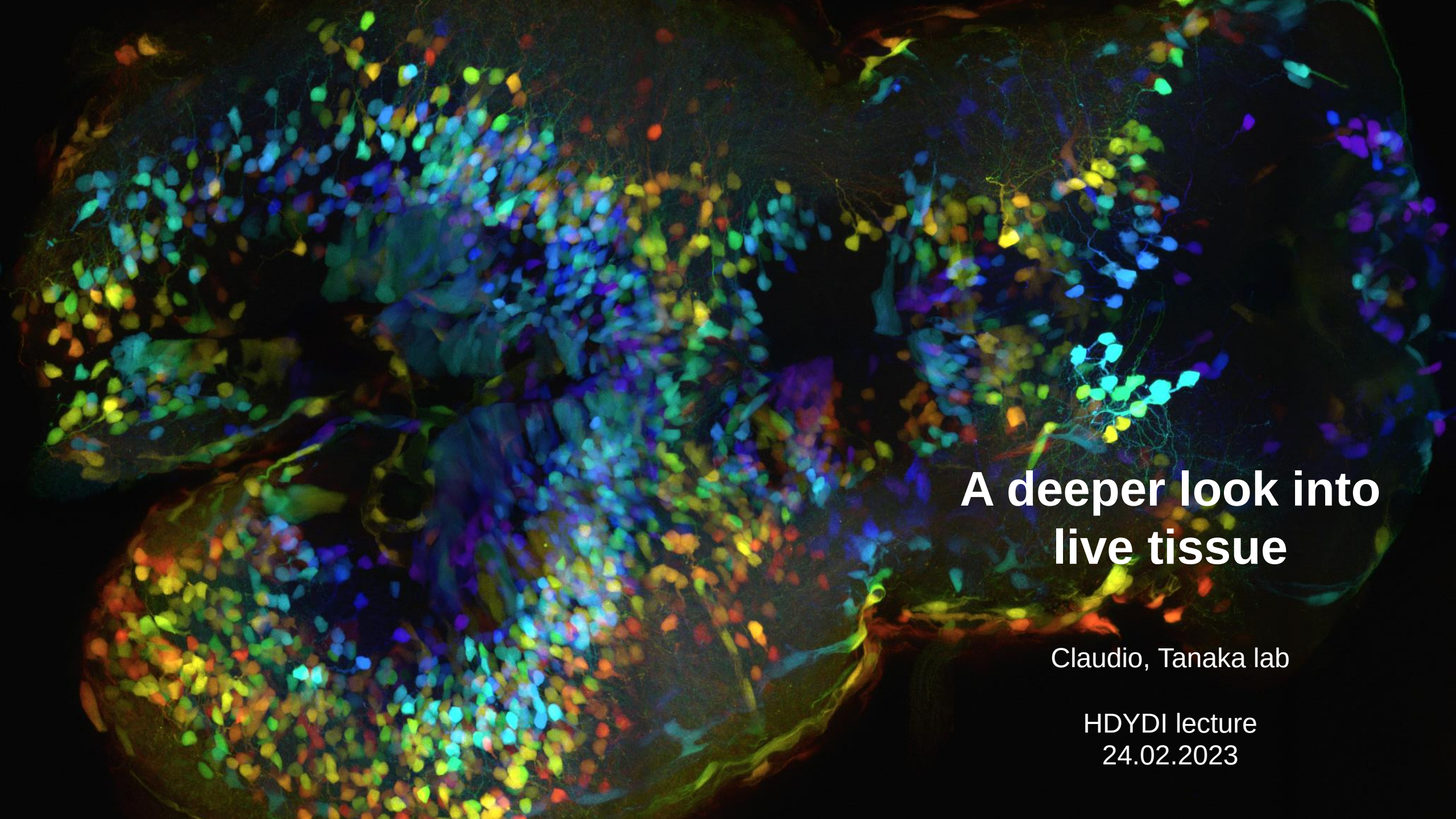
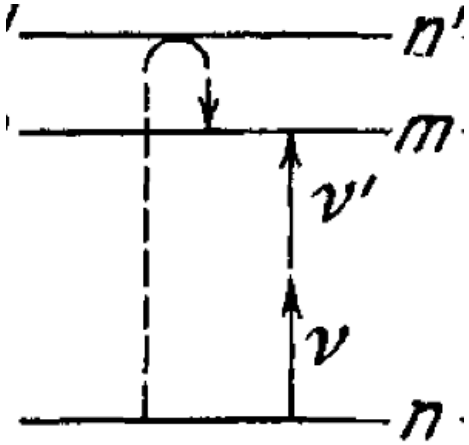


A deeper look into live tissue

Claudio, Tanaka lab

HDYDI lecture
24.02.2023

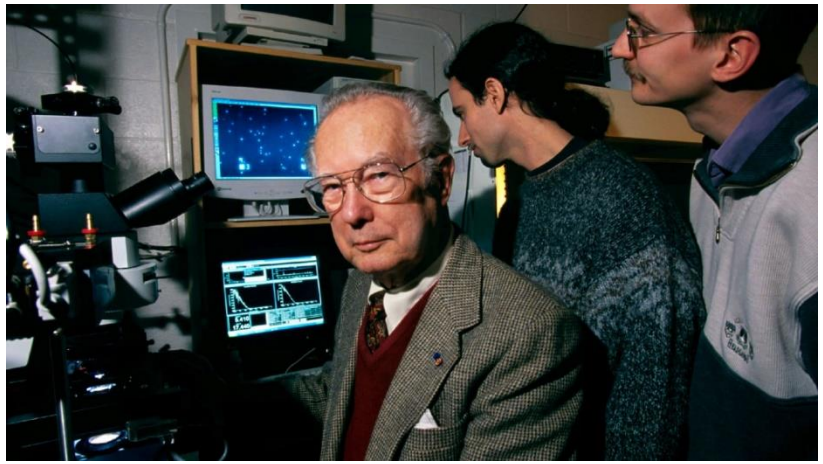
The origins of two-photon microscopy



Maria Goppert-Mayer, 1931.

Theoretical prediction of two-photon absorption by atoms in her doctoral thesis.

Göppert-Mayer, M., *Über Elementarakte mit zwei Quantensprüngen.* Ann. Phys., **401**: 273-294, (1931).

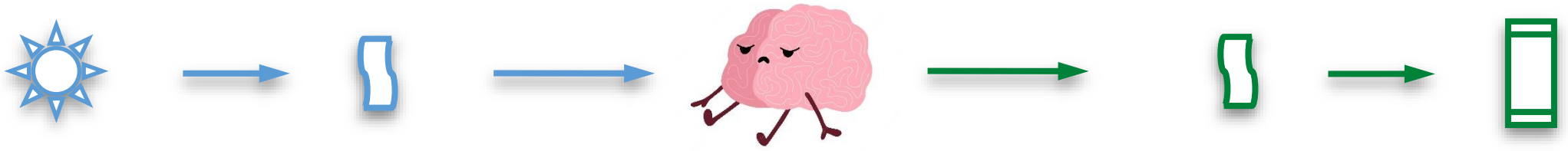


Watt Webb lab at Cornell, 1990.

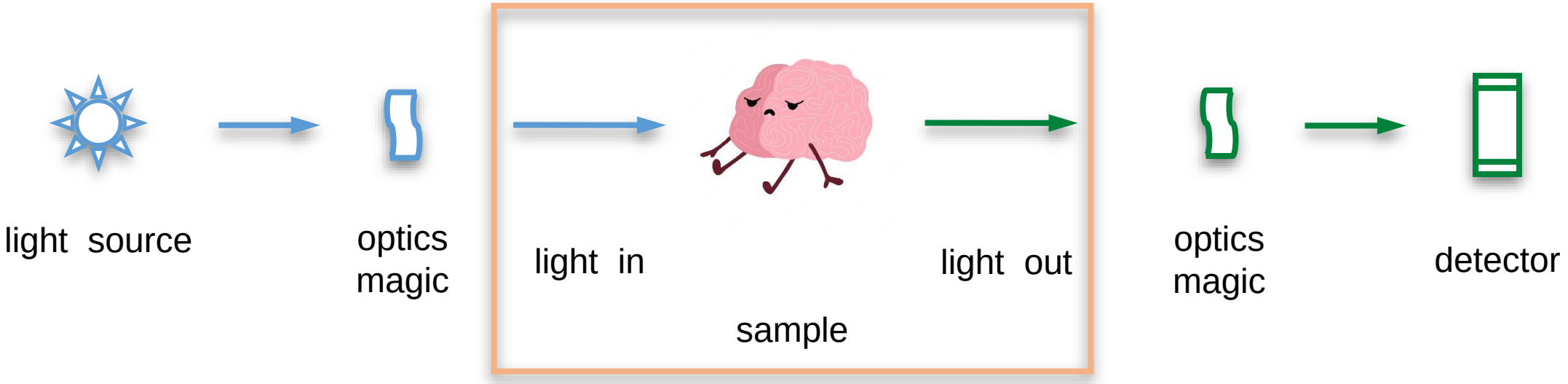
Development of two-photon absorprion microscopy in biological specimens (live cultured pig kidney cells).

Denk, W., J. H. Strickler, W. W. Webb., *Two-Photon Laser Scanning Fluorescence Microscopy*. Science **248**: 73-76 (1990).

Basic principle of microscopy

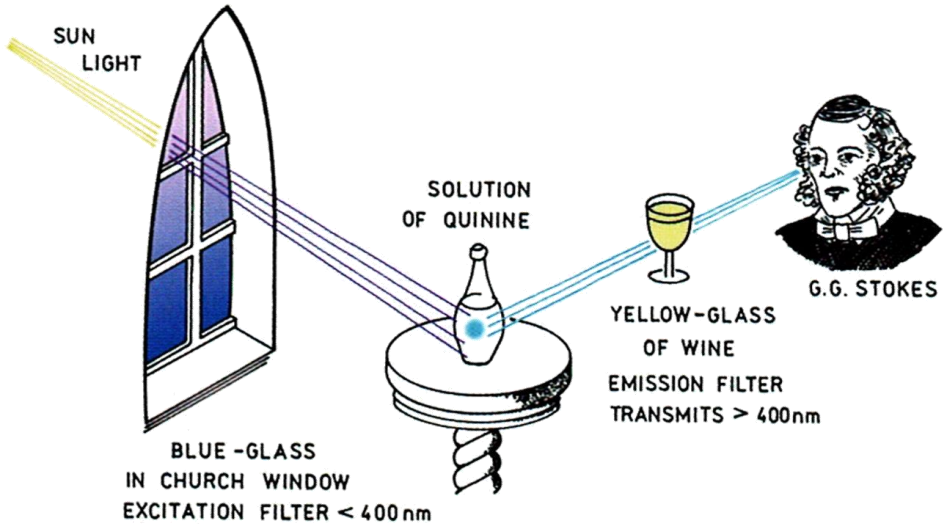


Basic principle of microscopy



Some properties of matter	… and their effects on light
Absorbance	Change in light intensity
Refractive index	Change in phase and direction of light
Optical rotation, birefringence	Change in propagation of polarized light
Fluorescence	Change in light colour
Fluorescence lifetime	Specific decay times of fluorescence signal
Interaction with other molecules	Energy transfer

History of fluorescence

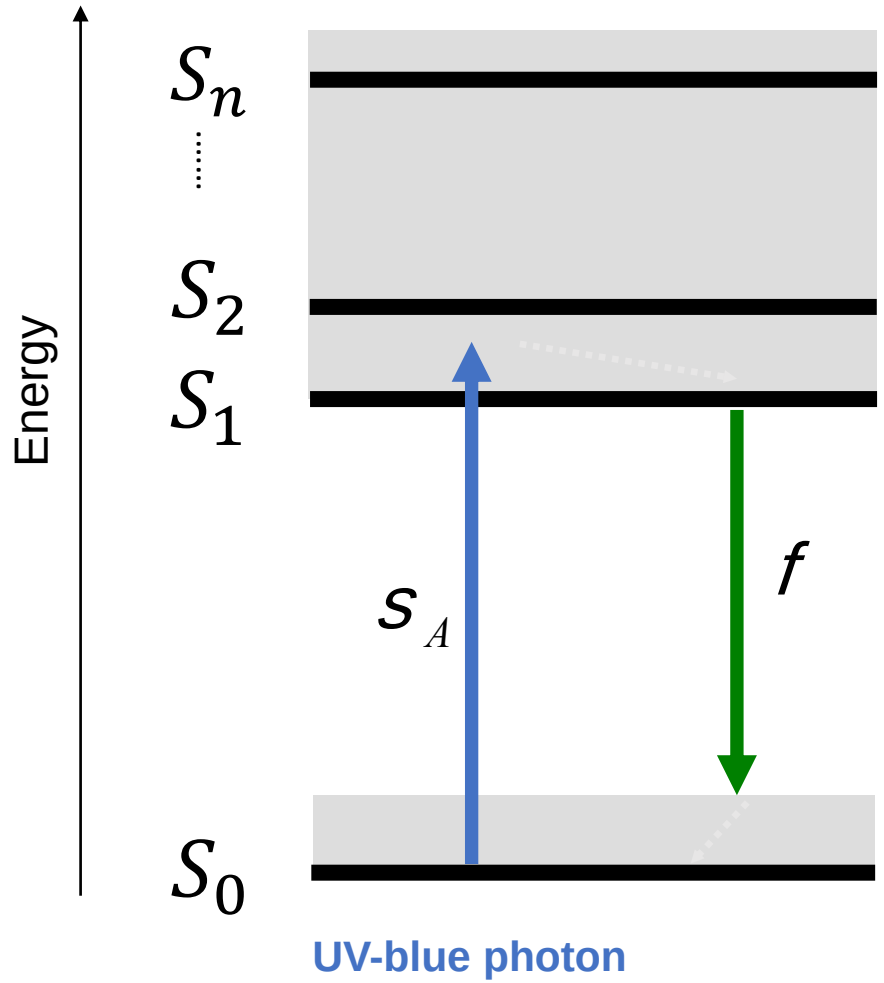


Extract from the original paper from Stokes (1852):

“The solution of quinine, though it appears to be perfectly transparent and colourless, like water, when viewed by transmitted light, exhibits nevertheless in certain aspects, and under certain incidences of the light, a beautiful celestial blue colour. It appears from the experiments of Sir John Herschel that the blue colour comes only from a stratum of fluid of small but finite thickness adjacent to the surface by which the light enters. After passing through this stratum, the incident light, though not sensibly enfeebled nor coloured, has lost the power of producing the same effect.”

Imaging deep into tissues of live animals

1-photon excited fluorescence



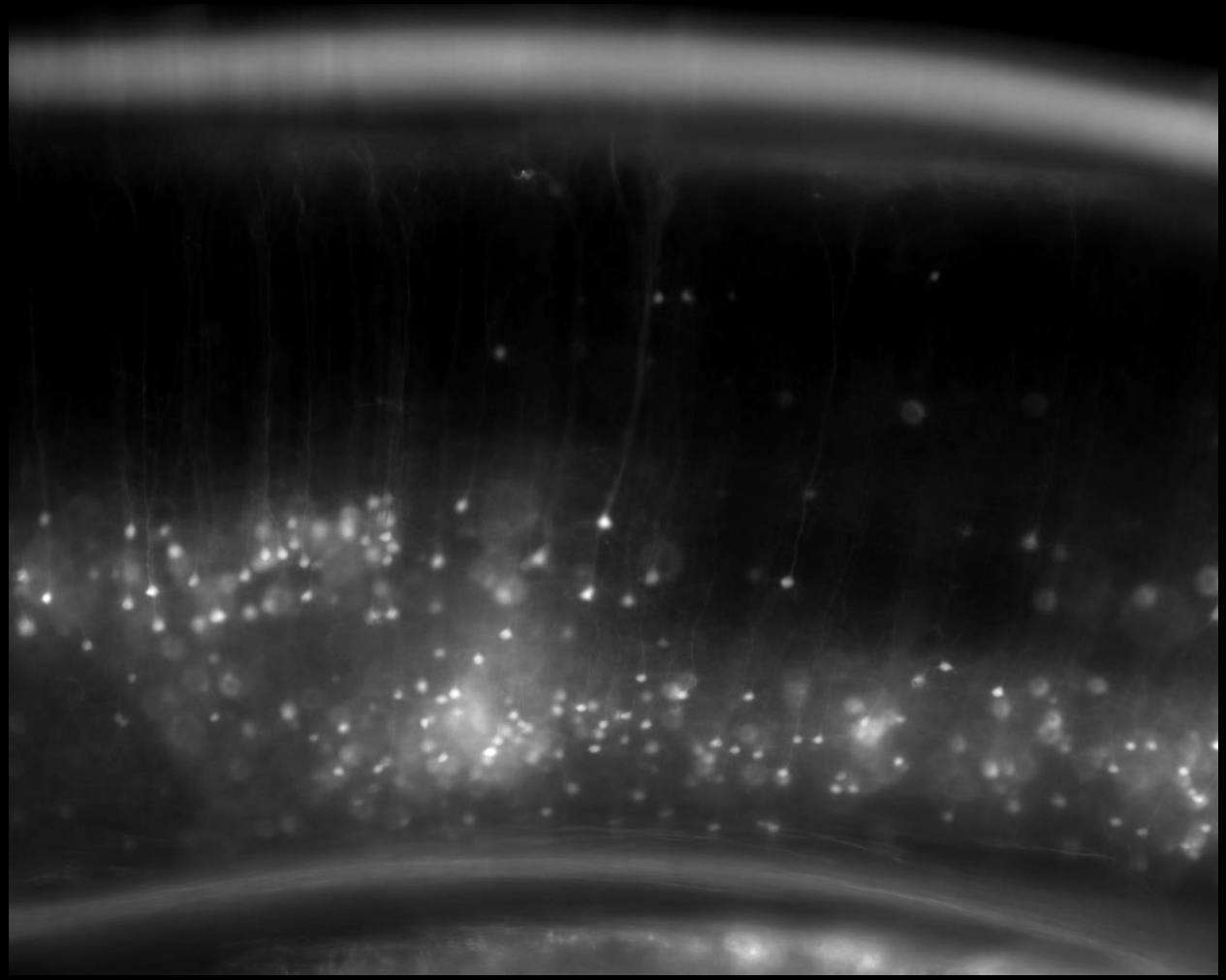
Fluorescence: $F \propto \phi\sigma_A I$

(linear)

ϕ: fluorescence quantum yield
σ_A: 1-photon absorption cross section

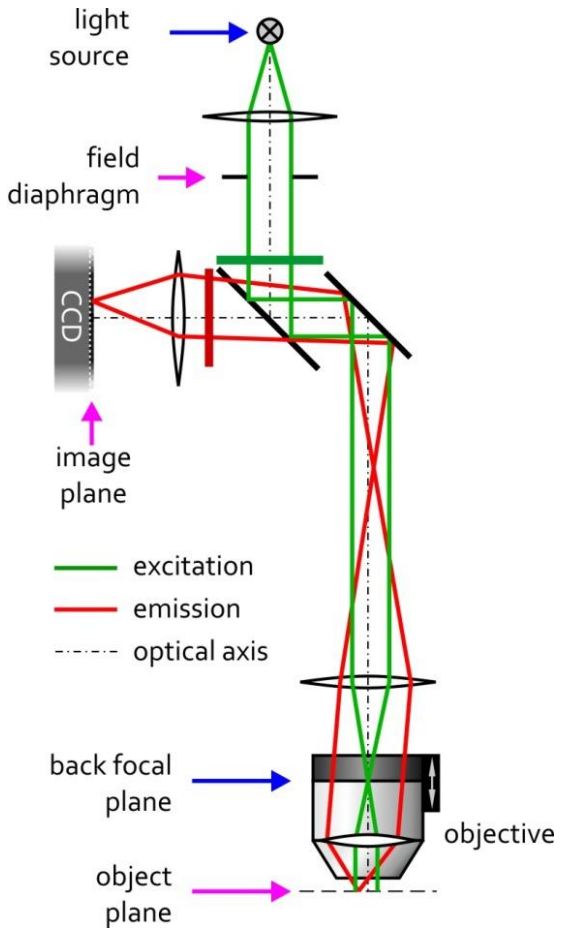
Epi-fluorescence, wide-field microscopy

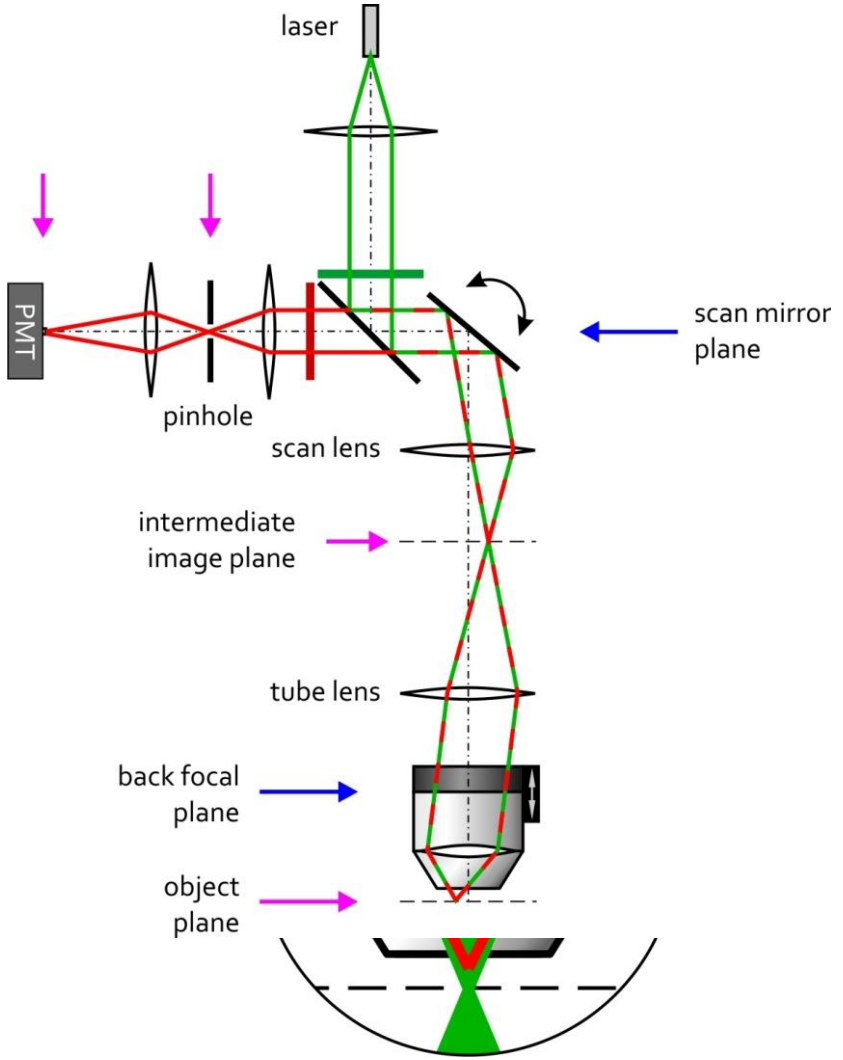
What bothers you in these images of images?



Removing out of focus light

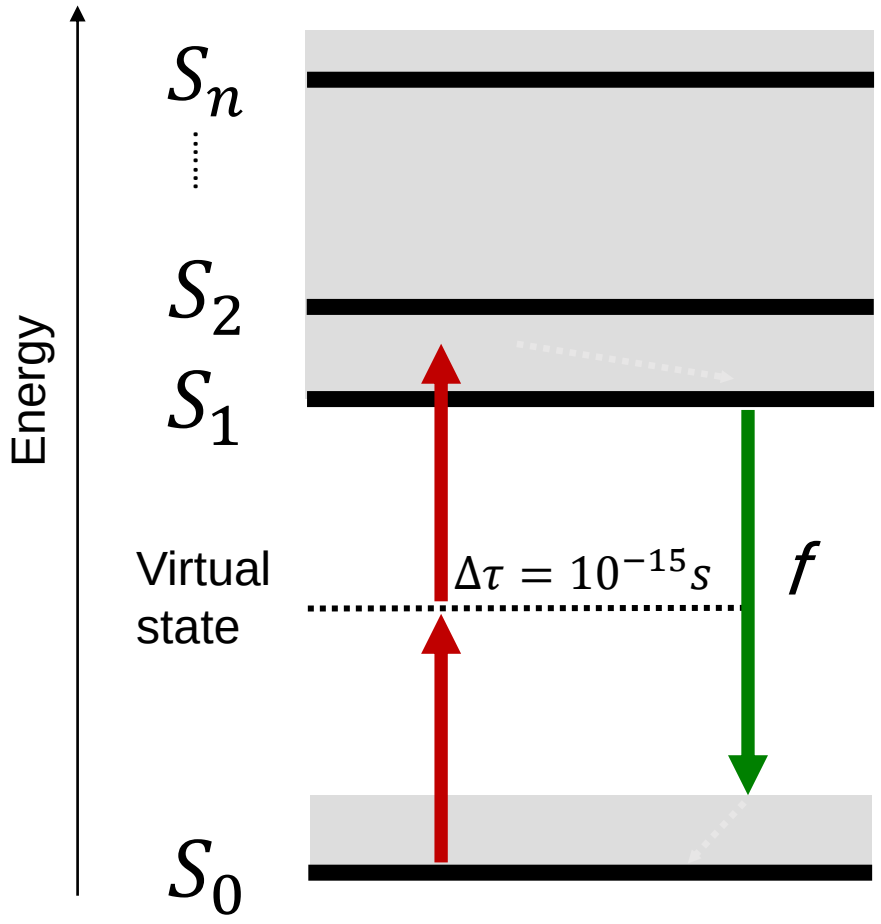
Solution? Confocal microscopy! Thanks to a pinhole we only detect light coming from the focal plane.





Imaging deep into tissues of live animals

2-photon excited fluorescence



Near Infra Red (700-1000nm) photons

Fluorescence: $F \propto \phi\sigma_{TPA}I^2$

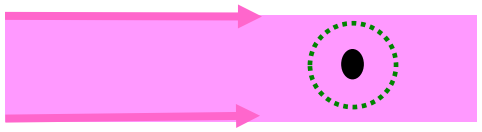
(non-linear)

ϕ: fluorescence quantum yield
σ_{TPA}: 2-photon absorption cross section

$$\sigma_{TPA} \propto \Delta\tau$$

Unit: Göppert-Mayer (GM) in honour of Maria

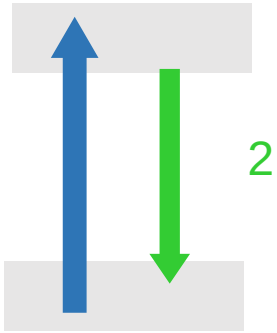
Exciting fluorophores with two photons



Single Rhodamine molecule

UV - visible

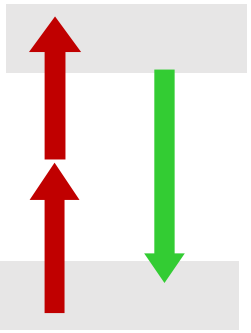
1mW @ 488nm
(2.5 10^{15} photon/s)



2 photon/s

IR

1mW @ 976nm
(5 10^{15} photon/s)

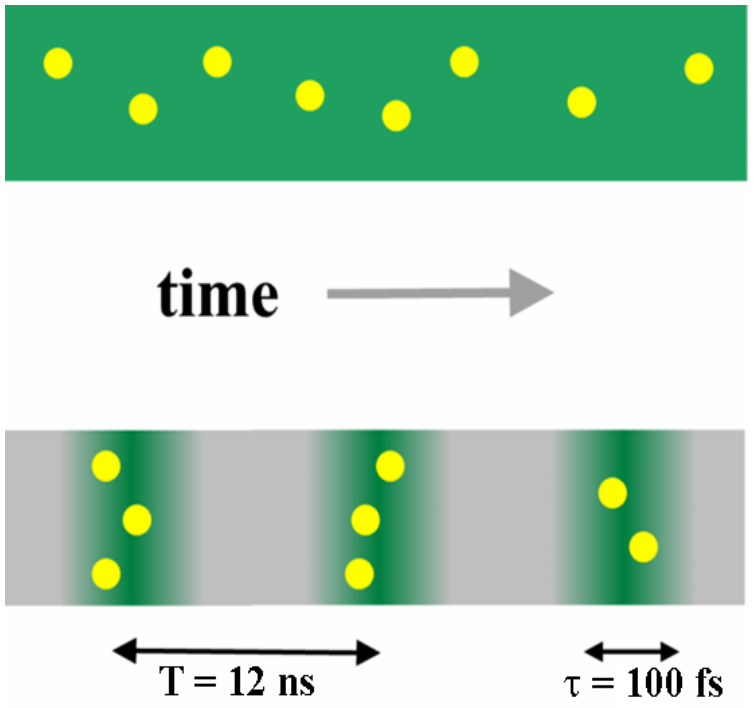


1.5 10^{-16} photon/s !!

Two-photon excitation is quite inefficient because of the very small cross-section.

The excitation power density need to be increased!

Solution, part 1: Temporal focusing

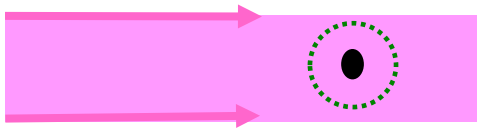


$$F \propto I^2$$

$$F \propto \frac{T}{\tau} I^2$$

two-photon enhancement: $\frac{T}{\tau} \sim 10^5$

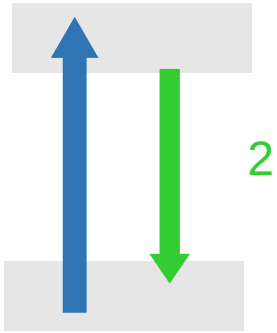
Exciting fluorophores with two photons



Single Rhodamine molecule

UV - visible

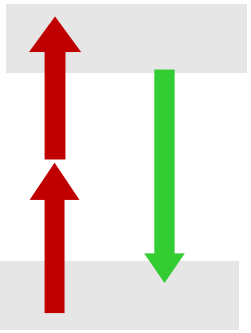
1mW @ 488nm
(2.5 10^{15} photon/s)



2 photon/s

IR

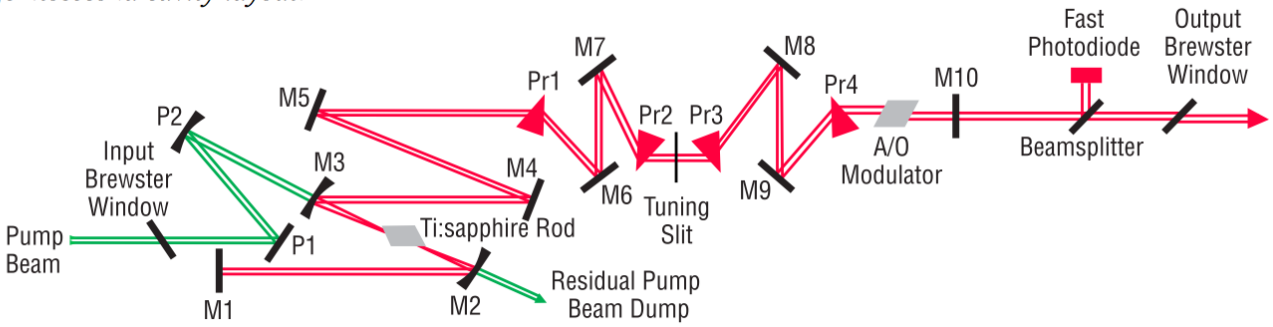
1mW @ 976nm
(5 10^{15} photon/s)

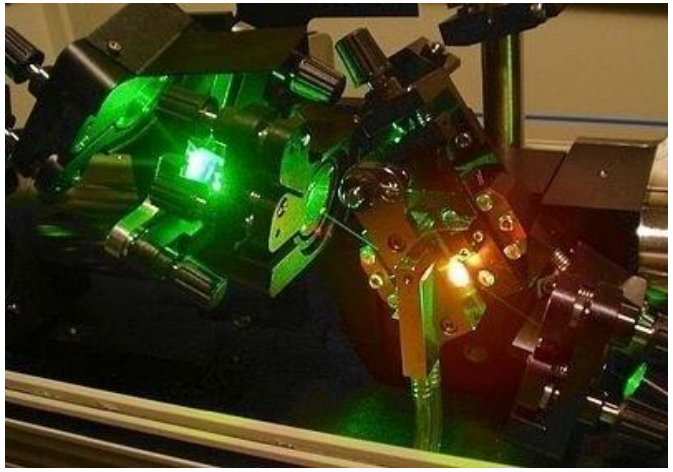


1.5 10^{-11} photon/s !!

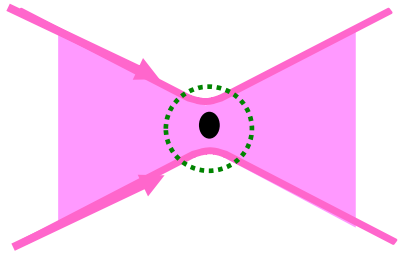
Typically used: Mode locked Ti:Sapphire Laser

Tsunami femtosecond cavity layout.





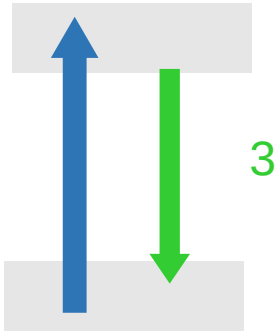
Exciting fluorophores with two photons



Single Rhodamine molecule

UV - visible

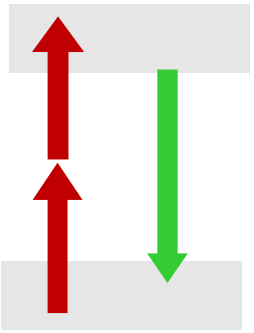
1mW @ 488nm
(2.5 10^{15} photon/s)



3 x 10^{7} photon/s

IR

1mW @ 976nm
(5 10^{15} photon/s)

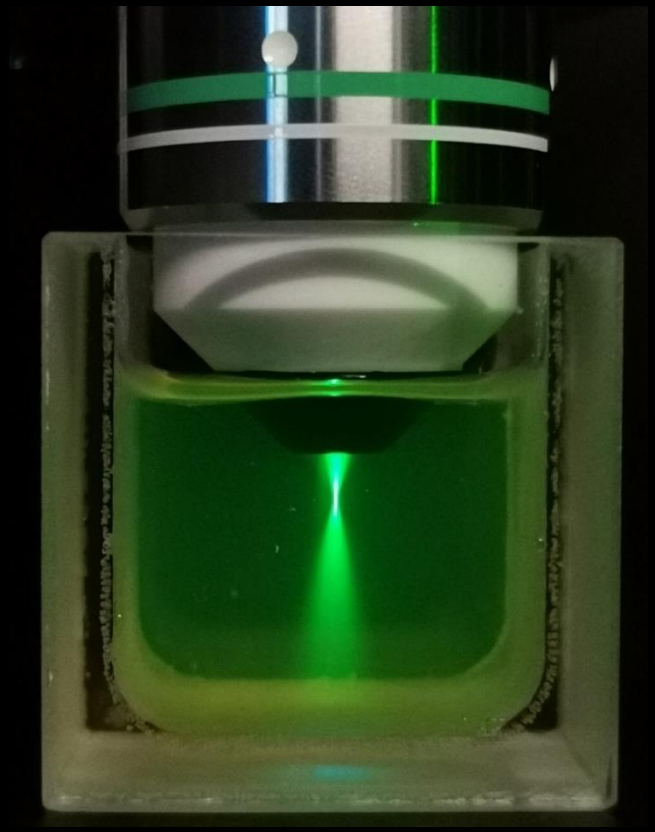


4 10^{3} photon/s

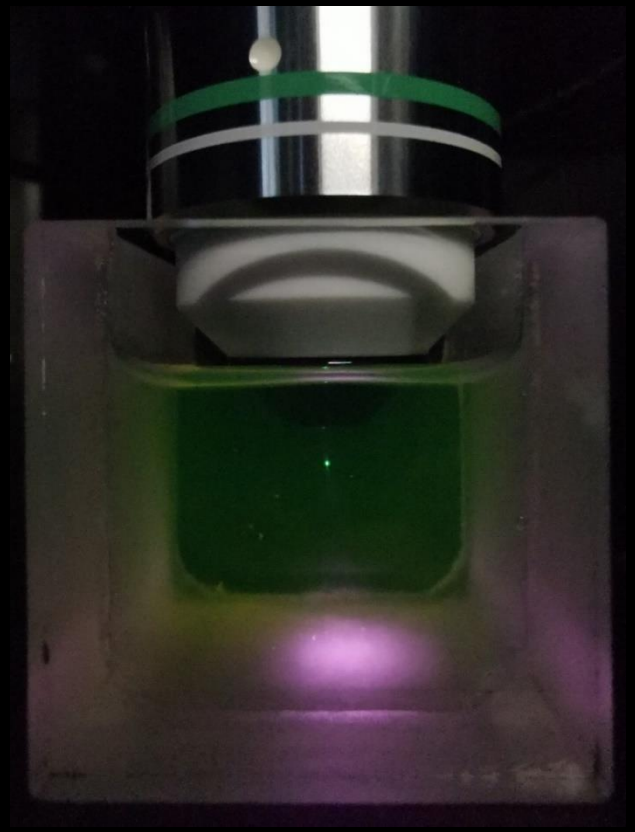
We have signal we can detect (signal-to-noise ratio).

Now we are in business!

Imaging into scattering media



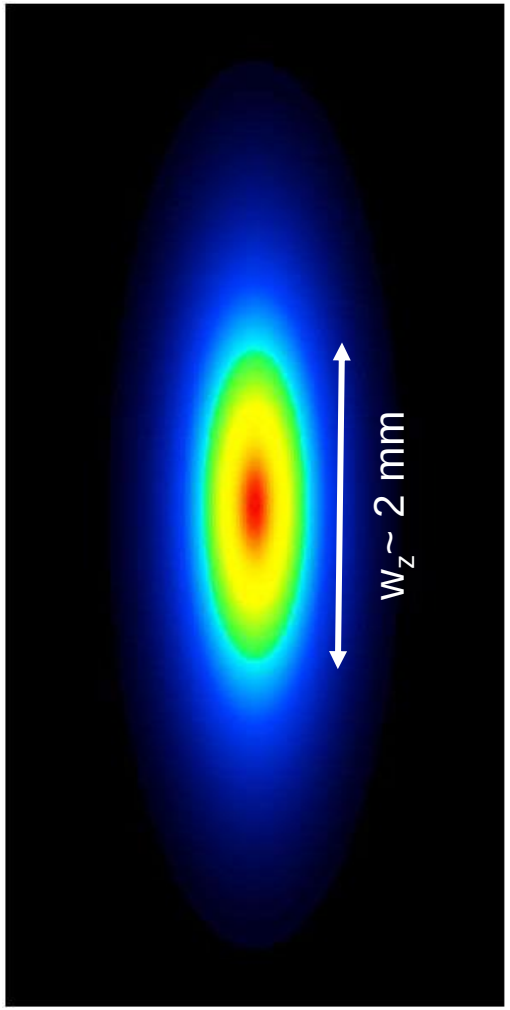
1-photon



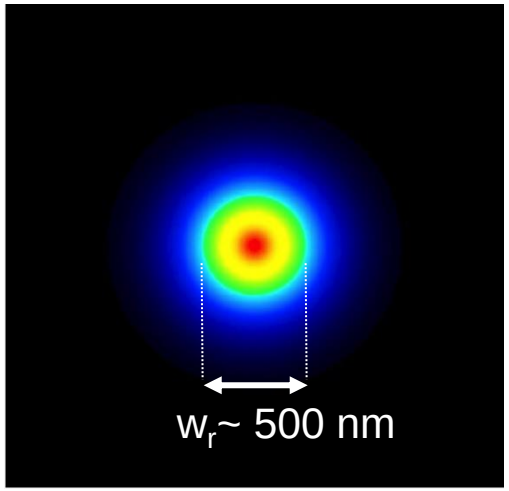
2-photon

Optical resolution of two-photon microscopy

Z (axial)



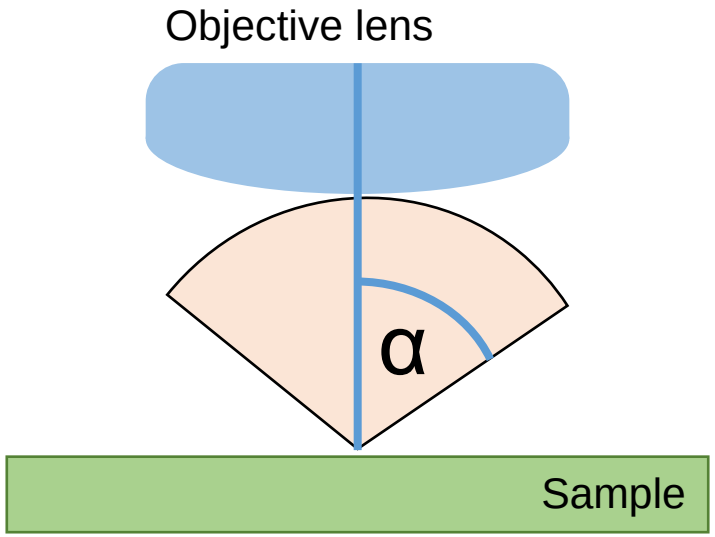
XY (radial)



Similar to standard microscopy:

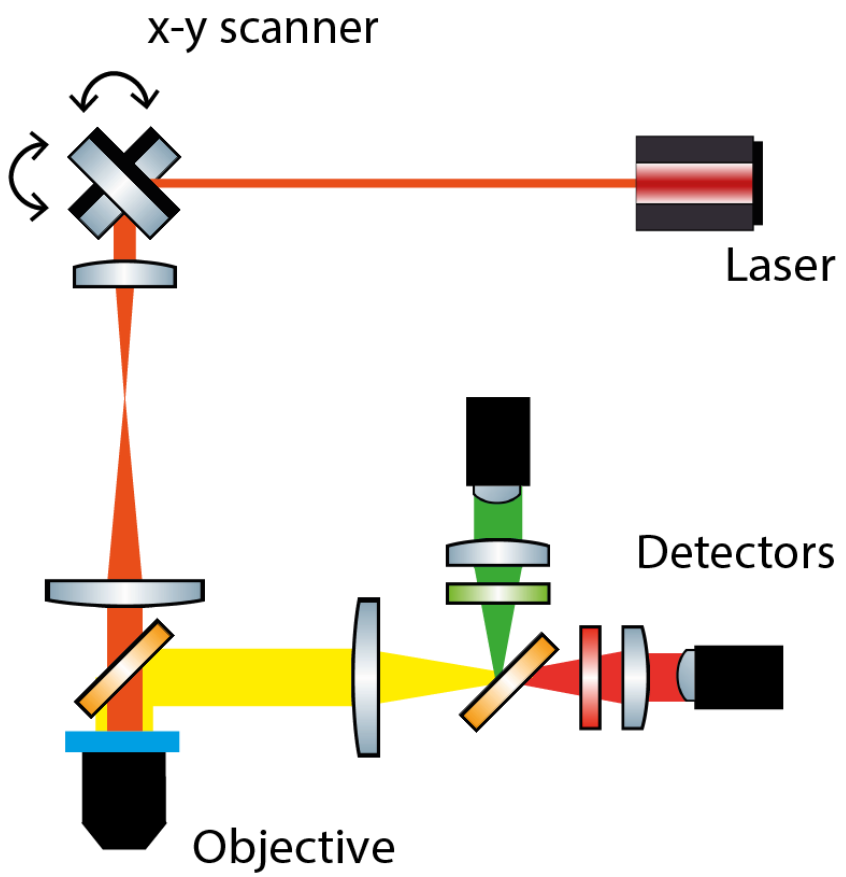
$$d_{LAT} = 0.61 \times \frac{\lambda}{NA}$$

$$NA = n \times \sin\alpha$$



Building a two-photon microscope

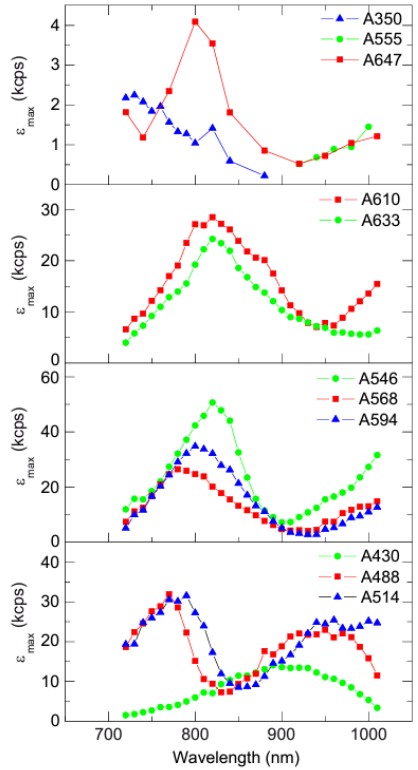
1. A high-power, pulsed (typically 100 fs, 80 MHz), infrared laser
2. A scanner to move the focused laser beam across the sample
3. An objective that focuses the beam onto the sample
4. Filters that separate emitted light from excitation
5. Wide-field detection proximal to the objective lens

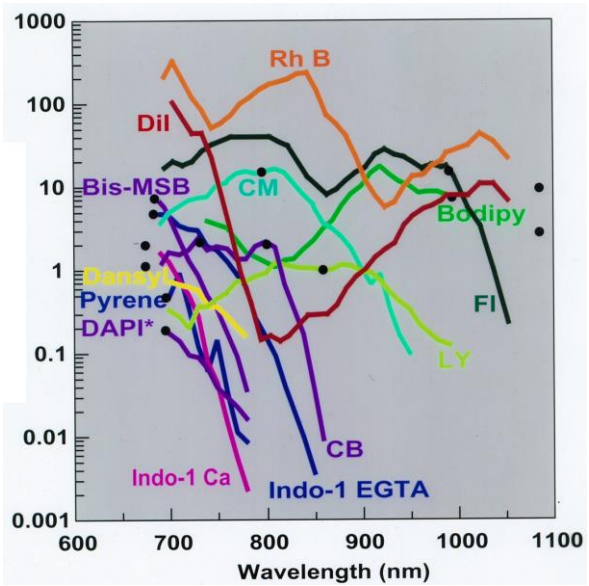


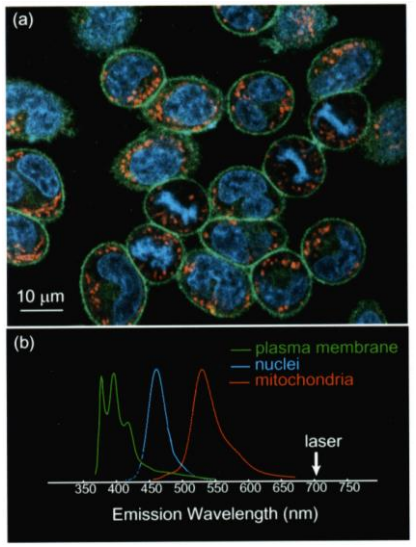
Fluorophores for 2-photon microscopy

Common fluorophores can be used for 2-photon microscopy, e.g.: AlexaFluor, Rhodamine, ATTO, Oregon, and the biologists favourite: DAPI.

Calculating their cross section is not trivial!







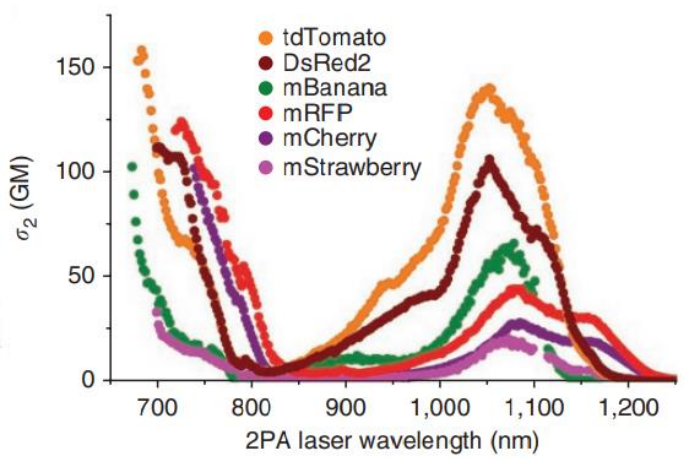
Overlap = 1 wavelength!

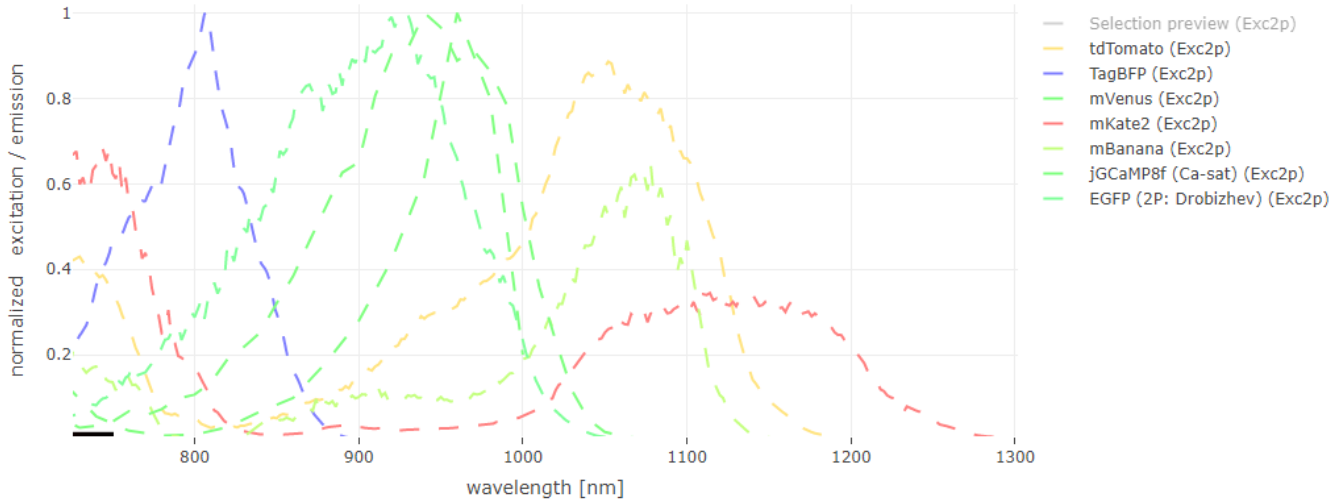
Xu, C., Williams, R.M., Zipfel, W. and Webb, W.W., *Multiphoton excitation cross-sections of molecular fluorophores*. Bioimaging, **4**: 198-207, (1996).

Mütze J, Iyer V, Macklin JJ, Colonell J, Karsh B, Petrášek Z, Schwille P, Looger LL, Lavis LD, Harris TD. *Excitation spectra and brightness optimization of two-photon excited probes*. Biophys J. 2012 Feb 22;**102**(4):934-44.

Fluorescent proteins for 2-photon microscopy

2-photon microscopy primarily targets *in-vivo* imaging. Significant effort has been made to use fluorescent proteins.





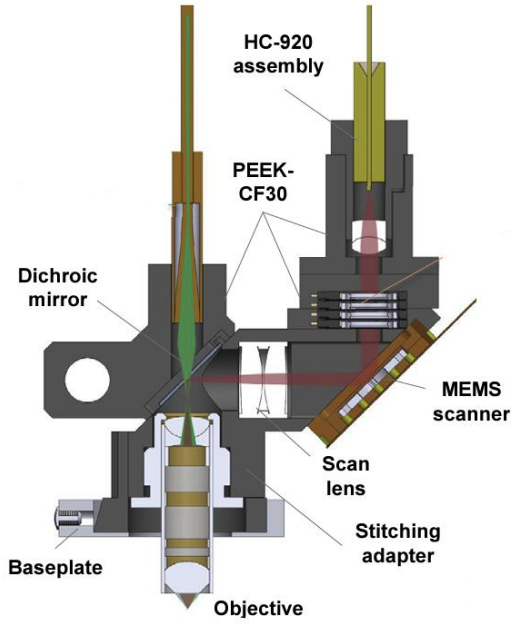
Drobizhev, M., Makarov, N., Tillo, S. et al. *Two-photon absorption properties of fluorescent proteins*. Nat Methods **8**, 393–399 (2011).

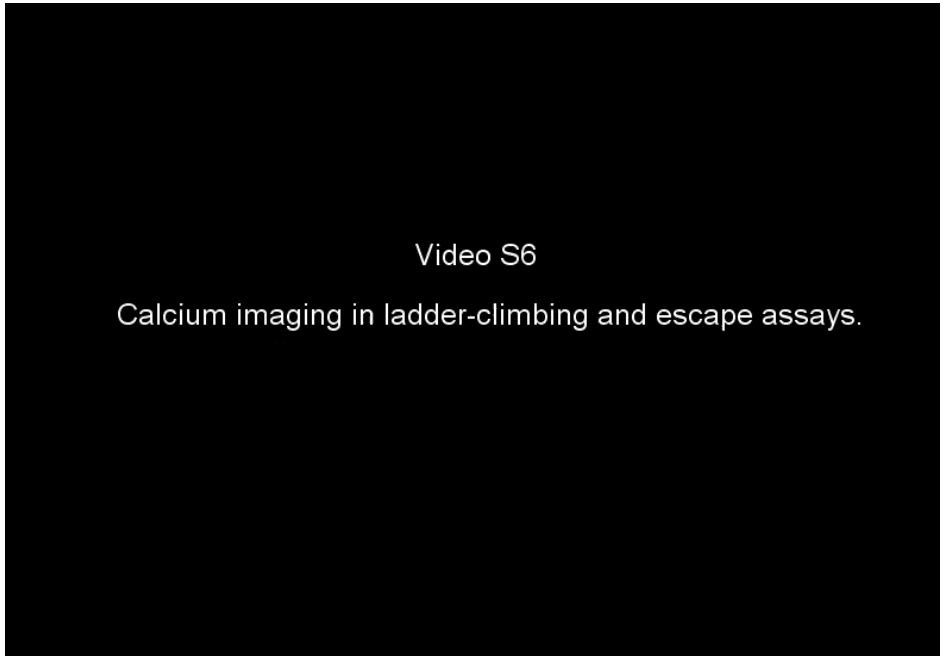
SpectraViewer of the Imaging Facility of the MPI for Brain Research, Frankfurt.

Further development: miniature 2-photon microscope

Development of a light-weight, headmounted, 2-photon miniscope for calcium imaging in freely moving mice.

MINI2P



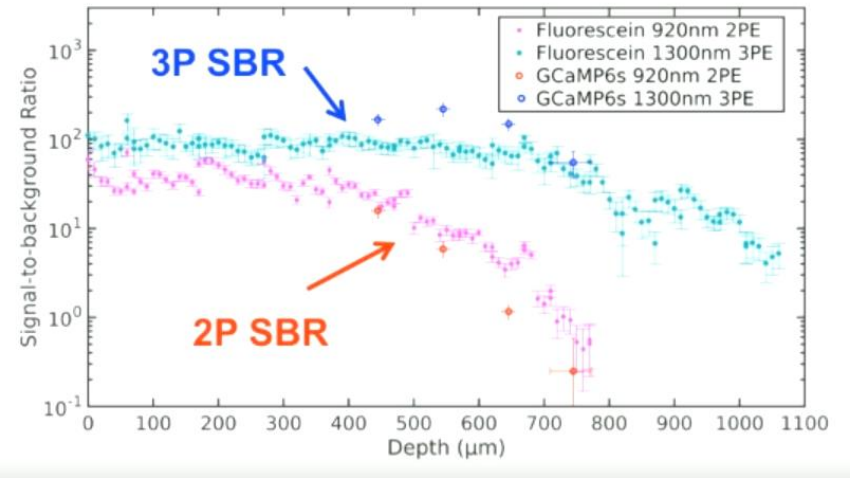


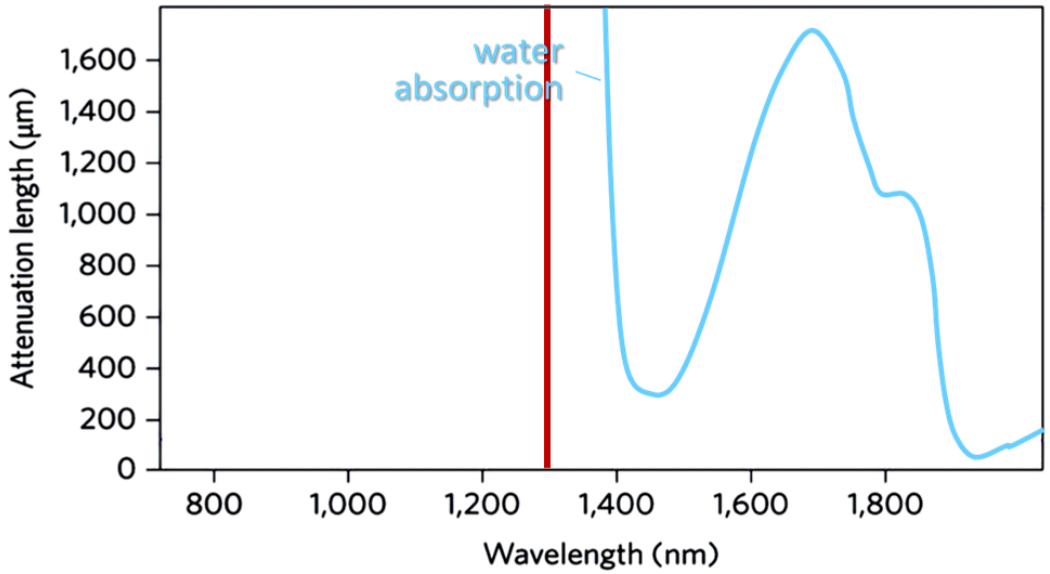
Wong W. et al. *Large-scale two-photon calcium imaging in freely moving mice*. Cell, **185**, 7, 1240-1256.e30, (2022).
https://github.com/kavli-ntnu/MINI2P_toolbox.

Further development: 1, 2,…3 photons

What if instead of 2 photons we employ a 3-photon excitation?

Larger wavelength leads to even less scattering and an even higher axial confinment. It leads to a dramatic increase in the signal to background at larger depths.

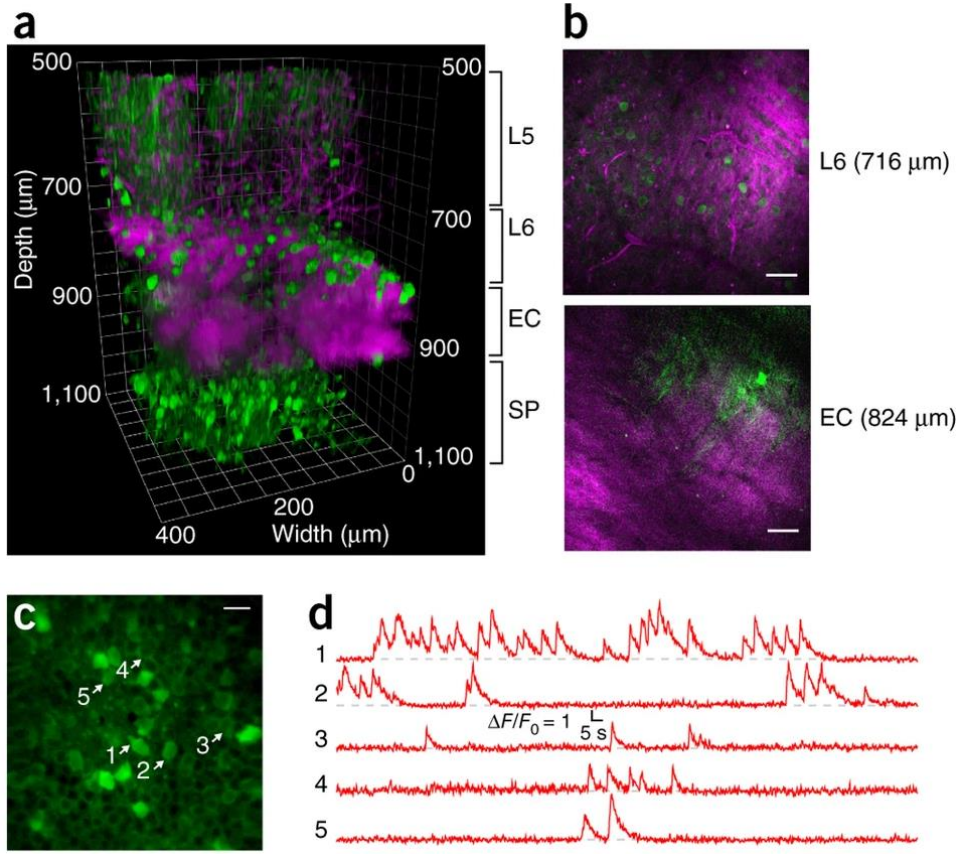




Horton, N., Wang, K., Kobat, D. et al. *In vivo three-photon microscopy of subcortical structures within an intact mouse brain*. Nature Photon **7**, 205–209 (2013).

Further development: 1, 2,…3 photons

Able to image up to more than 1 mm deep into live tissue. In the mouse cortex all the way to below the white matter!

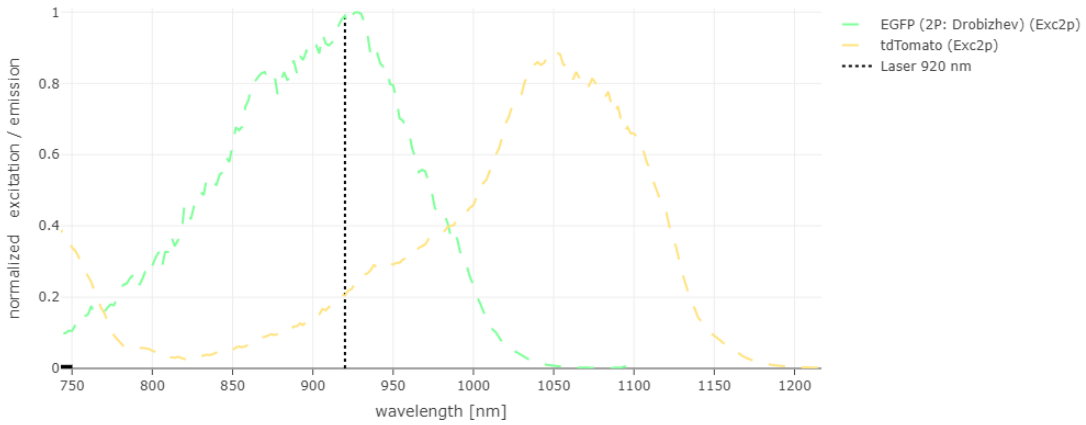


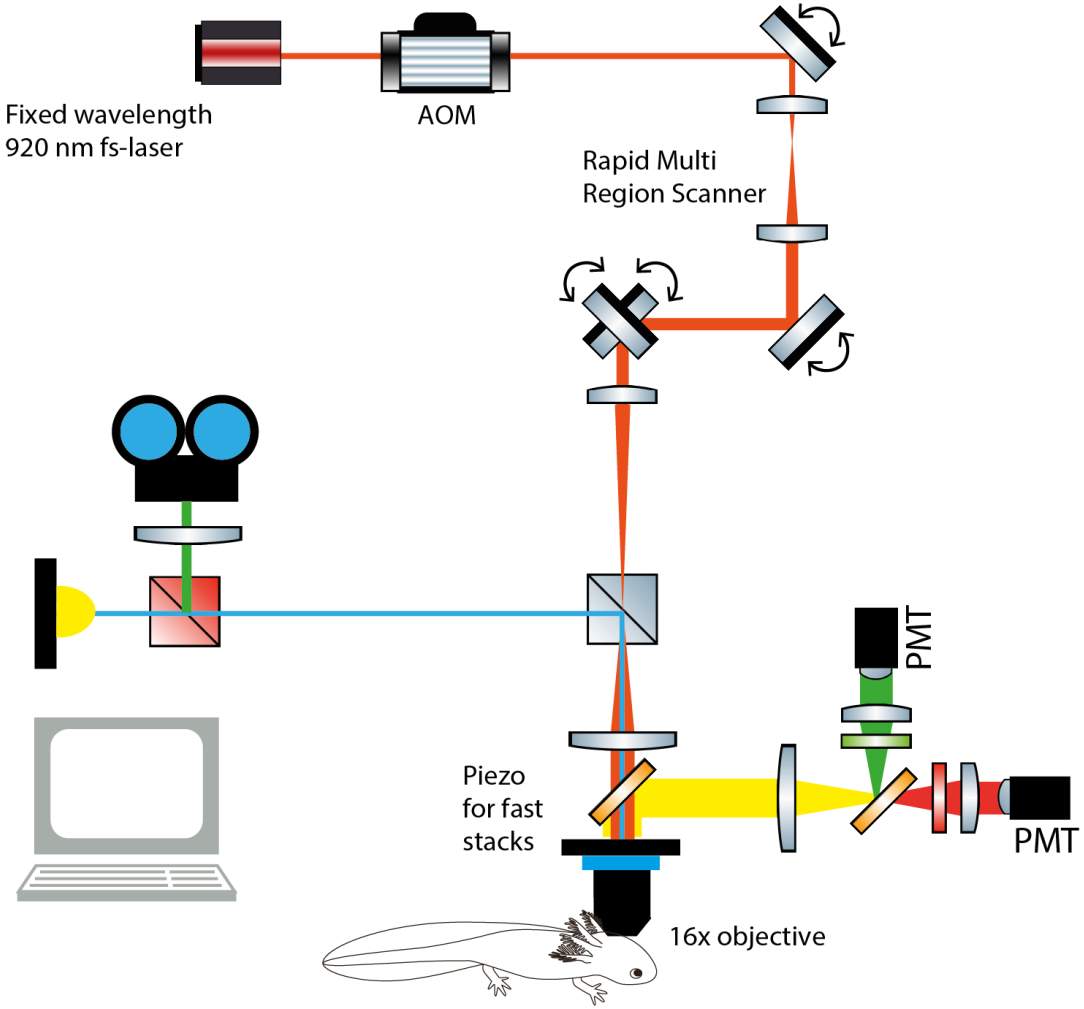
Ouzounov, D., Wang, T., Wang, M. et al. *In vivo three-photon imaging of activity of GCaMP6-labeled neurons deep in intact mouse brain.* Nat Methods **14**, 388–390 (2017).

A two-photon microscope for imaging deep into axolotl tissues

High-power laser:

920 nm and 2W of peak power for efficient excitation of GFP-like and Td-Tomato proteins





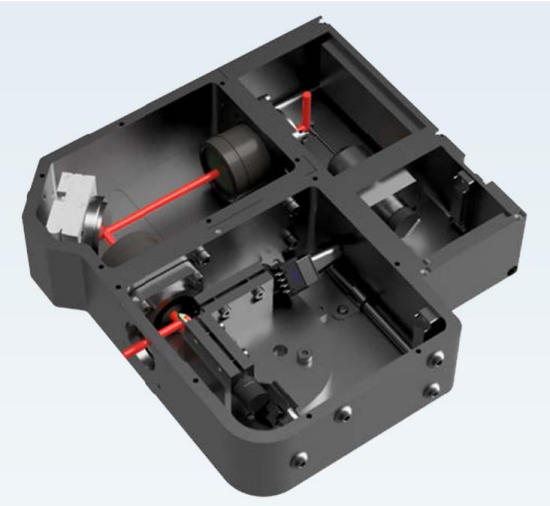
A two-photon microscope for imaging deep into axolotl tissues

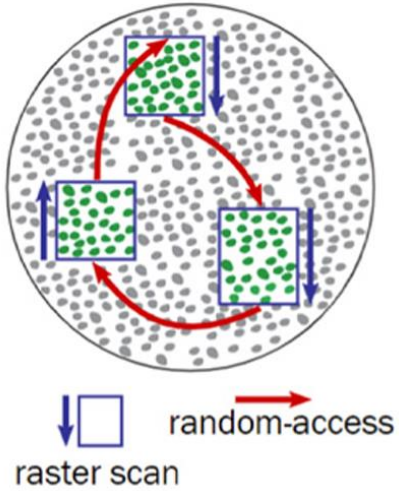
High-power laser:

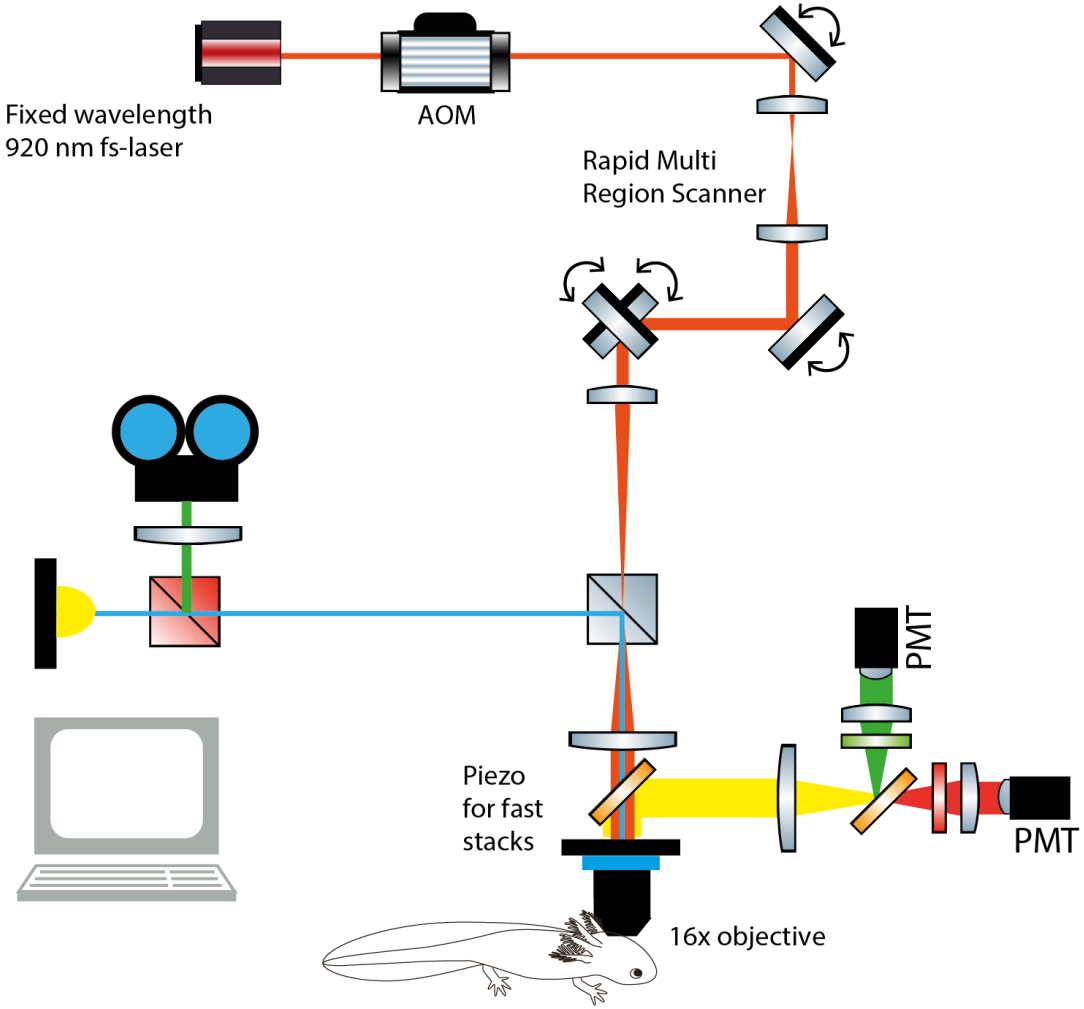
920 nm and 2W of peak power for efficient excitation of GFP-like and Td-Tomato proteins

Rapid multi region scanning:

Scanning system comprising of a resonant scanner and two galvo mirrors for arbitrary scanning within the field-of-view







A two-photon microscope for imaging deep into axolotl tissues

High-power laser:

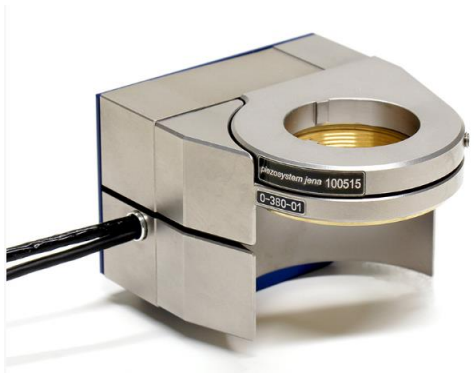
920 nm and 2W of peak power for efficient excitation of GFP-like and Td-Tomato proteins

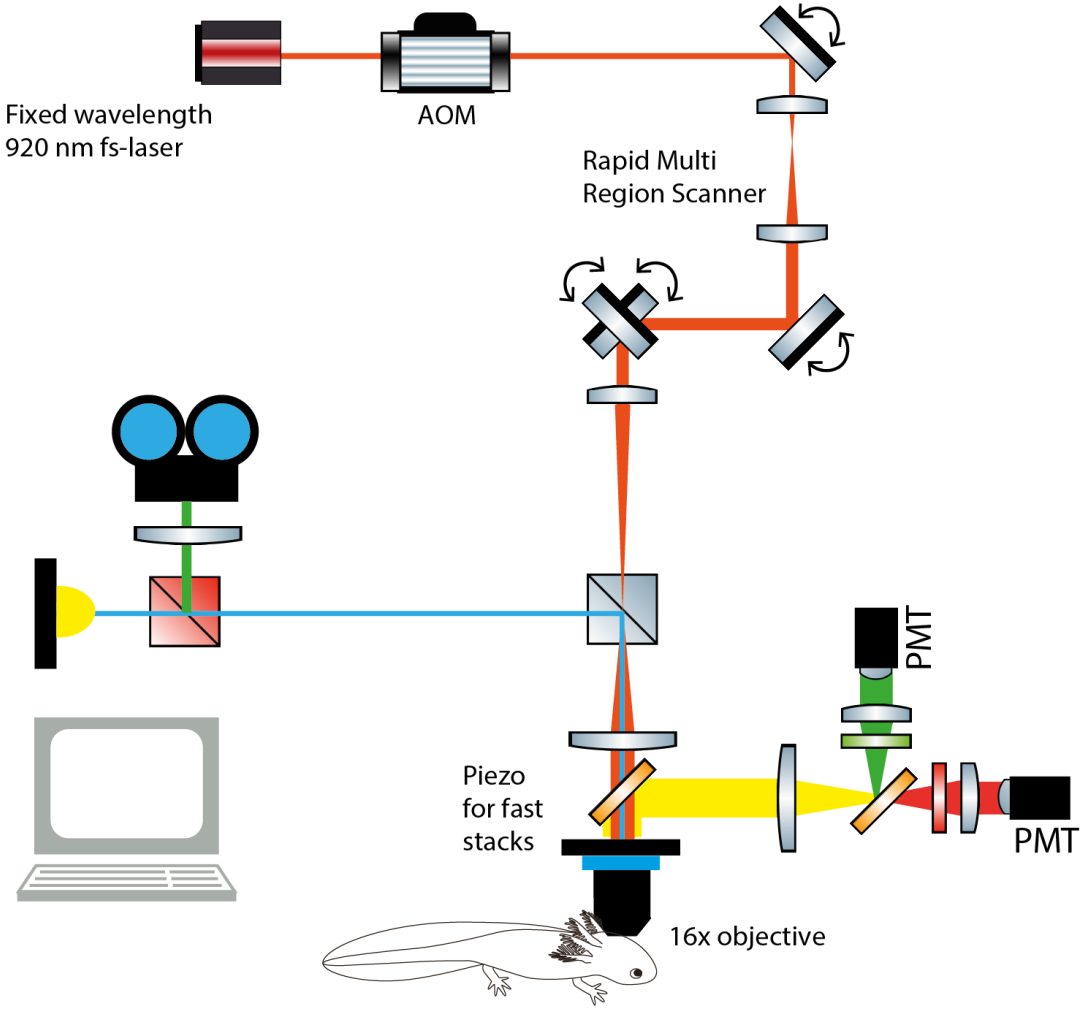
Rapid multi region scanning:

Scanning system comprising of a resonant scanner and two galvo mirrors for arbitrary scanning within the field-of-view

Fast volumetric imaging:

Objective piezo for fast axial scanning over 600 µm

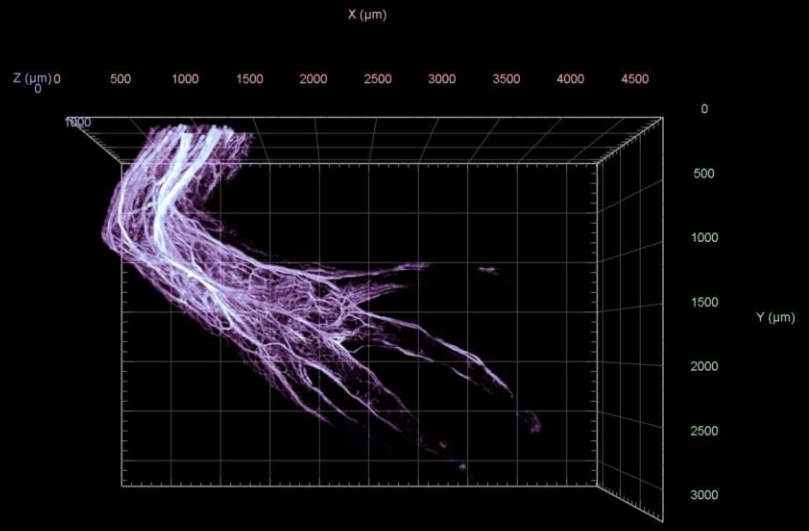




Overcoming tissue clearing

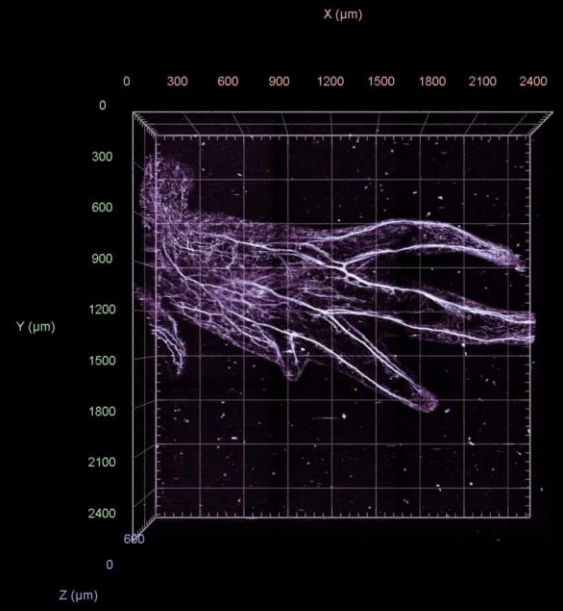
Fixed, CUBIC-cleared, stained

Lightsheet imaging



Fixed

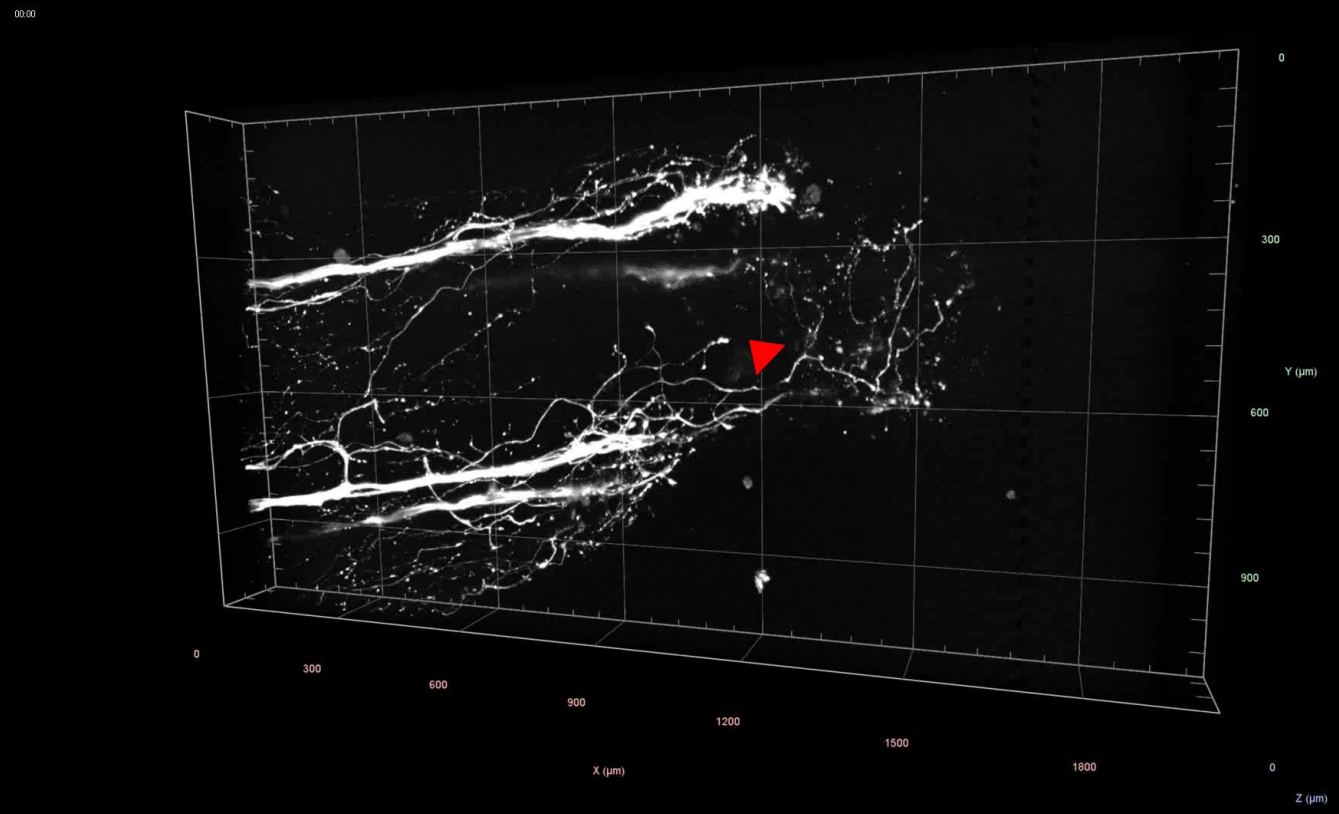
2-photon imaging



ß3-Tubulin endogenous fluorescence

Structural *in-vivo* imaging

Long-term imaging of neuronal projections in a live axolotl blastema (finger) for over 8 hours, 7 days post amputation.

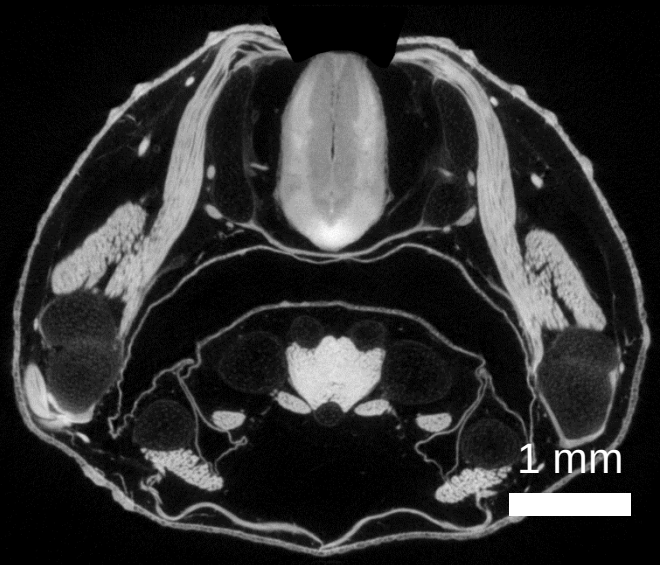


ß3-Tubulin endogenous fluorescence

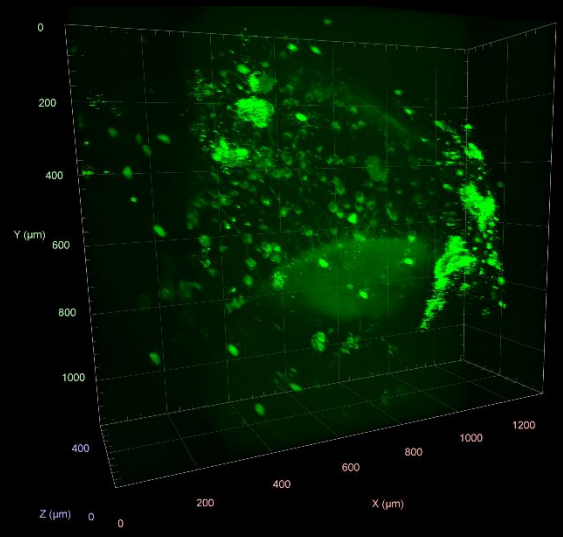
Functional imaging in the optic tectum of axolotls

Functional imaging of the optic tectum in live axolotls

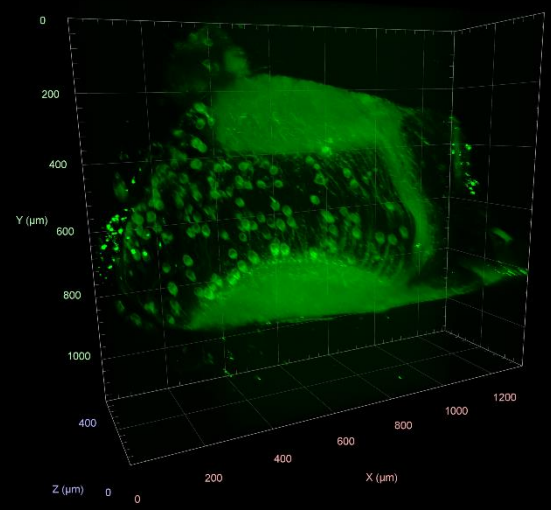
Opening of cranial window.



Through skin and skull



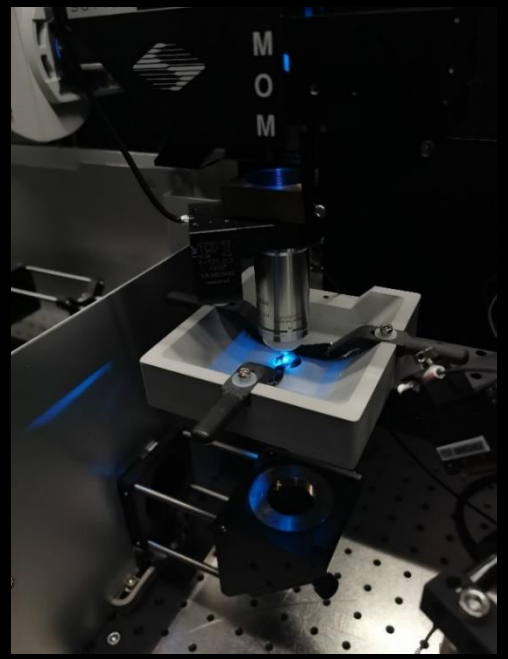
Cranial window

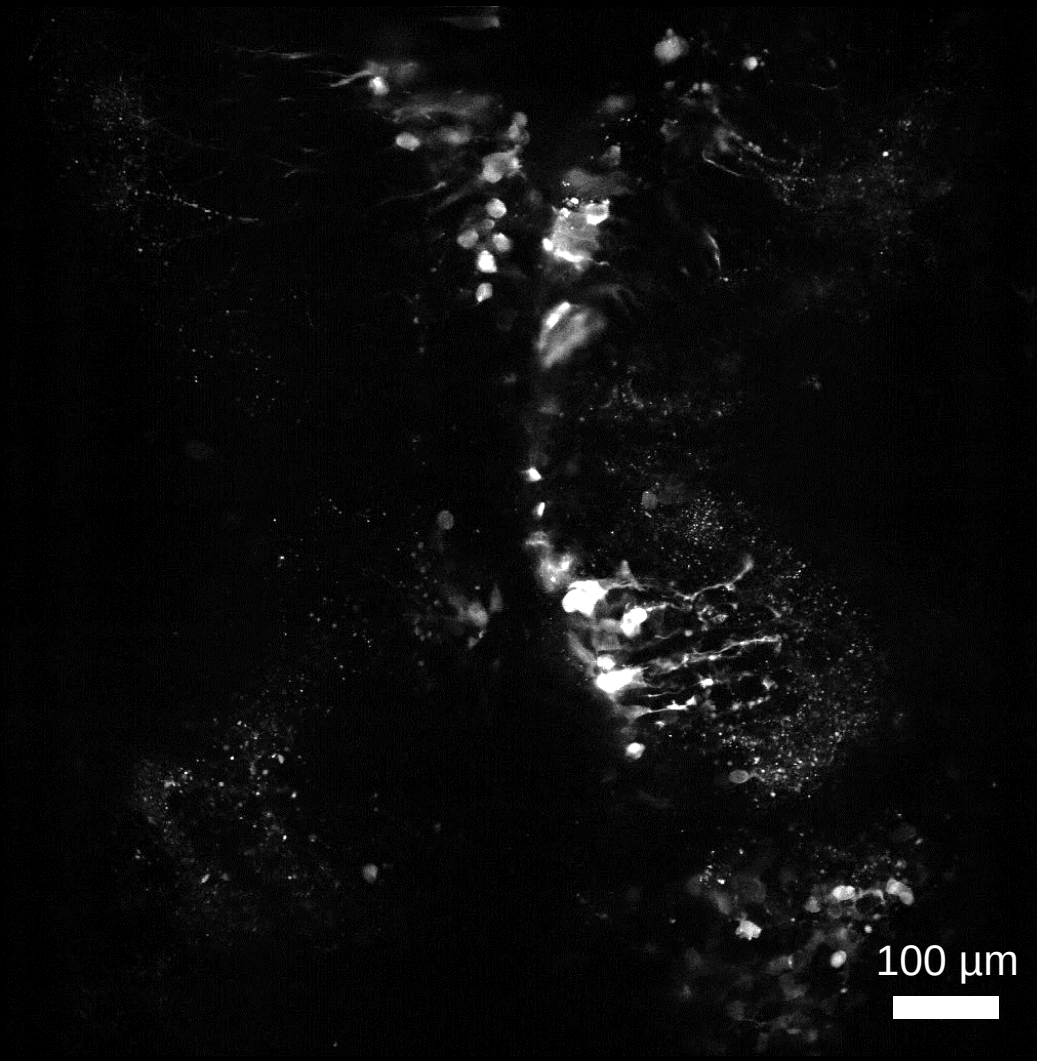


Functional imaging of the optic tectum in live axolotls

Imaging of neuronal activity in the optic tectum. Imaged at 5 Hz.

Custom 3D
printed mount

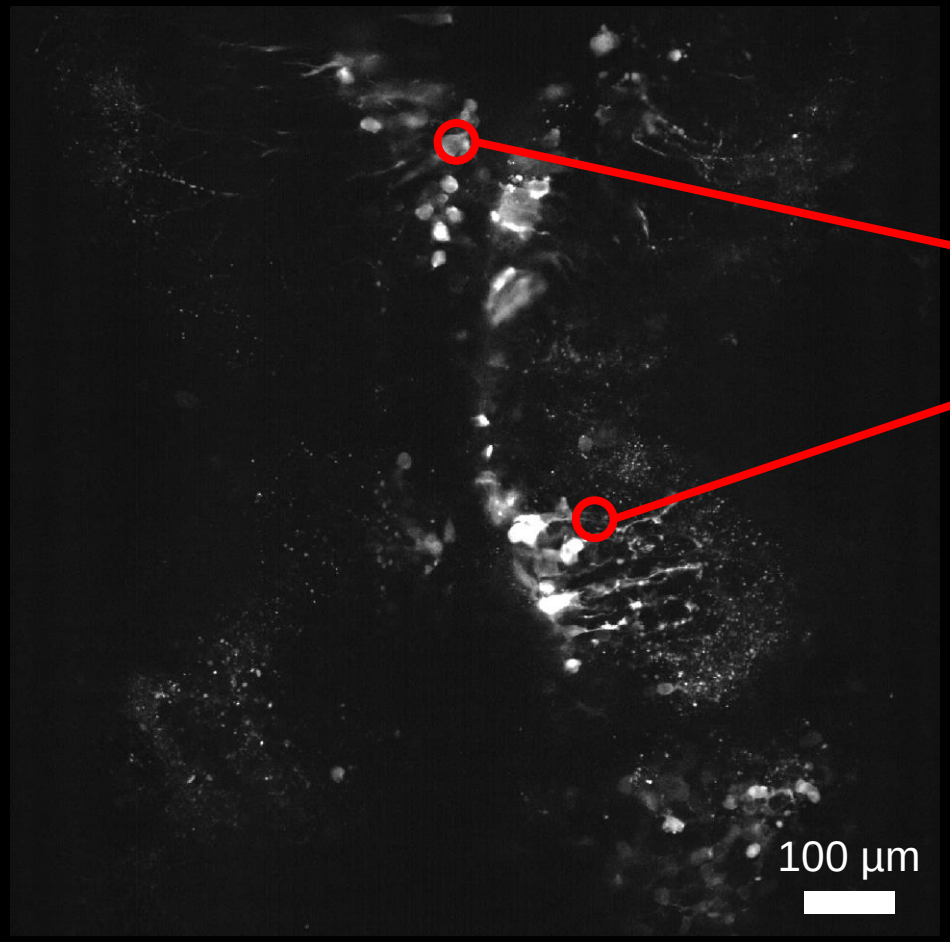




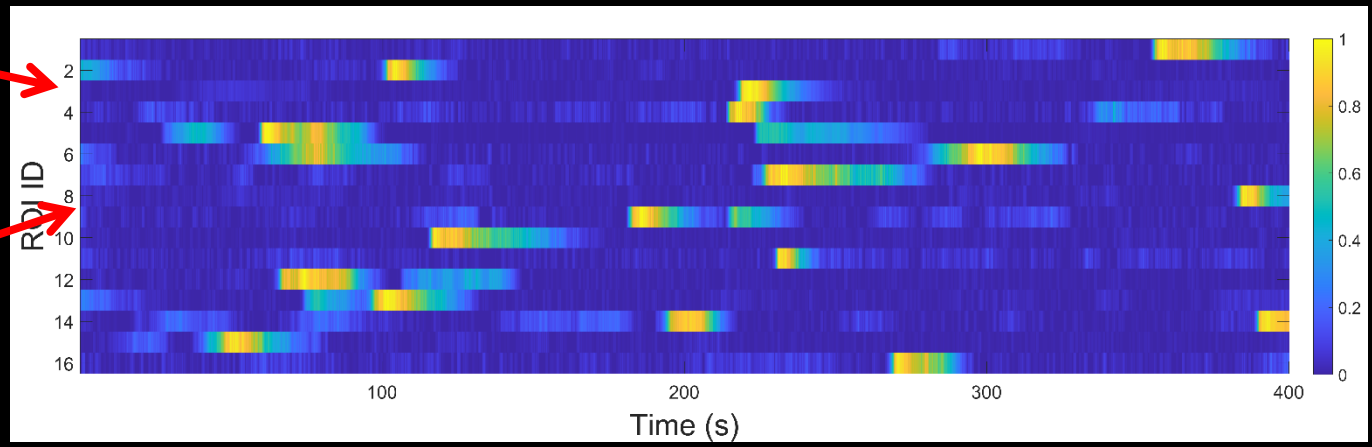
Plotting calcium traces

Further analysis on deconvolved calcium traces for spike detection

Raw trace

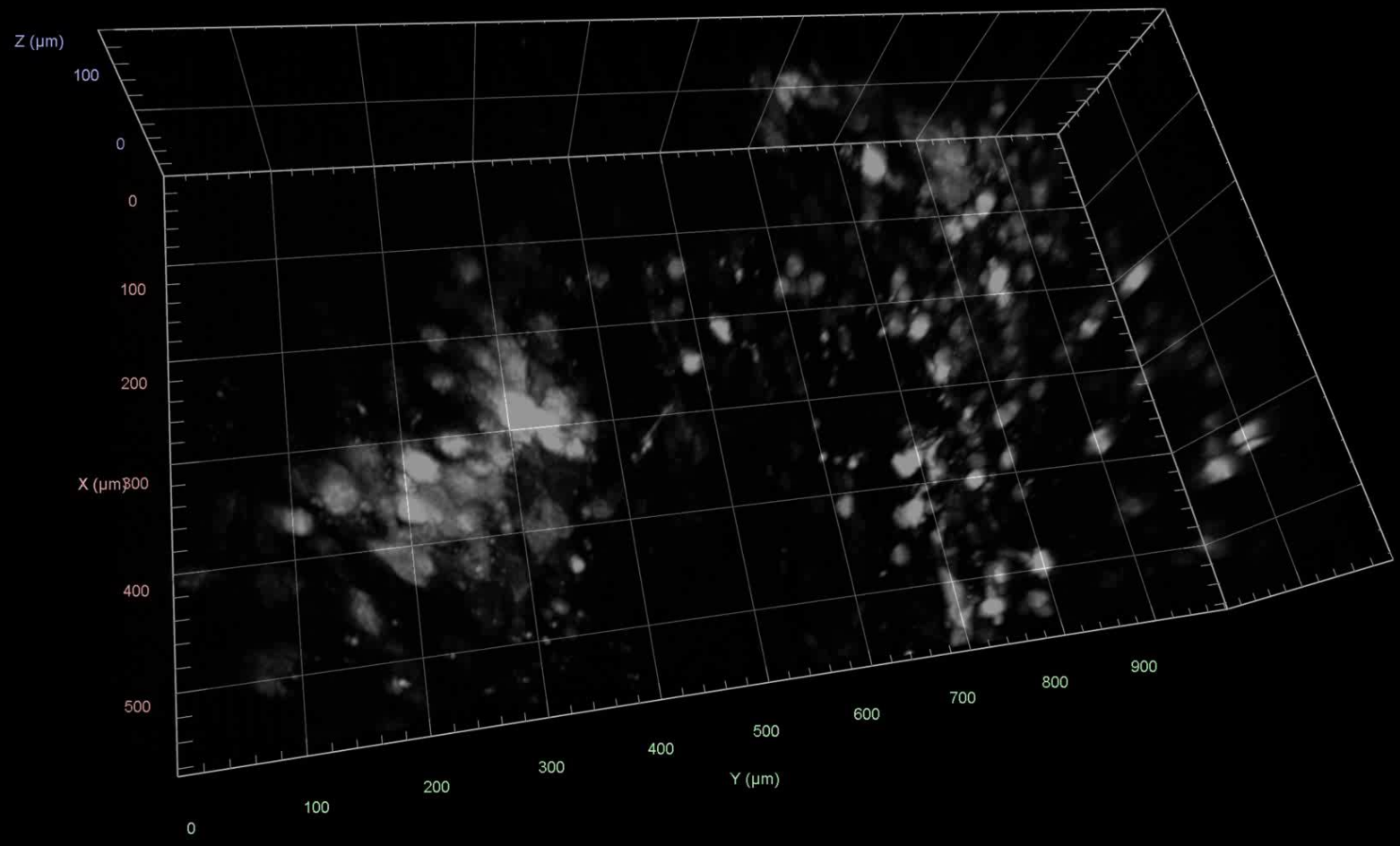


Deconvolved traces with moving average over 5 s



Functional imaging in 3D

GCaMP8s activity in the optic tectum. 20 planes, 10 µm apart and imaged at 1 Hz.



Summary

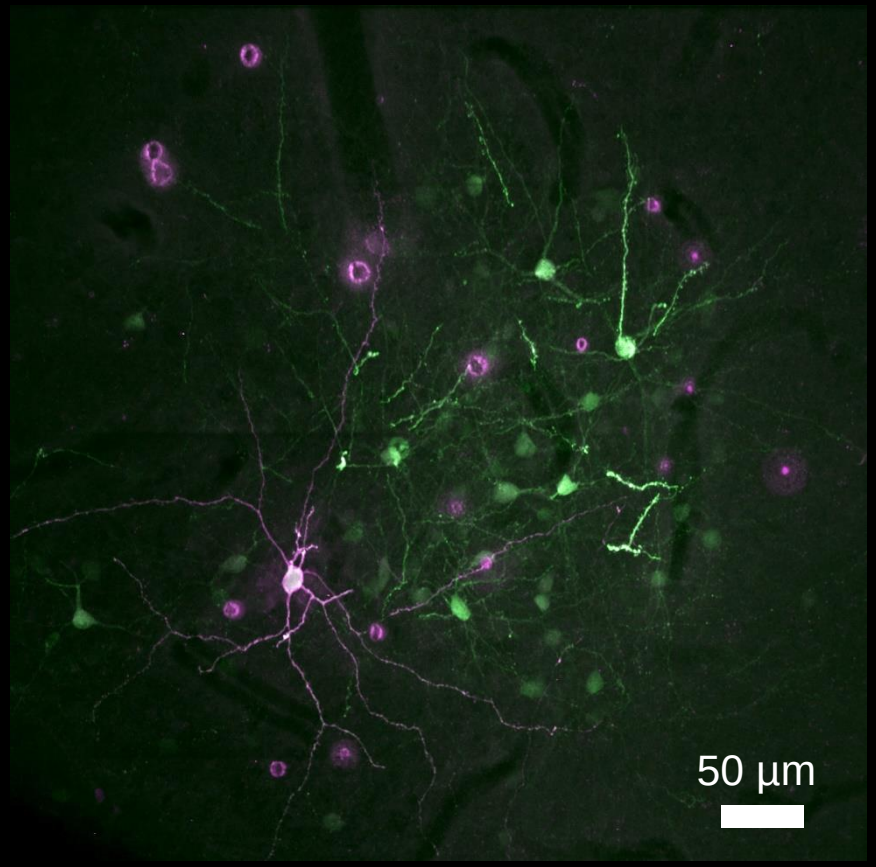
A multi-photon microscope for imaging deep into the tissue of live animals or specimens:

- Dual colour imaging (Green: e.g. EGFP, mVenus, GCaMP and Red: e.g. Td-Tomato)

Td-Tomato

GFP

Bearded dragon pallium

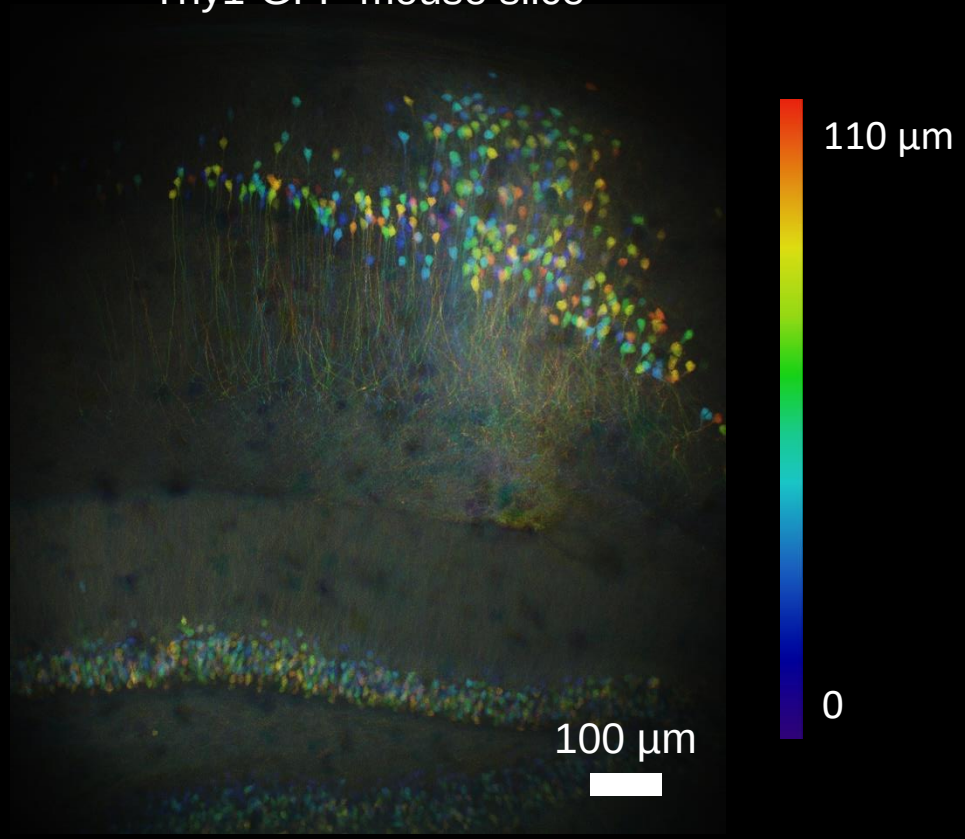


Summary

A multi-photon microscope for imaging deep into the tissue of live animals or specimens:

- Dual colour imaging (Green: e.g. EGFP, mVenus, GCaMP and Red: e.g. Td-Tomato)
- Multi-region scanning

Thy1-GFP mouse slice



Lab tour and hands-*OFF* session

Time slot	People
16:00-16:30	Alison Deyett Balazs Erdi Kevin Doppelmayer Jeanne Fesselet
16:30-17:00	Joonsun Lee Dunja Rokvic Leonie Adelmann Federico Scaramuzza
17:00-17:30	Theresa Sommer David Mörsdorf Daniel Velikov