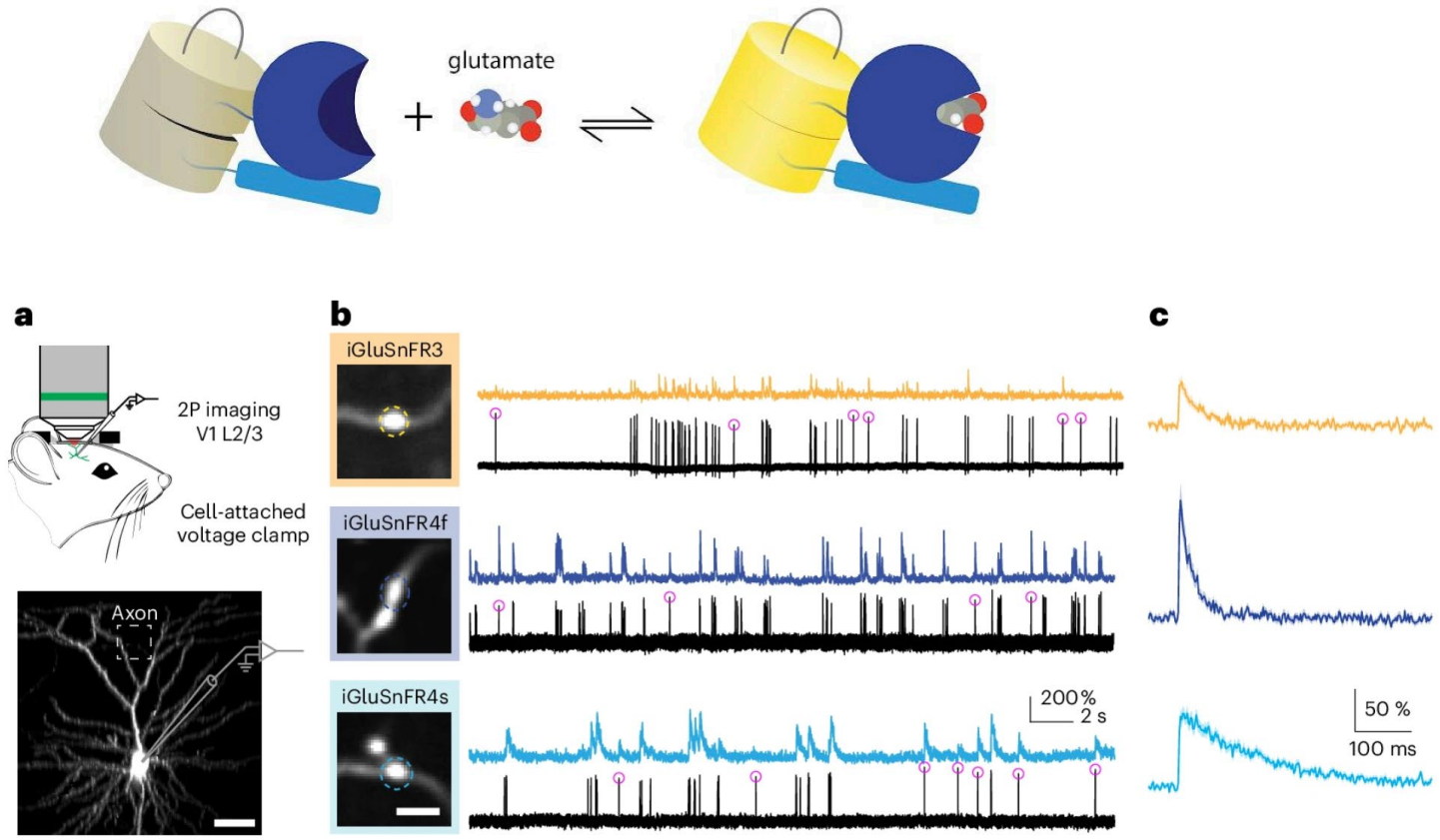




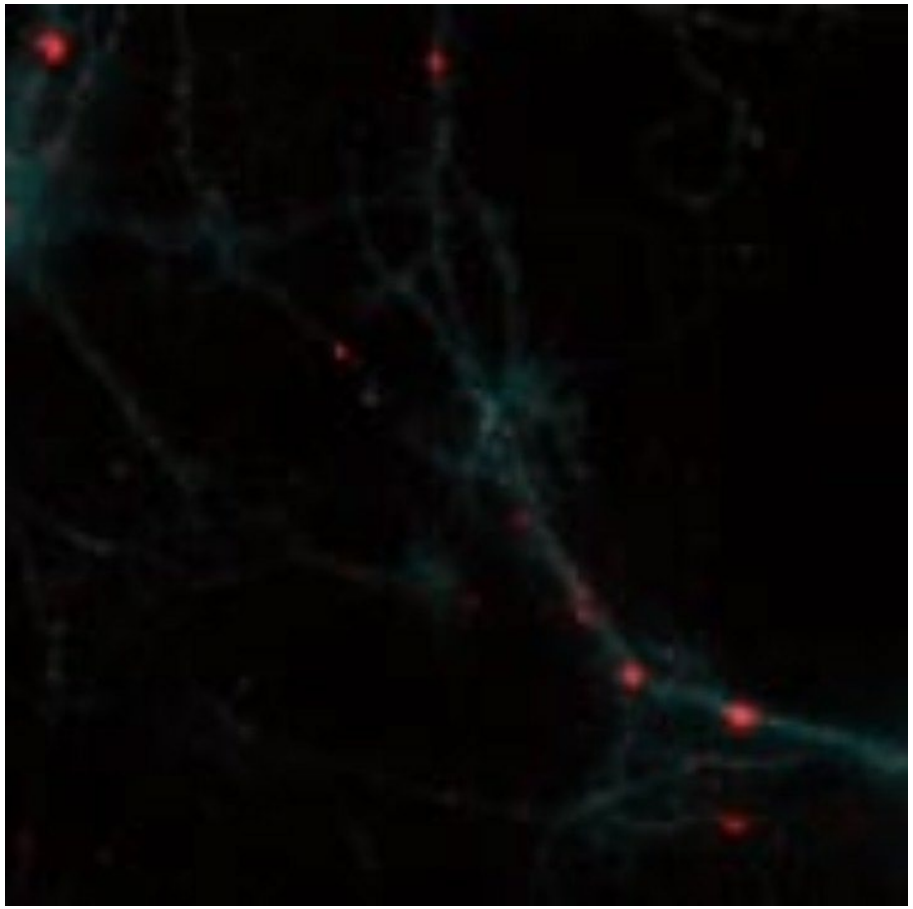


Inoue et al. (2019) *Cell*

Two-photon glutamate imaging with *iGluSnFR3*



(Aggarwal et al., 2026
https://doi.org/10.1038/s41592-025-02965-z)

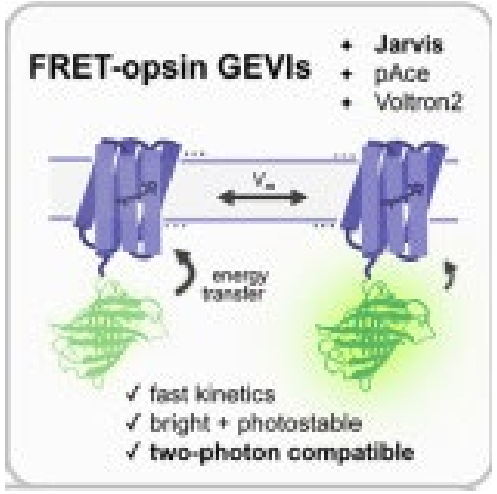


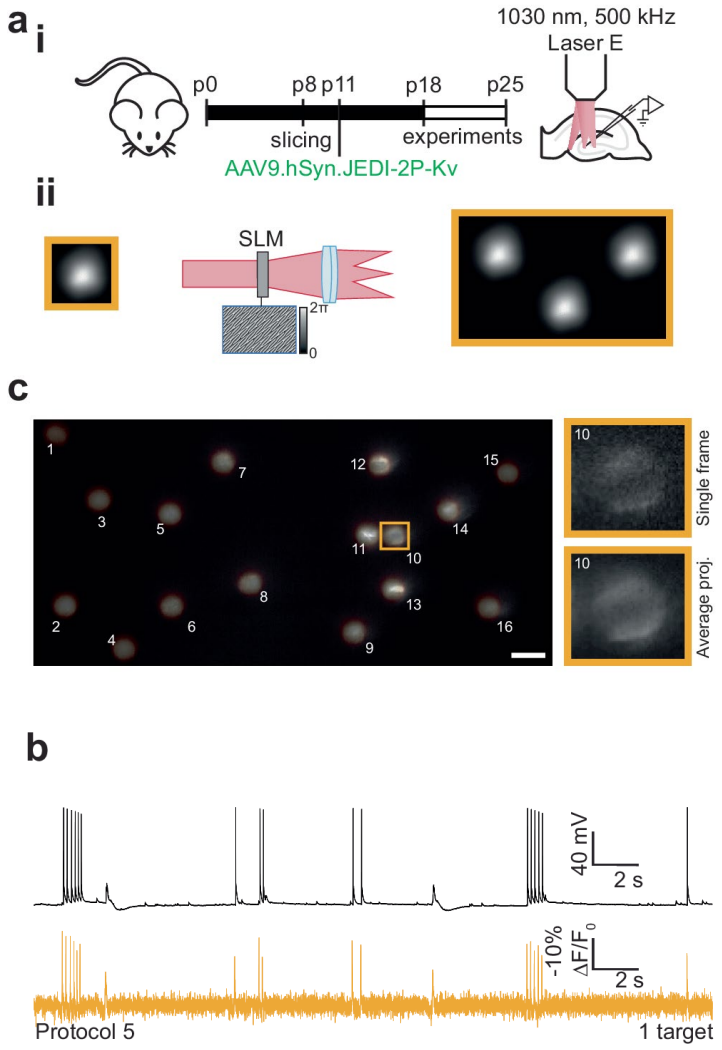
*SLAP2 microscope recording iGluSnFR3 activity (synaptic activity in red, structure in cyan) from the cell body and dendrites of a Layer 2/3 neuron in mouse motor cortex. These dendrites were imaged at **220 Hz.***

Allen Institute; https://vimeo.com/1015679273/d9a03585be

Two photon voltage imaging

- Hurdles:
  - Development of GEVI suitable for 2P excitation
    - E.g. JEDI-2P (Liu et al, 2022, *Cell*), Jarvis (Grimm et al, 2026, *Neuron*), pAce (Kannan et al, 2022, *Science*)
  - Acquisition Speed: Voltage signals require frame rates of hundreds of Hz to 1-2 kHz
    - Random access and scan-less (holographic) systems are being developed
  - "Photon budget": Trade off between signal-to-noise, acquisition speed

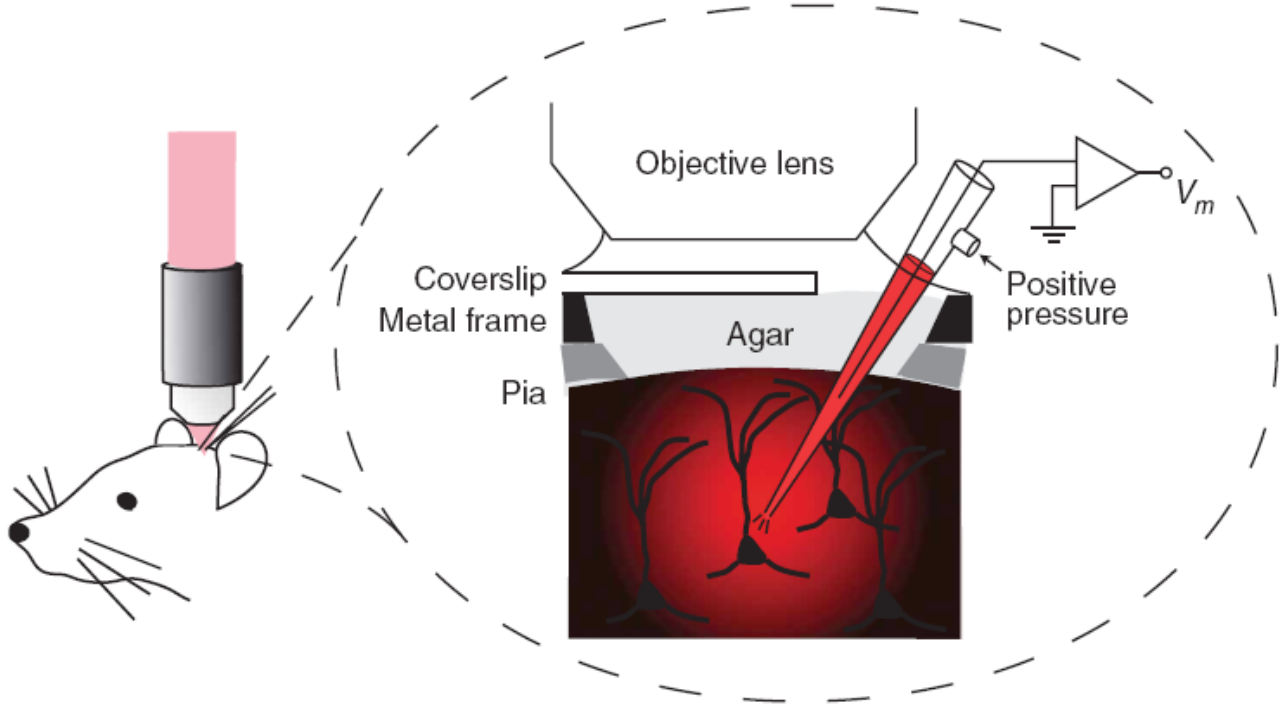




Sims et al, 2026 Nature Communications

TWO-PHOTON GUIDED ELECTROPHYSIOLOGY

Shadow patching and targeted patching under *in vivo* 2P

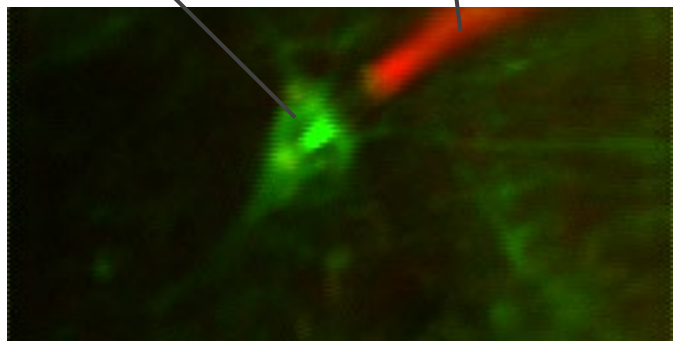


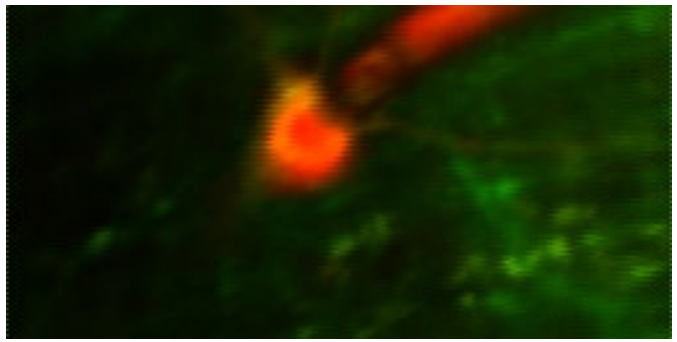
Kitamura, 2008

Pipette filled with red dye (AlexaFluor 594)

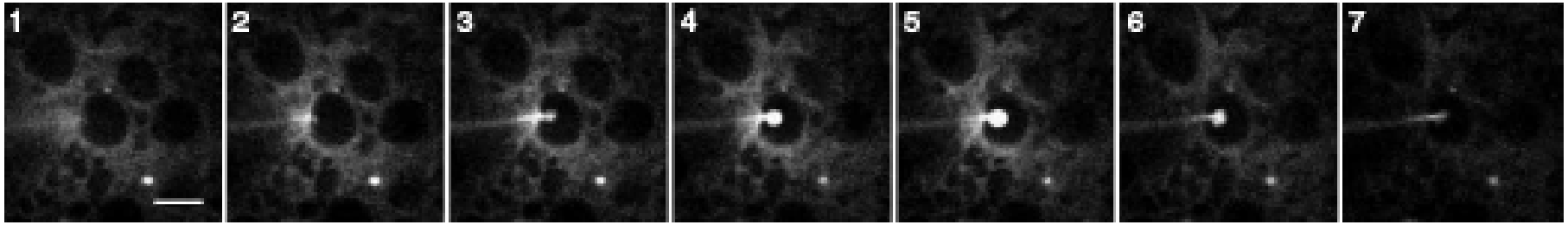
Cell with green fluorophore (GCaMP)

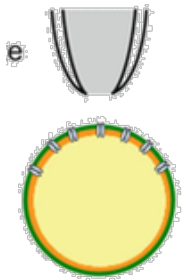






A.B.Wong





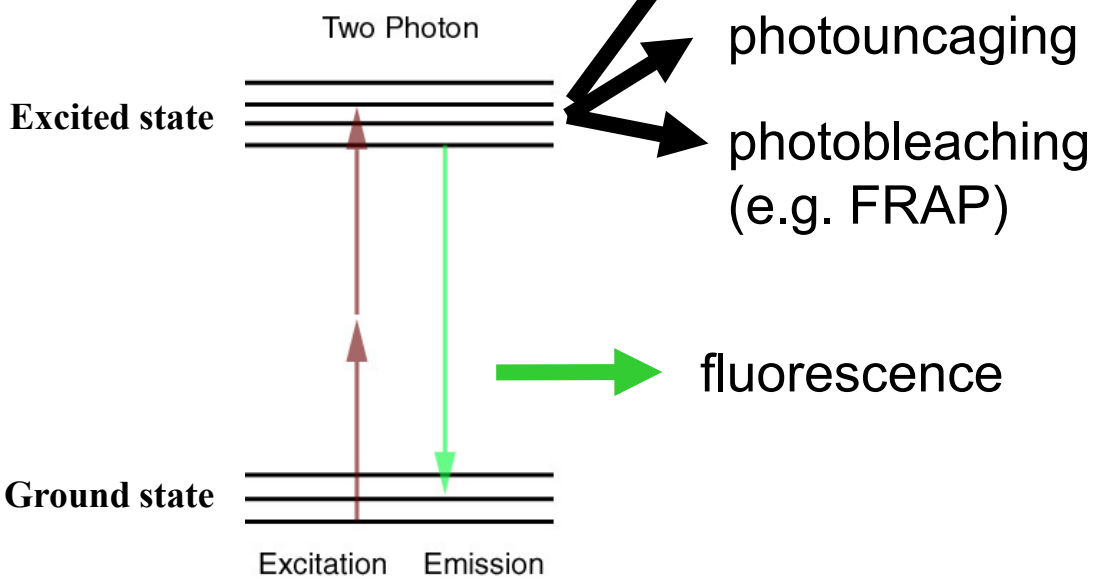




FRAP, photo-uncaging, *in vitro* applications, etc.

BEYOND FLUORESCENCE & IMAGING

Localized Photo-stimulation

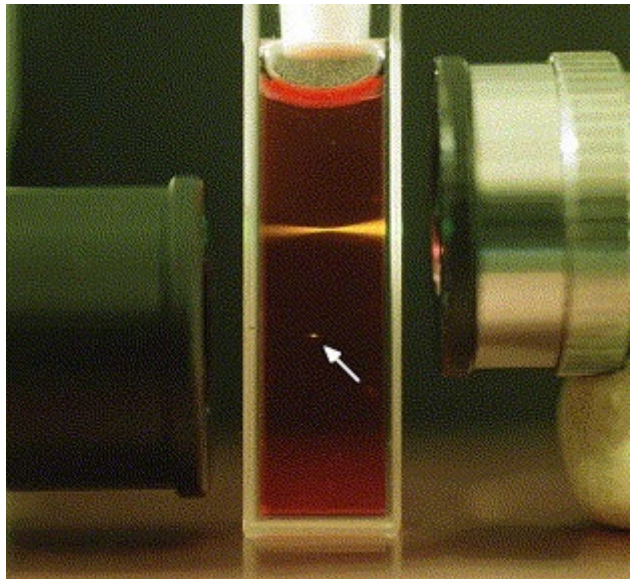


photoactivation
(e.g. optogenetics)

photouncaging

photobleaching
(e.g. FRAP)

fluorescence



Nobel Prize in Physiology or Medicine 2026

Karl Deisseroth

“for their discoveries concerning light-gated ion channels and optogenetics”



Ill. Niklas Elmehed © Nobel Prize Outreach

Peter Hegemann

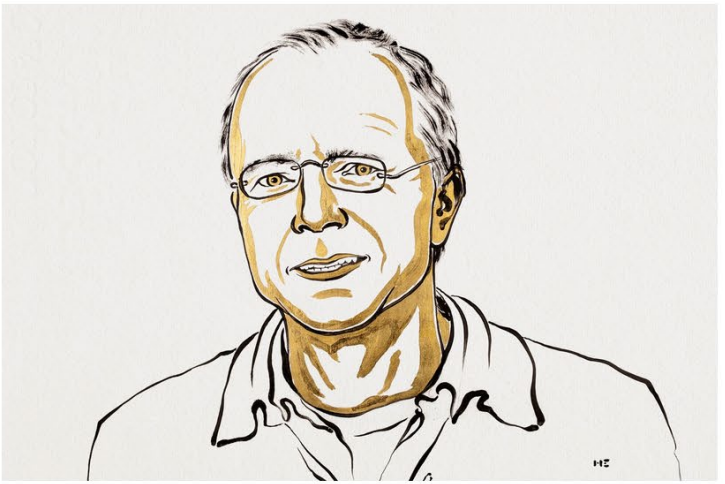
“for their discoveries concerning light-gated ion channels and optogenetics”



Ill. Niklas Elmehed © Nobel Prize Outreach

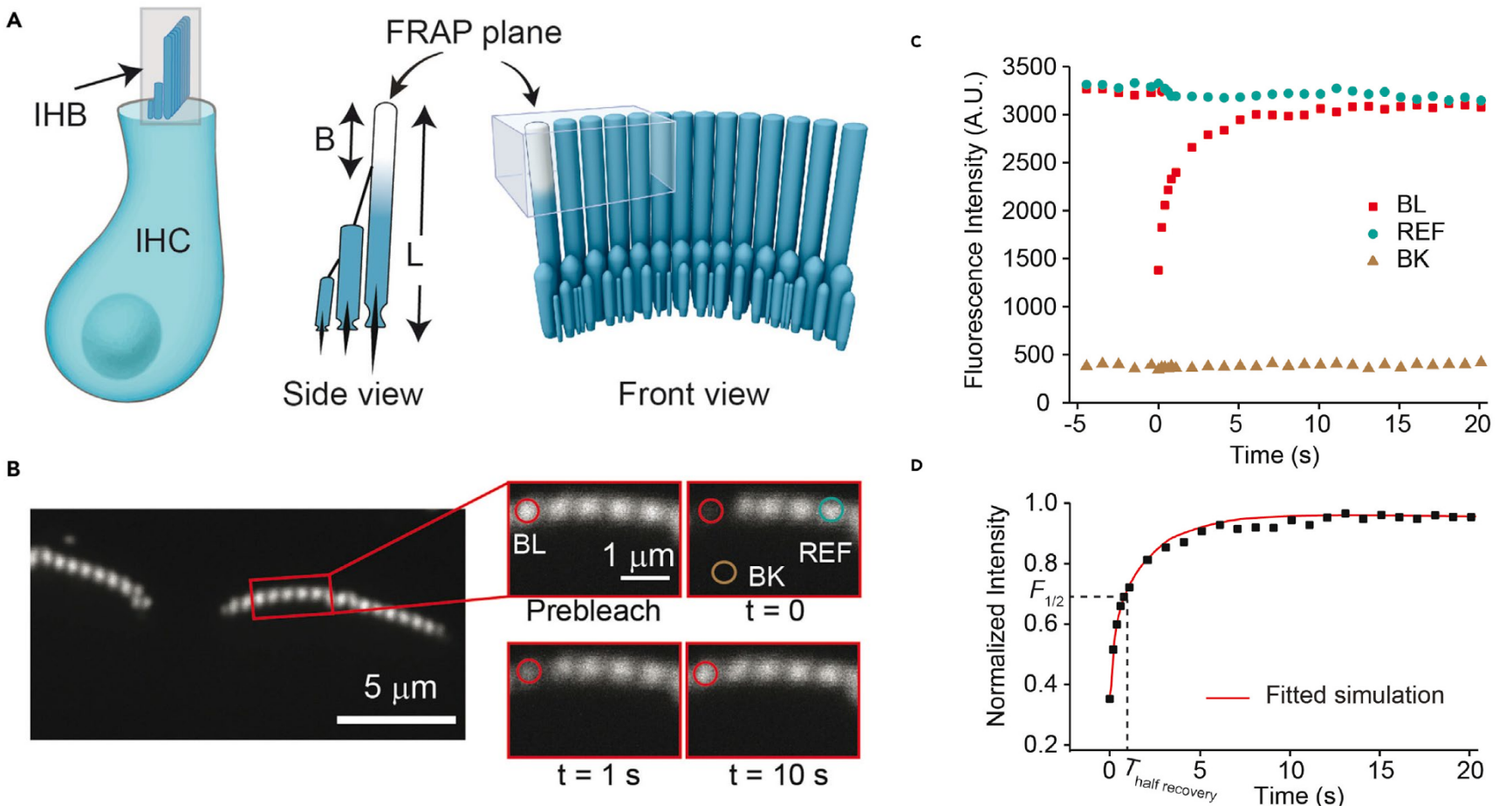
Georg Nagel

“for their discoveries concerning light-gated ion channels and optogenetics”



Ill. Niklas Elmehed © Nobel Prize Outreach

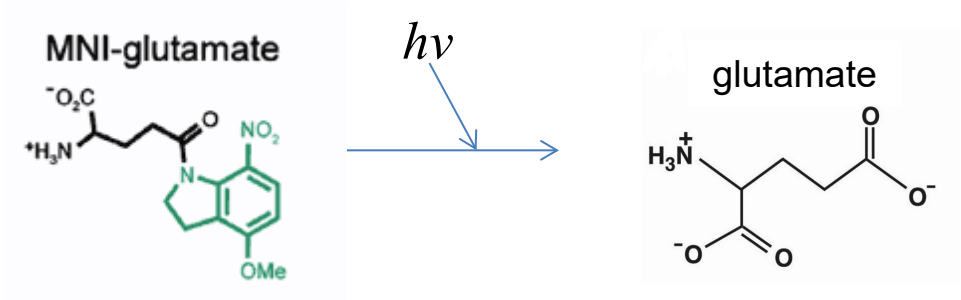
Subcellular FRAP experiment: Stereocilia of rat inner hair cells



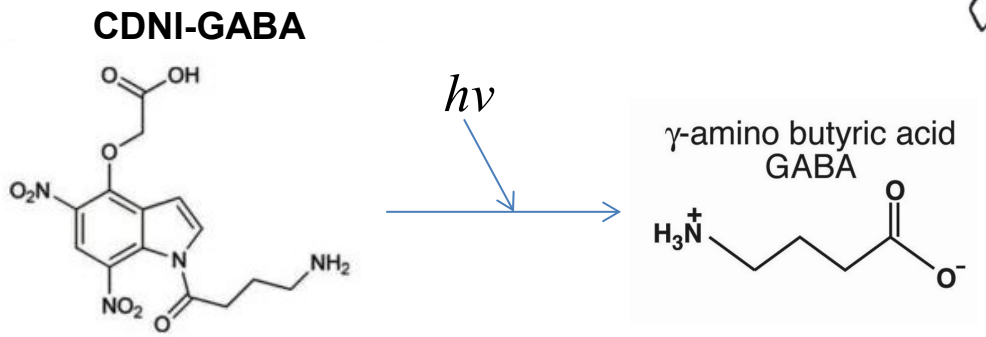
FRAP: fluorescence recovery after photobleaching
IHC: inner hair cell
IHB: IHC bundle
Dye used: di-3-ANEPPDHQ (lipophilic dye)
Wavelength: 860nm (2P)

George SS, Steele CR and Ricci AJ (2021).

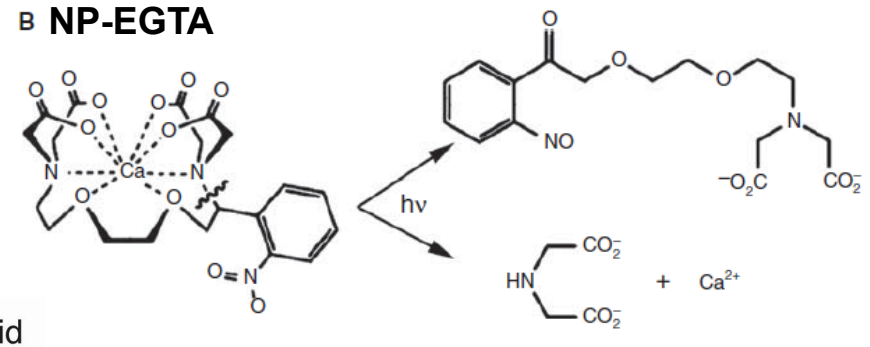
Photo-uncaging of calcium & neurotransmitters



Matsuzaki et al 2001



Matsuzaki et al 2010

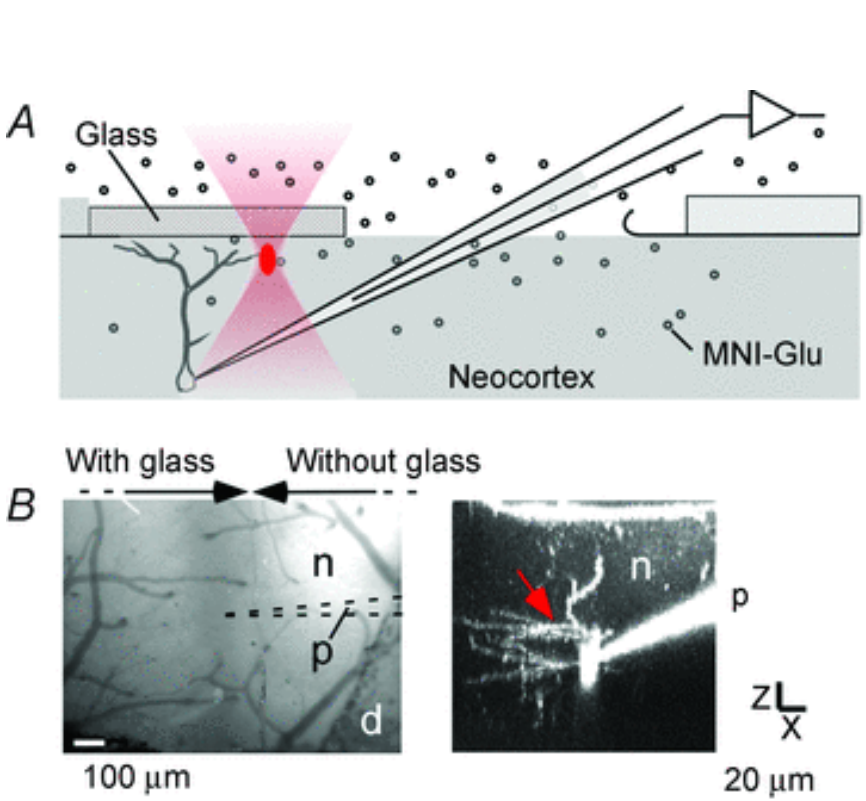


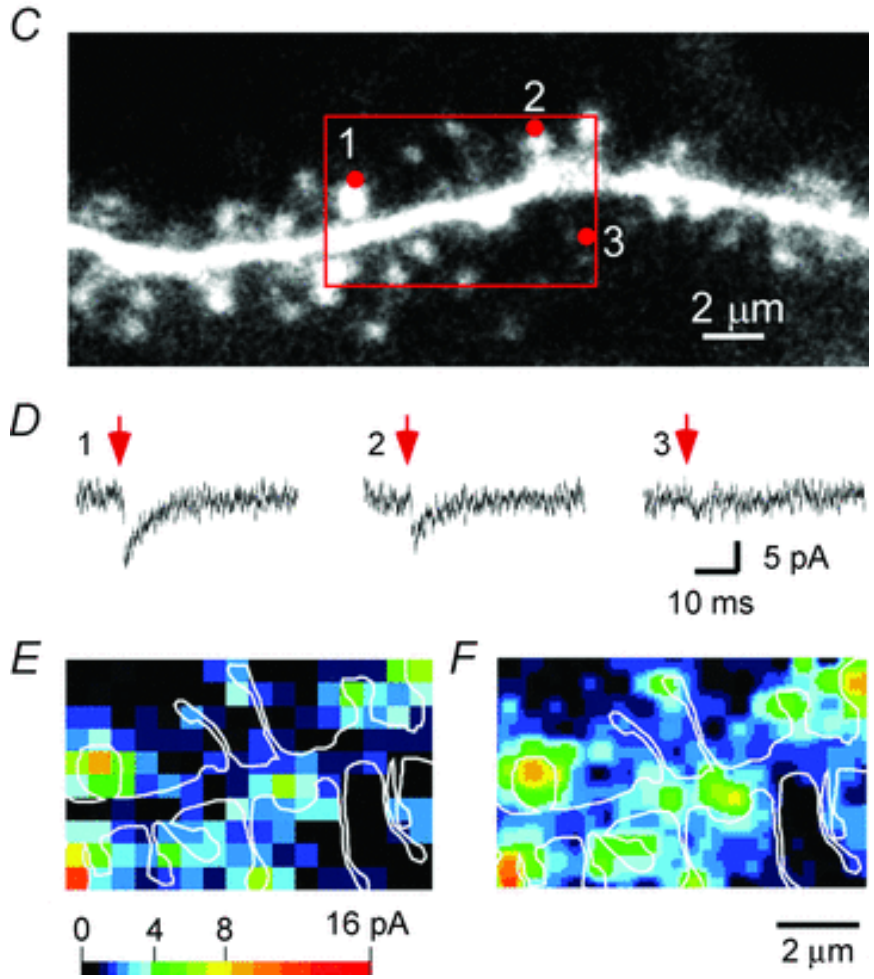
Free Ca^{2+} ion

MNI-glutamate: 4-Methoxy-7-nitroindolinyl-caged-L-glutamate

CDNI-GABA: 4-carboxymethoxy-5,7-dinitroindolinyl γ-aminobutyric acid

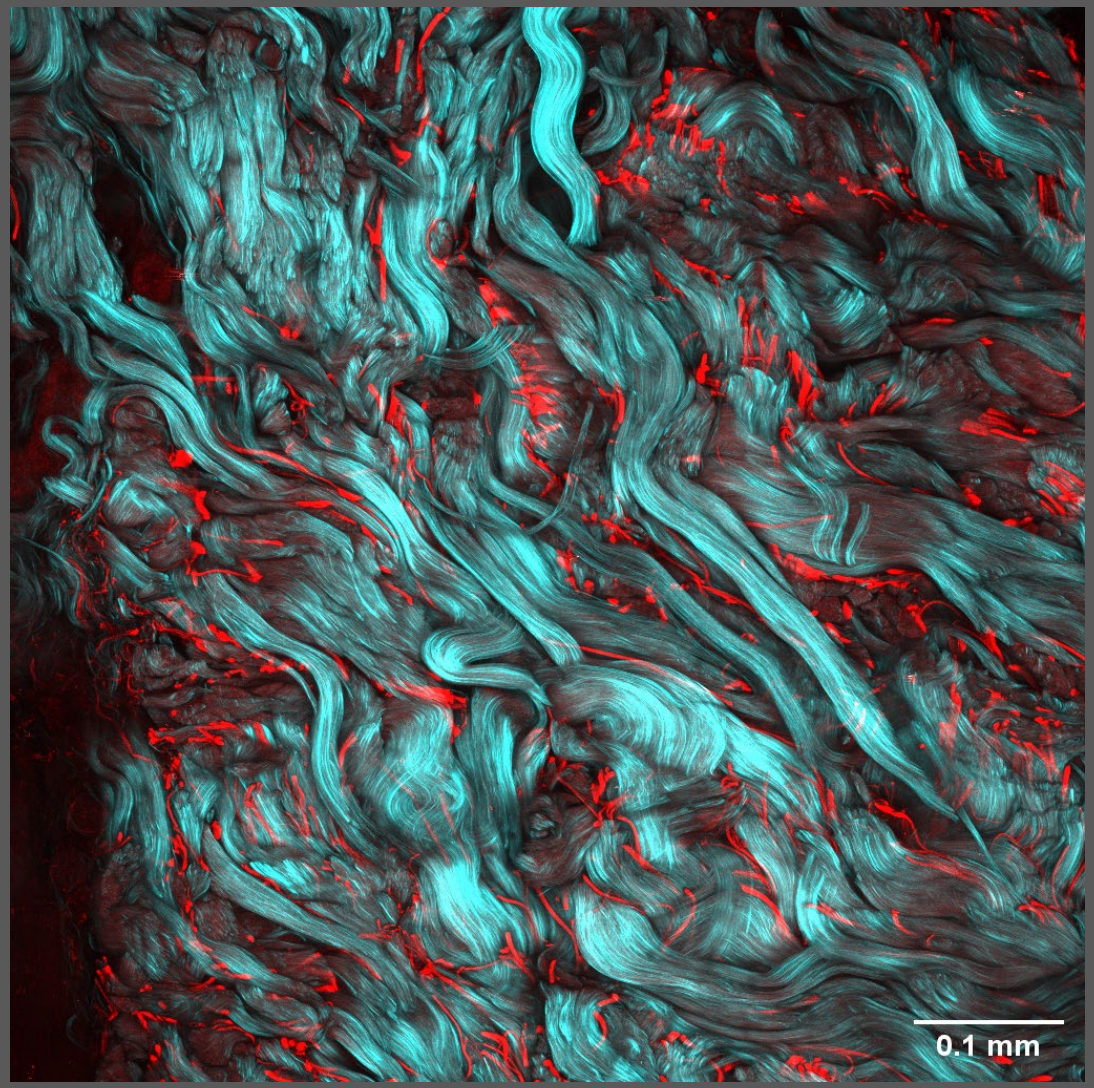
In vivo two-photon uncaging of glutamate

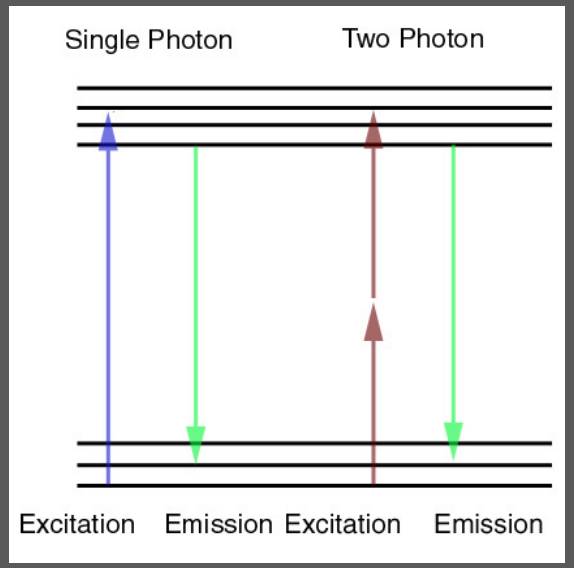




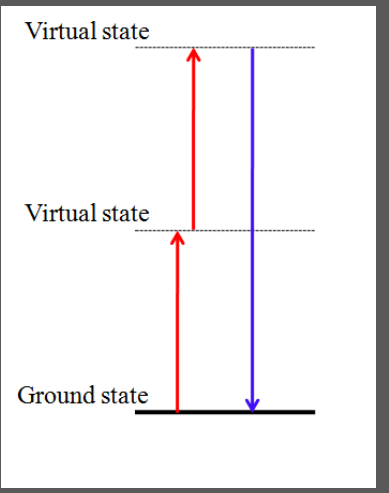
Noguchi et al, 2011

Label free imaging: Second Harmonic Generation (SHG) imaging





Second Harmonic Generation



Collagen and elastin in skin tissue
880 nm excitation
Collagen (SHG) and elastin (2PE fluorescence)
Maximum projection
Gert-Jan Kremers, Bram van Eerden, Erasmus MC

Applications of a multiphoton microscope

- Imaging thick samples
- *In vivo* imaging
- Spatially-confined photomanipulation
  - FRAP, photo-uncaging, optogenetics, etc
- *In vitro biophysics:* Fluorescence correlation spectroscopy (FCS)
- Other relating imaging techniques
  - Three-photon imaging
    - *Longer wavelength = less scatter = deeper, brighter*
  - Second harmonic generation (SHG) imaging
    - Material with regular structure, e.g. collagen

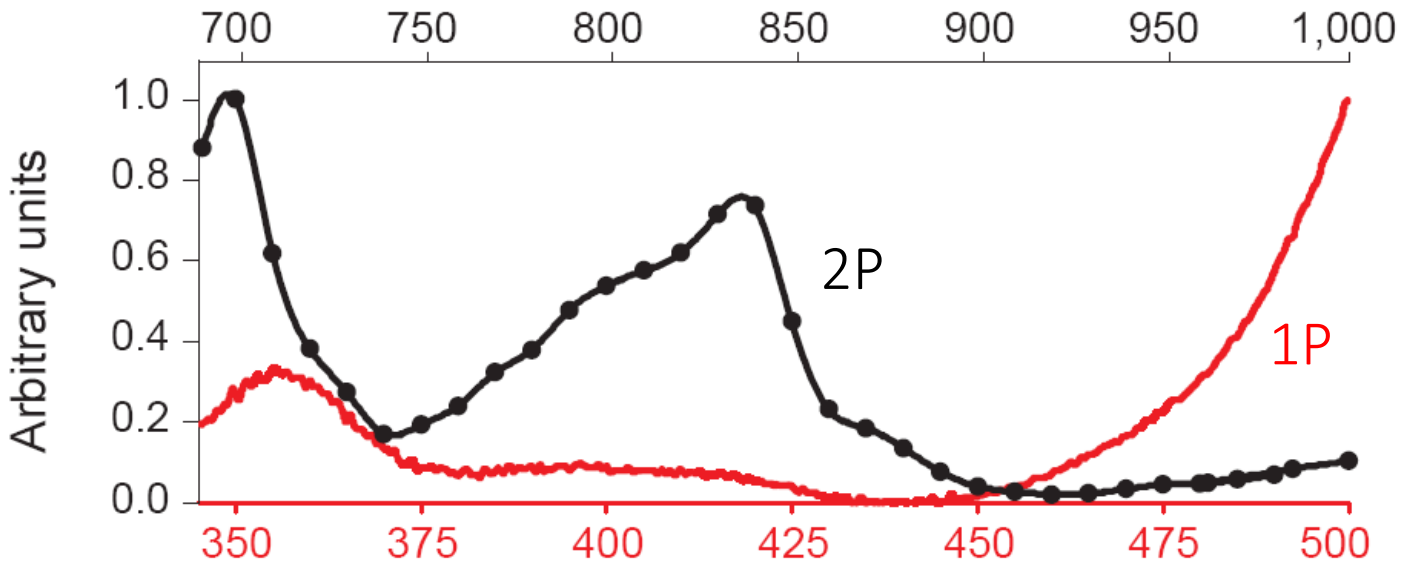
What you would need to think about.

PRACTICAL CONSIDERATIONS

Practical considerations when using two-photon microscopy

- Absorption spectra of fluorophores

One- and Two-Photon Absorption Spectrum of Rhodamine B

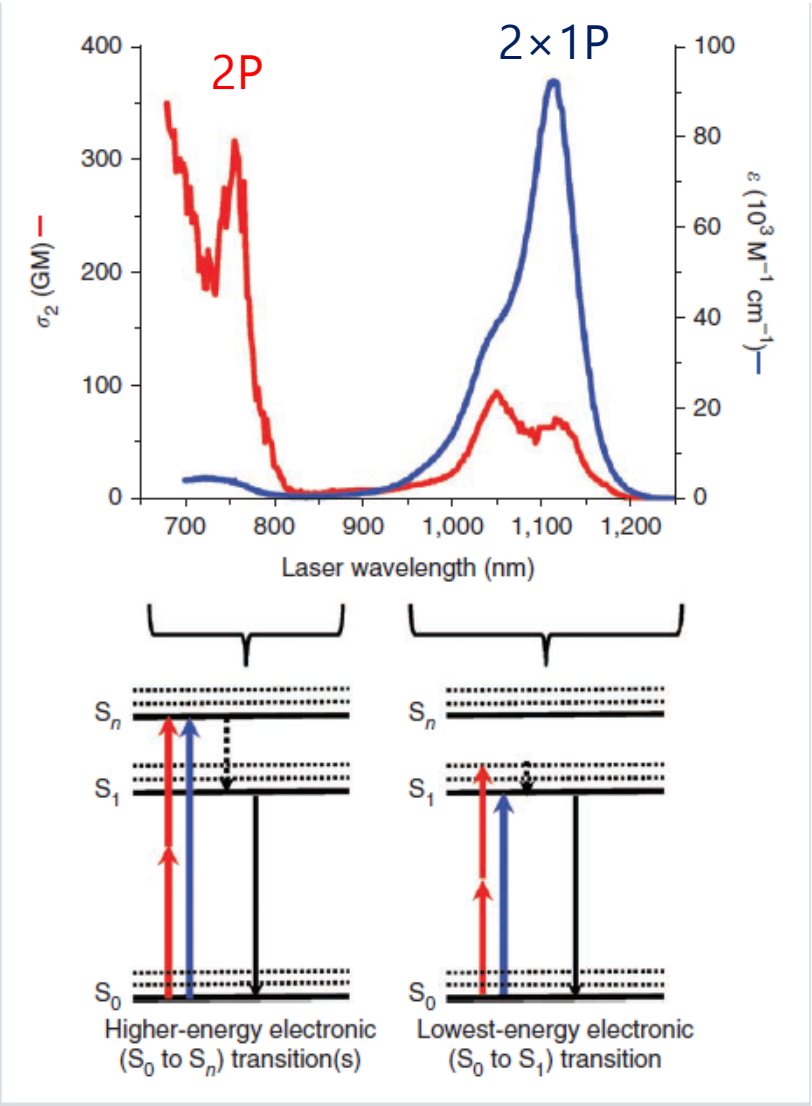


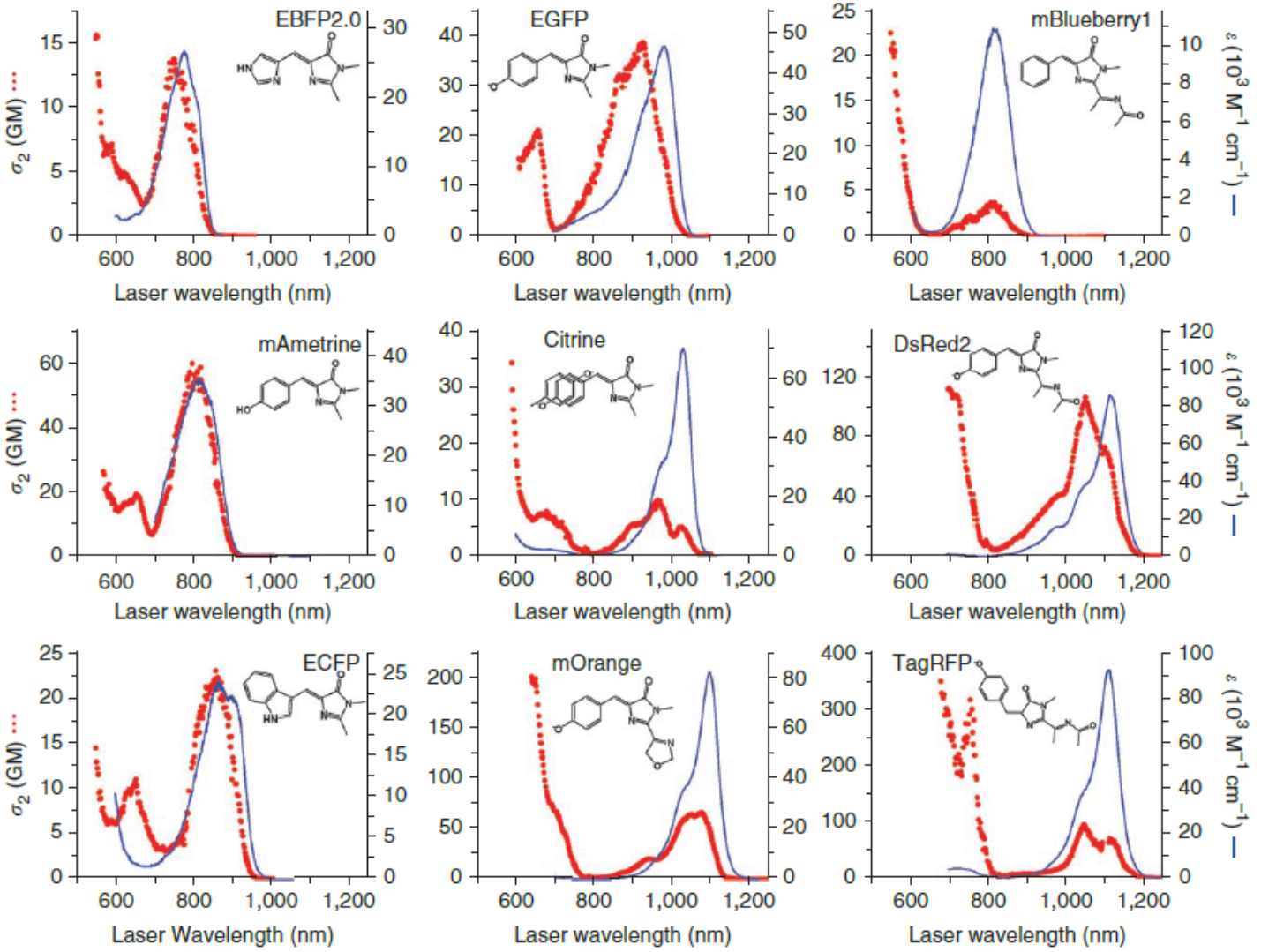
$2P \neq 2 \times 1P$!

From: Zipfel et al, 2003

Two-Photon Absorption Spectrum: 2P ≠ 2×1P

TagRFP

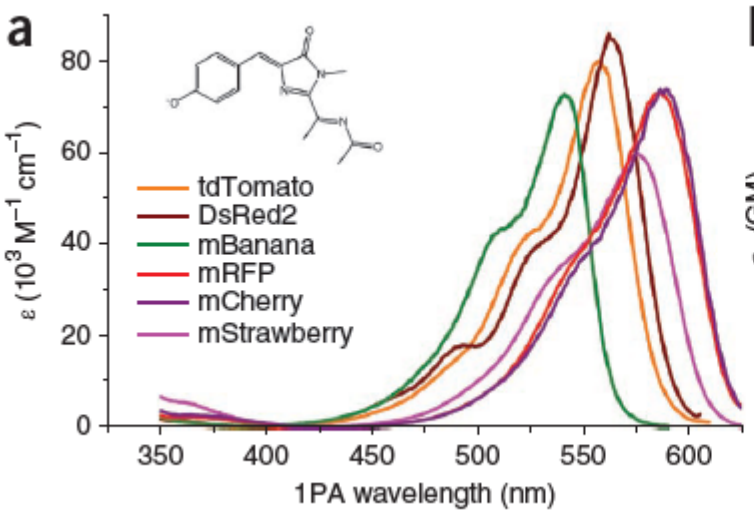




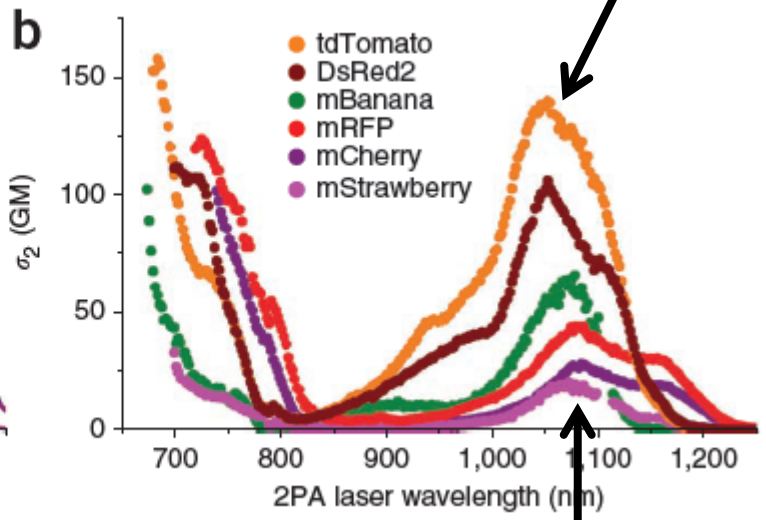
From Drobizhev et al 2011 *Nat Methods*

Some dyes may not be suitable for two-photon excitation

Similar 1P absorption

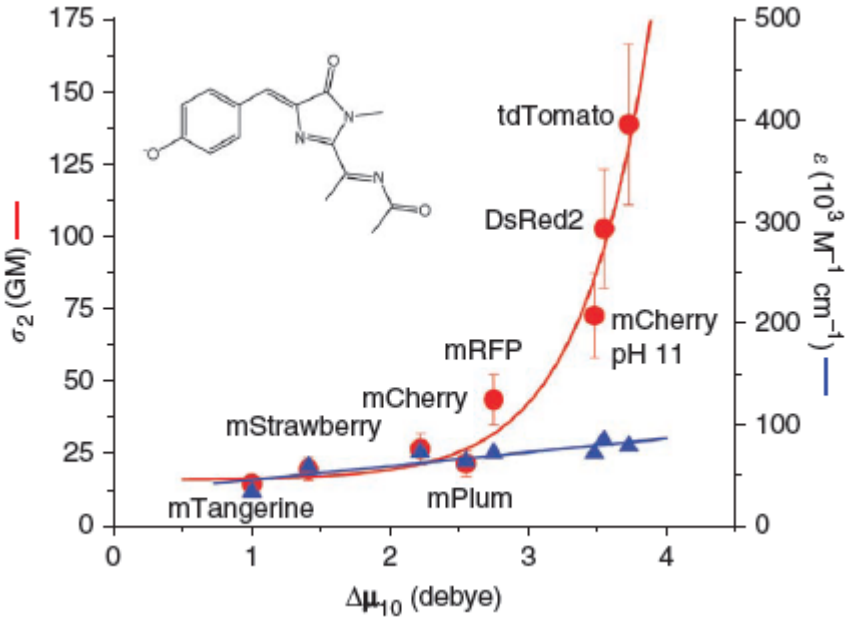


tdTomato absorbs really well



mStrawberry absorbs horribly

Two-photon absorption is highly sensitive to the electric field in the protein environment.



From Drobizhev et al 2011 *Nat Methods*

Reference material for two-photon excitation spectra

SpectraViewer WebApp

- **(by Scientific Computing Facility at Max-Planck Institute for Brain Research)**
  https://public.brain.mpg.de/shiny/apps/SpectraViewer/
  includes 2P (and some 3P) spectra of common organic and protein fluorophores

Multiphoton Characterization Resource

- By Dept of Microbiology and Cell Biology at Montana State University
- https://www.montana.edu/mbi/multiphoton_laboratory.html
- Help characterize multiphoton properties of (new) fluorescent probes

Some Primary literature

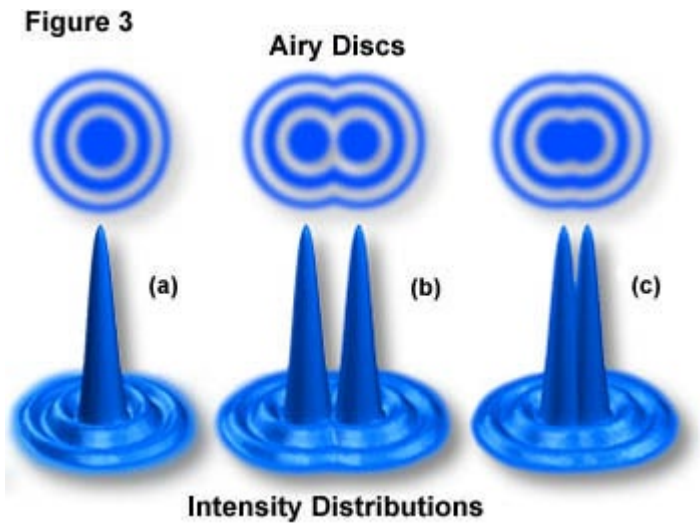
- Bestvater F, Spiess E, Stobrawa G, Hacker M, Feurer T, Porwol T, Berchner-Pfannschmidt U, Wotzlaw C, Acker H (2002) Two-photon fluorescence absorption and emission spectra of dyes relevant for cell imaging. Journal of microscopy 208:108–115.
- Drobizhev M, Makarov NS, Tillo SE, Hughes TE, Rebane A (2011) Two-photon absorption properties of fluorescent proteins. Nature Methods 8:393–399.
- Drobizhev M, Tillo S, Makarov NS, Hughes TE, Rebane A (2009) Absolute Two-Photon Absorption Spectra and Two-Photon Brightness of Orange and Red Fluorescent Proteins. J Phys Chem B 113:855–859.
- Fisher JAN, Salzberg BM, Yodh AG (2005) Near infrared two-photon excitation cross-sections of voltage-sensitive dyes. Journal of Neuroscience Methods 148:94–102.
- Mütze J, Iyer V, Macklin JJ, Colonell J, Karsh B, Petrášek Z, Schwille P, Looger LL, Lavis LD, Harris TD (2012) Excitation Spectra and Brightness Optimization of Two-Photon Excited Probes. Biophysical Journal 102:934–944.
- Xu C, Williams RM, Zipfel W, Webb WW (1996) Multiphoton excitation cross-sections of molecular fluorophores. Bioimaging 4:198–207.

Practical considerations when using two-photon microscopy

- Absorption spectra of fluorophores
  - $2P \neq 2 \times 1P$!
  - Combining fluorophores requires careful considerations

- Resolution

Resolution



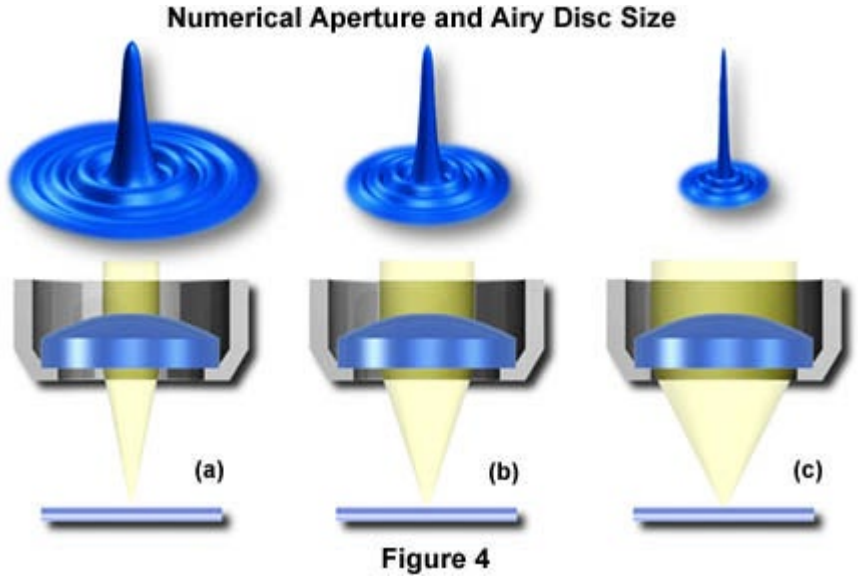


Figure 4

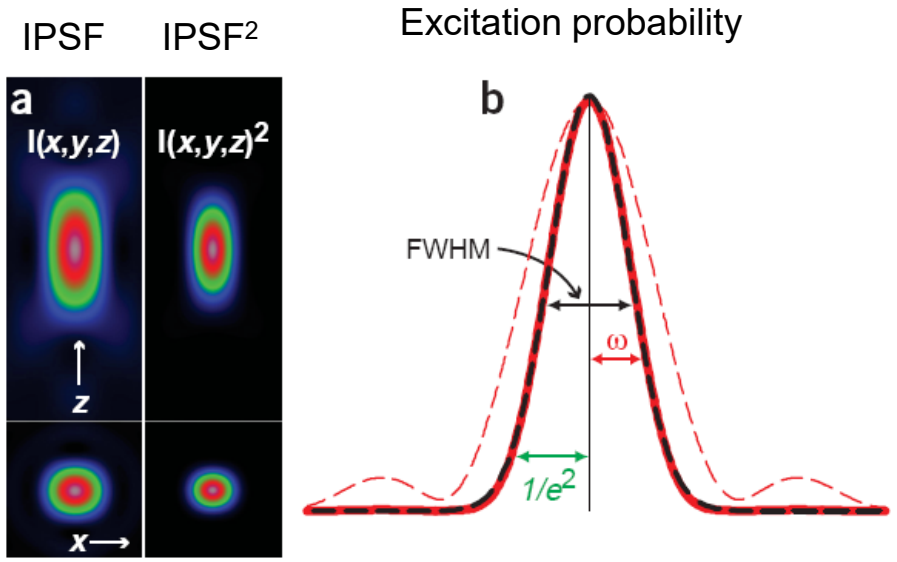
$$R \approx \frac{\lambda}{2\ \mathrm{NA}}$$

Abbe diffraction limit

Reminder
λ: wavelength of light
NA: numerical aperture,
(NA = $n \sin\theta$)
n: refractive index of medium
θ: half angle of light cone

http://micro.magnet.fsu.edu/primer/anatomy/numaperture.html

Illumination Point Spread Function (IPSF) in 2P microscopy



c

$$\omega_{xy} = \begin{cases} \dfrac{0.320\lambda}{\sqrt{2}\,NA} & NA \le 0.7 \\ \dfrac{0.325\lambda}{\sqrt{2}\,NA^{0.91}} & NA > 0.7 \end{cases} \qquad \omega_z = \frac{0.532\lambda}{\sqrt{2}}\left[\frac{1}{n-\sqrt{n^2-NA^2}}\right]$$

FWHM: full width at half maximum
NA: numerical aperture
GM: Göppert-Mayer
ω_{XY}: diffraction-limited lateral 1/*e* radii of IPSF²
ω_{XY}: diffraction-limited axial 1/*e* radii of IPSF²

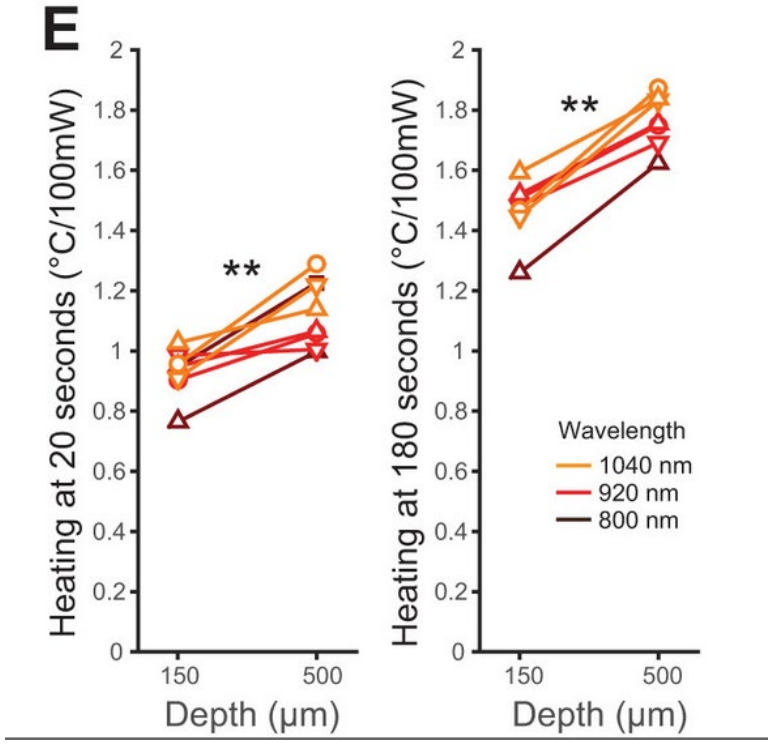
From: Zipfel et al, 2003

Practical considerations when using two-photon microscopy

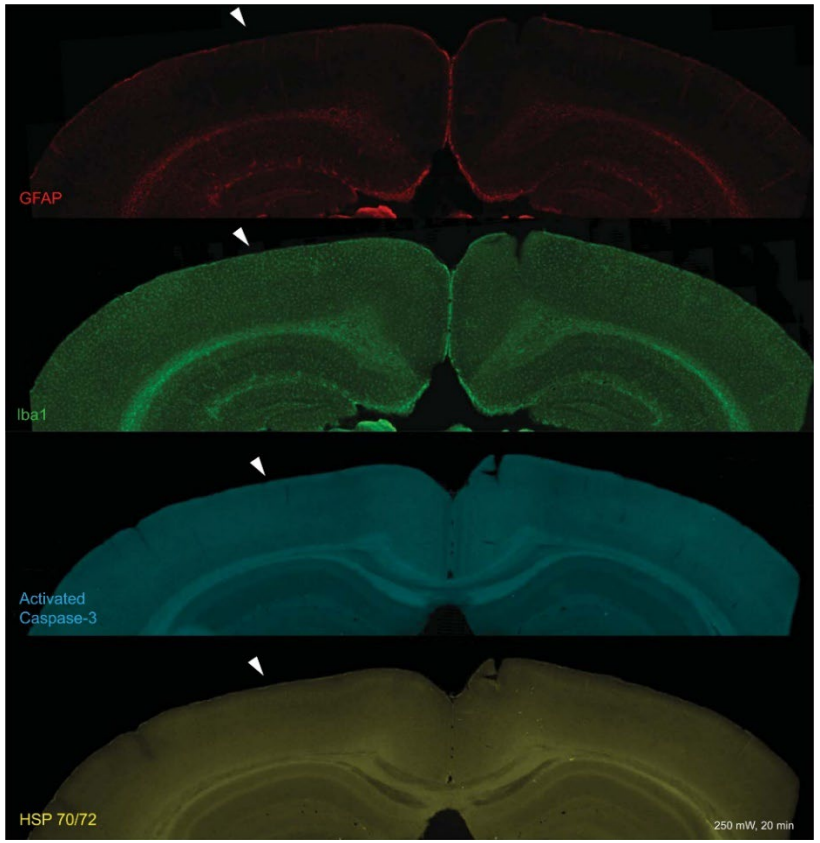
- Absorption spectra of fluorophores
  - $2P \neq 2 \times 1P$!
  - Combining fluorophores requires careful considerations

- Resolution
  - Theoretically: longer excitation wavelength => worse resolution
  - *But* in practice:
    - 1P excitation: more *s*cattering => PSF degradation
    - Confocal pinhole is bigger than the theoretical zero
    - Better light collection with 2-photon microscope (no pinhole)

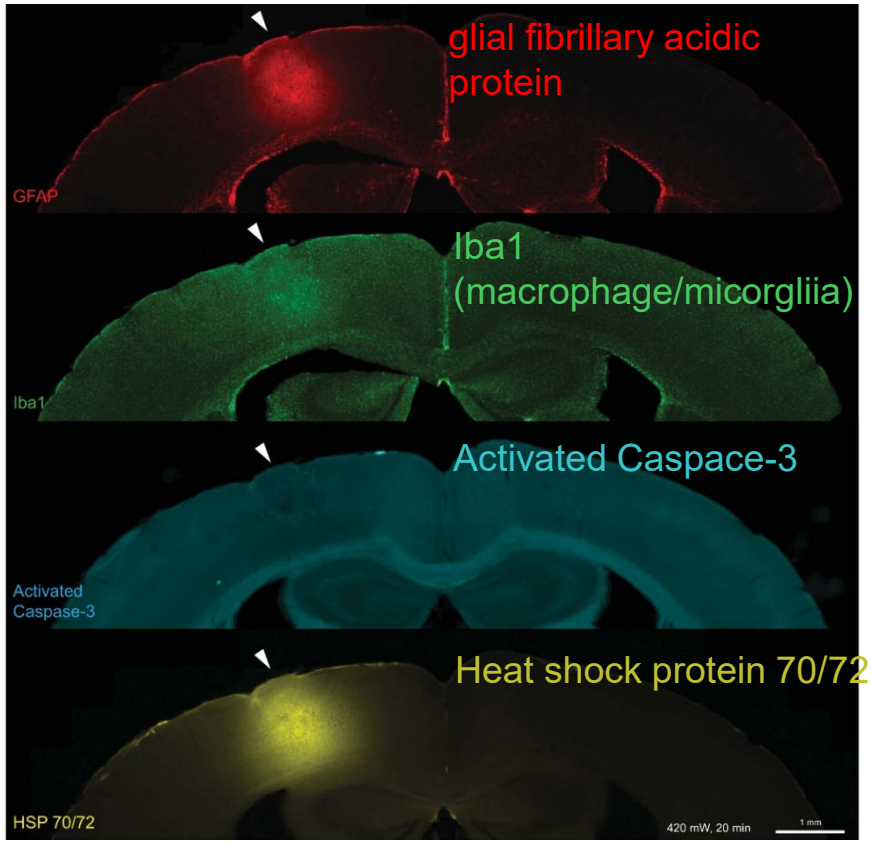
Heat damage caused by two photon imaging



250 mW, 20 min, 920 nm



420mW, 20min, 920 nm



"Continuous illumination of a 1-mm² area produced a peak temperature increase of ~1.8°C/100 mW."

Podgorski K and Ranganathan G (2016)

Practical considerations when using two-photon microscopy

- Absorption spectra of fluorophores
- Resolution

- Heat damage
  - High power infrared light (10's – 100's mW) may heat up the tissue

- Price
  - Special laser needed: e.g. Ti-Sapphire laser
  - [new dev] Cheaper, fixed-wavelength lasers (920nm, 1040 nm, etc.)

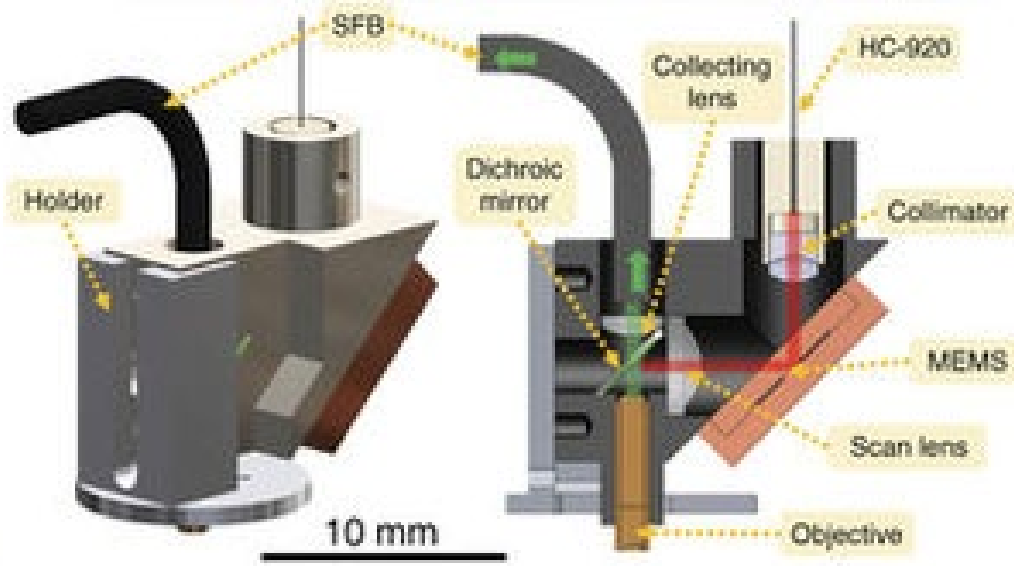
- Speed
  - Laser-scanning microscope is speed limiting
  - *But see:* random access scanning, scanless excitation (temporal focusing)

- Non-linear bleaching
  - Two-photon excitation bleaches faster with increasing intensity (Patterson & Piston, 2000; Drummond et al, 2002)

Building on the basics; Breaking some of the limitations

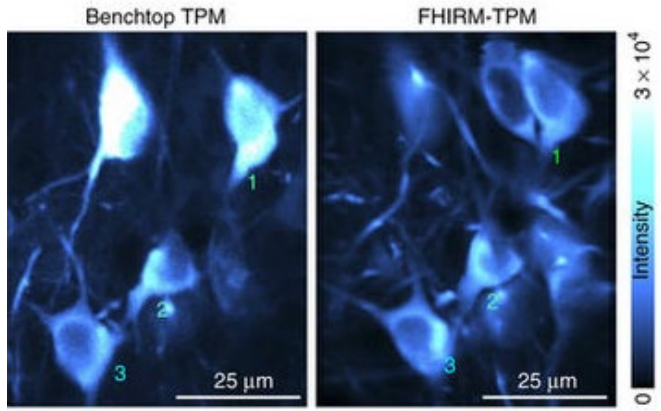
ADVANCED MULTIPHOTON MICROSCOPY

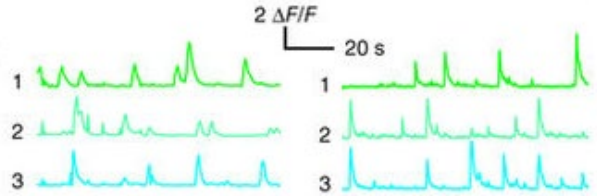
Miniaturization of microscope





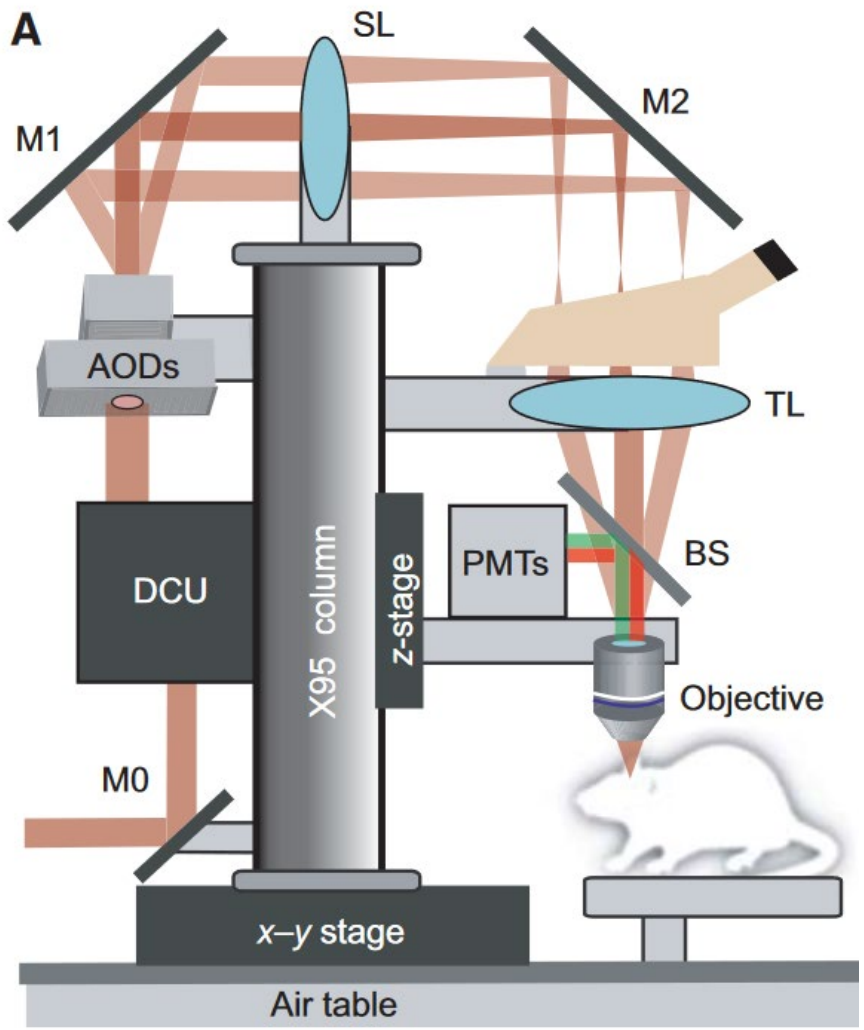


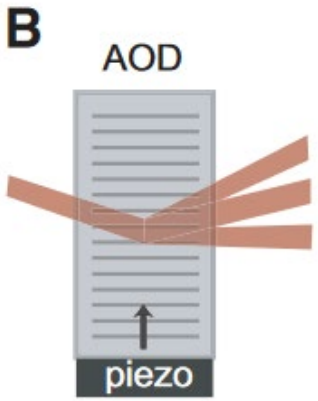




Zong et al 2017 *Nat Meth*

Fast, “random access” scanning using acousto-optic deflector (AOD)



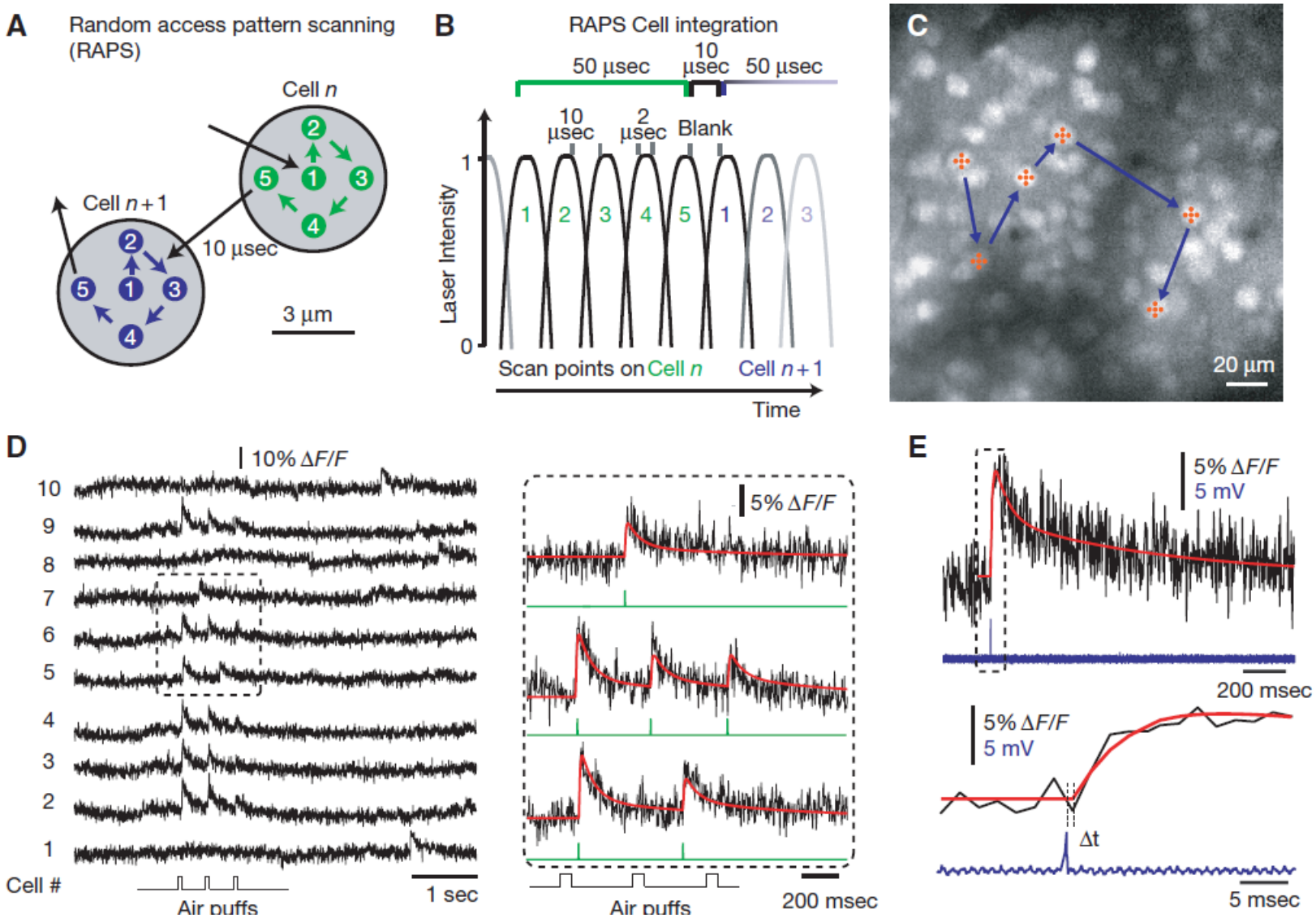


- A sound wave (MHz-range) in solid material, deflecting the laser beam by a small angle.
- Modulation of the frequency changes the deflection angle

*“The main advantage of using AOD laser scanners is the possibility to **rapidly shift the focal excitation spot** between any two positions within the scanning field within only a few microseconds (10 µsec in our setup).”*

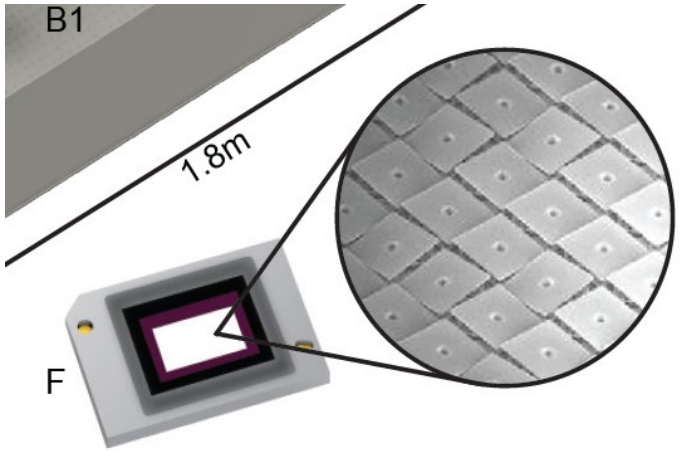
Grewe & Helmchen 2014
Cold Spring Harb Protoc

Fast, “random access” scanning using acousto-optic deflector (AOD)

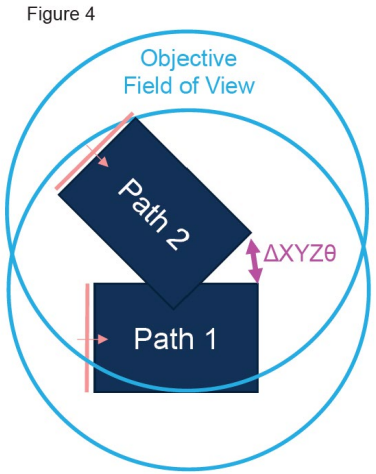


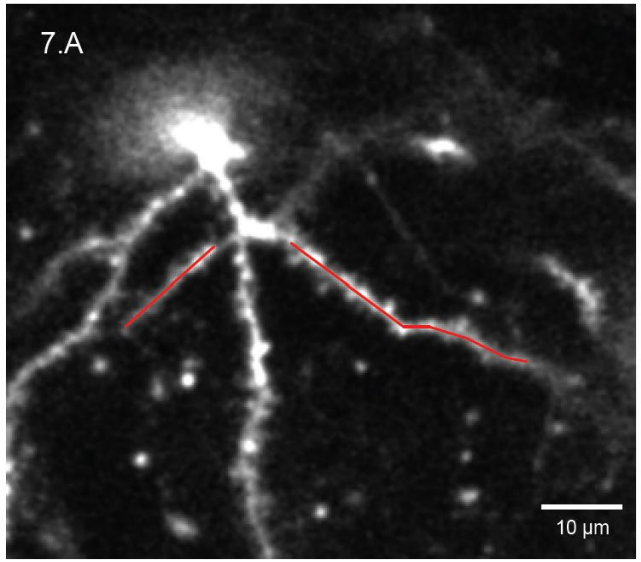
Grewe & Helmchen 2014 *Cold Spring Harb Protoc*

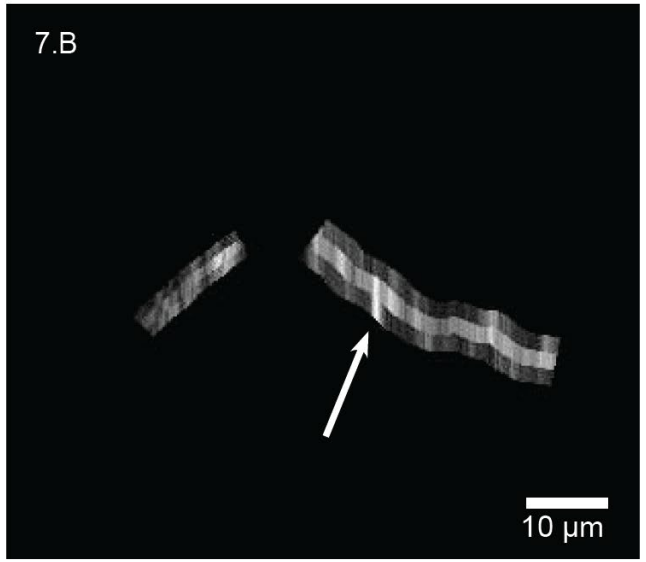
Scanned Line Angular Projection (SLAP2)



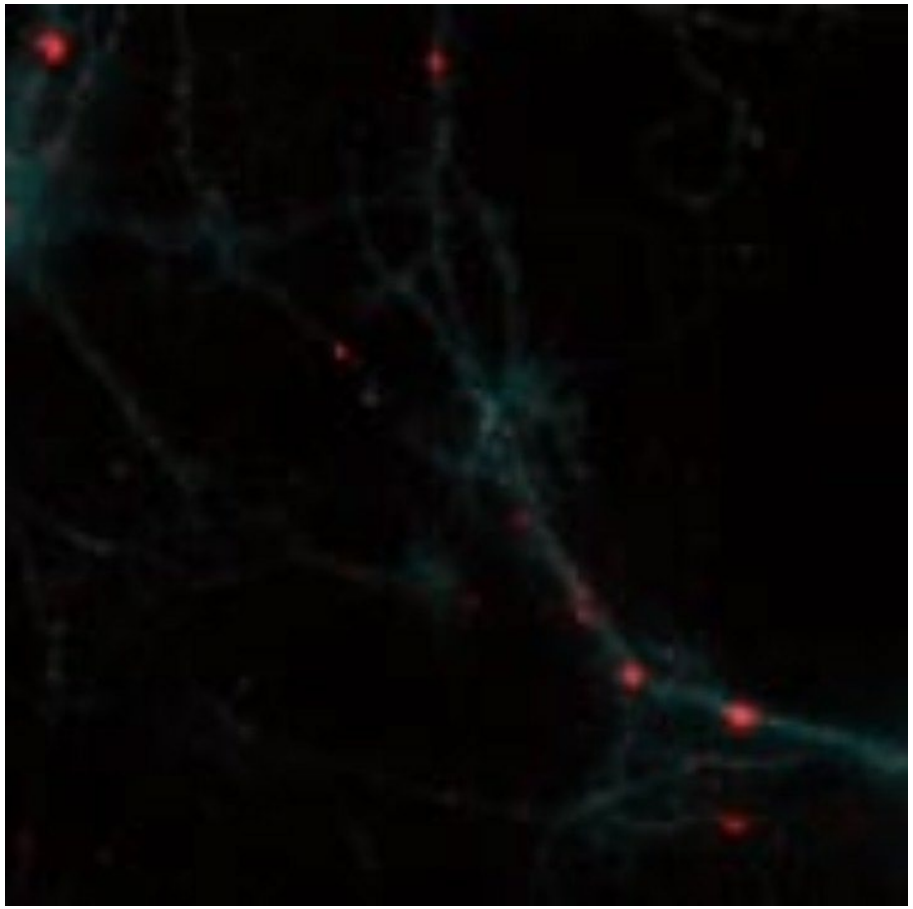
Digital Micromirror Device (DMD)







JAINDL et al, 2023
(https://www.mbfbioscience.com/app/uploads/2023/07/SLAP2_Research-Poster.pdf)

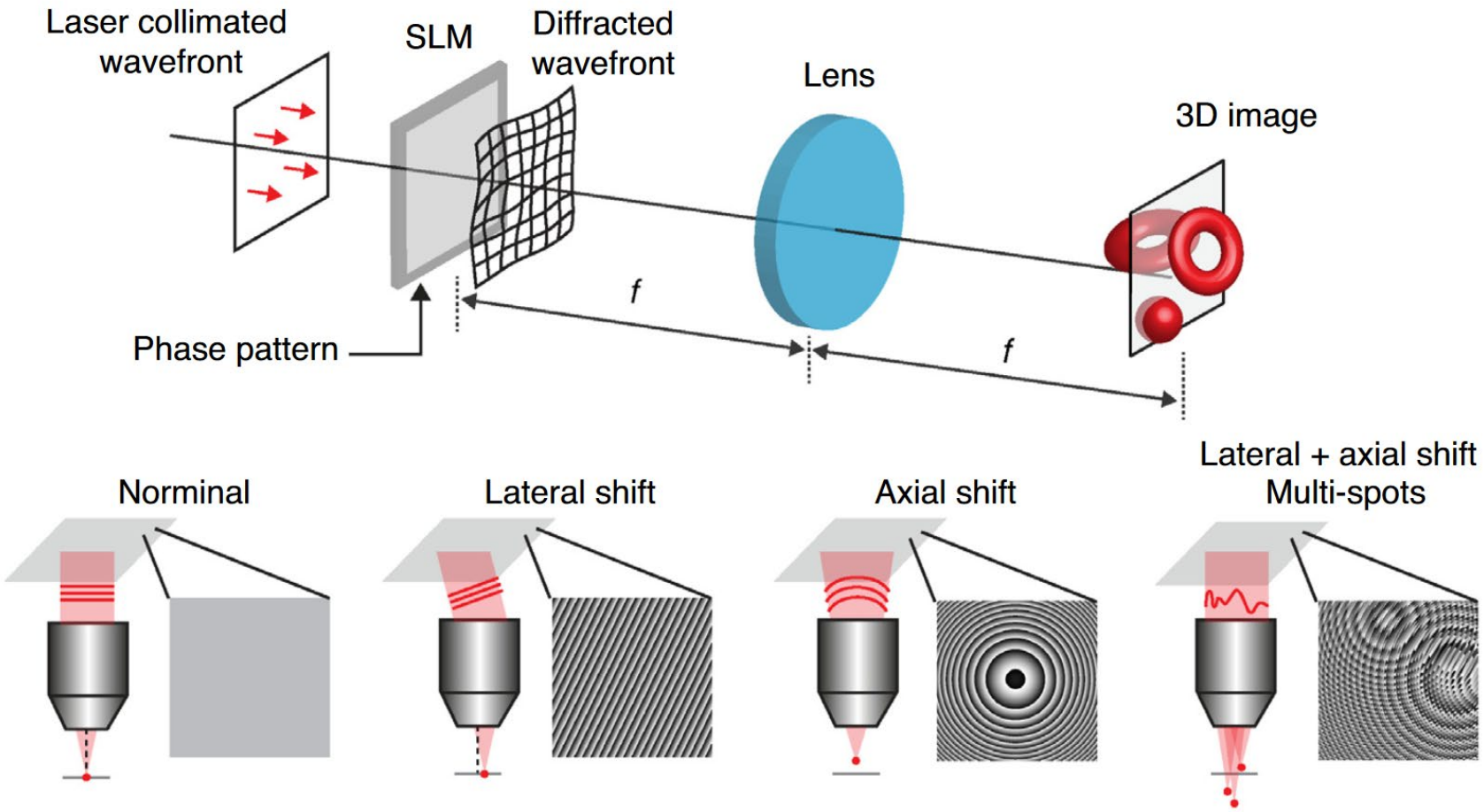


*SLAP2 microscope recording iGluSnFR3 activity (synaptic activity in red, structure in cyan) from the cell body and dendrites of a Layer 2/3 neuron in mouse motor cortex. These dendrites were imaged at **220 Hz.***

Allen Institute; https://vimeo.com/1015679273/d9a03585be

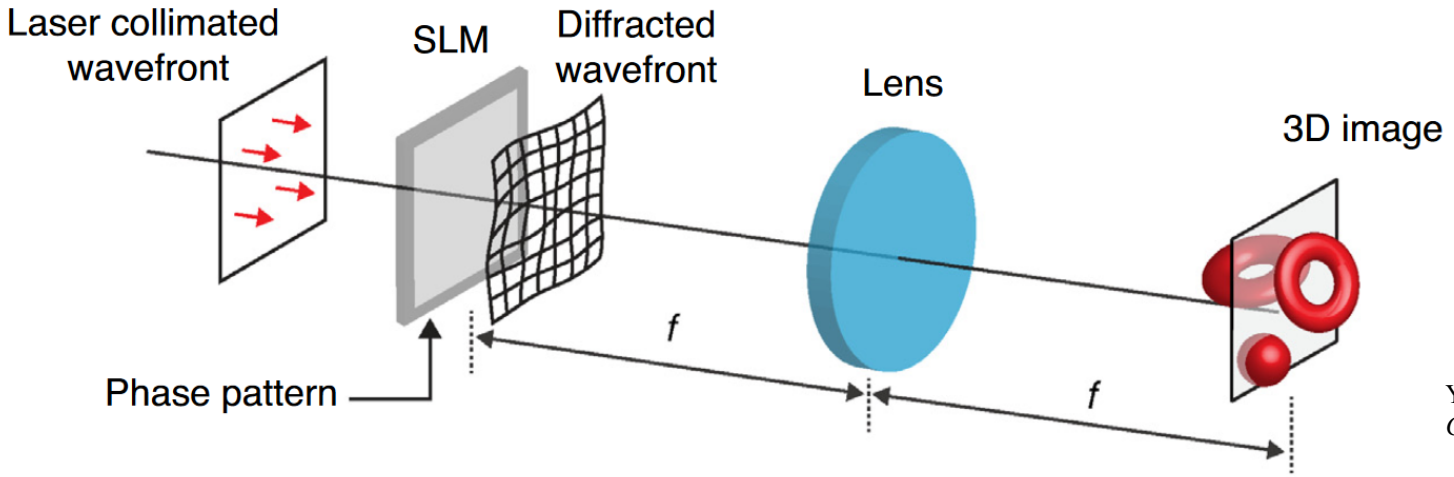
Computer-generated Holographic (CGH) illumination

SLM: spatial–light modulator

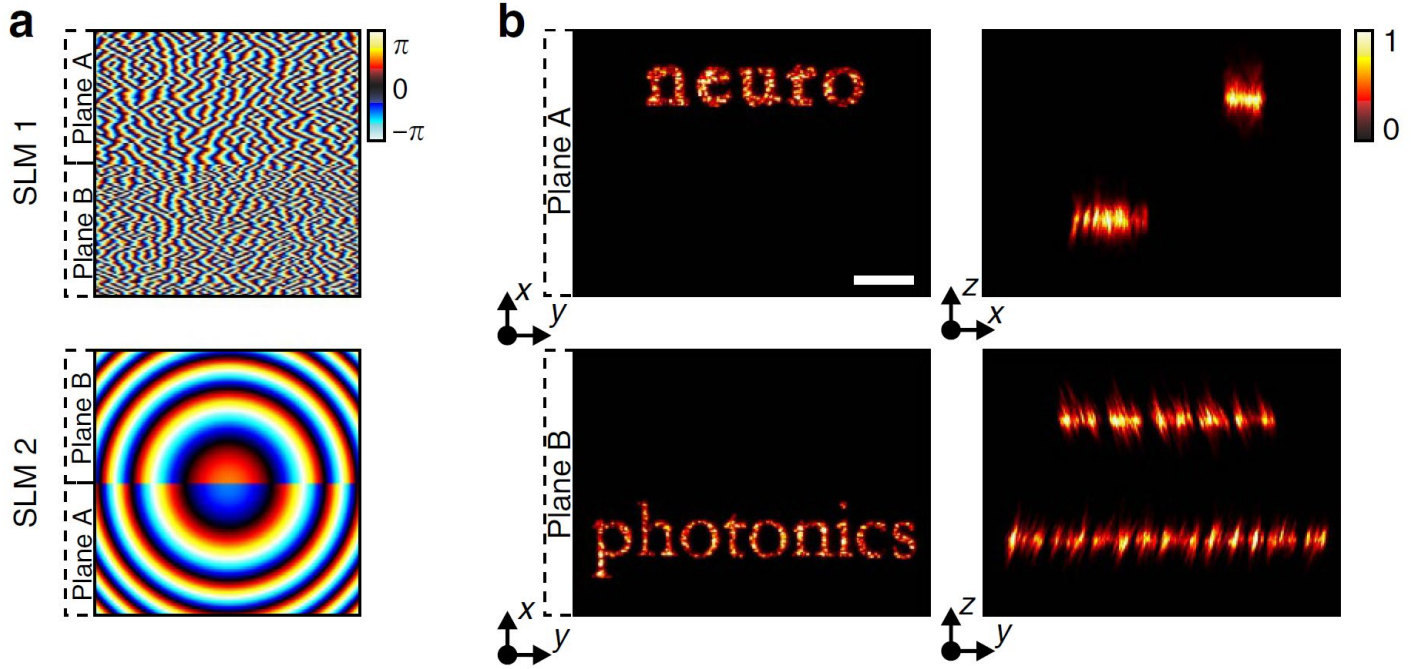


Yang & Yuste (2018)
Curr Op in Neurobiol

Computer-generated Holographic (CGH) illumination



Yang & Yuste (2018)
Curr Op in Neurobiol



Hernandez et al.
(2015) *Nat Comm*

To test your understanding ~~vs my personal opinion~~

CASE STUDIES

Would you use multiphoton microscopy?

- You have fixed, immunostained tissue (brain, kidney, heart, etc.) with very good clearing (e.g. with iDISCO)
  - Probably not necessary

Would you use multiphoton microscopy?

- You have a cell culture and would like to do live cell imaging.
  - Probably not necessary; but consider photodamage for long-term imaging

Would you use multiphoton microscopy?

- You have a **3D** cell culture and would like to do live cell imaging.
  - Probably worth trying

- You have a cell culture and would like to do live cell imaging of **subcellular compartments**.
  - Resolution:, super-resolution, confocal, 2P, epifluorescence
  - Speed: epifluorescence, confocal/2P/STED, PALM/STORM

- You have a cell culture and would like to perform photostimulation/FRAP at subcellular resolution
  - Good idea.
  - Are you cells flat? Then maybe confocal is good enough

Would you use multiphoton microscopy?

- You want to look at morphology of neurons in the brain *in vivo*
  - Good idea.
- You want to look at activity of neurons in the brain *in vivo*
  - Good idea, but there are alternatives
- You want to look at activity of neurons in a **very large area** of the brain *in vivo*
  - Check your scanning speed and **Nyquist!**
- More complicated cases…
  - Let’s discuss about it with OIC and us.

Some references:

- Ustione A, Piston DW (2011) **A simple introduction to multiphoton microscopy**. Journal of Microscopy 243:221–226. https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2818.2011.03532.x

- Kitamura K, Judkewitz B, Kano M, Denk W, Häusser M (2008) Targeted patch-clamp recordings and single-cell electroporation of unlabeled neurons in vivo. Nature methods 5:61–67. https://www.nature.com/articles/nmeth1150
- Svoboda K, Yasuda R (2006) Principles of Two-Photon Excitation Microscopy and Its Applications to Neuroscience. Neuron 50:823–839. http://www.sciencedirect.com/science/article/pii/S0896627306004119
- Wilt BA, Burns LD, Wei Ho ET, Ghosh KK, Mukamel EA, Schnitzer MJ (2009) Advances in light microscopy for neuroscience. Annu Rev Neurosci 32:435–506. https://www.ncbi.nlm.nih.gov/pubmed/19555292

- [*Acousto-Optical Deflection*] Grewe & Helmchen 2014 Cold Spring Harb Protoc https://www.ncbi.nlm.nih.gov/pubmed/24890212; https://doi.org/10.1101/pdb.prot081778
- [*Temporal focusing*] Hernandez O, Papagiakoumou E, Tanese D, Fidelin K, Wyart C and Emiliani V (2016). Three-dimensional spatiotemporal focusing of holographic patterns. *Nat Commun* ***7: 11928*** ***https://www.ncbi.nlm.nih.gov/pubmed/27306044***

Question?