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In vivo imaging in awake animals

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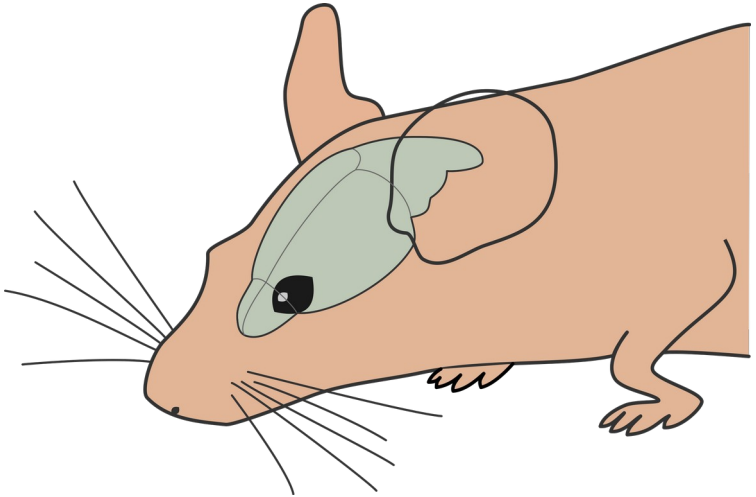
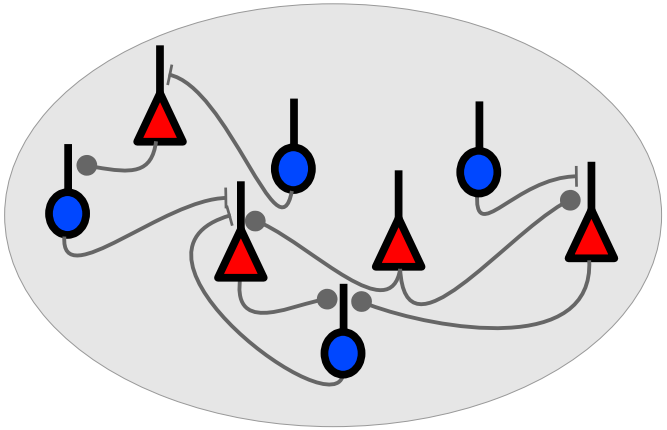
Aim

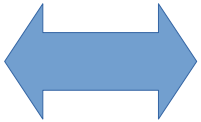
- study brain activity during relevant tasks – tasks which the brain has evolved and optimized to deal with
- explore brain function in its natural environment
- record (neural activity) from the brain of an *alive, awake* animal performing a task



Major challenge in neuroscience

neural circuits



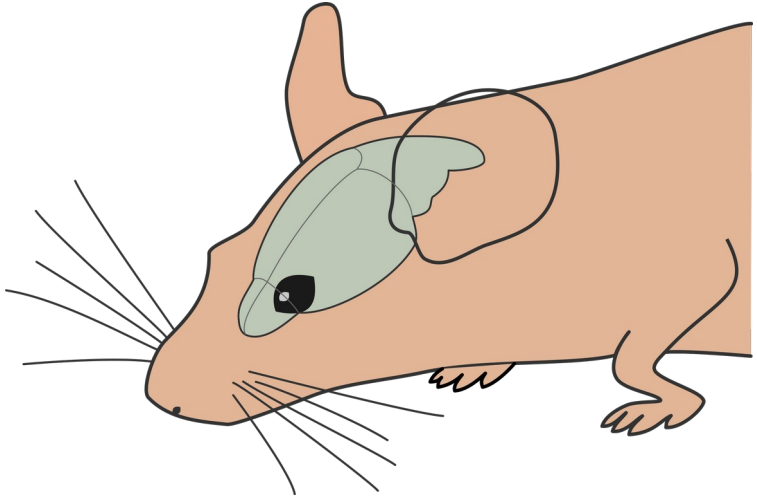


behavior

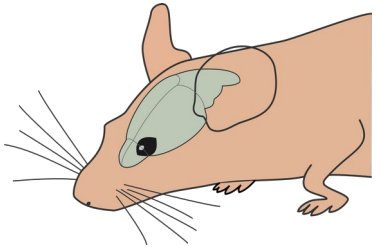
How do neural circuits encode, store, modify and retrieve information?

Technical challenges

- access region/neurons of interest
- assure animal's health and well-being
- make the animal perform a task
- perform stable recordings



Outline of the talk



1. Basics of *in vivo* imaging
   - parts list for imaging experiment
   - challenges of deep tissue imaging
   - 1- vs. 2-photon imaging
2. Considerations of *in vivo* imaging in awake animals
   - sensory modalities studied
   - practical implementation : head-fixed vs. 'freely' moving
   - virtual reality systems
   - calcium vs. voltage imaging
3. Examples from ongoing research
   - Cerebellum and motor control
   - Presubiculum and head-direction neurons

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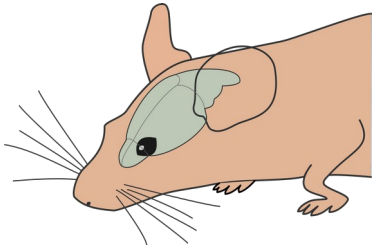

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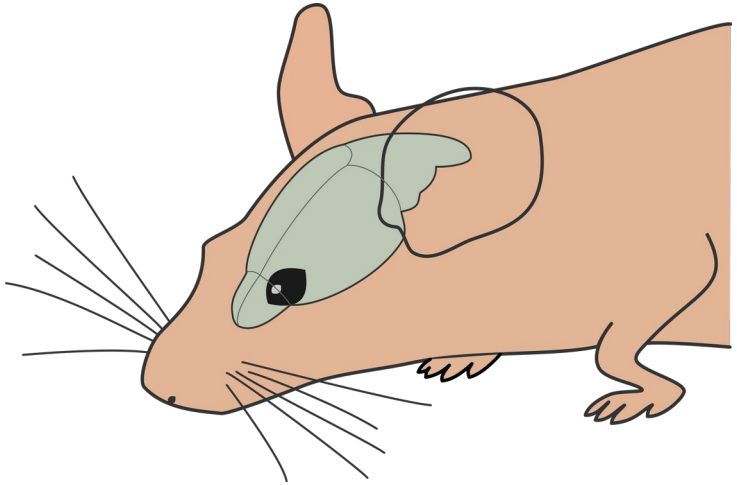
General parts list for *in vivo* imaging

Which general parts do we need if we want to record neural activity optically ?



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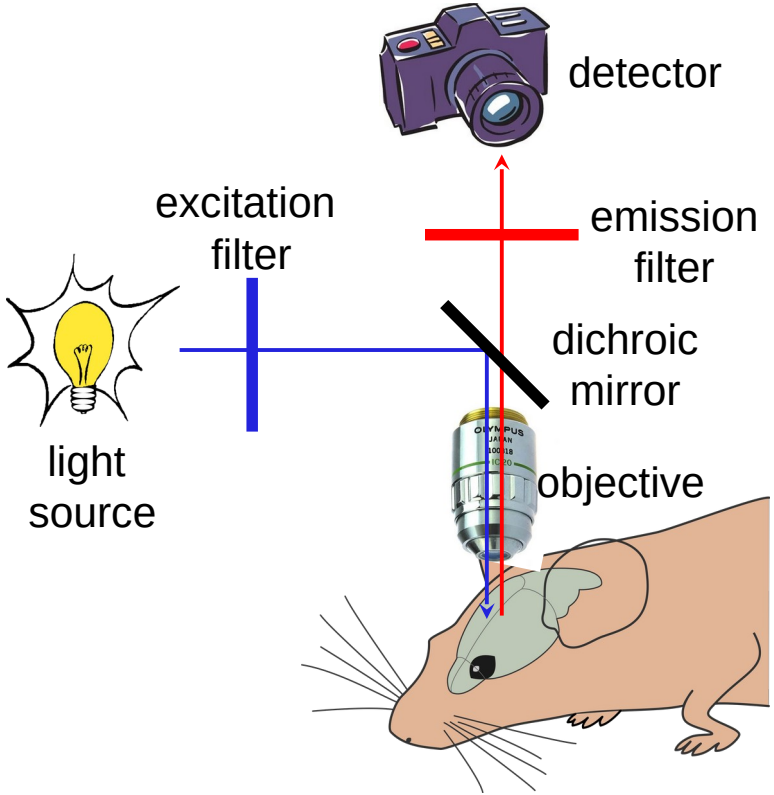
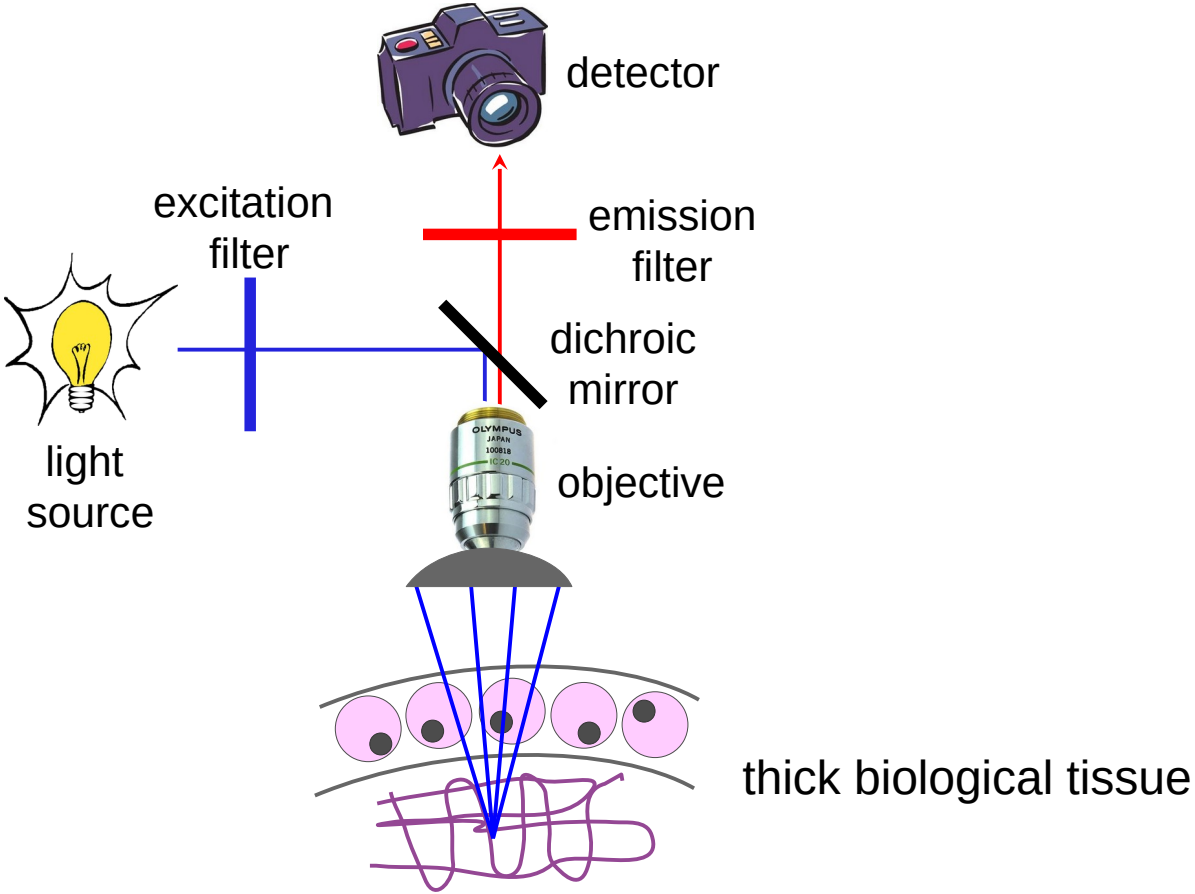
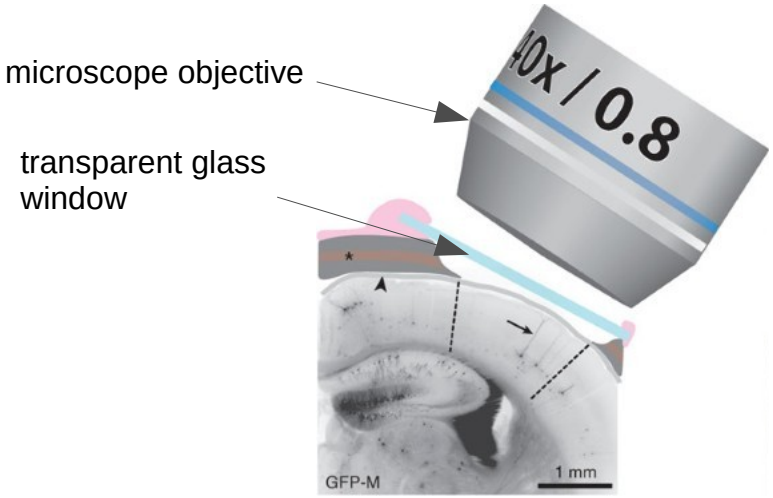


- **Light source**: LED, laser, mercury vapor lamp,...
- **Excitation filter**: enables to select a specific excitation range.
- **Dichroic mirror**: reflects wavelengths that are under/above a cutoff value and transmit wavelengths above this value.
- **Objective** : focuses light on region of interest
- **Sample** : structure labeled with fluorophore
- **Emission filter**: enables to select fluorescent photons in a given range.
- **Detector**: camera, PMT, eye,...

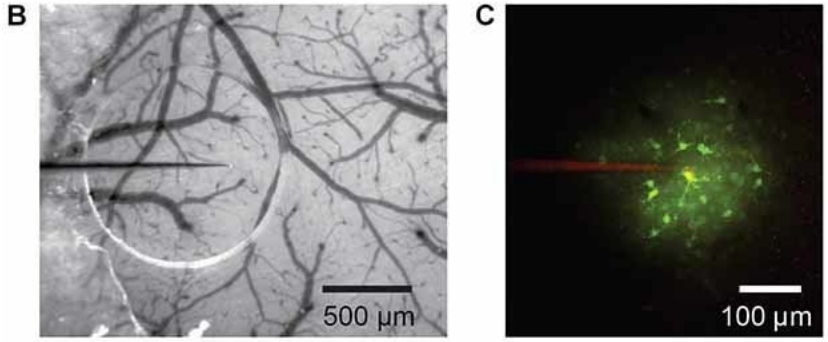
Challenge: optical access to tissue to be imaged

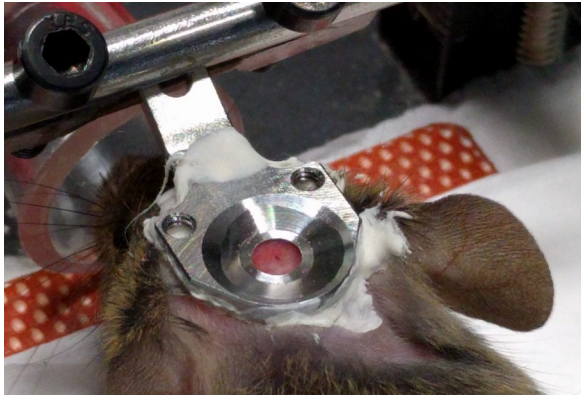


Optical access through chronic window

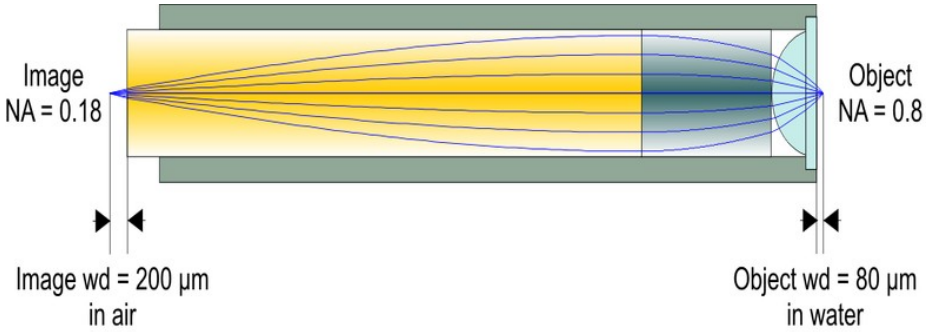


- Transparent window implanted in place of skull over region of interest :
  maximal achievable imaging depth up to 600-800 µm with 2-photon imaging; and 200 µm with 1-photon imaging
- bone thinning can provide sufficient visibility





Improved access to deep tissue with GRIN lens



- GRIN lens : glass gradient refractive index lens probe (microendoscopes)
- provides optical access to deep (and not so deep) structures in particular for one photon imaging
- **Disadvantage** : induces damage to more superficial structures (btw. the tissue to be imaged and the brain surface) as the physical object has to be inserted

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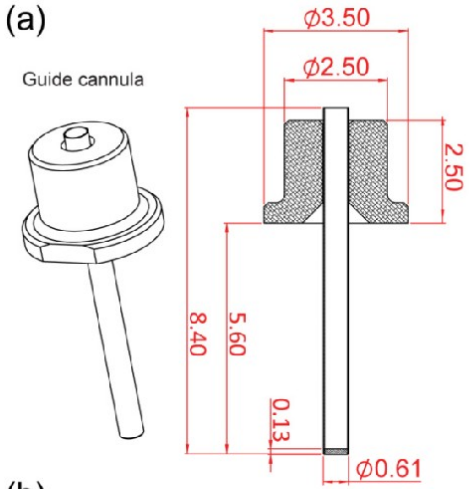

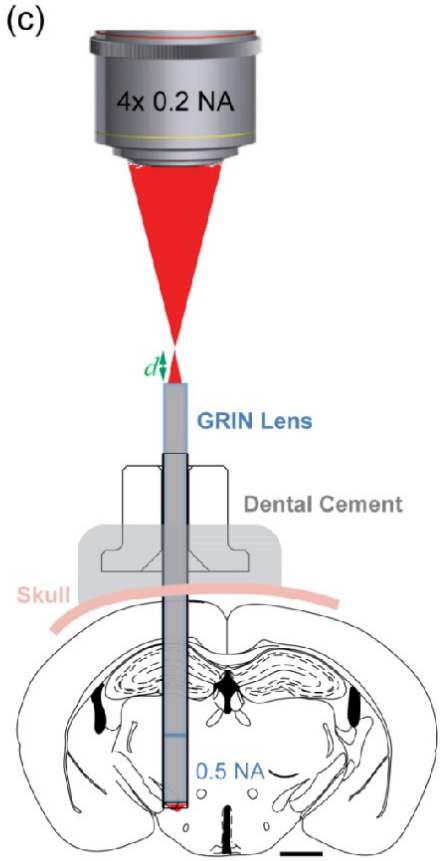

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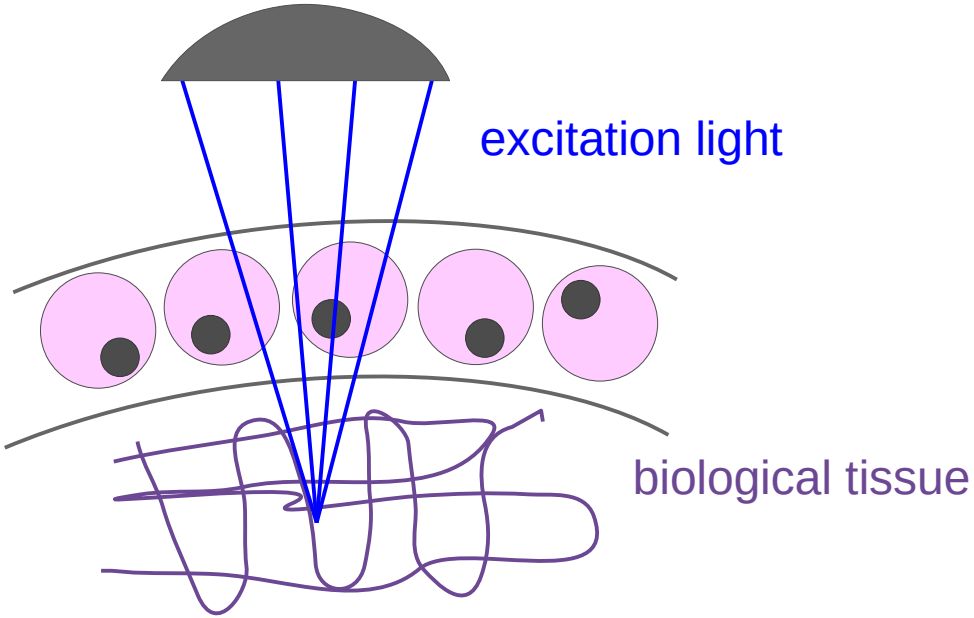
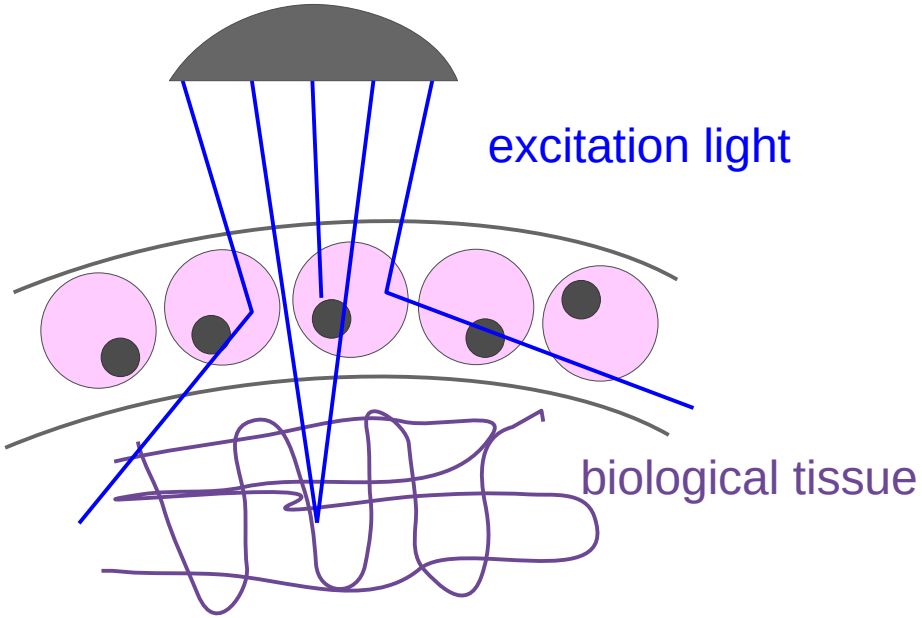
Imaging of thick biological tissue



Ideal case

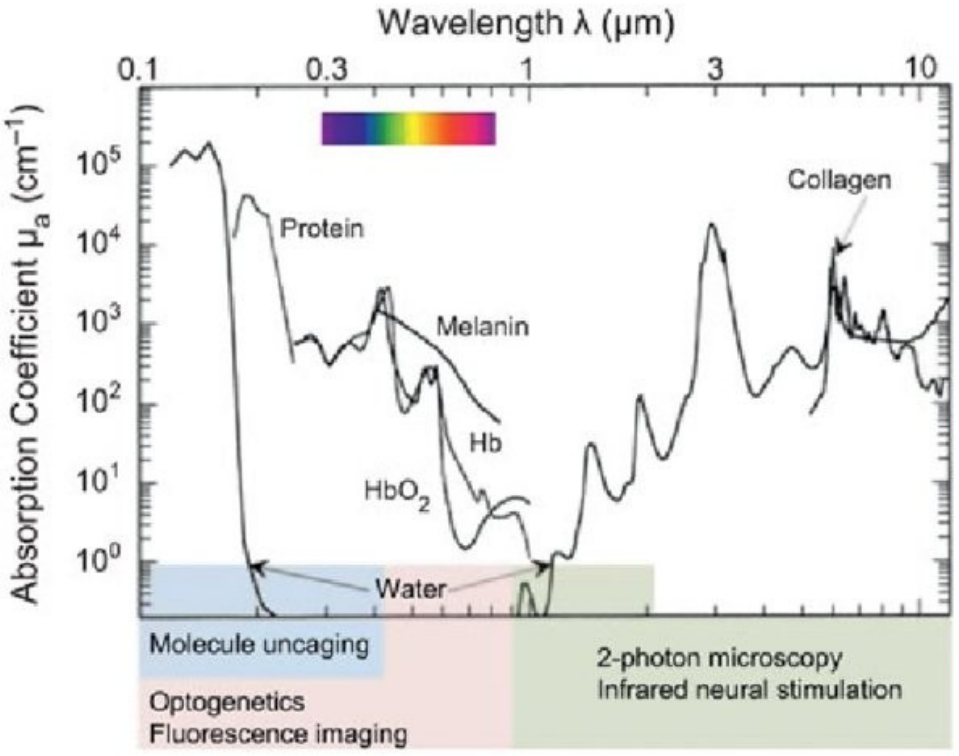
What limits imaging depth ?



Realistic case in thick biological tissue

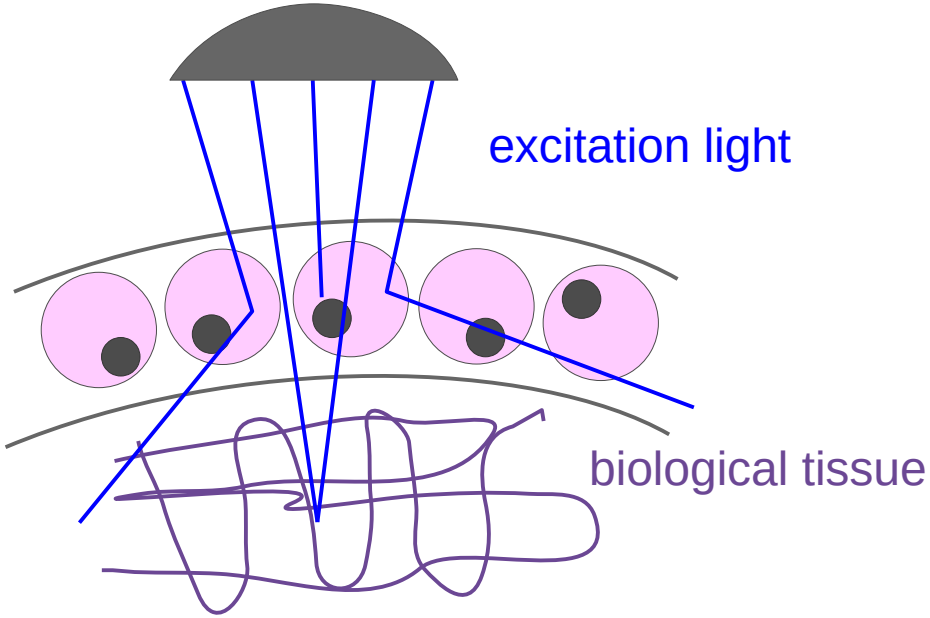
- *Absorption* : light is absorbed and converted into energy by molecules
- *Scattering* : light is diverted by molecules in different directions

Absorption coefficient in biological tissue



- absorption coefficient : logarithmic measure for the distributed absorption in a medium
- absorption coefficient in biological tissue varies greatly over the visible spectrum

Also scattering is wavelength dependent

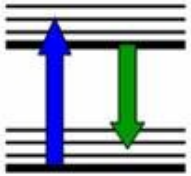


- the amount of light scattered scales as $1/\lambda^4$ (Raleigh scattering)
- Imaging in the near-infrared minimizes both absorption and scattering

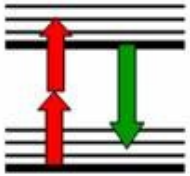
Fluorescence induced by 1- or 2-photons

fluorophore

1-photon excitation

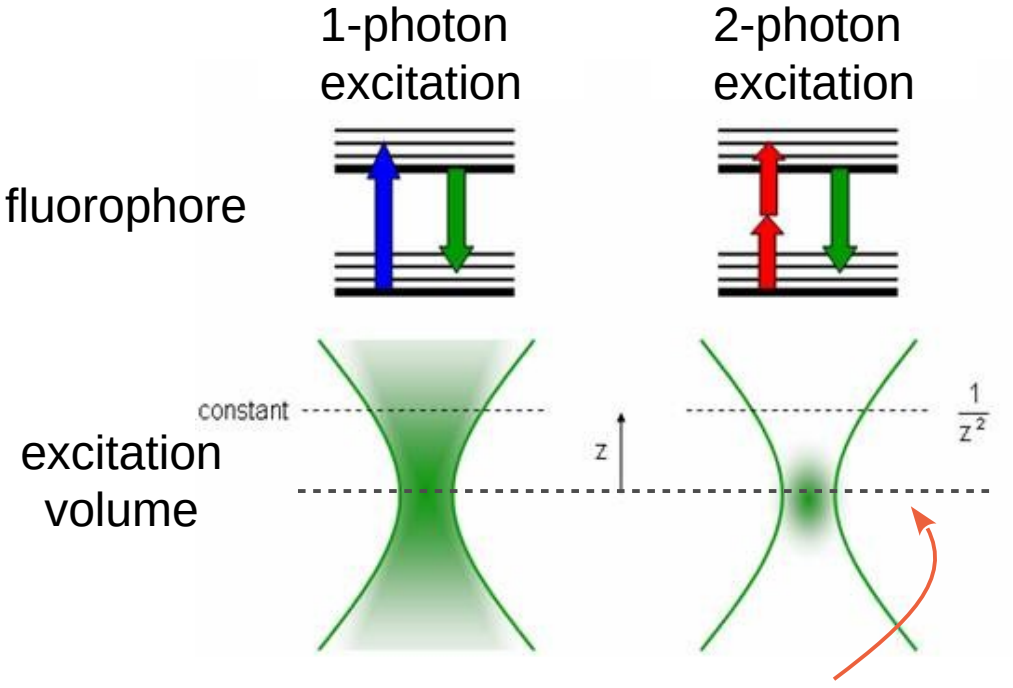


2-photon excitation



- Fluorescence: emission of light by the fluorophore that has absorbed light; emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation

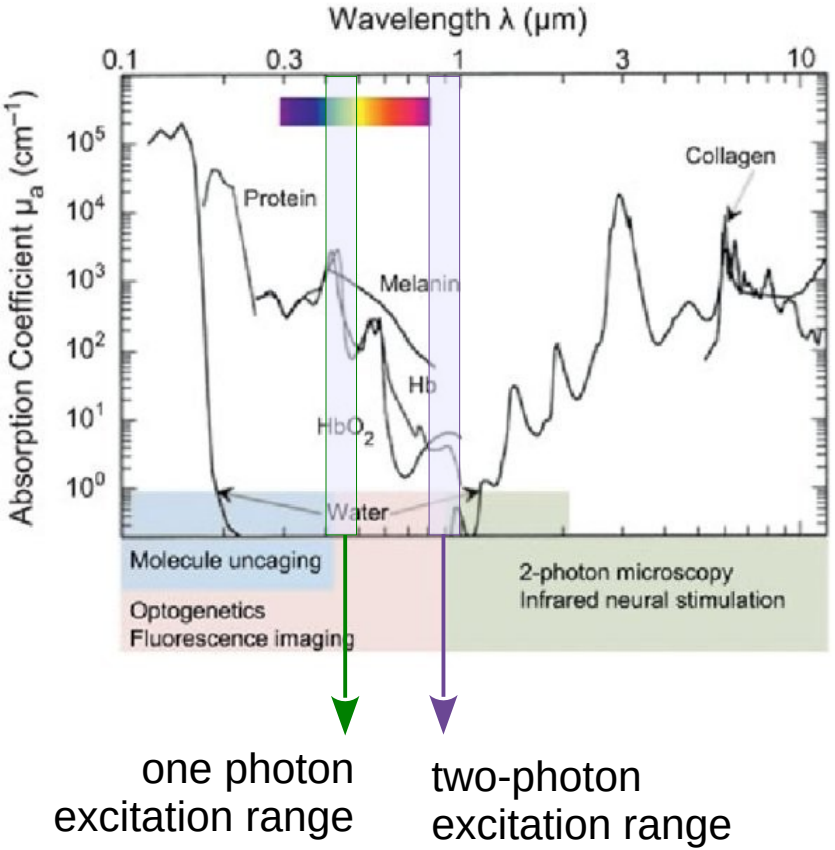
One photon vs. 2-photon fluorescence : resolution



[z is distance from focal plane]

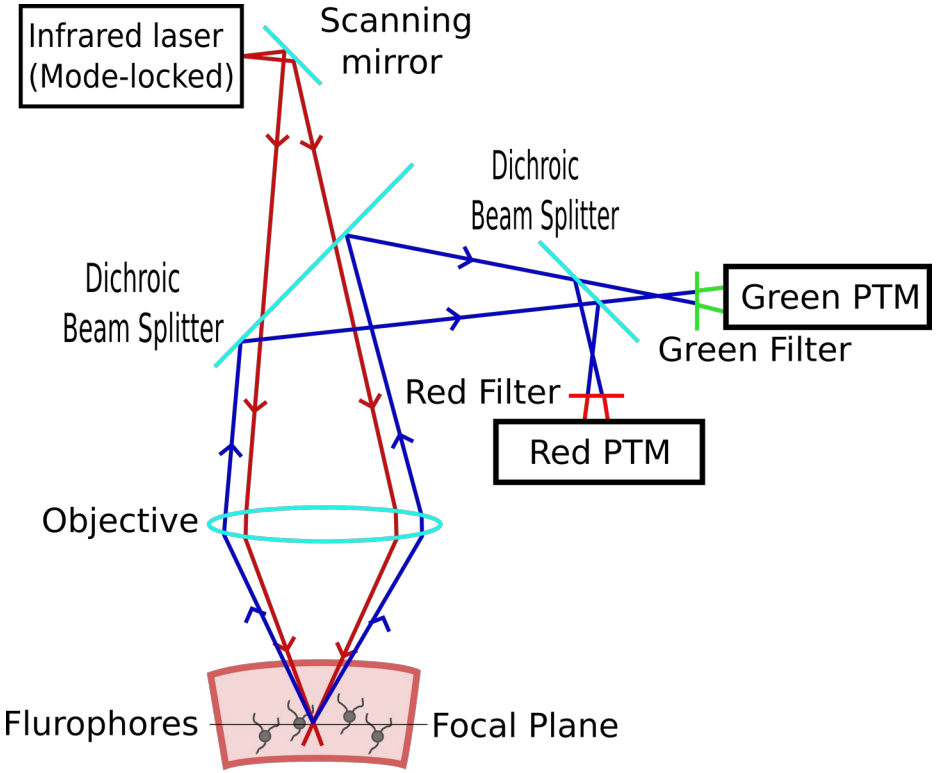
- excitation volume/fluorescence is confined to the focal center of the laser beam
- both photons must arrive nearly simultaneous (< 1 fs)
- fluorescence falls off as ~$1/z^2$, while it falls off as $1/z$ with single photon excitation
  → 3D-imaging with out-of-focus background rejection similar to a confocal microscope
  → much higher spatial resolution can be achieved

One photon vs. 2-photon fluorescence : absorption



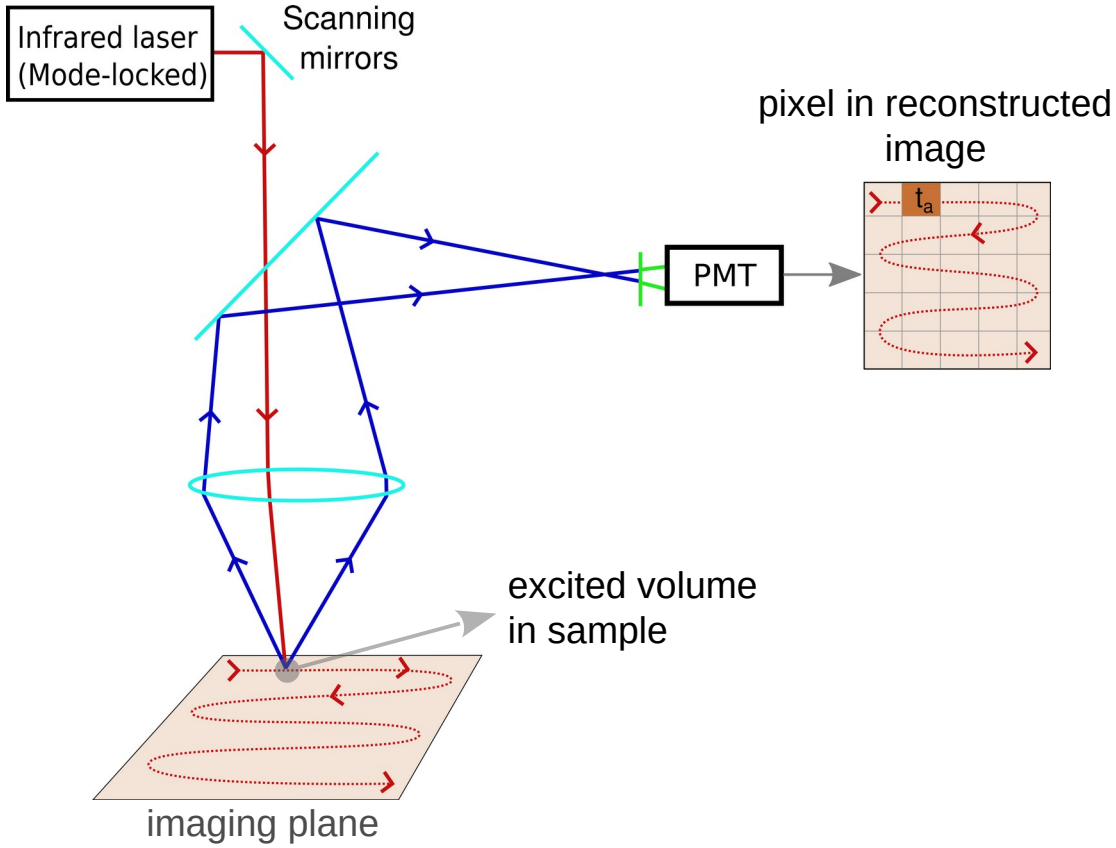
- commonly used fluorescent dyes have excitation spectra in the 400 to 500 nm range → wavelengths used to excite the same dyes with two-photons tend to be between about 800 and 1000 nm
- infrared light can penetrate deeper in biological tissue due to little absorption
- commonly used: titanium-sapphire tunable laser of wavelength 650 nm-1100 nm

Parts list for **2-photon** *in vivo* imaging



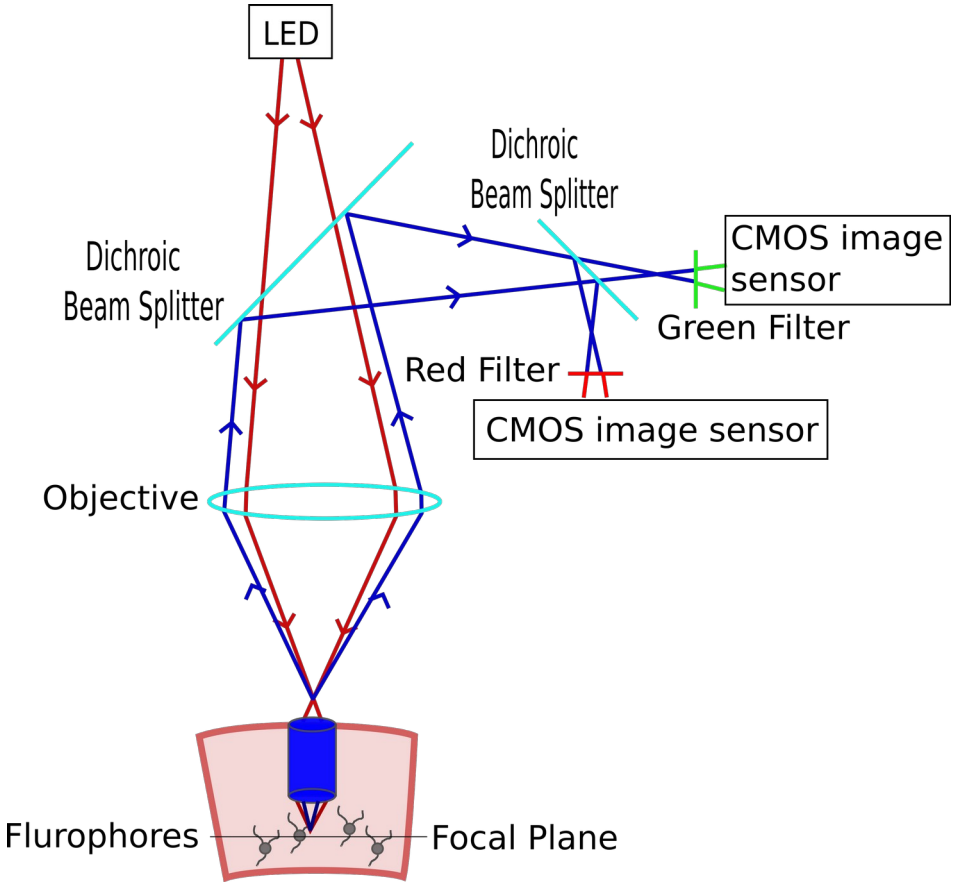
- **Light source**: laser producing light pulses on the order of femtoseconds (10^{-15} s)
- **Excitation filter**: not required since laser produces single wavelength
- **Scanning mirrors:** directs/scans the laser beam over the sample
- **Dichroic mirror**
- **Objective**: focuses light on region of interest
- **Sample**: structure labeled with fluorophore
- **Emission filter**: enables to select fluorescent photons in a given range.
- **Detector**: PMT

2-photon imaging : functioning



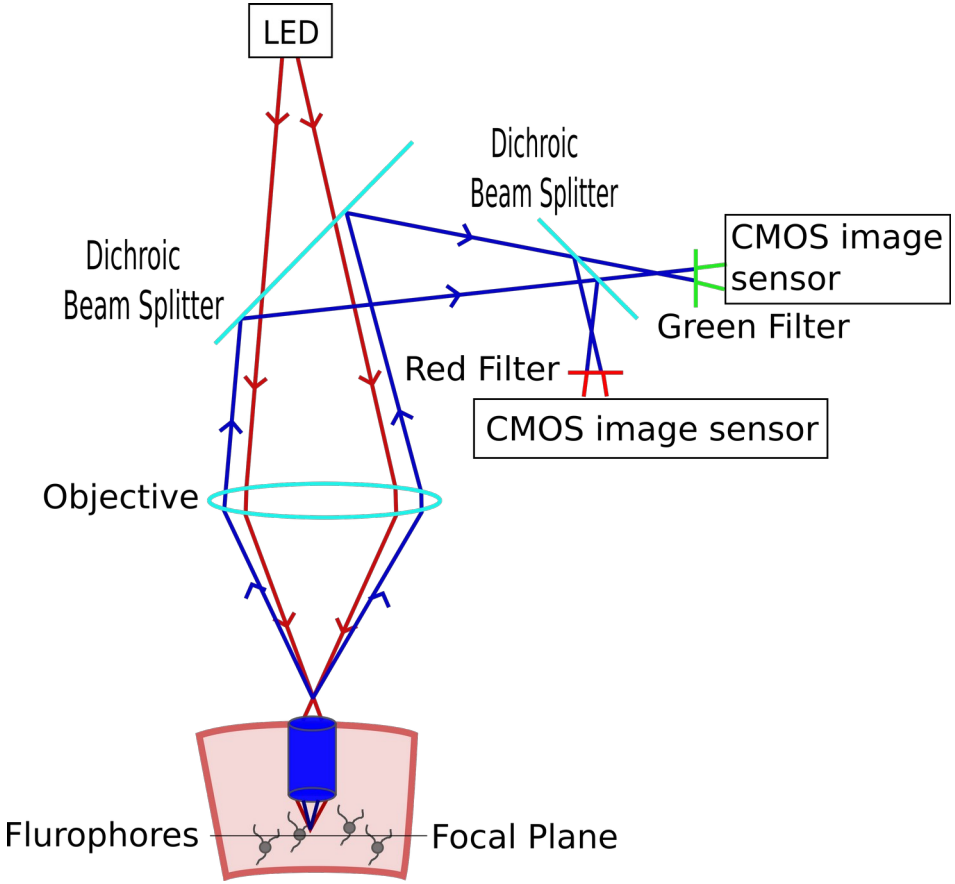
- A small excitation volume is excited by the laser light – defines resolution
- All fluorescent light is collected from the sample (indirect and direct light)
- Scanning mirrors move the laser beam across the imaging region – sequential acquisition of image (typical frame rate 30 Hz)

Parts list for **1-photon** *in vivo* imaging



- **Light source**: LED producing continuous light of a given wavelength
- **Excitation filter**: not required since LED produces single wavelength
- **Dichroic mirror**
- **Objective**: focuses light on region of interest
- **Grin lens:** provides access to deep tissue
- **Sample**: structure labeled with fluorophore
- **Emission filter**: enables to select fluorescent photons in a given range.
- **Detector**: CMOS image sensor (fast, energy-efficient camera)

1-photon imaging : functioning



- Entire sample is illuminated and imaged at once (no scanning of the laser beam)
- Each point in field of view is imaged onto a specific point on the sensor surface
- CMOS image sensor collects photons during the entire exposure time of an image

Comparison : 1 vs 2-photon imaging

1-photon (epifluorescence) imaging

- each pixel is sampled during the entire imaging duration – more signal photons can be collected
- entire image is sampled simultaneously simplifies motion correction
- full commercially available solutions
- lightweight and portable system, does not restrict application and animal behavior

Advantages

2-photon imaging

- near-infrared light minimizes both absorption and scattering – greater depth of imaging
- small excitation volume results in reduced phototoxicity and dye bleaching
- high spatial resolution – no out-of-focus light
- easy separation between excitation and emission light

Comparison : 1 vs 2-photon imaging

Disadvantages

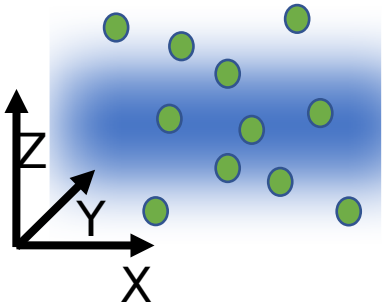
1-photon (epifluorescence) imaging

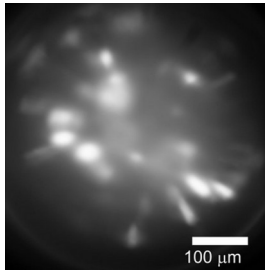
- poor resolution makes it impossible to image neurites or spines; out of focus fluorescent signal (from neuropil)
- insertion of GRIN lens destroys neural tissue above the region to be imaged
- phototoxicity and dye bleaching problematic due to constant illumination

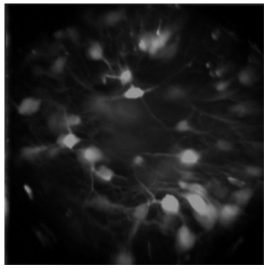
2-photon imaging

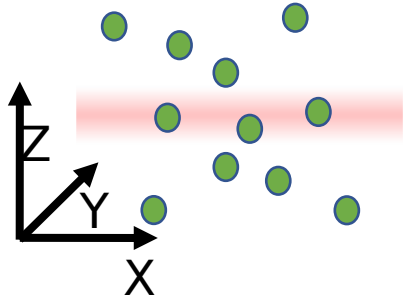
- lasers needed are expensive, large, complicated and consume a lot of power
- complete commercially available systems are pricey
- limited photon counts per pixel and limited imaging speed (in particular for voltage imaging)
- line-by-line image acquisition can lead to distortion due to motion
- requires (most of the time) head-fixation of the animal (but see new developments)

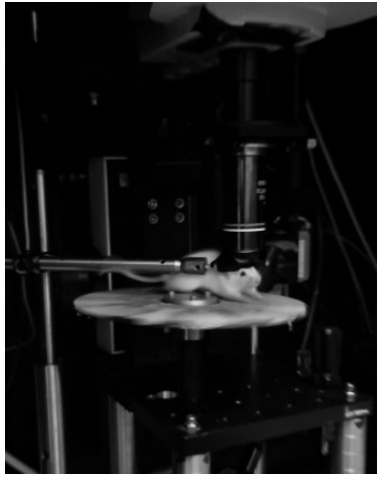
1p vs 2p



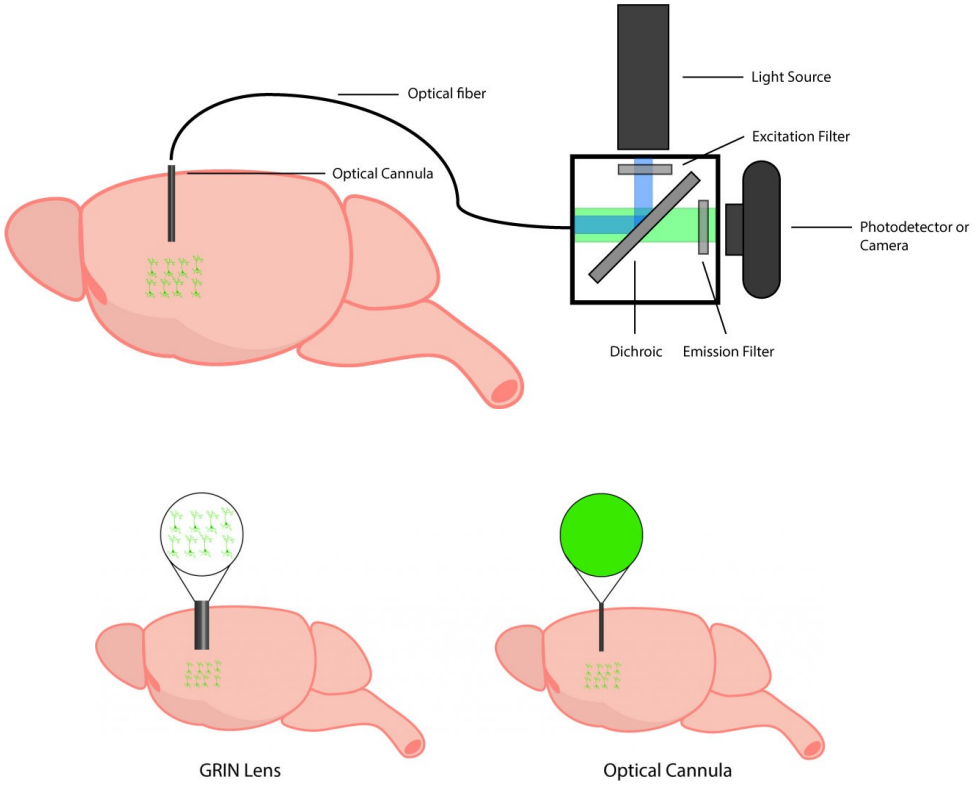






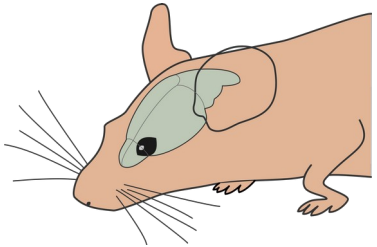


Fiber photometry



- excitation light and fluorescence signal is transmitted through the optical fiber (or cannula)
- optical cannula collects cumulative/combined signal from all neurons
  -> gives access to population-level neural activity – no cellular resolution
- typically used in combination with 1-photon imaging

Outline of the talk



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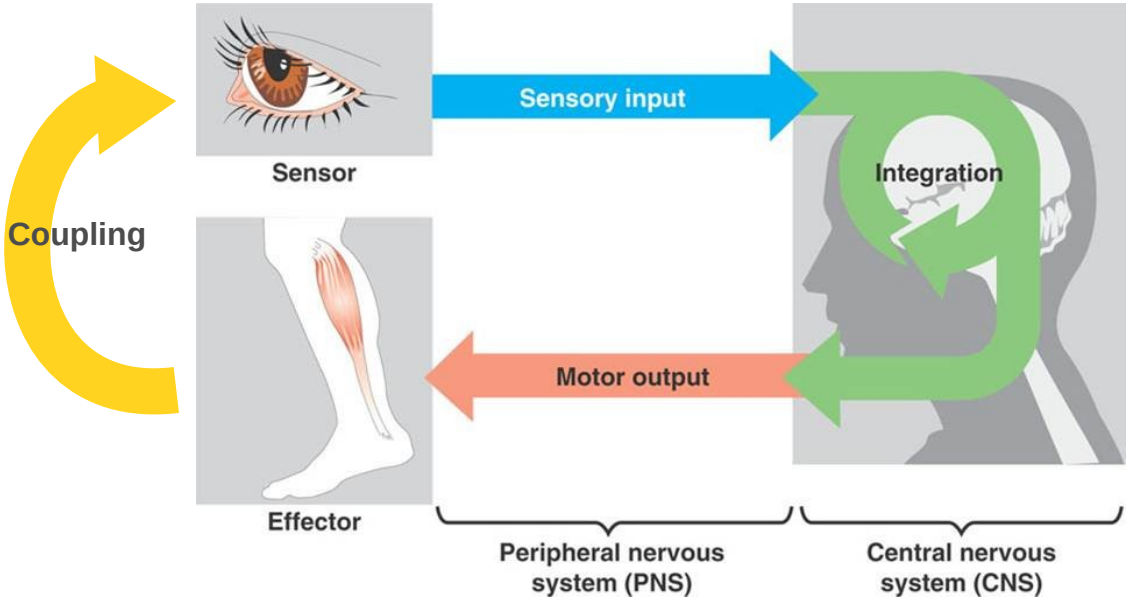
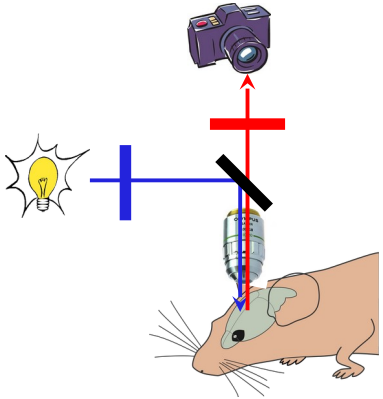
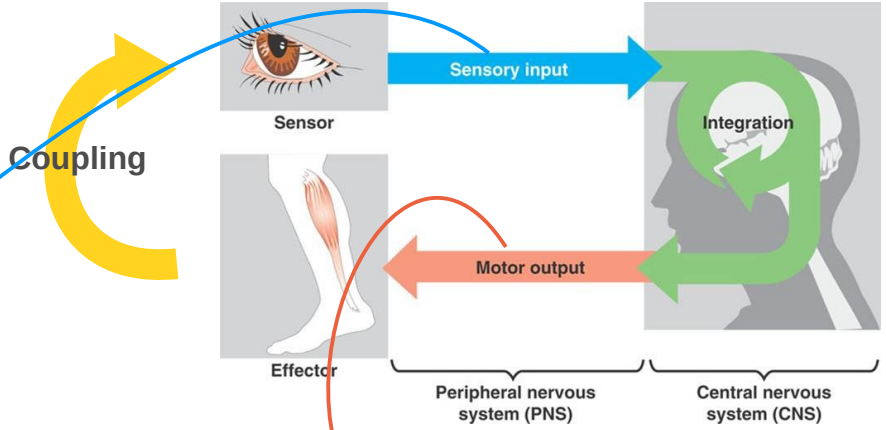
Rational behind *in vivo* experiments



- **goal** : naturalistic behaviors, where one's actions determine sensory stimulation
- **initially** : *in vivo* approaches focused on sensory perception (passive stimulation of single sensory modality)
- **however** : sensorimotor processing varies with behavioral state/output
- **interactive setting** : study sensoriomotor interactions with the outside world

Feasibility of *in vivo* imaging experiments

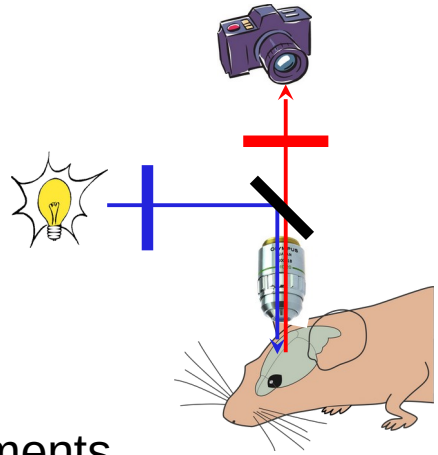




- **sensory stimuli :**
  - easy to implement : touch (whisker), vision (static), smell, taste, sound
  - difficult : vision (dynamic), equilibrium (vestibular)

- **motor output** :
  - easy : licking, paw/arm movement, gaze, whisking
  - difficult : locomotion

Stability btw. imaging system and imaging tissue



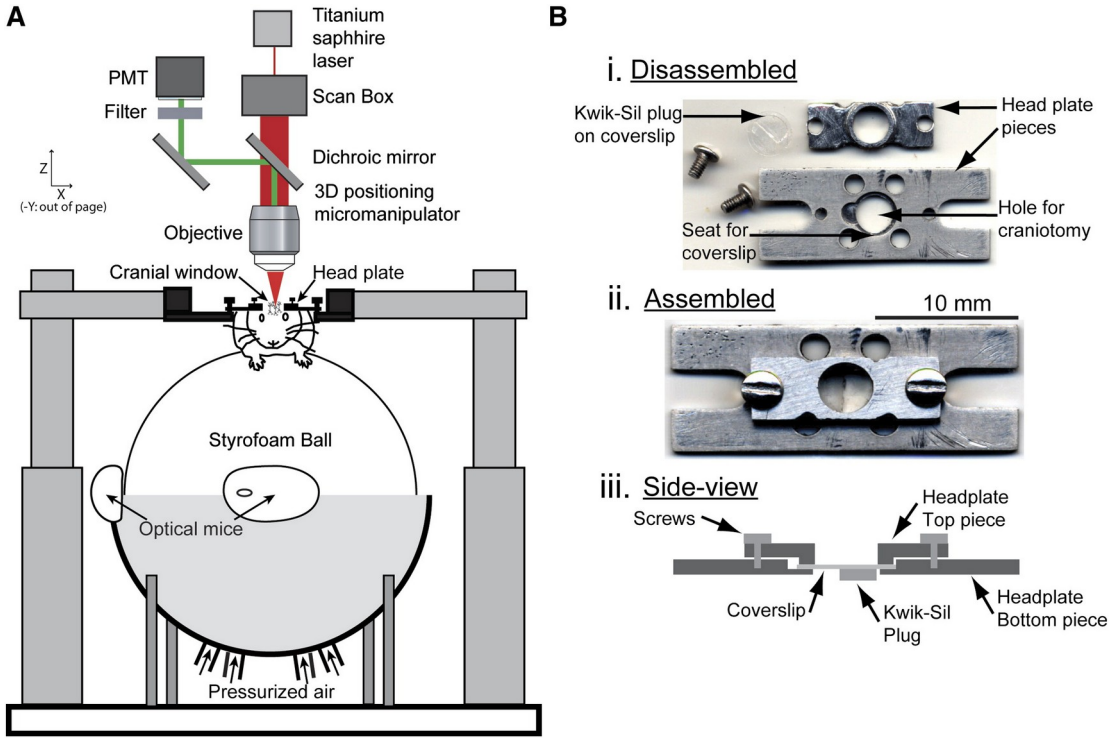
1) Minimize relative movements between animal to be imaged and the microscope

→ fix the animal head under the microscope

2) Place (parts of) microscope on the head of the animal, i.e., microscope moves with the animal

→ miniaturize imaging system

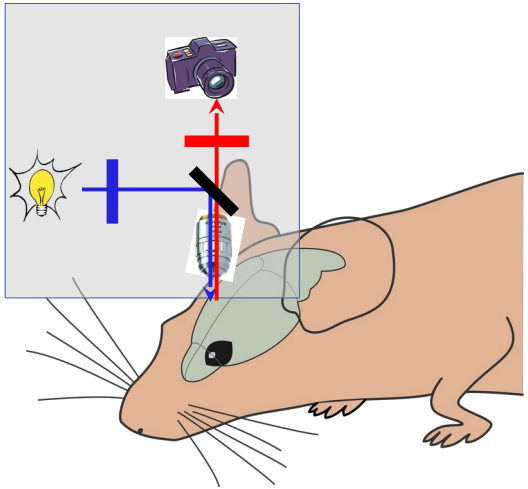
Most 2-p imaging experiments use head-fixation



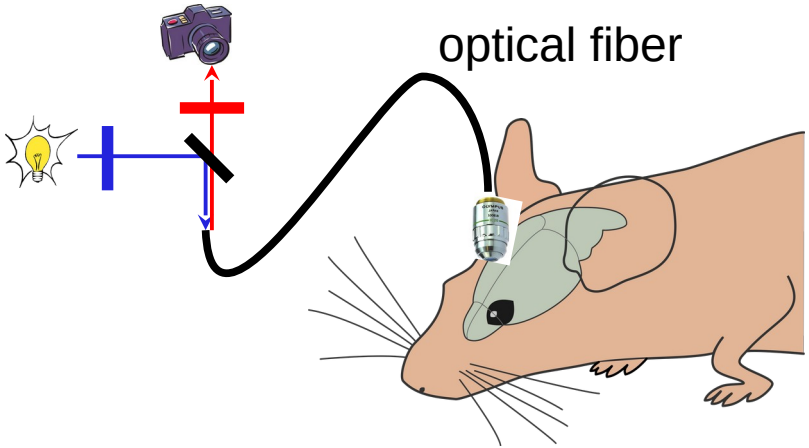
- Minimizes relative movements between animal – to be imaged – and the microscope
- adapter – headplate – is implanted on the animal's head to allow for solid and repeated fixation in the experimental setup
- allows to study sensorimotor integration for many sensorimotor modalities

[Dombeck *et al.* Tank, Neuron 2007]

'Freely' moving animal solutions



- miniaturized microscope mounted on animals head
- feasible for epifluorescence imaging

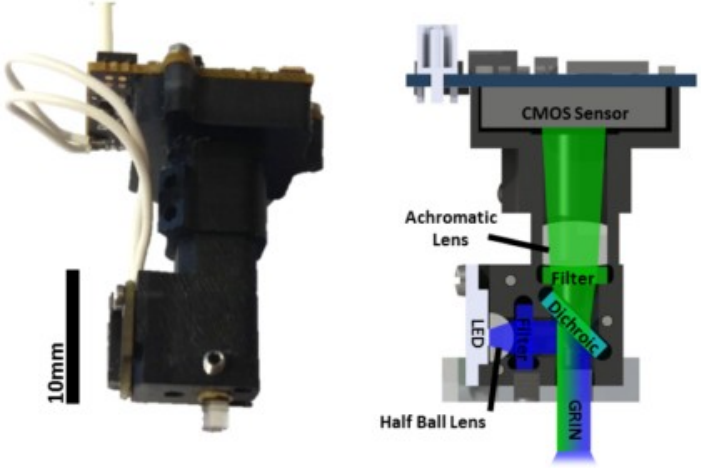


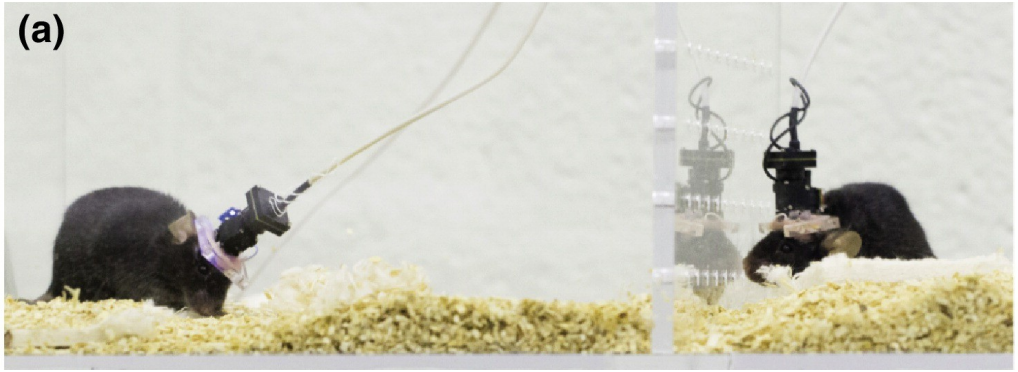
- flexible optical fiber connects static microscope parts (light source/detector) and animal-mounted optics
- allows for 2-photon imaging in 'freely' moving animals

Head-mounted wide-filed epifluorescence (1-p imaging)

miniscope

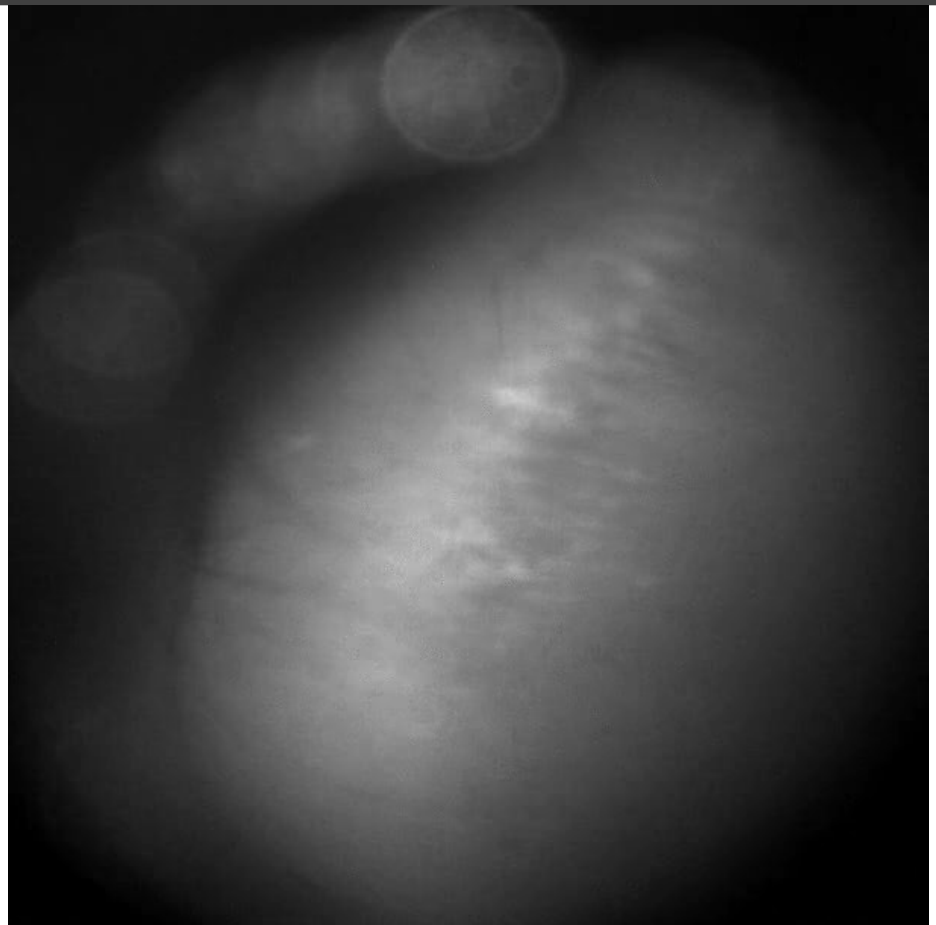
weight ~ 2g



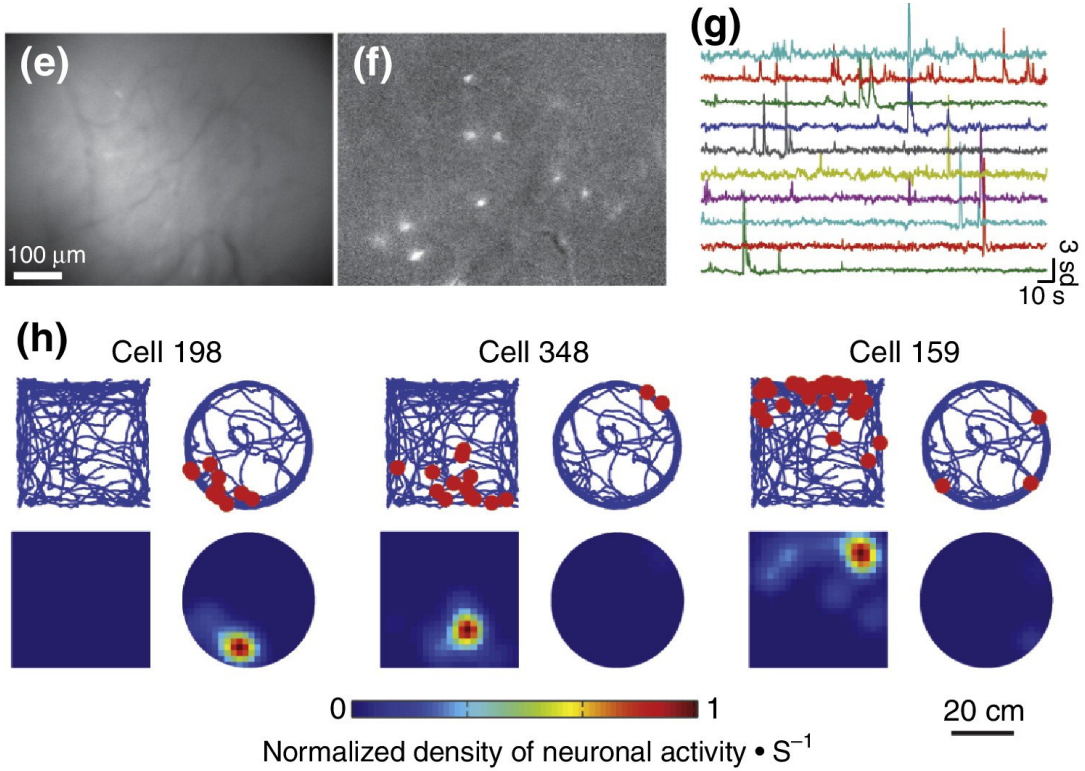


[Ziv & Ghosh, *Current Opinion in Neurobiol* 2015]

Hippocampal Ca dynamcis in behaving mice



Hippocampal Ca dynamcis in behaving mice



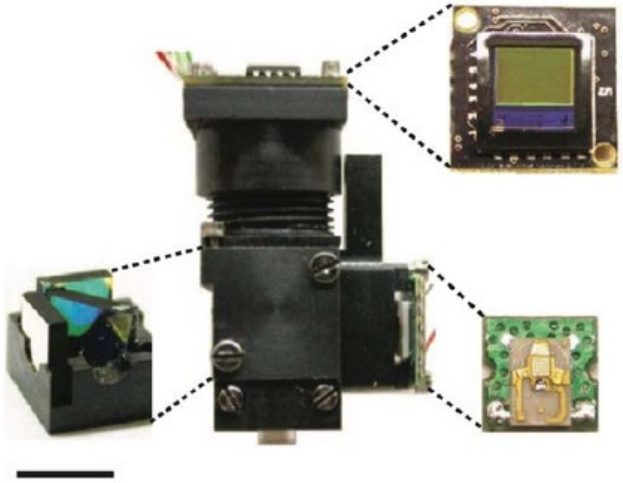
[Ziv & Ghosh, *Current Opinion in Neurobiol* 2015]

- epifluorescence imaging of pyramidal cells in CA1 region of the hippocampus
- cells in this region feature place-cells : cell which fire when animal enter a particular place in environment

1p miniscopes : from origin to open-source

Origins

Mark Schnitzer lab,
Stanford University

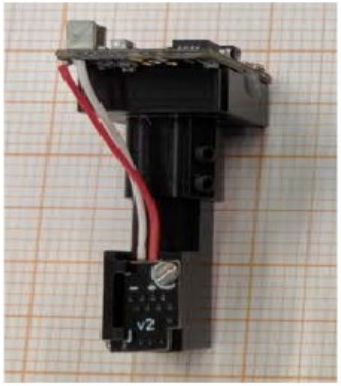


Commercial

Inscopix

Original cost: 100.000 $

Open-source (DIY)



UCLA Miniscope

Cost: <1500 $

Different commercial and open-source miniscopes

commercial solutions

Inscopix

Doric lenses

open-source miniscopes

FinchScope

Dim: 10 x 6 x 21 mm
Wired: 1.8 gram
Wireless: ~ 4 gram
FOV: 880 x 600 µm
Frame Rate: 30 Hz
Focus: turret
DAQ: Arduino
Software: MacOS

miniScope

Dim: 12 x 12 x 20 mm
Wired: 2.4 gram
FOV: 1.1 x 1.1 mm
Frame Rate: 10 Hz
Focus: turret
DAQ: Opal Kelly
Software: Win & Mac

UCLA Miniscope

Dim: 16.5 x 13 x 22.5 mm
Wired: ~ 3 gram
Wire-free: 4.5 gram
FOV: 700 x 450 µm
Frame Rate: 60 Hz
Focus: linear slider
DAQ: custom PCB
Software: Win

CHEndoscope

Dim: 15.9 x 17 x 32.5 mm
Wired: 4.5 gram
FOV: ~ 500 µm across
Frame Rate: 20 Hz
Focus: turret
DAQ: direct to PC
Software: Win & Linux

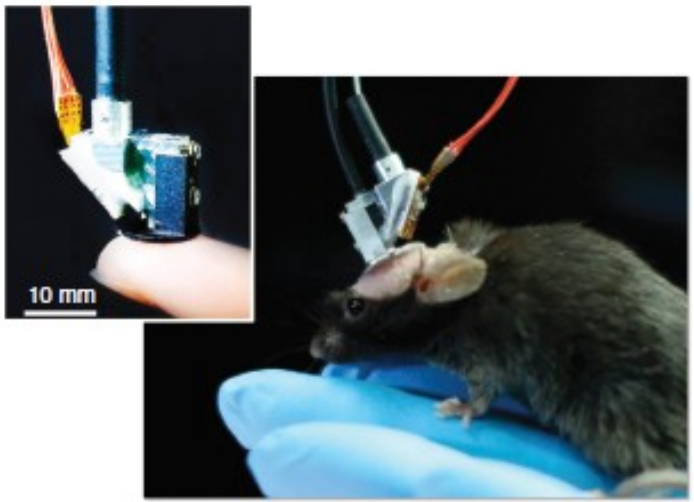
NINscope

Dim: 11 x 11 x 18 mm
Wired: 1.6 gram
FOV: 00 µm
Frame Rate: 30-120 Hz
Focus: linear slider
DAQ: direct to PC
Software: Mac, Win & Linux
Built-in: G-sensor, opto-LED

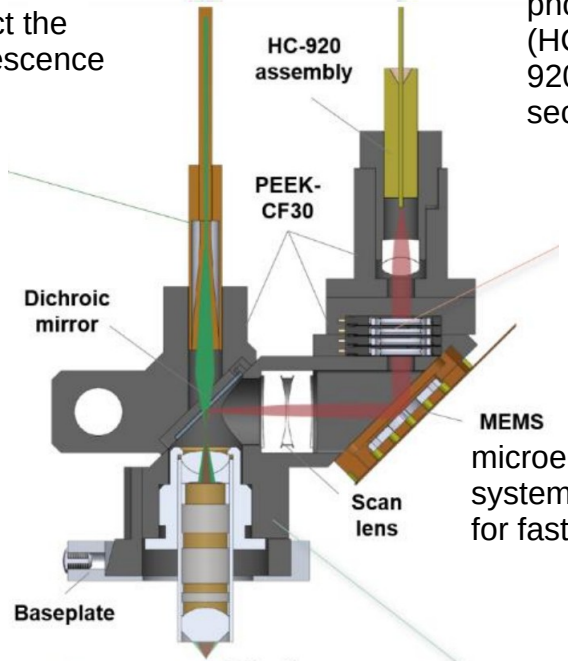
Aharoni and Hoogland, Frontiers in Cell. Neurosci. 2019

2p-laser scanning fiber-coupled microscope

Freely-moving 2p recording – the challenge of miniaturizing heavy technology



supple fiber bundle to collect the fluorescence

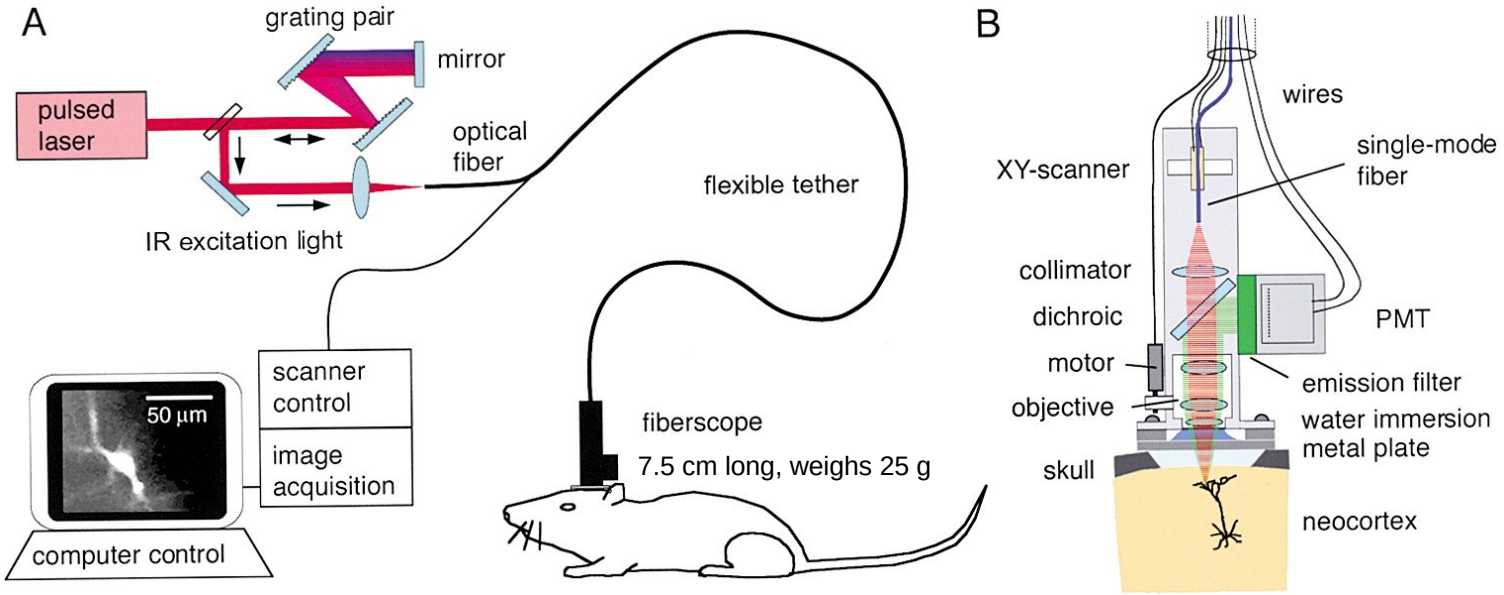


a hollow-core photonic-crystal fiber (HC-920) to deliver 920-nm femto-second laser pulses

microelectromechanical systems (MEMS) scanner for fast point scanning

[Zong *et al*. *Cell* 2022]

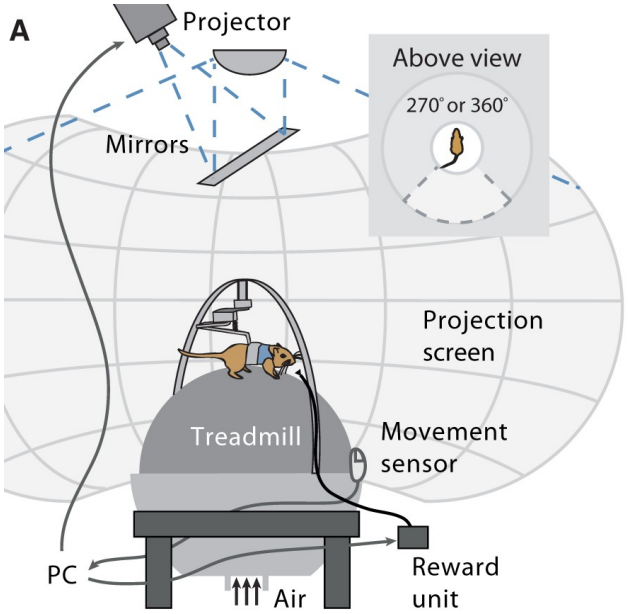
2p-laser scanning fiber-coupled microscope

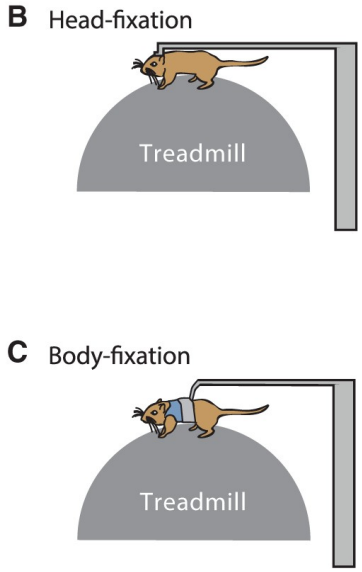


[Helmchen et al. *Neuron* 2001]

- light source at remote location from the animal
- spatial resolution : scanning mirrors and detector in fiberscope on the animal's head, or multi-core fiber
- challenges : dispersion in the excitation fiber, image distortion, inflexible optical cables

Virtual reality (VR) systems : visual VR

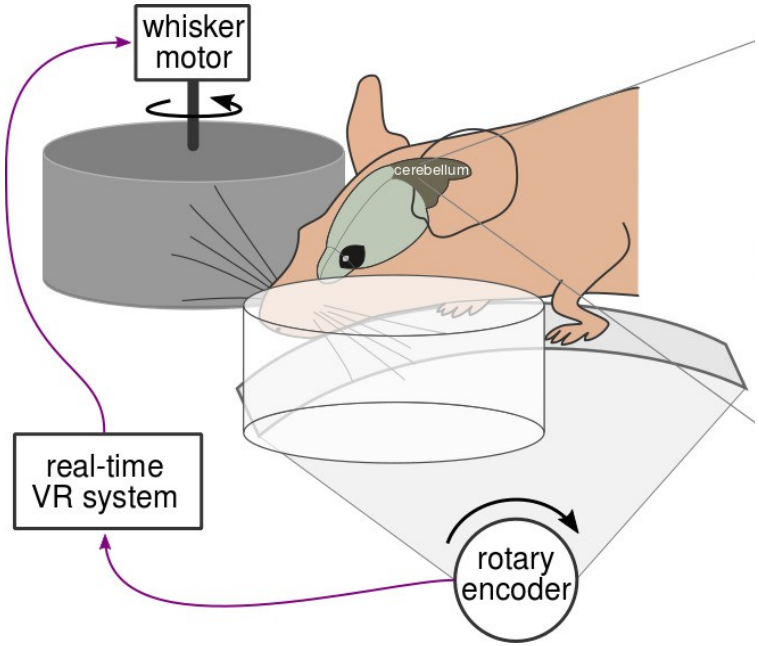




- creating a sensorimotor loop between locomotion and visual feedback (i.e. optical flow linked to movement)
- animal is restrained, animals paw movement is recorded and controls sensory stimulation
- https://www.youtube.com/watch?v=1DJOTEDBA2c

[Thurley & Ayaz, *Current Zoology* 2017]

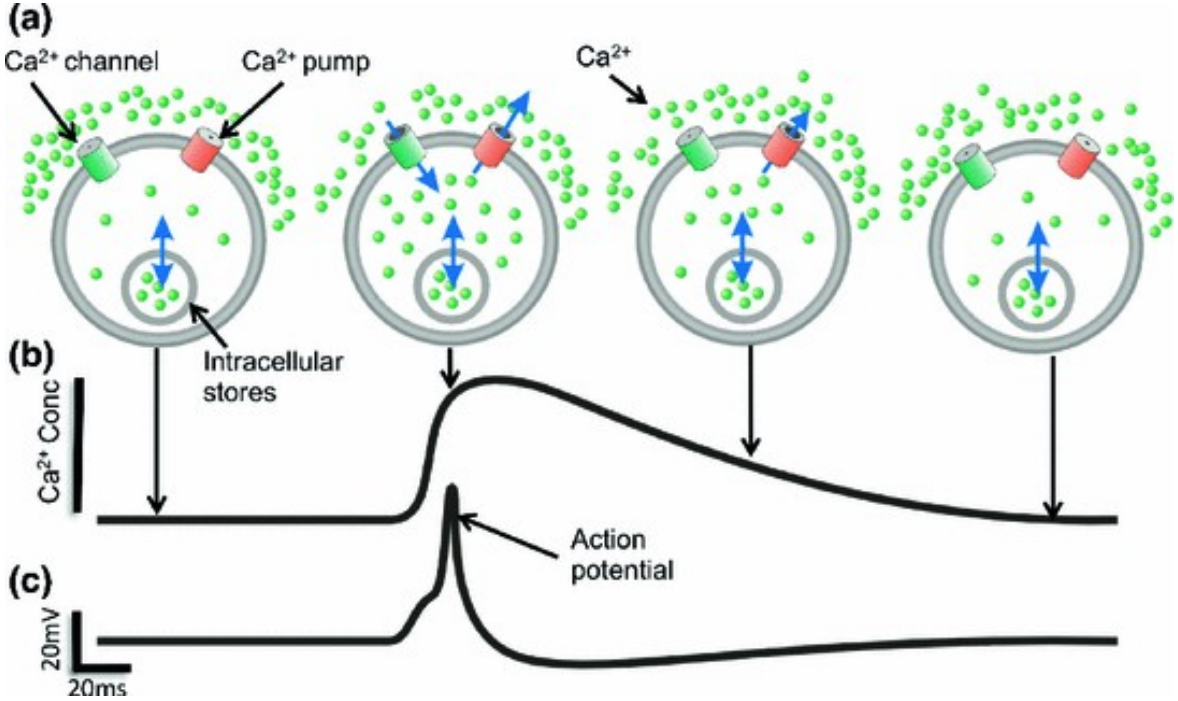
Virtual reality (VR) systems : tactile VR



[Stell *unpublished* 2019]

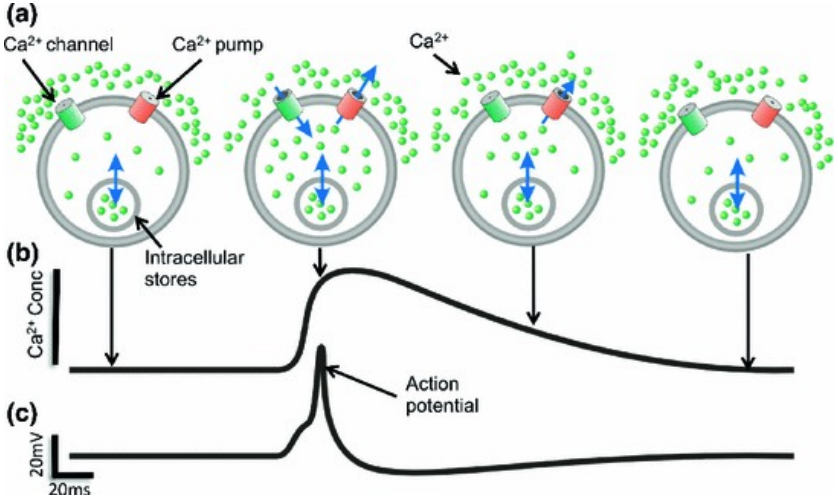
- creating a sensorimotor loop between locomotion and tactile feedback (i.e. mechanic stimulation linked to movement)
- animal is restrained, animals paw movement is recorded and controls rotation of whisker wheels

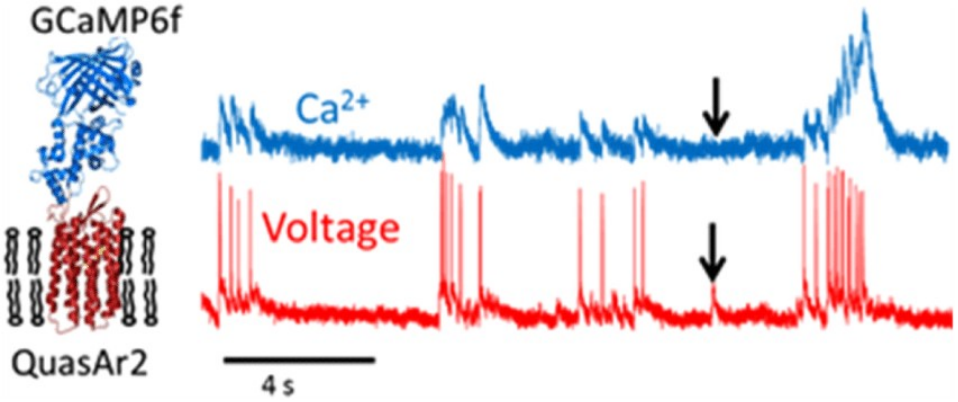
Calcium vs. voltage imaging



- membrane potential depolarizations induce calcium transients
- calcium is a proxy of neural activity
- calcium transients are much longer (~100 ms) than membrane potential depolarizations (~2 ms)

Calcium vs. voltage imaging





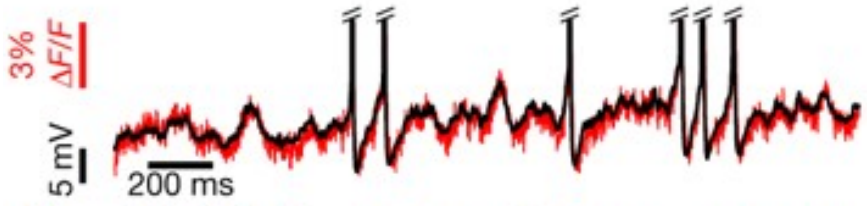
Calcium imaging

- genetically encoded calcium indicators (GECIs) report calcium trace
- Uses nuclear calcium signal as proxy for neuronal activity

Voltage imaging

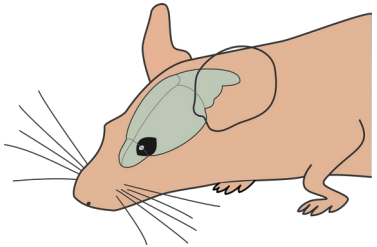
- genetically encoded voltage indicators (GEVIs, e.g. QuasAr, ASAP) report directly transmembrane voltage
- located in cell membrane

Challenges of voltage imaging



- Requires high-speed microscopes due to short duration of action potentials (~2 ms)
- Photobleaching due to frequent, high intensity illumination
- Requires good membrane trafficking of fluorophores
- Requires exceptionally bright fluorescence due to fewer fluorescent proteins in field of view (volumne vs. surface)

Outline of the talk

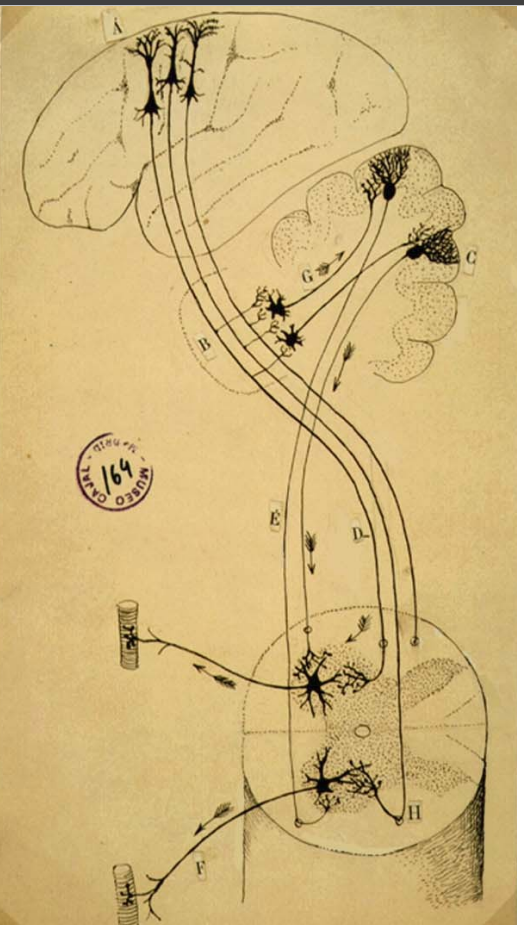


1. Basics of *in vivo* imaging
   - parts list for imaging experiment
   - challenges of deep tissue imaging
   - 1- vs. 2-photon imaging

2. Considerations of *in vivo* imaging in awake animals
   - sensory modalities studied
   - practical implementation : head-fixed vs. 'freely' moving
   - virtual reality systems
   - calcium vs. voltage imaging

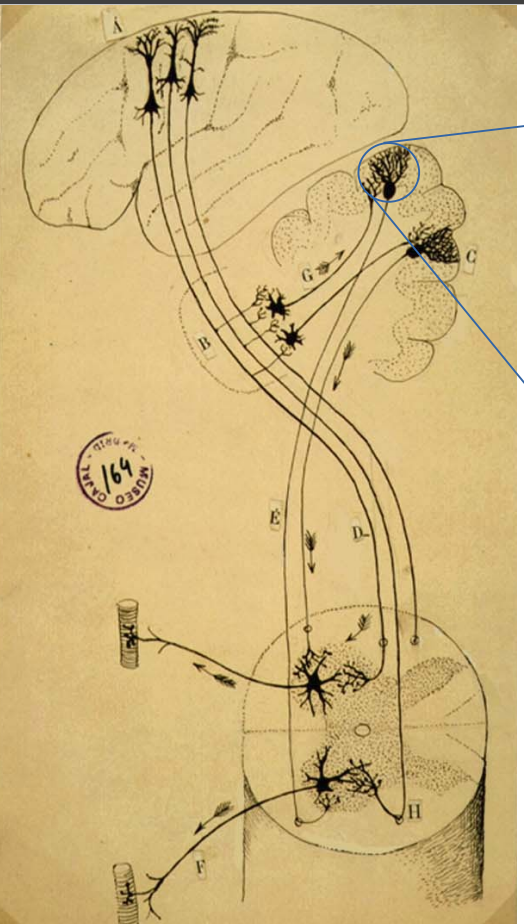
3. Examples from ongoing research
   - Cerebellum and motor control
   - Presubiculum and head-direction neurons

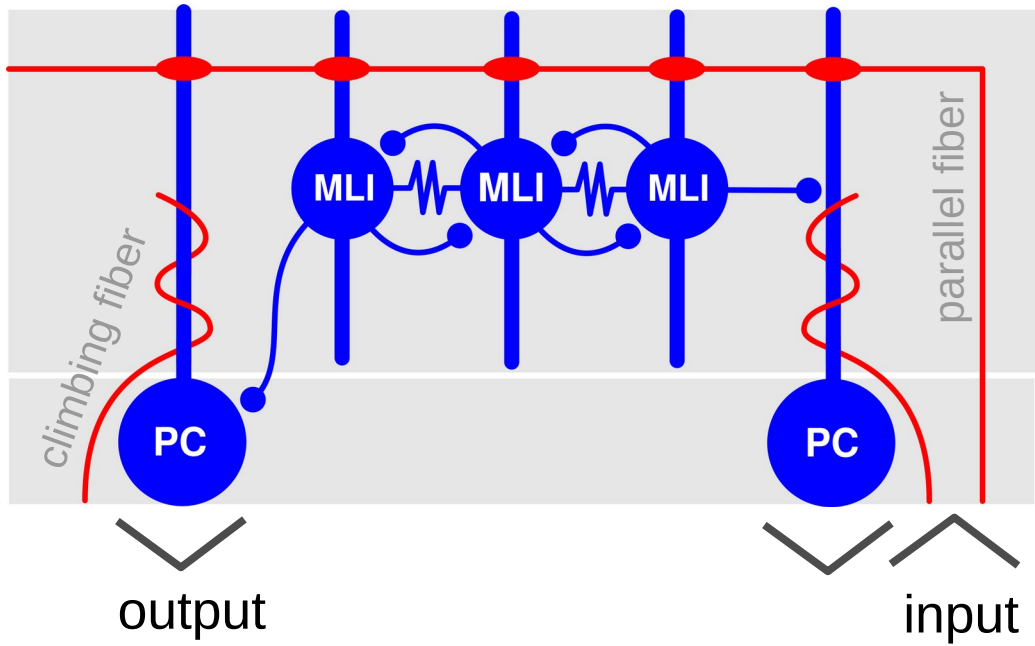
Ongoing project in the lab : Cerebellum and locomotion



- motor neurons in the spinal cord receive inputs from motor cortex and the cerebellum
- neurons in the cerebellum encode motor variables
- role of the cerebellum in motor control unclear

Cerebellar cortex molecular layer interneuron network *in vivo*





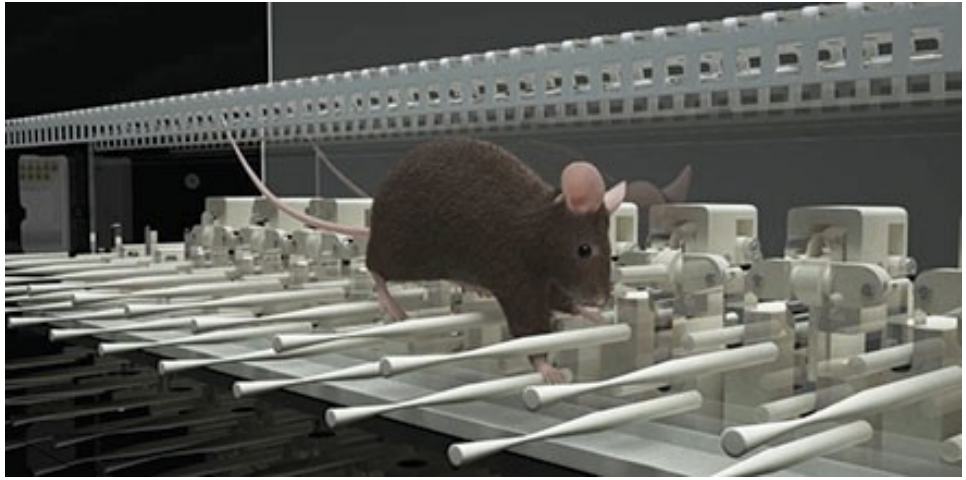
MLI ... molecular layer interneuron

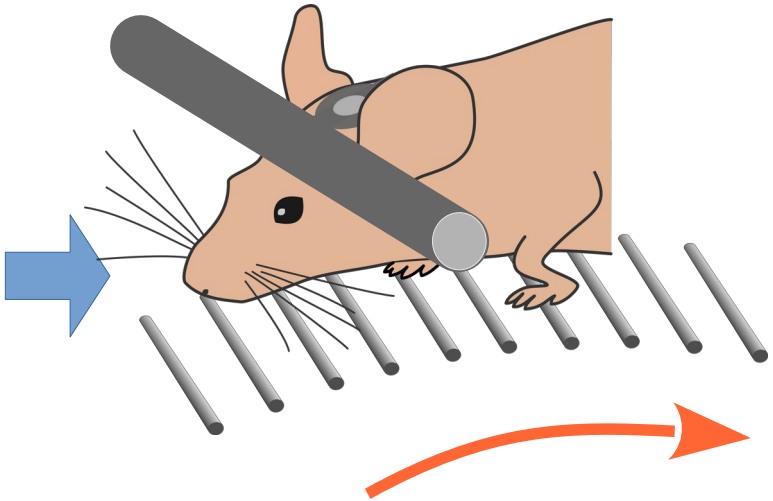
PC ... Purkinje cell

Task to study motor coordination on cellular level

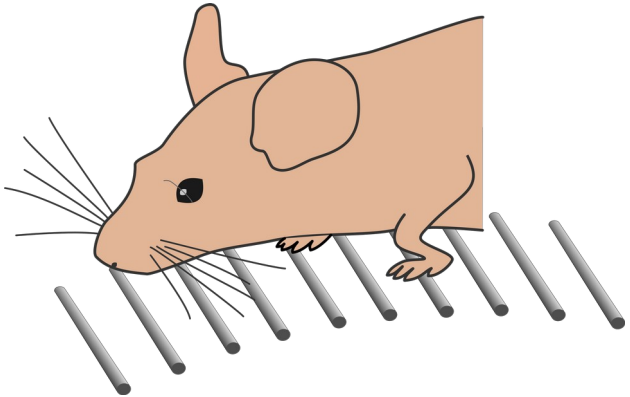
Acquisition of a
complex motor task
in head-fixed animal

Erasmus Ladder | Noldus

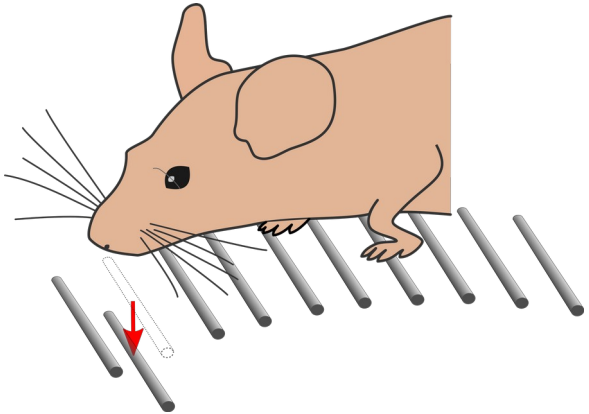




Task to study motor coordination on cellular level

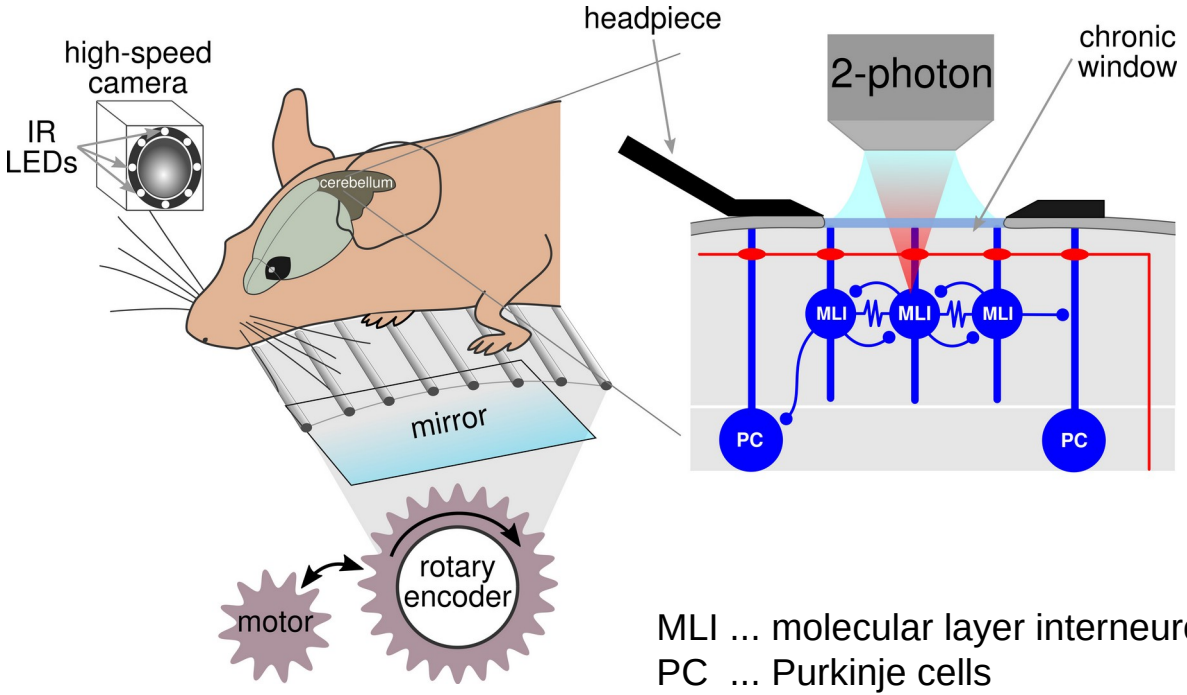


1) acquisition of a complex motor task



2) adaptation of the motor plan to a sudden environmental change

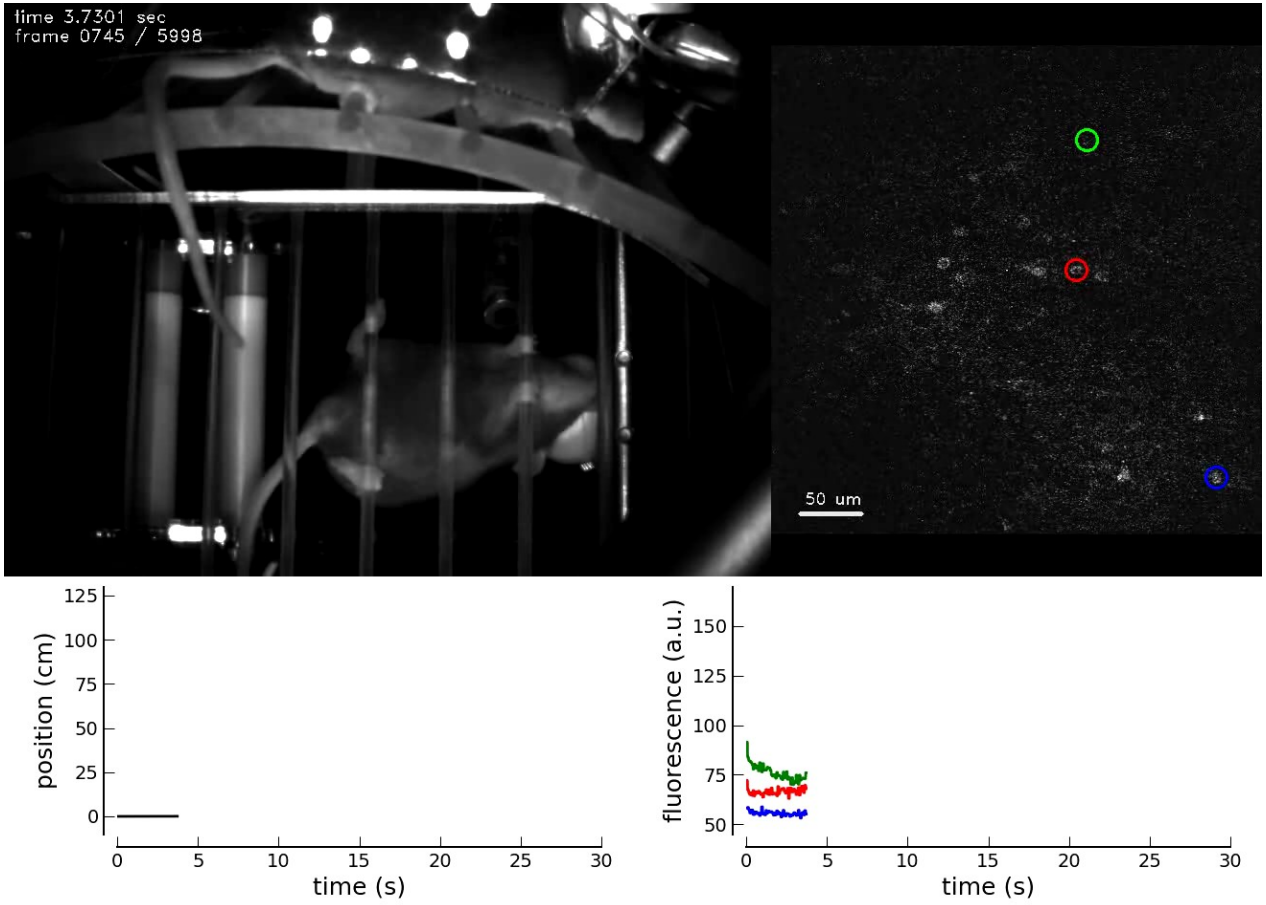
Experimental methods and setup



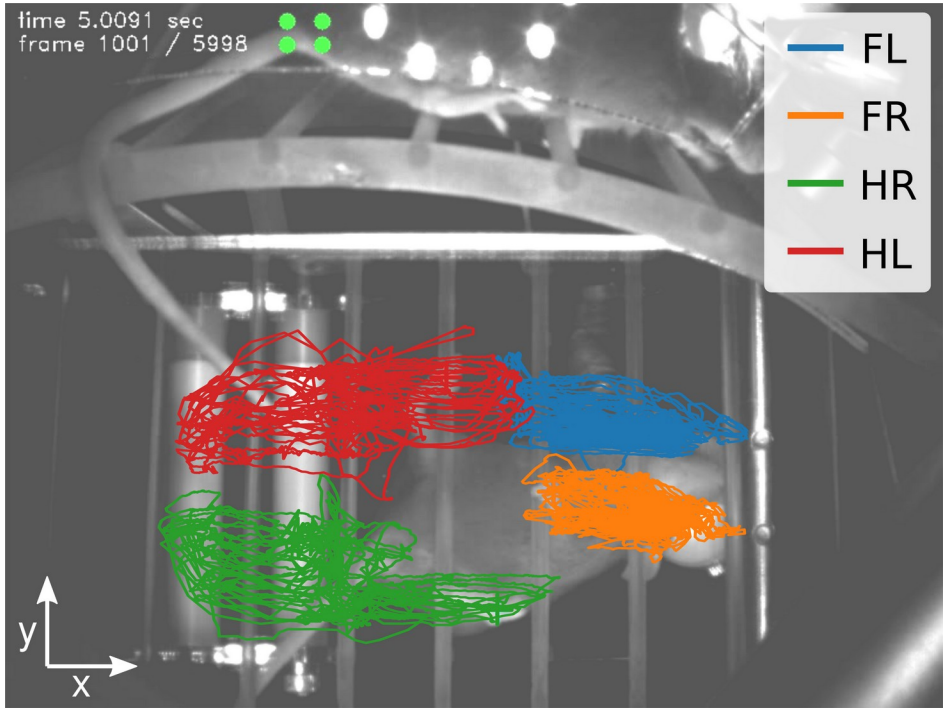
MLI ... molecular layer interneurons
PC ... Purkinje cells

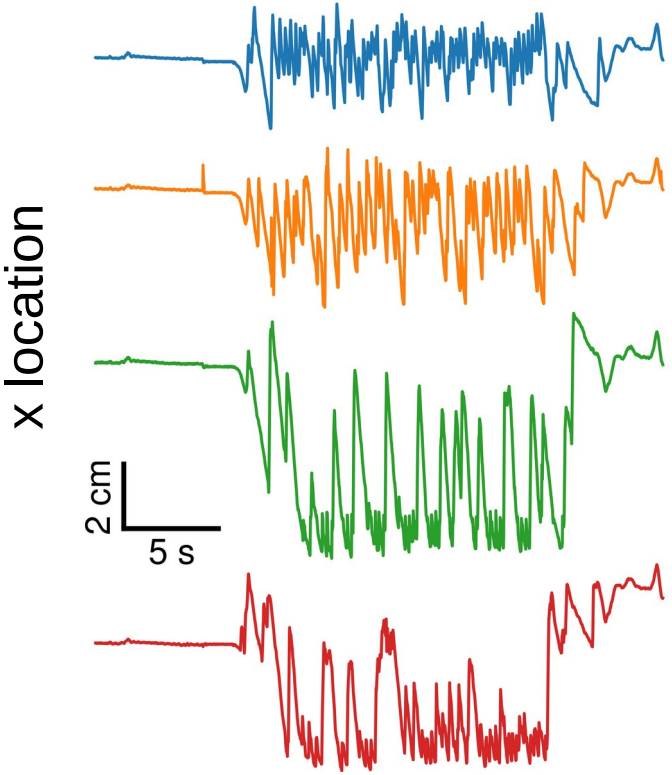
- calcium imaging from molecular layer interneurons (MLIs)
- lobule IV/V in Vermis
- GCaMP6f is expressed through transgenic approach : reporter mouse GCaMP6f-Tigre x promoter mouse PV-Cre

Mouse walking on treadmill with bars (rungs)



Extraction of paw trajectories with DeepLabCut

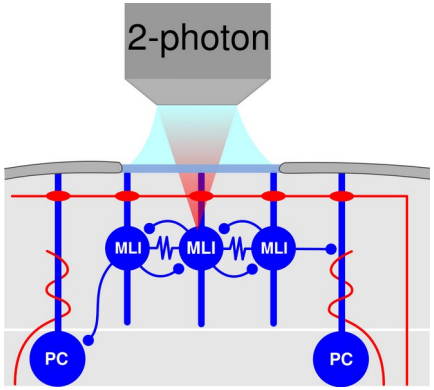


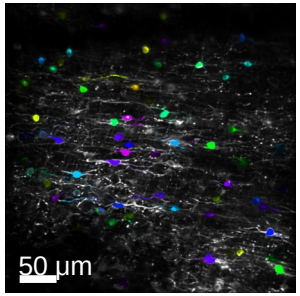


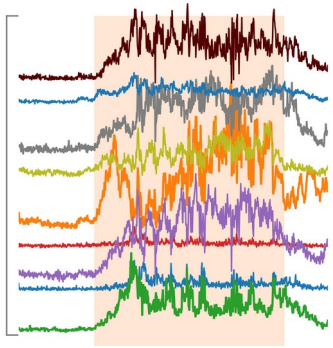
[Mathis *et al. Nat Neurosci* 2018]

Question: Link btw. calcium activity and locomotion?

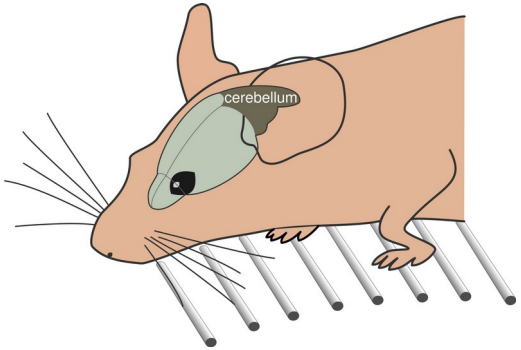
Interneuron activity

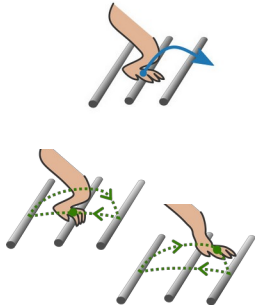


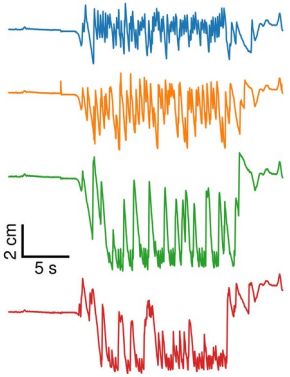




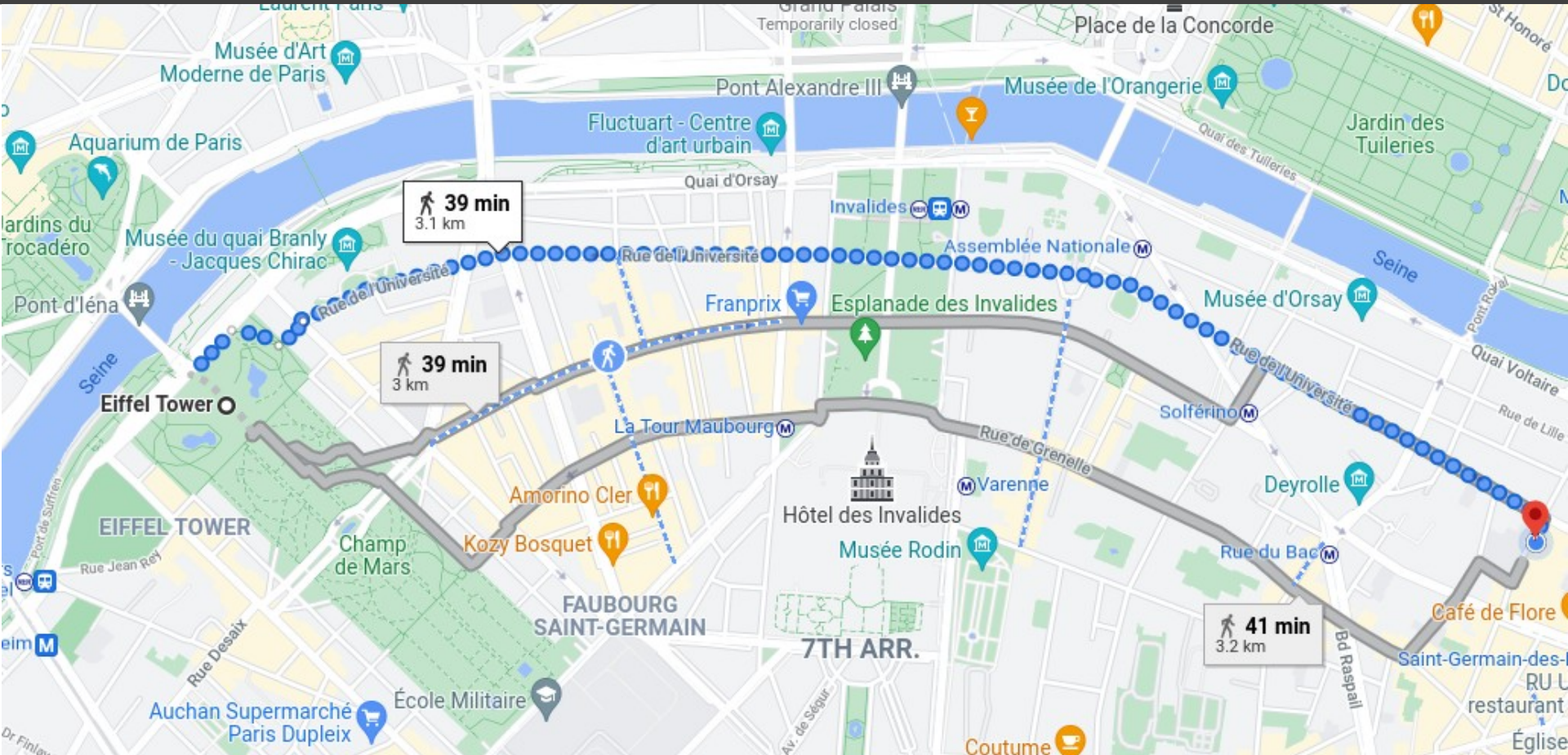
Motor behavior





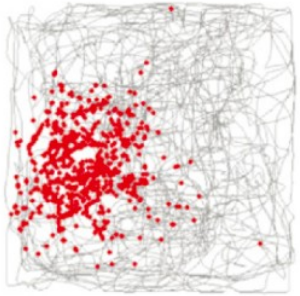


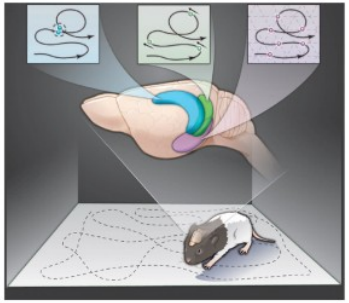
Investigating neural circuits for orientation



Cells and circuits coding for space

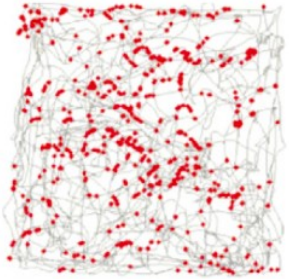
Place cells
hippocampus

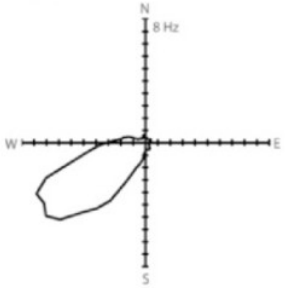




O'Keefe et Nadel 1978

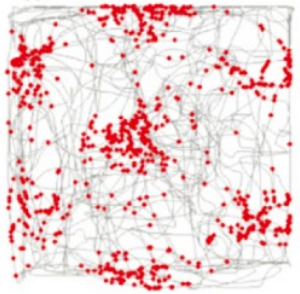
Head direction cells
presubiculum

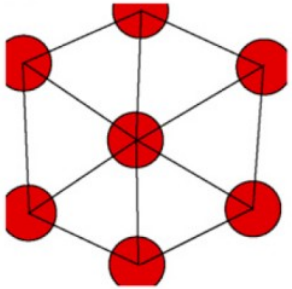




Taube et al. 1990

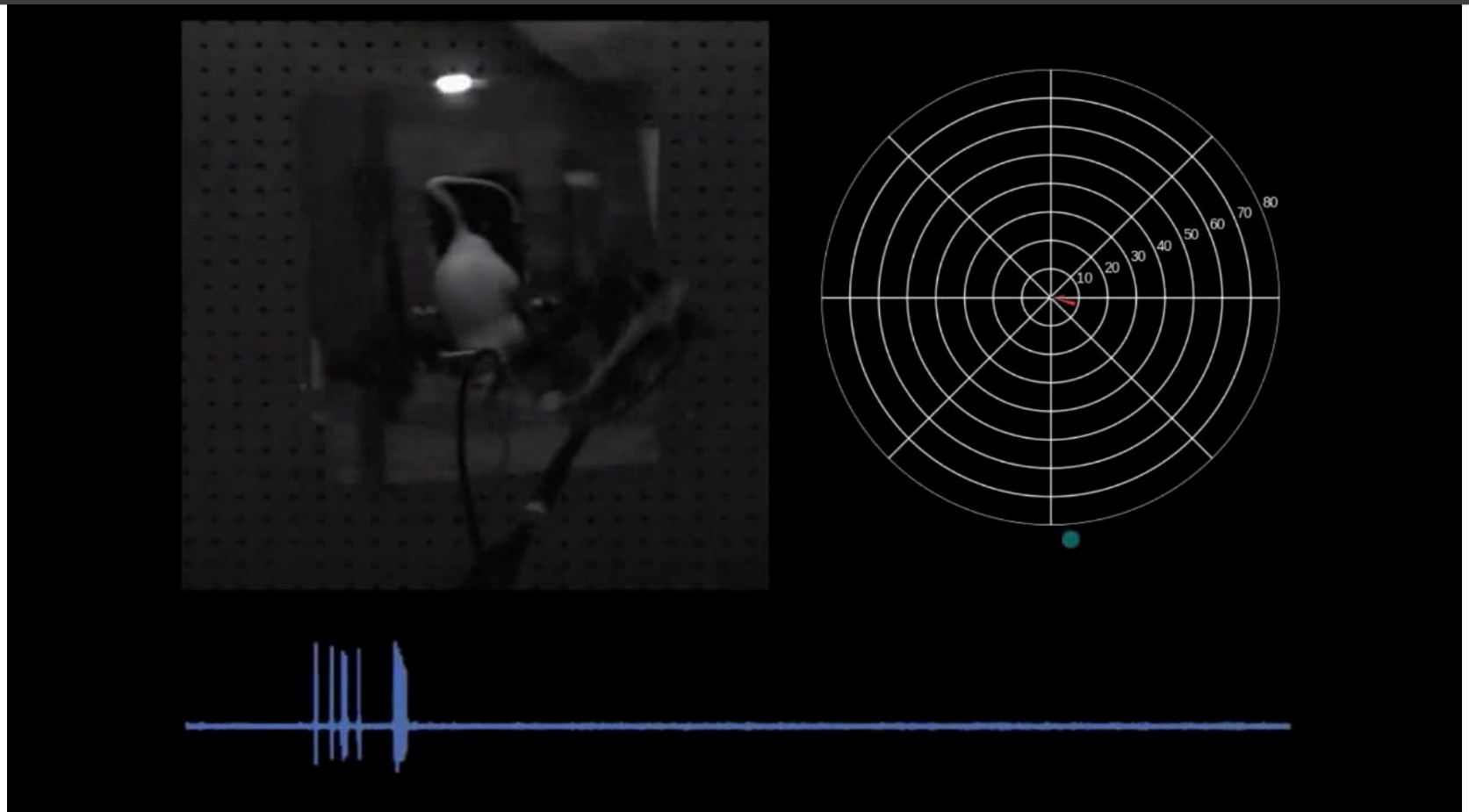
Grid cells
entorhinal cortex



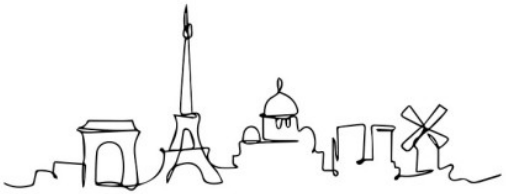


Fyhn et al., 2004

Head-direction neurons in the presubiculum



Presubiculum integrates vestibular and visual inputs



Visual inputs

Visual cortical areas

Thalamic and brain stem areas

Presubiculum

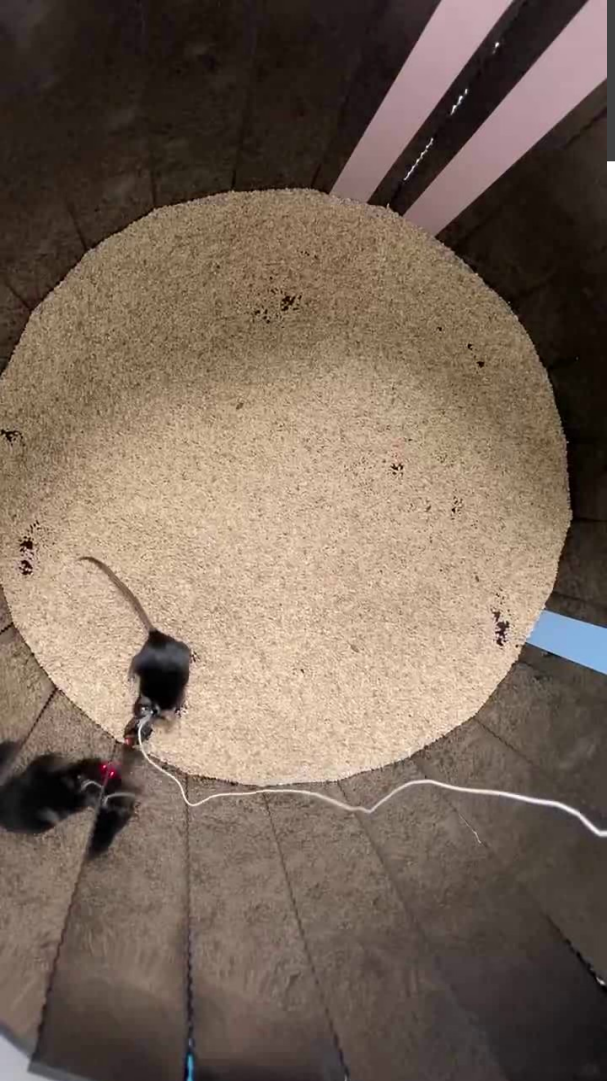
head-direction cells

Vestibular inputs

Question :

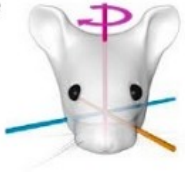
→ How is the head-direction signal encoded by populations of neurons in the Presubiculum ?

Calcium imaging in presubiculum

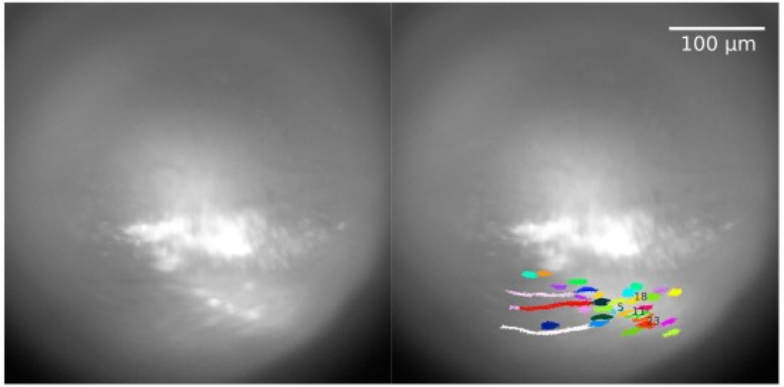


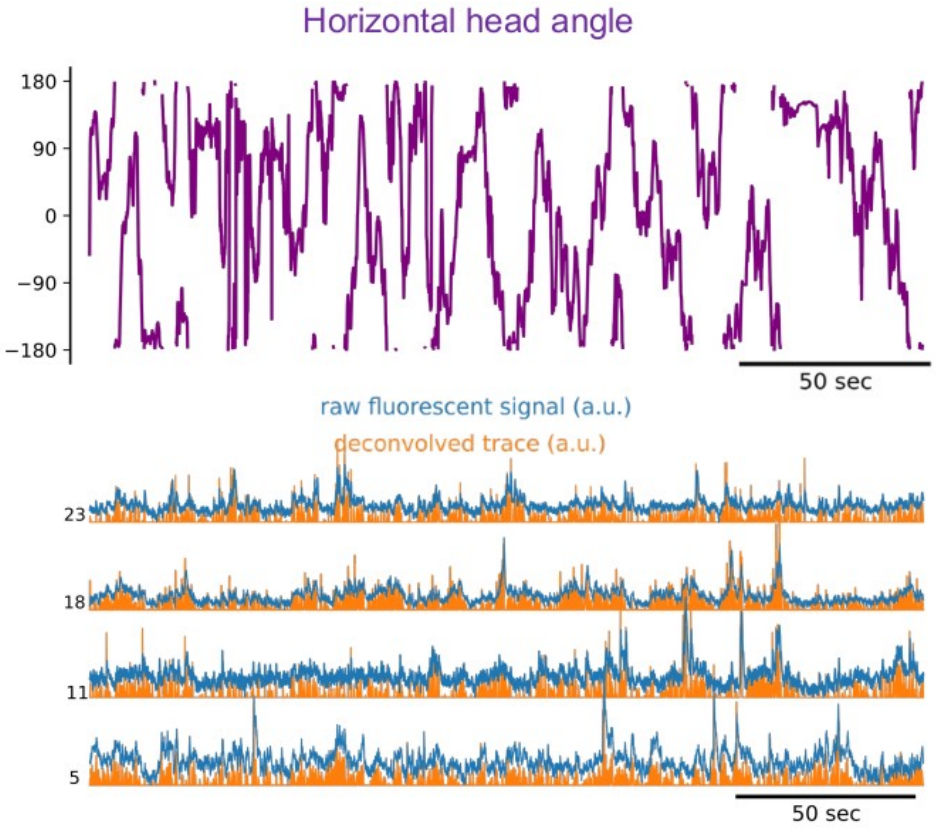
Calcium imaging in presubiculum

- Horizontal head angle

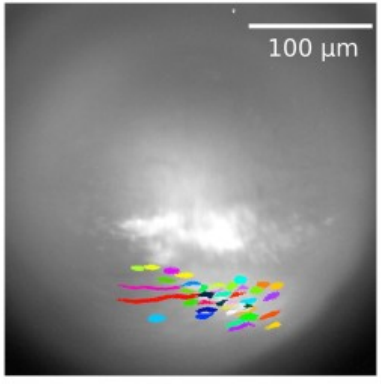


- Calcium imaging

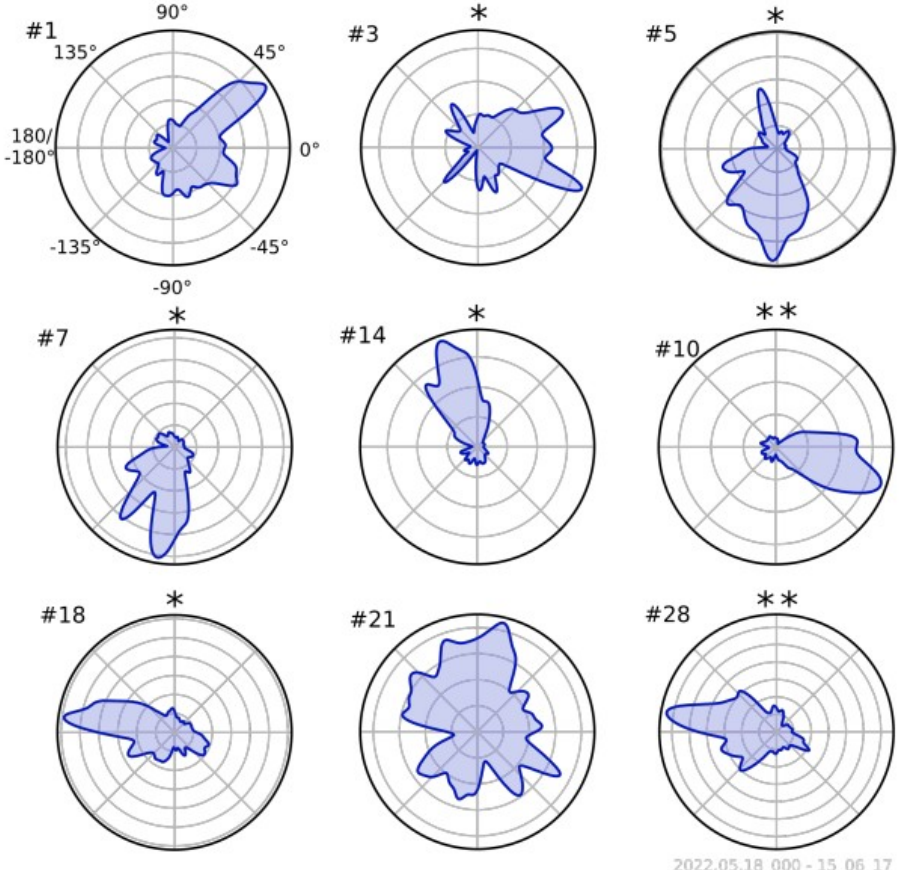




Experiments with miniscope : head-direction neurons



- 41 ROIs in total, 29 significantly HD tuned



In vivo imaging as tool to study sensorimotor integration