SAINTS-PERES
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In vivo imaging in awake animals

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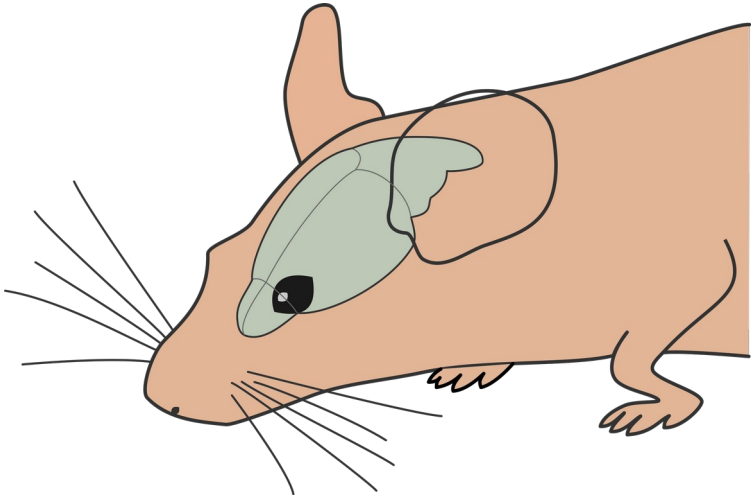
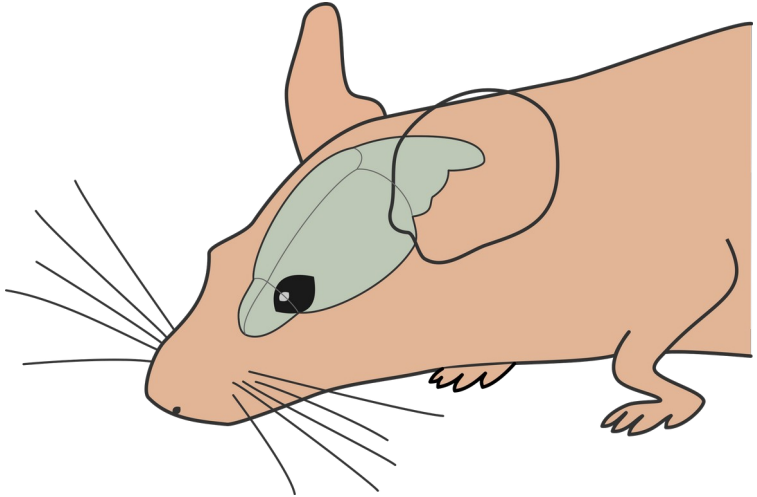
Aim

- 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

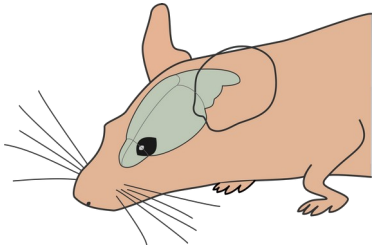


Challenges

- 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 : optical access, head-fixed vs. 'freely' moving
   - virtual reality systems
   - movement artifacts
   - calcium vs. voltage imaging
3. Cerebellum and motor control

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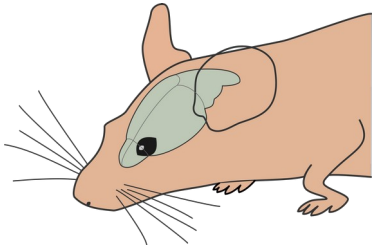

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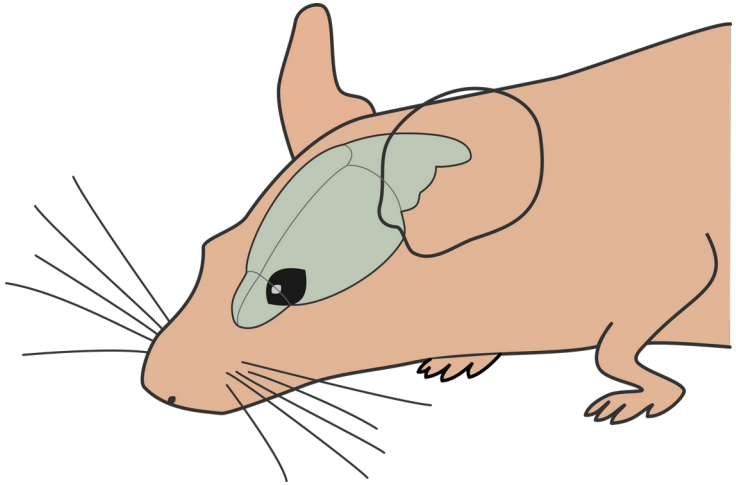
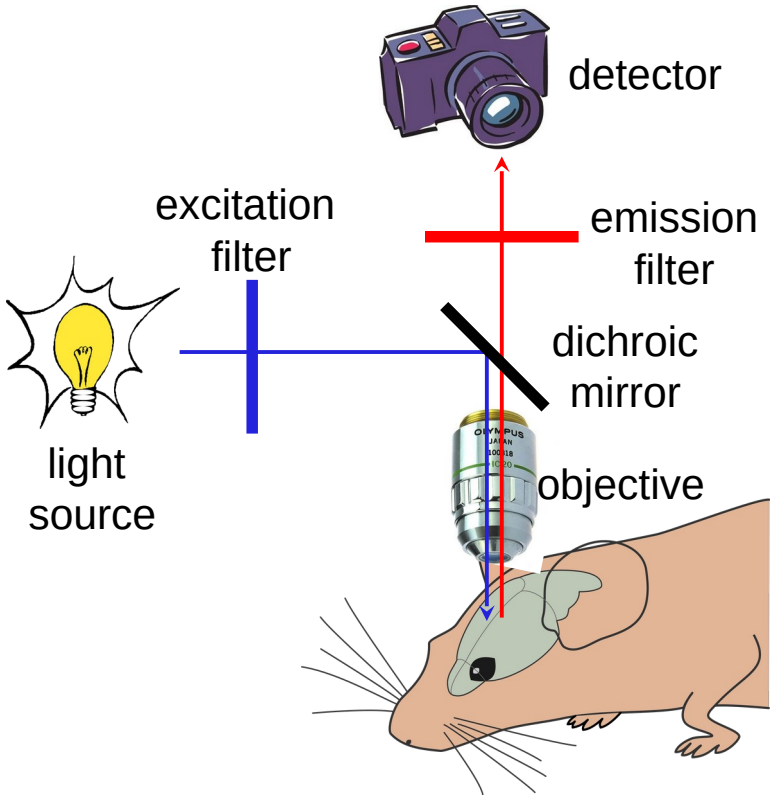
General parts list for *in vivo* imaging

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

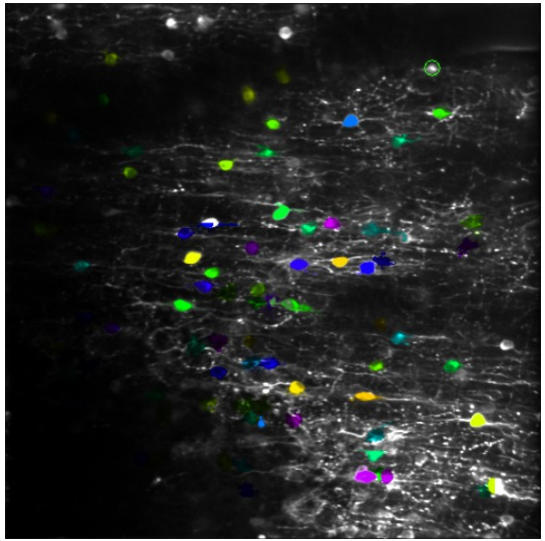


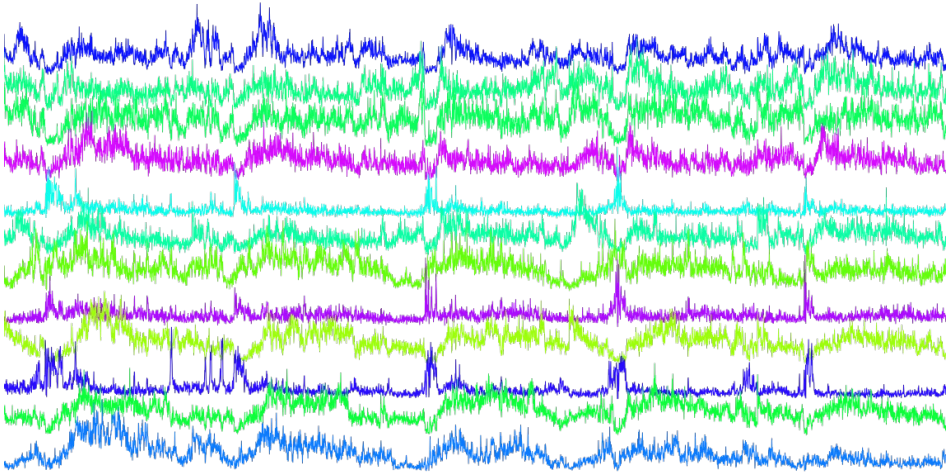
General parts list for *in vivo* imaging



- **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,...

Current method of choice : Calcium imaging using GECIs



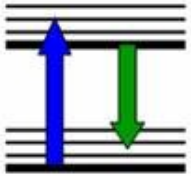


- Genetically encoded calcium indicators (GECIs) can be targeted to specific neuron populations
- Calcium transients serve as proxy readout of neural activity
- Non-invasive and repeatable means to measure neural activity from large populations of neurons

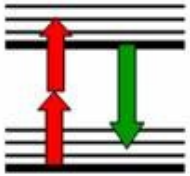
Fluorescence induced by 1- or 2-photons

fluorophore

1-photon excitation

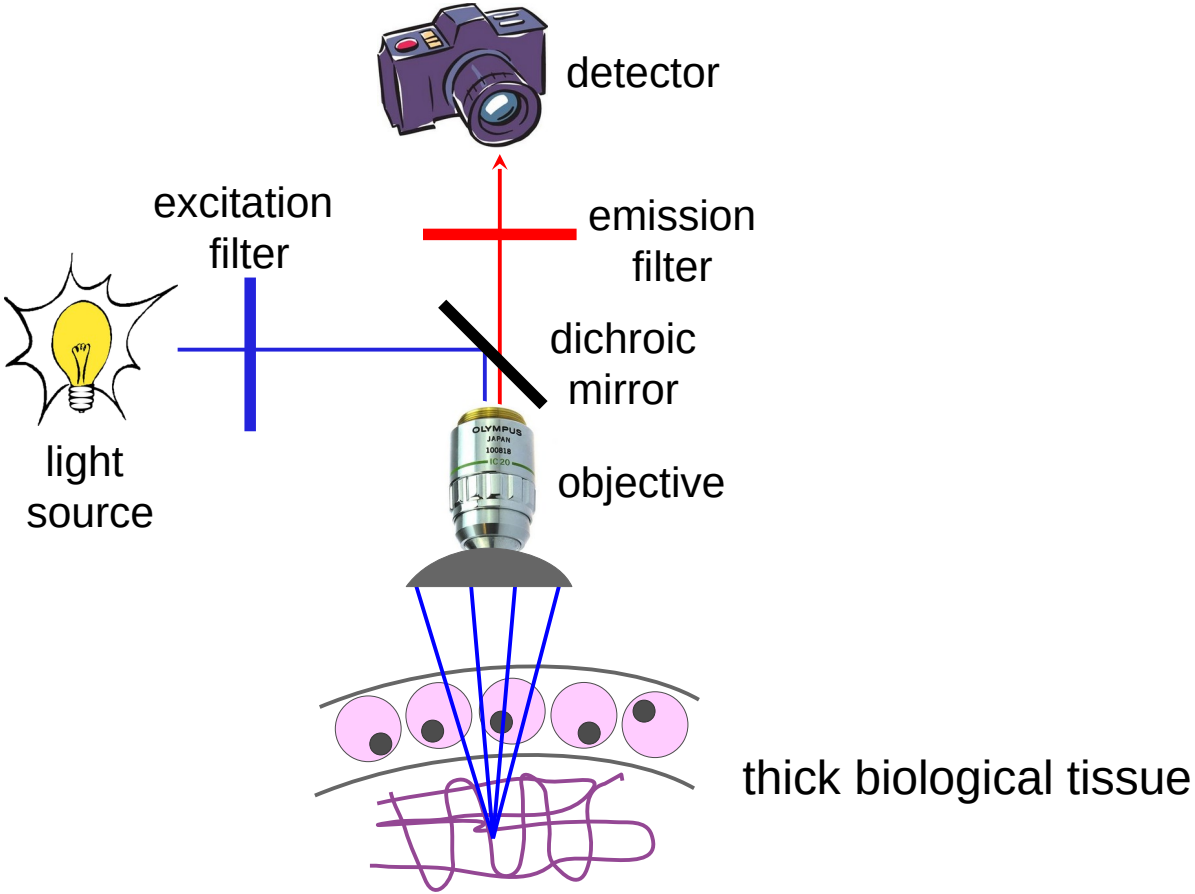


2-photon excitation

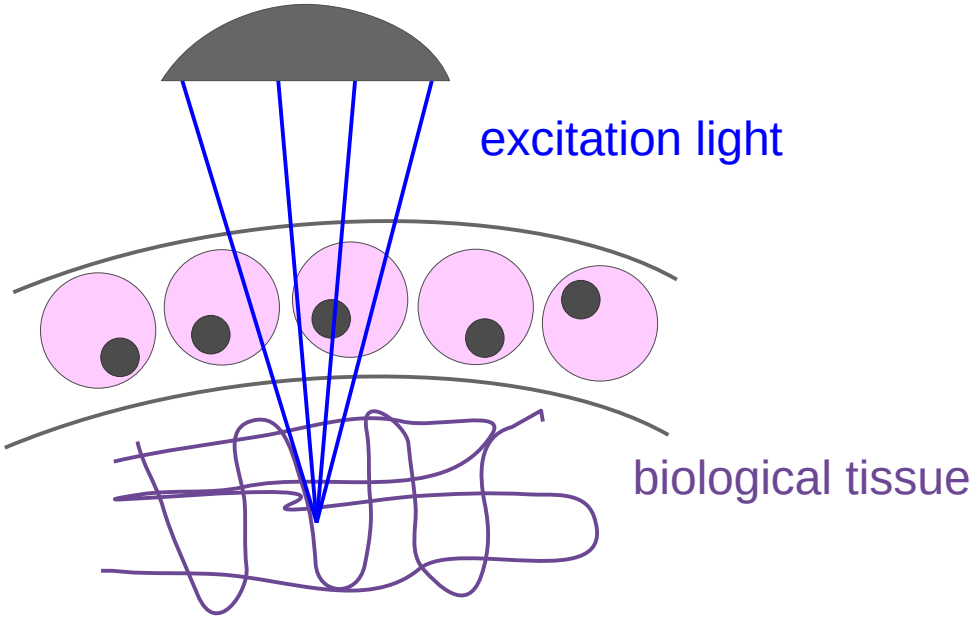


- Fluorescence: emission of light by the fluorophore that has absorbed light; emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation

Challenge: optical access to tissue to be imaged

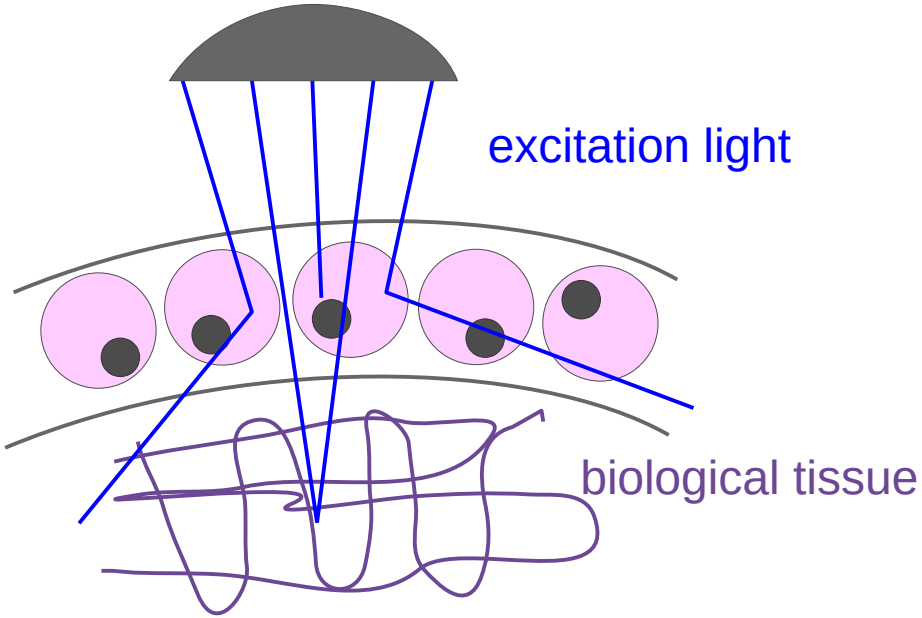


Imaging of thick biological tissue



Ideal case

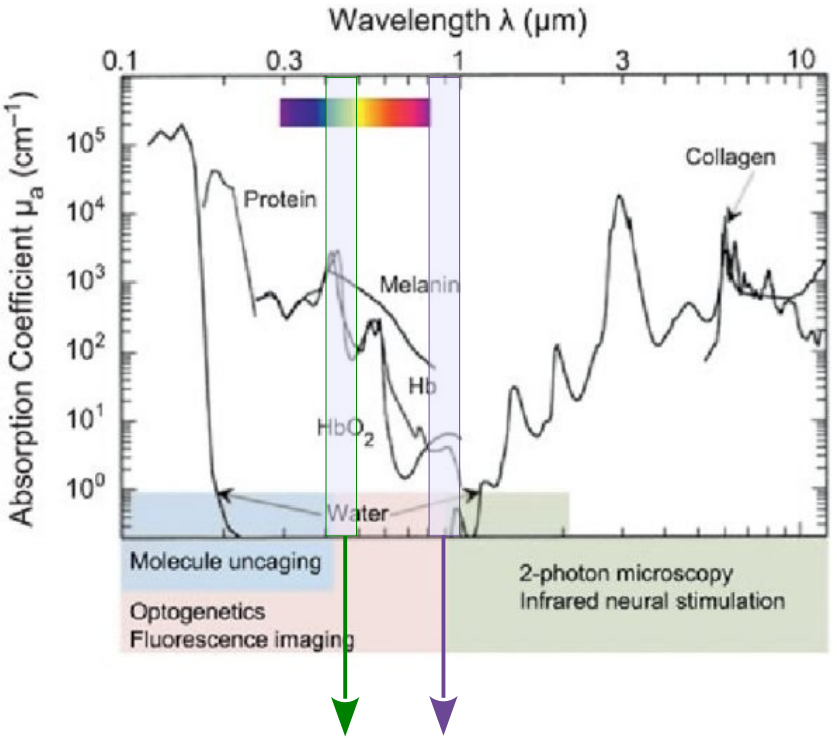
What limits imaging depth ?



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

One photon vs. 2-photon fluorescence : absorption

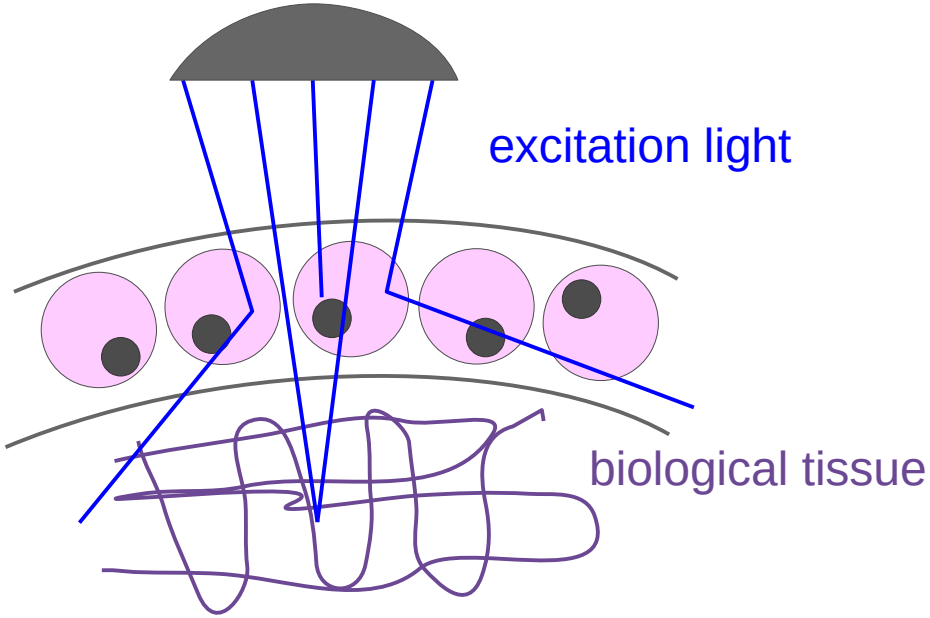


one photon excitation range

two-photon excitation range

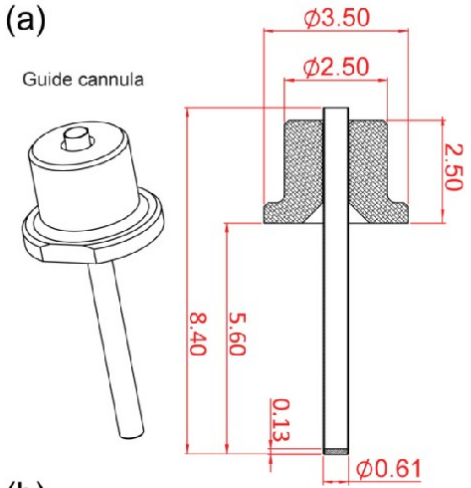
- commonly used fluorescent dyes have excitation spectra in the 400 to 500 nm range → wavelengths used to excite the same dyes with two-photons tend to be between about 800 and 1000 nm
- infrared light can penetrate deeper in biological tissue due to little absorption
- commonly used: titanium-sapphire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

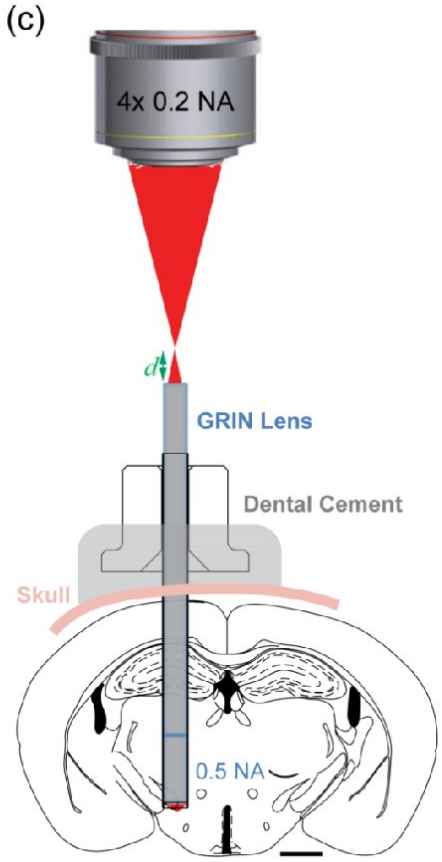
One photon vs. 2-photon fluorescence : scattering



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

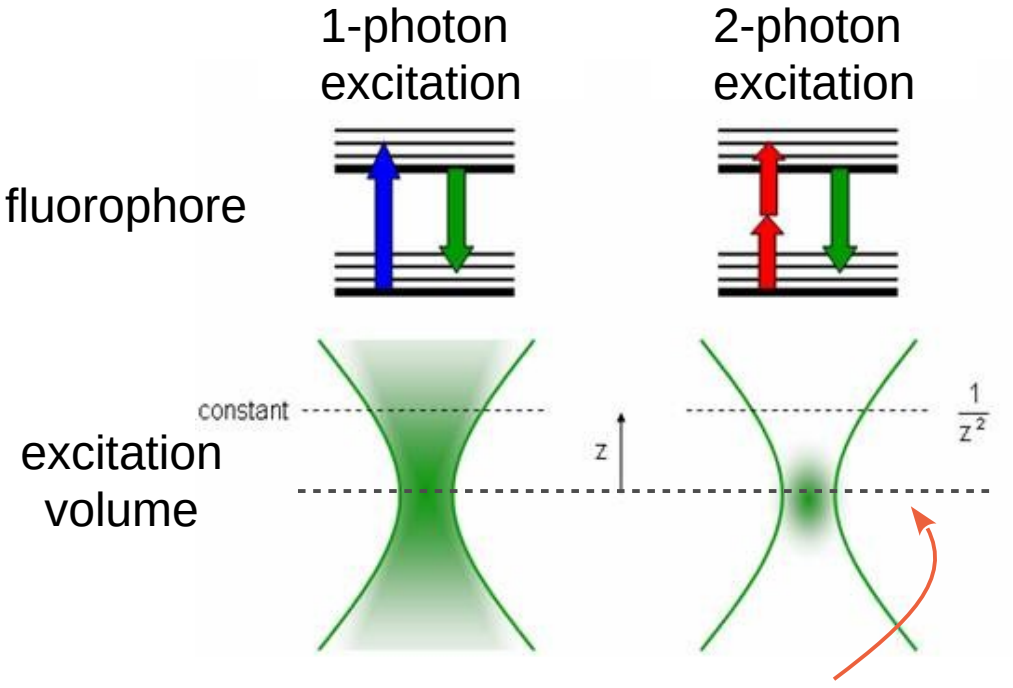
Improved access to deep tissue with GRIN lens





- 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

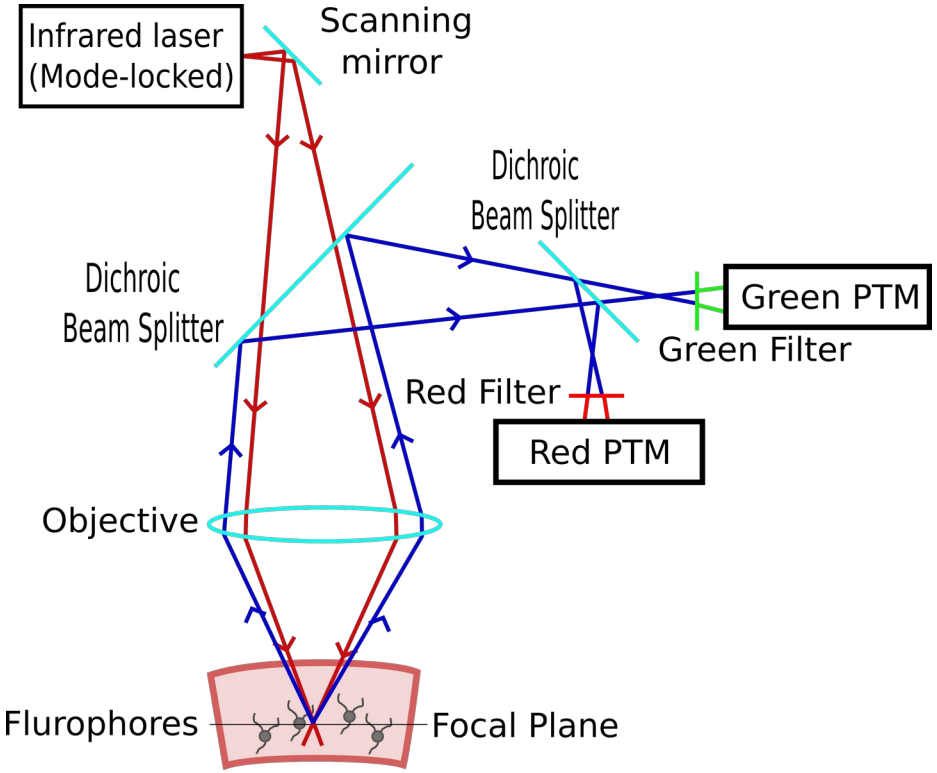
One photon vs. 2-photon fluorescence : resolution



[z is distance from focal plane]

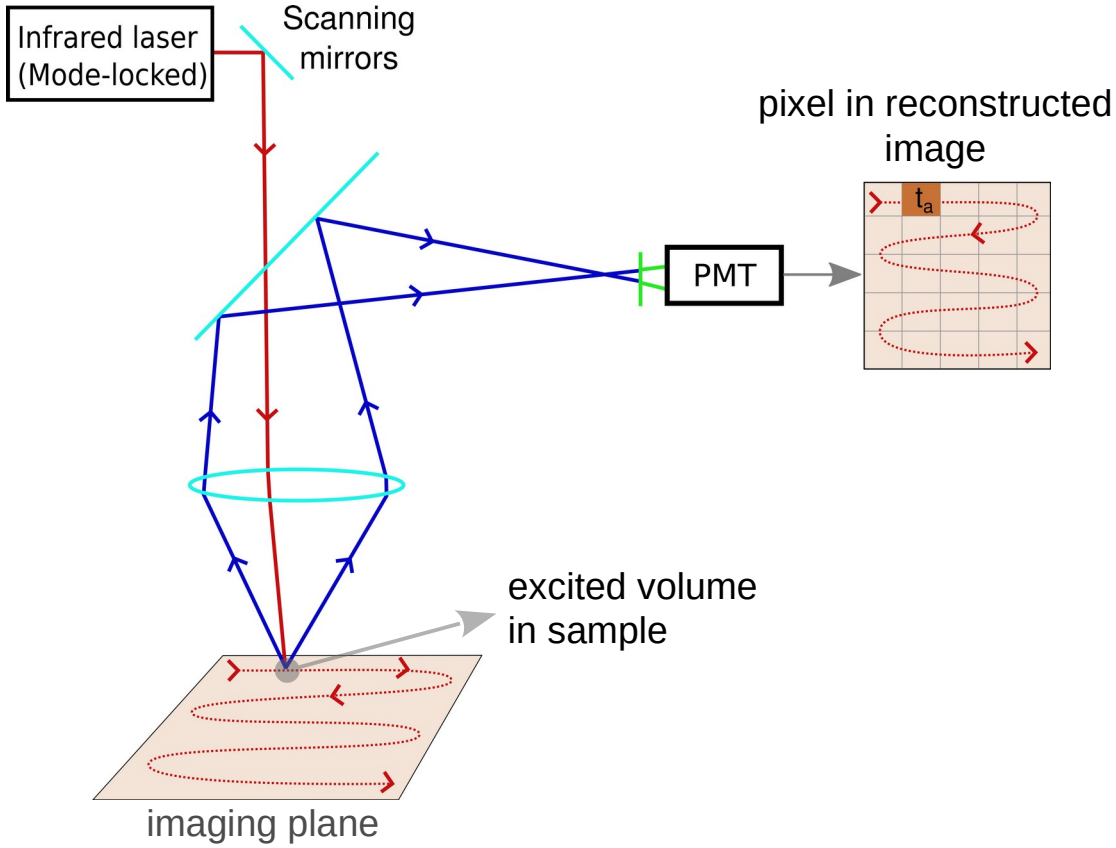
- excitation volume/fluorescence is confined to the focal center of the laser beam
- both photons must arrive nearly simultaneous (< 1 fs)
- fluorescence falls off as ~$1/z^2$, while it falls off as 1/z with single photon excitation
  → 3D-imaging with out-of-focus background rejection similar to a confocal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

Parts list for 2-photon *in vivo* imaging



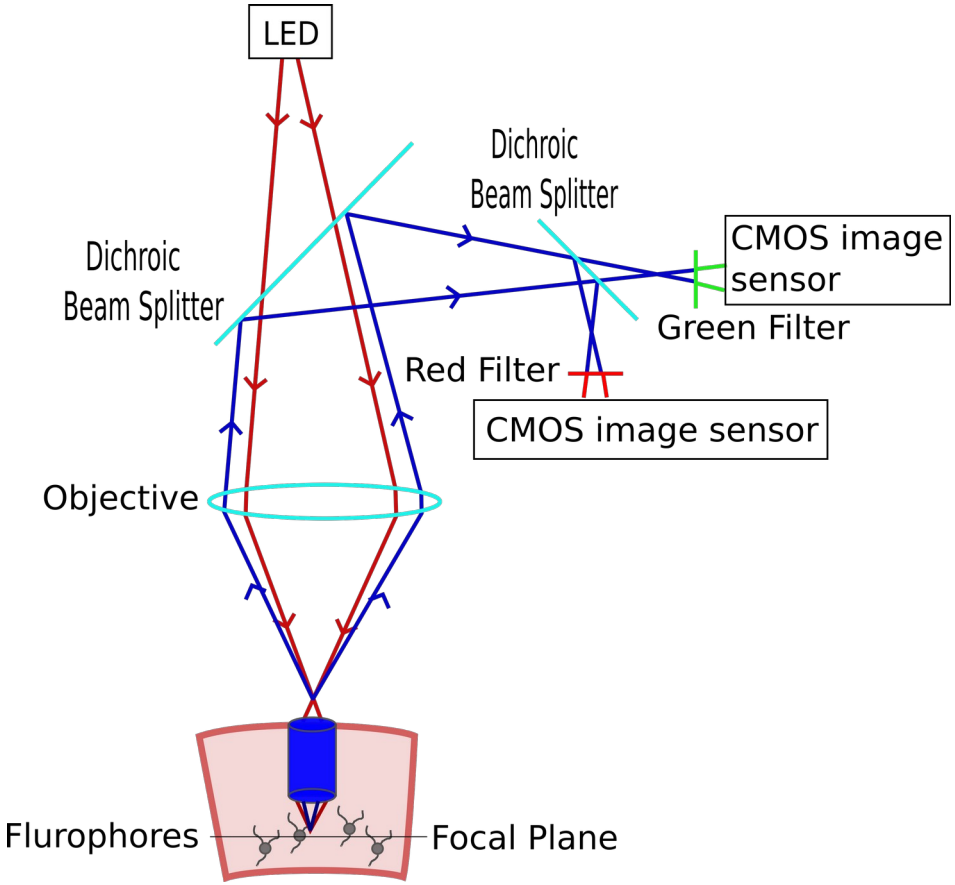
- **Light source**: laser producing light pulses on the order of femtoseconds (10^{-15} s)
- **Excitation filter**: not required since laser produces single wavelength
- **Scanning mirrors:** directs/scans the laser beam over the sample
- **Dichroic mirror**
- **Objective**: focuses light on region of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

2-photon imaging : functioning



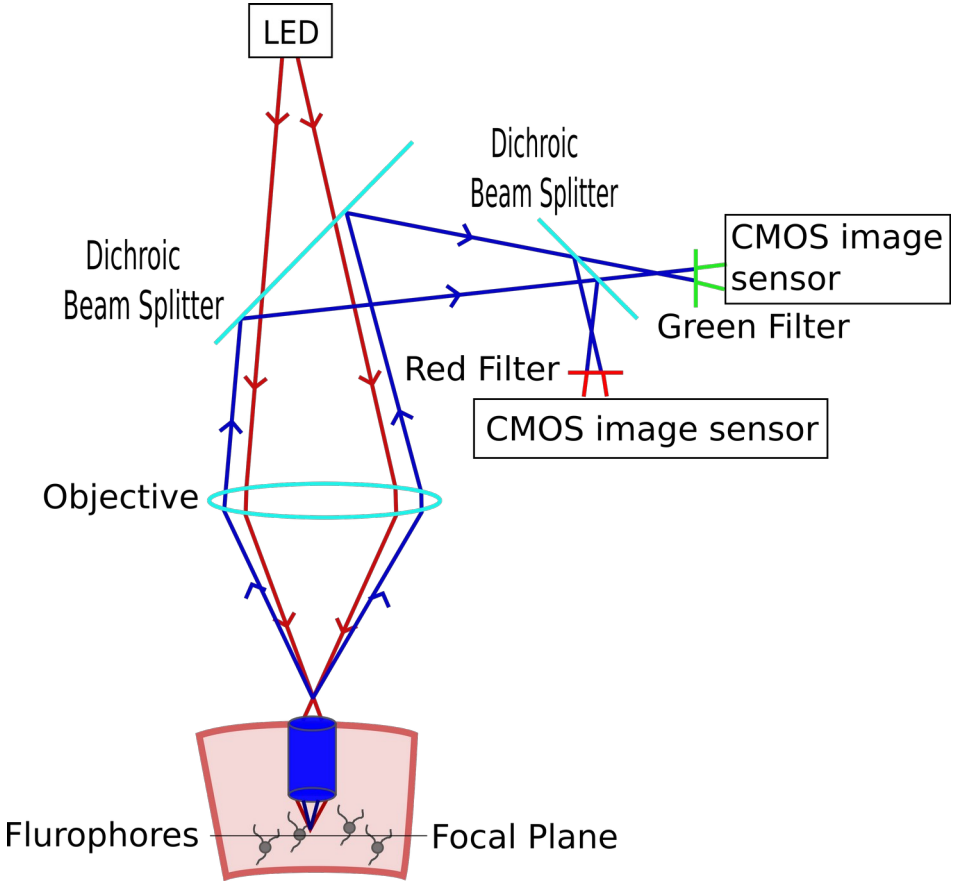
- A small excitation volume is excited by the laser light – defines resolution
- All fluorescent light is collected from the sample (indirect and direct light)
- Scanning mirrors move the laser beam across the imaging region – sequential acquisition of image (typical frame rate 30 Hz)

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 photos 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

1-photon (epifluorescence) imaging

- poor resolution makes it impossible to image neurites or spines
- insertion of GRIN lens destroys neural tissue above the region to be imaged

Disadvantages

2-photon imaging

- lasers needed are expensive, large, complicated and consume a lot of power
- no complete commercially available systems
- 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 head-fixation of the animal (but see new developments)

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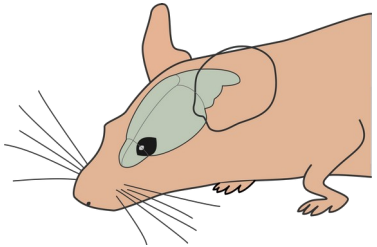

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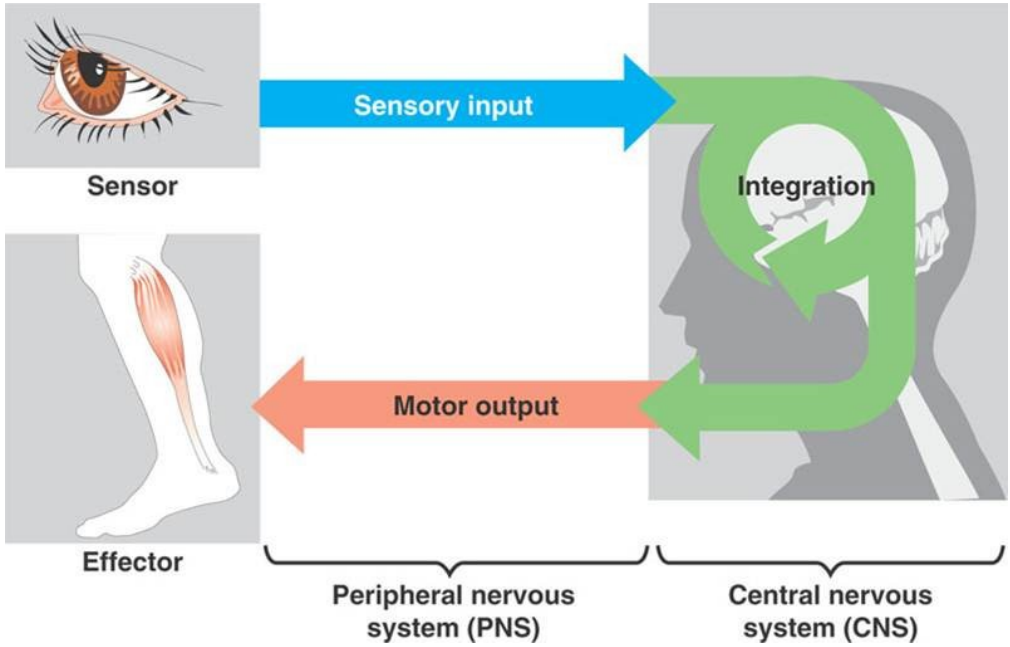
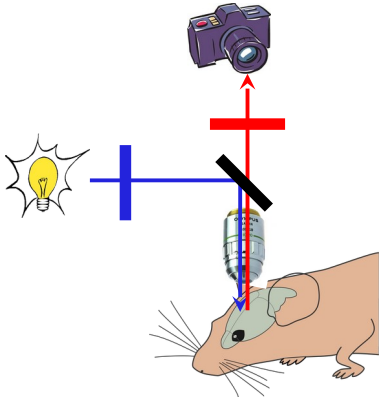
3. Cerebellum and motor control

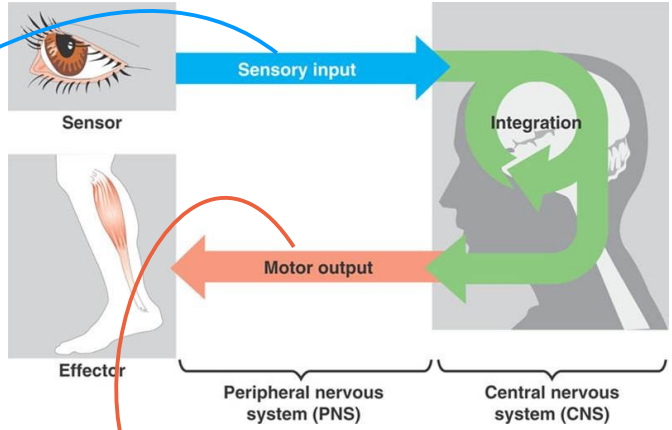
Rational behind *in vivo* experiments



- **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** : sensorimtor processing varies with behavioral state/output
- **interactive setting** : study sensoriomotor interactions with the outside world

Feasibility of *in vivo* imaging experiments

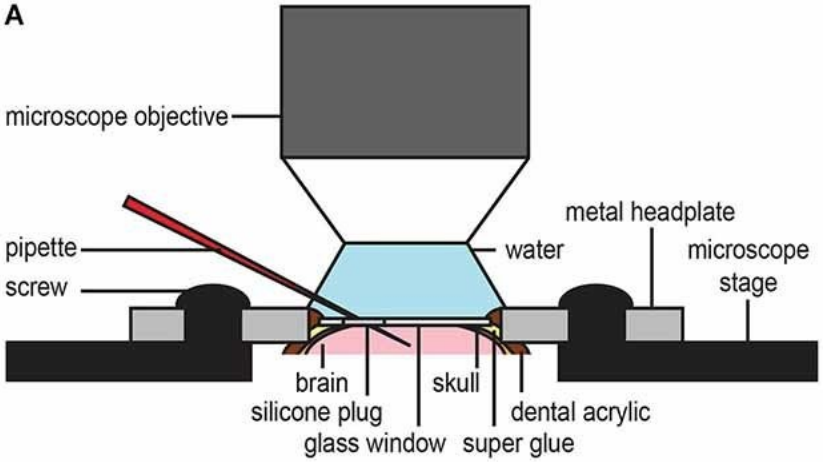


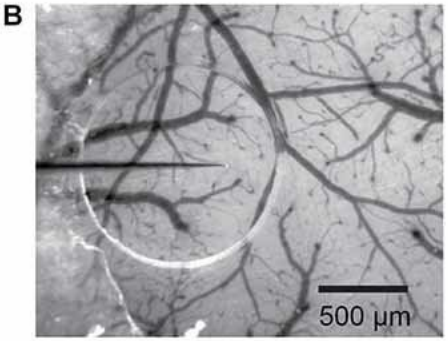


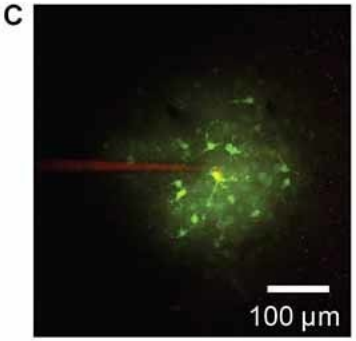
- **sensory stimuli :**
  - easy to implement : touch (whisker), vision (static), smell, taste, sound
  - difficult : vision (dynamic), equilibrium (vestibular)

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

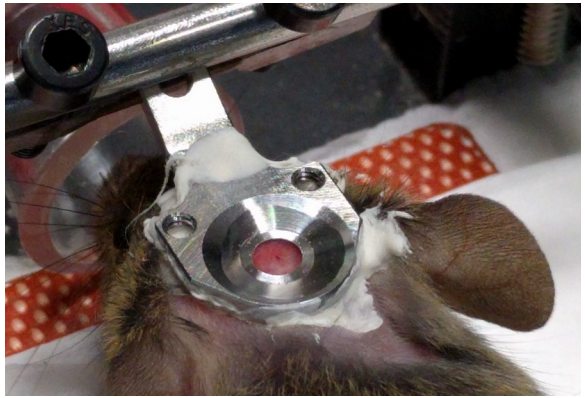
Optical access through chronic window



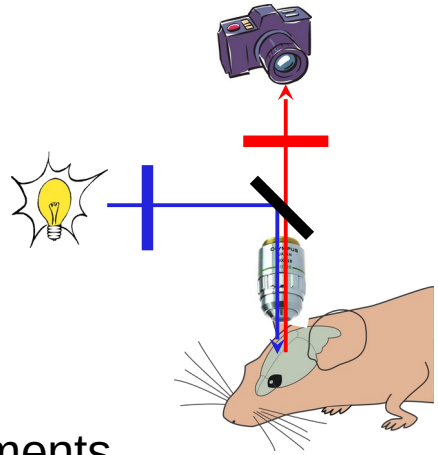




- Transparent window implanted in place of skull over region of interest
- bone thinning can provide sufficient visibility
- access port can allow for additional electrode access



Assure stability btw. imaging system and imaging tissue



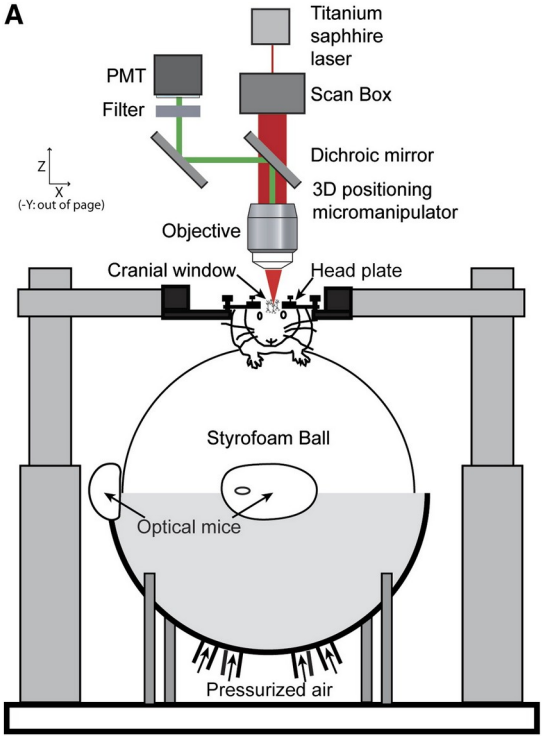
1) Minimize relative movements between animal to be imaged and the microscope

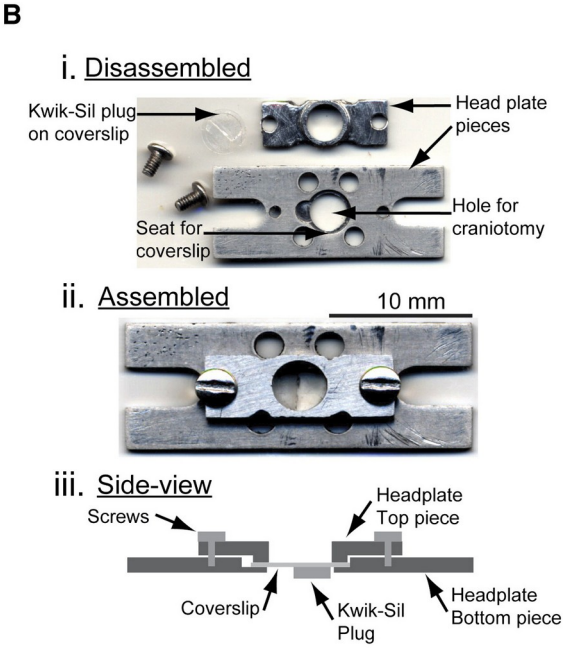
→ fix the animal head under the microscope

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

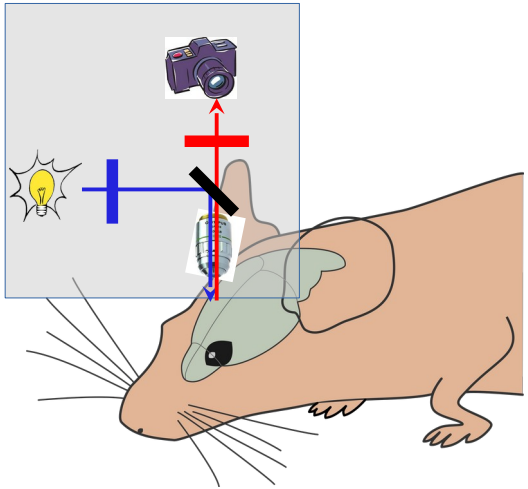




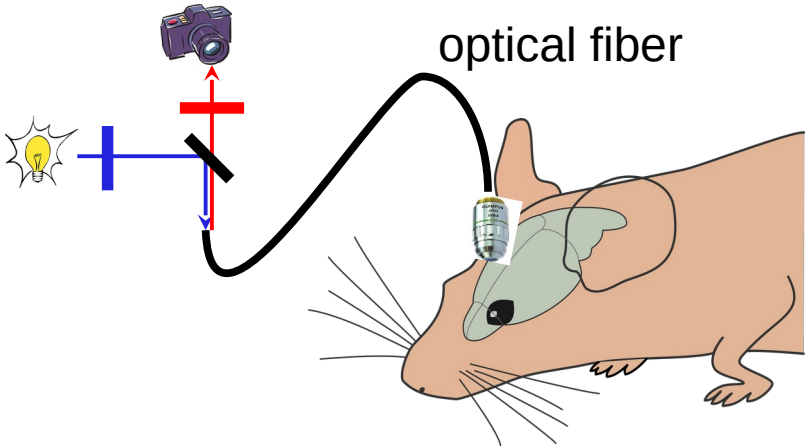
[Dombeck *et al.* Neuron 2007]

- 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

'Freely' moving animal solutions



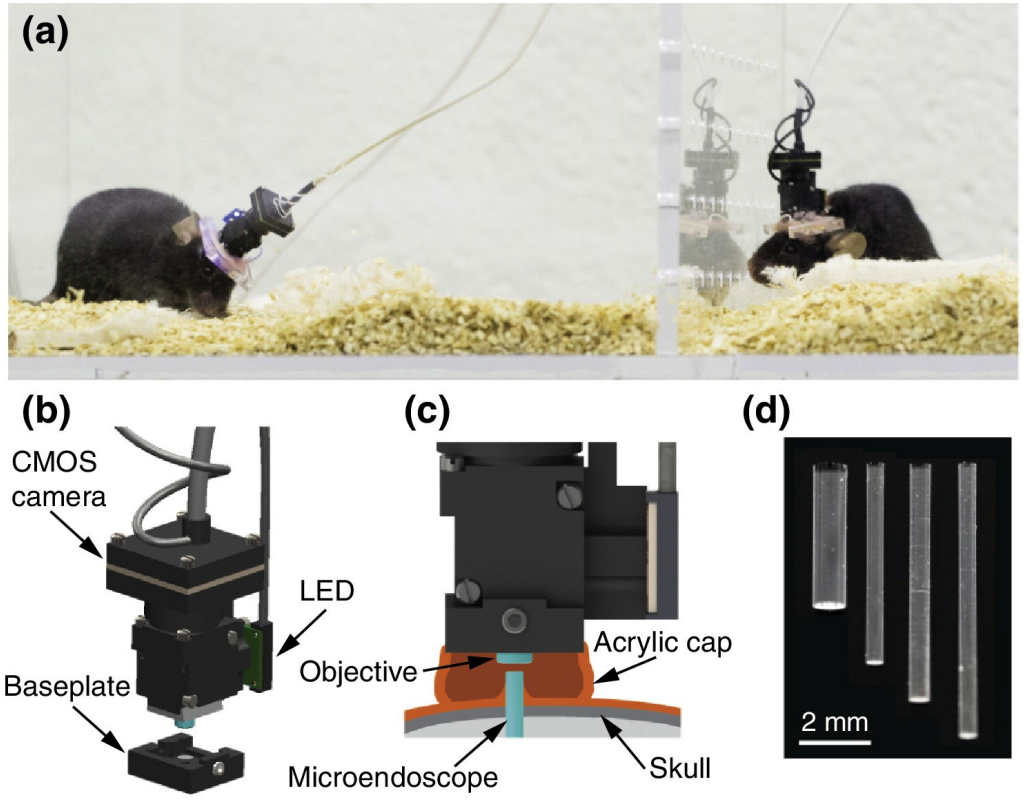
- miniaturized microscope mounted on animals head
- feasible for epifluorescence imaging



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

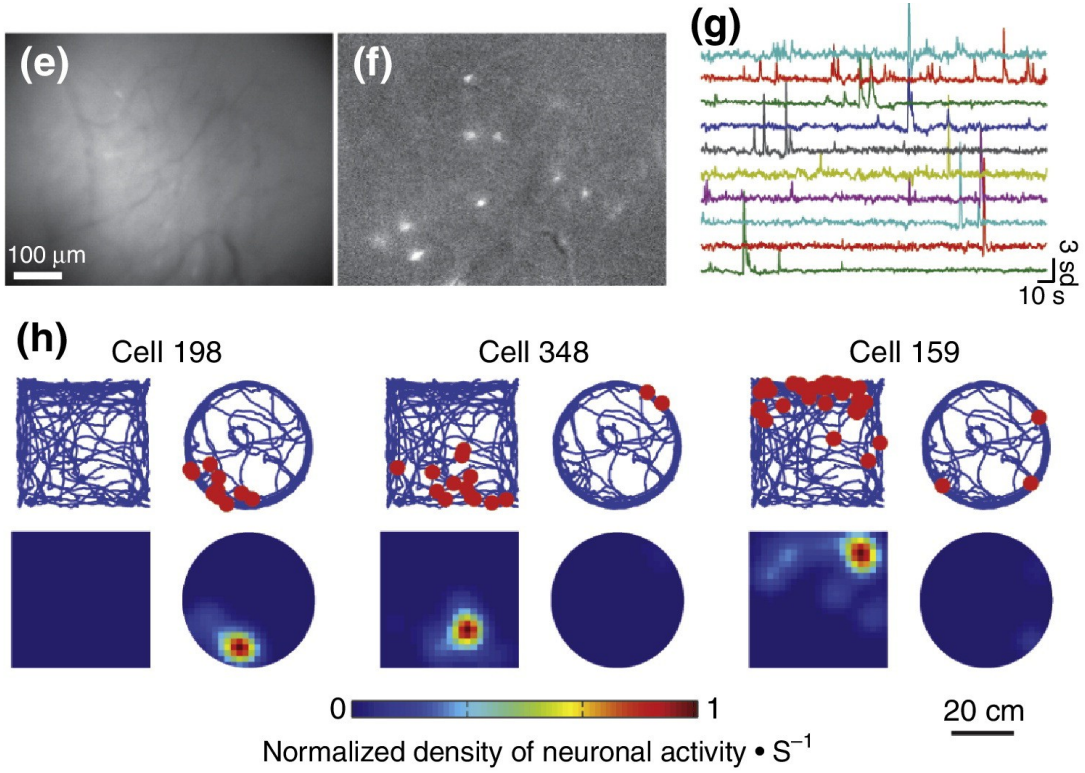
Head-mounted wide-filed epifluorescence (1-p imaging)

weight ~ 2g



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

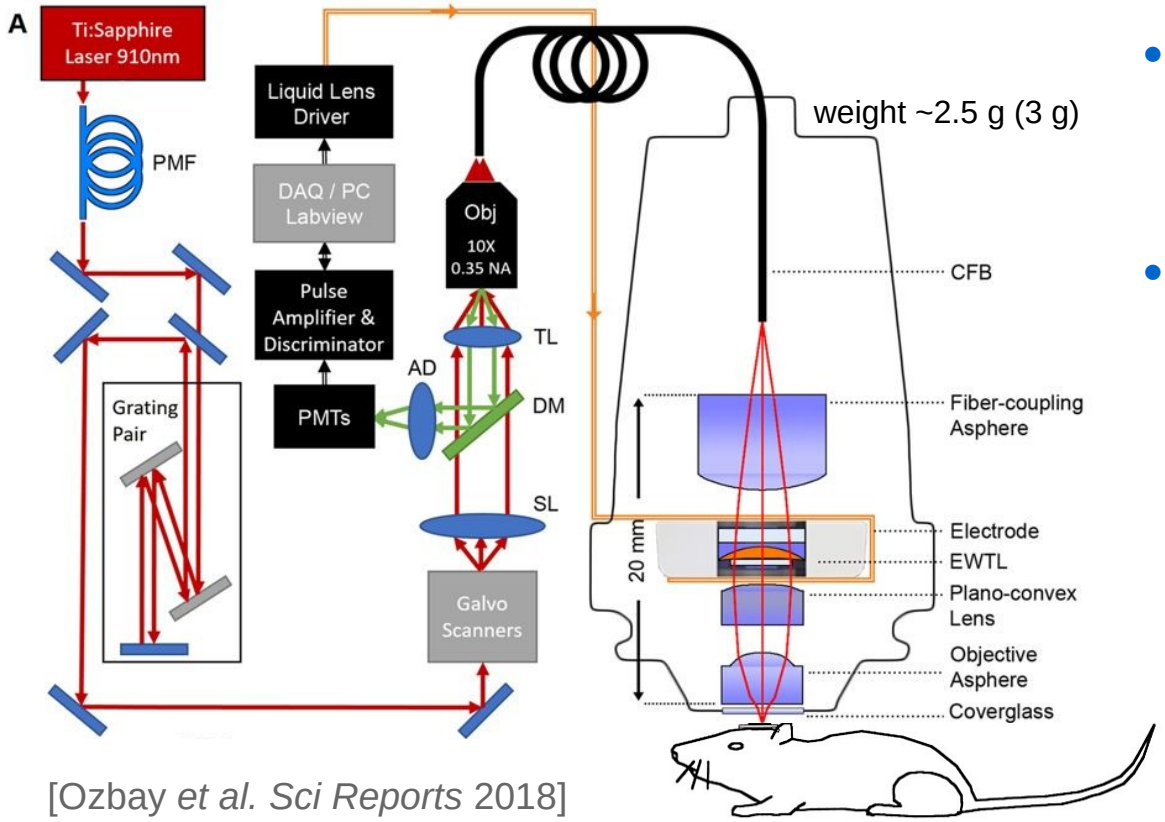
Hippocampal Ca dynamcis in behaving mice



[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

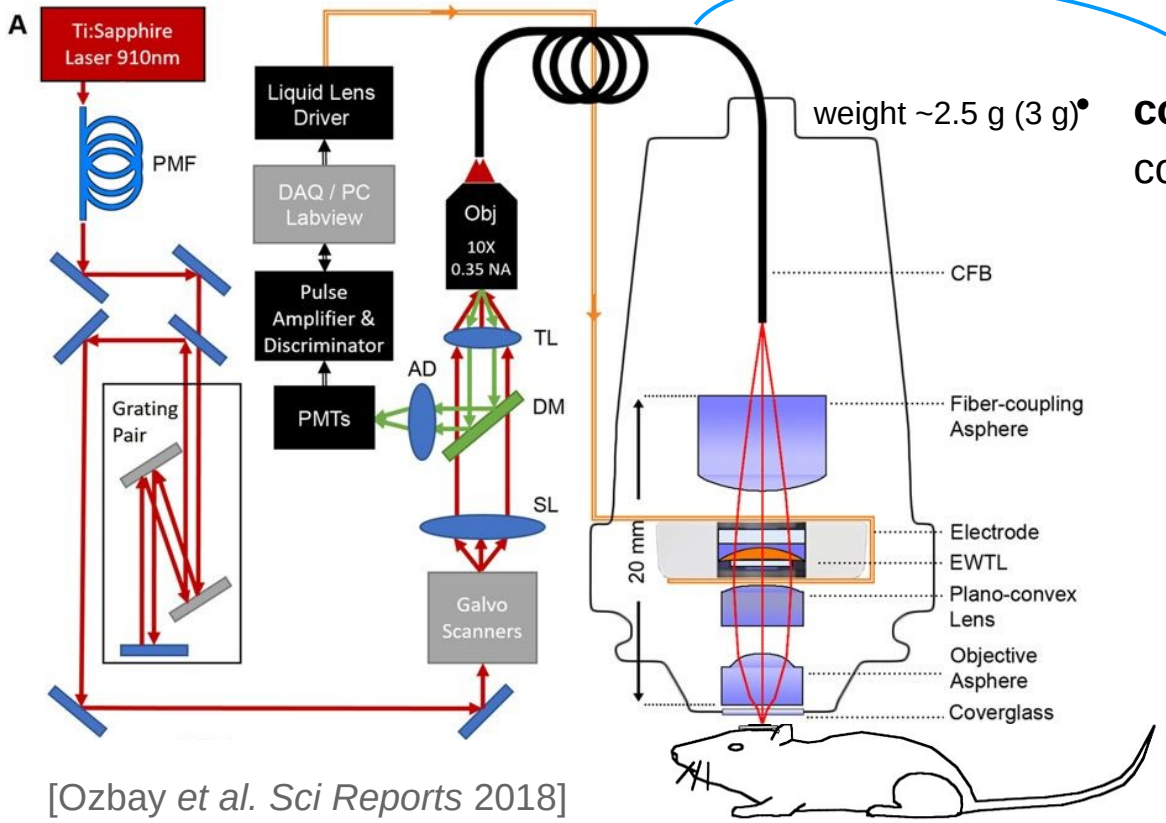
2p-laser scanning fiber-coupled microscope



[Ozbay *et al. Sci Reports* 2018]

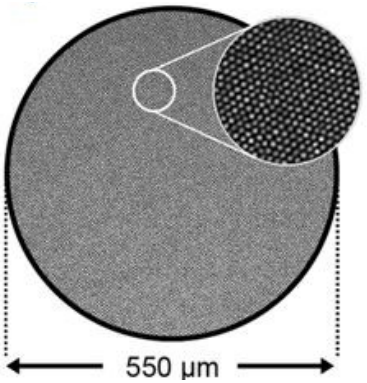
- light source, scanning mirrors and detector at remote location from the animal
- excitation and emission light transmitted through coherent fiber bundle: preserves spatial information of excitation

2p-laser scanning fiber-coupled microscope



[Ozbay *et al. Sci Reports* 2018]

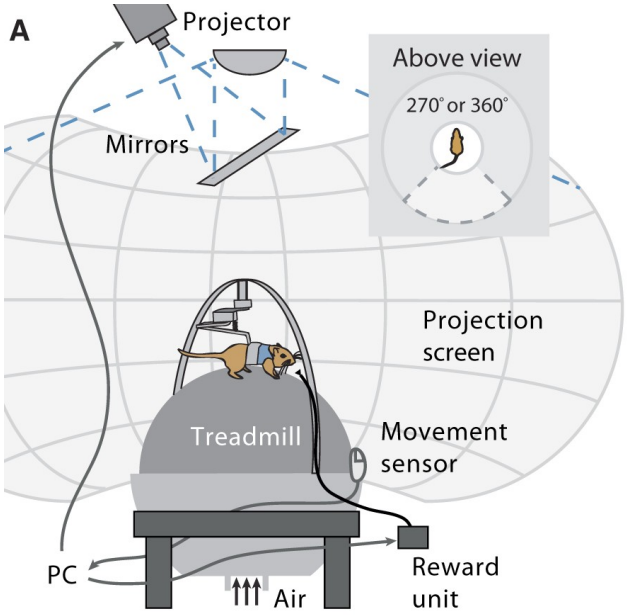
coherent fiber bundle : ~15,000 cores, core diameter ~2.9 µm, spacing ~4.5 µm

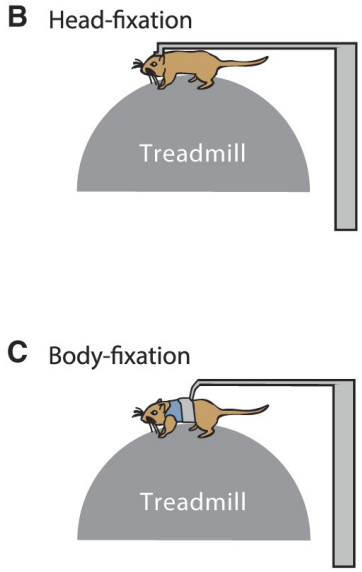


2p-laser scanning fiber-coupled microscope: 2

Video

Virtual reality (VR) systems : visual VR

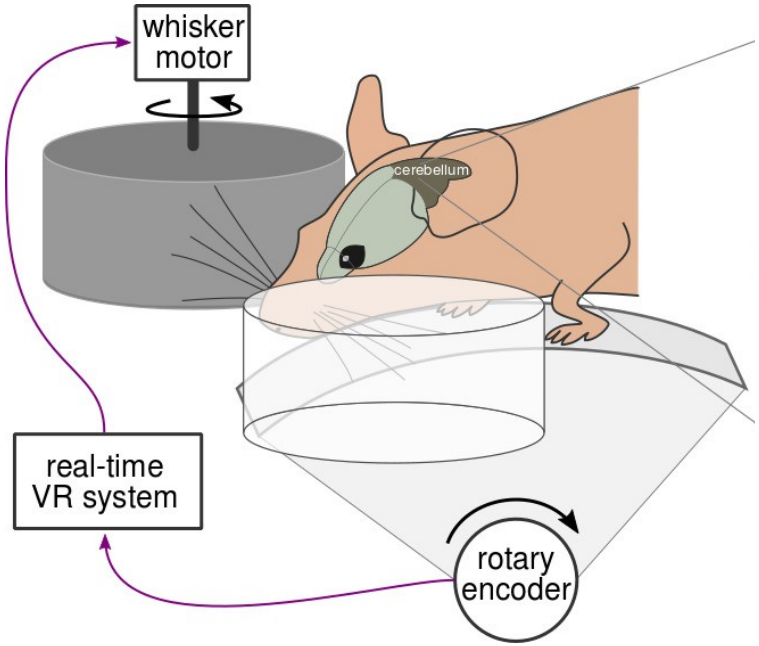




[Thurley & Ayaz, *Current Zoology* 2017]

- 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

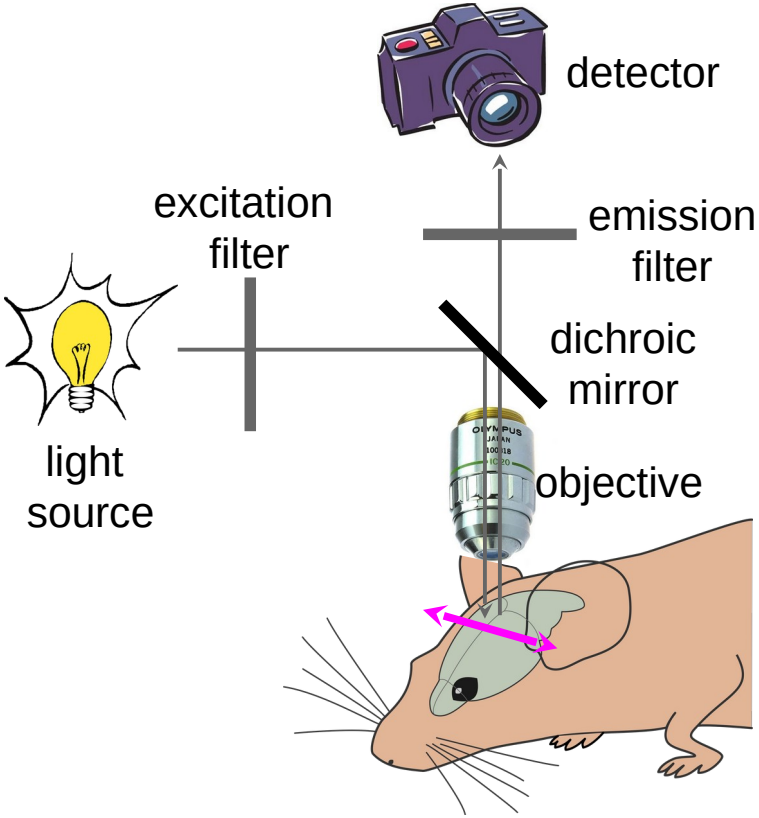
Virtual reality (VR) systems : tactile VR



[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

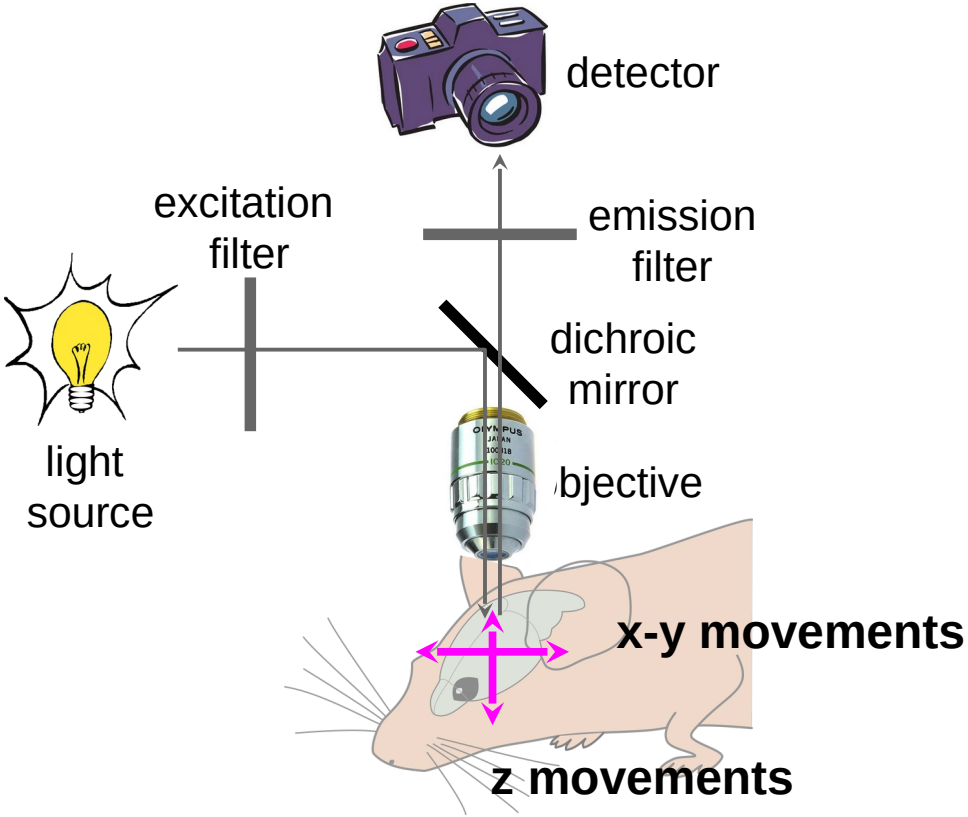
Challenge : movement artifacts



Reasons for relative movements

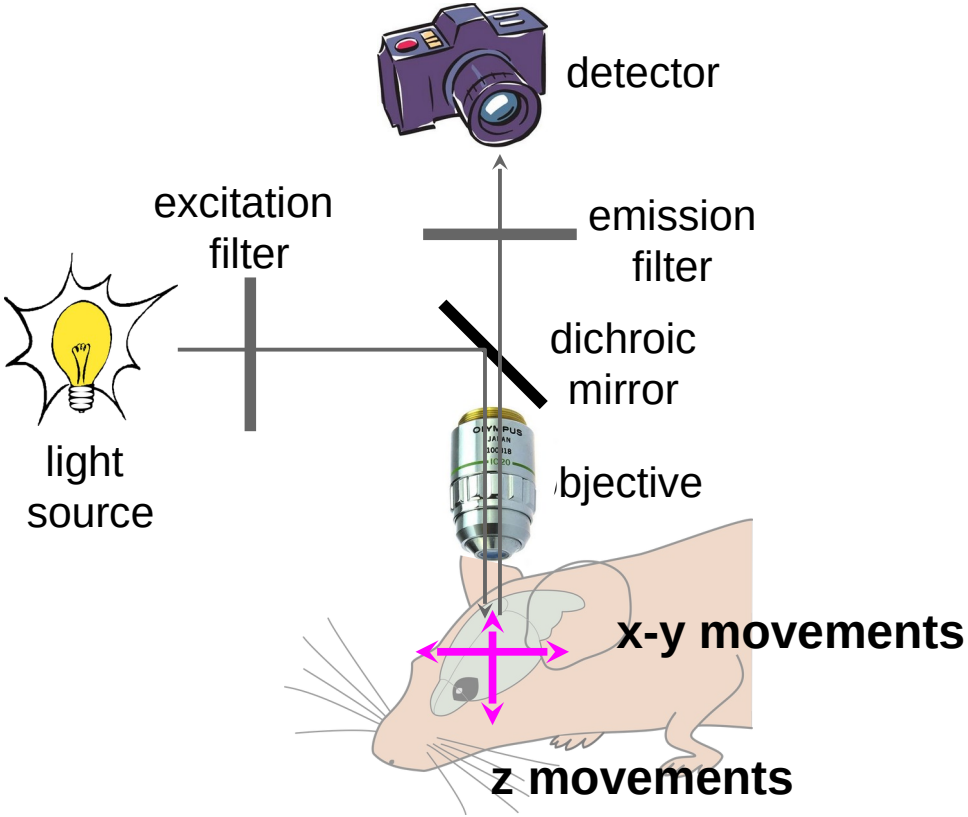
- imperfect head-fixation
- movement from respiration
- movement from heartbeat/blood flow
- animal movement translated to the brain

Image registration : tackle movement artifacts



- x-y displacements – displacement within the focal plane – can be corrected in post-hoc analysis
- z-displacements cannot be corrected

Image registration : tackle movement artifacts



- x-y displacements – displacement within the focal plane – can be corrected in post-hoc analysis
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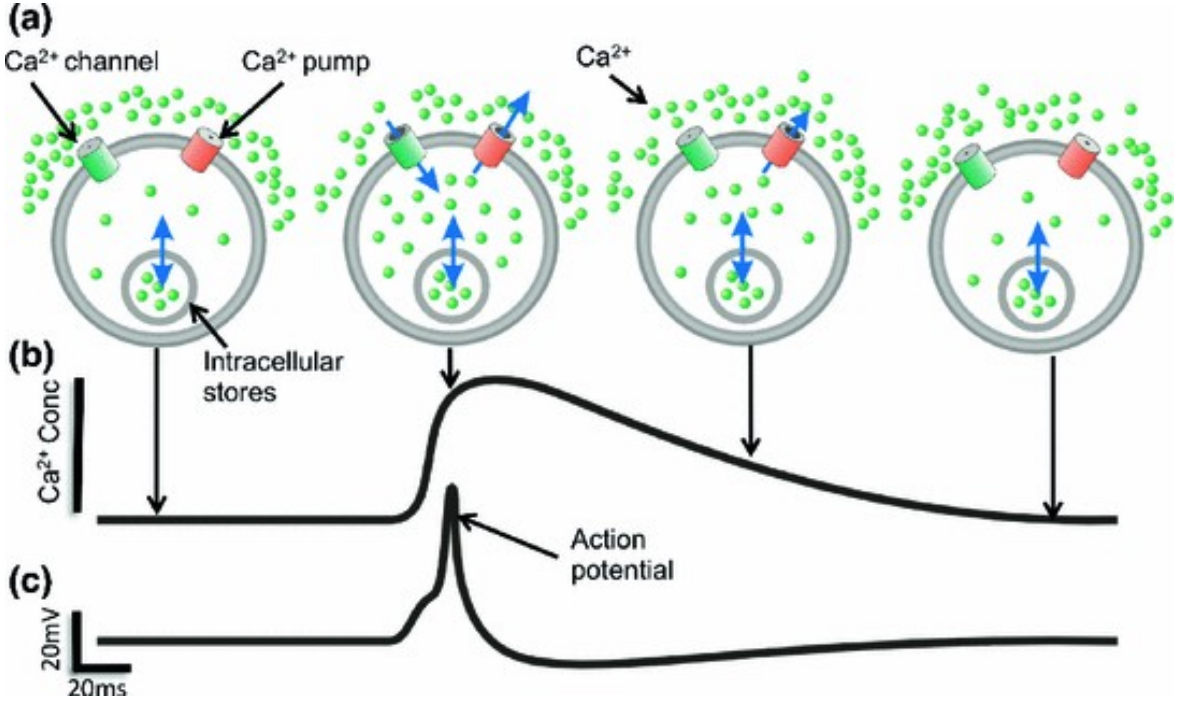
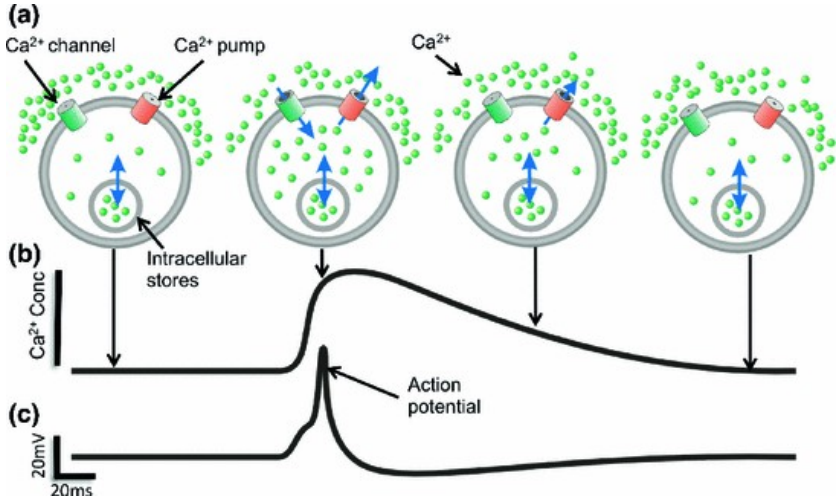
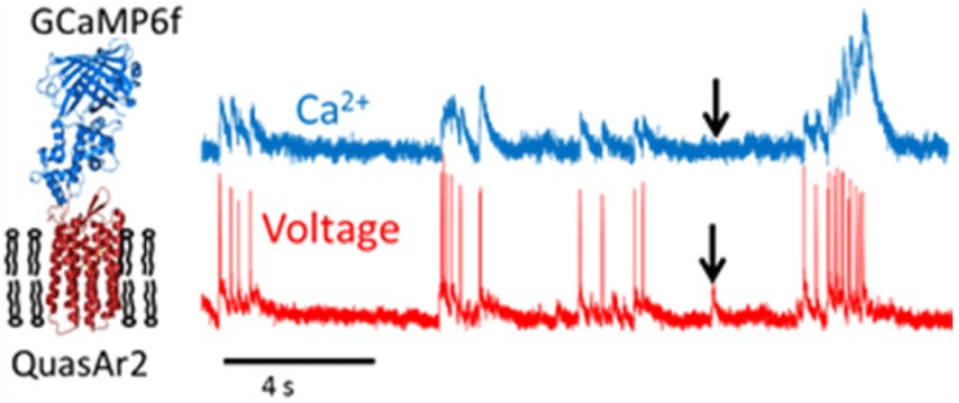
Calcium vs. voltage imaging



- 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





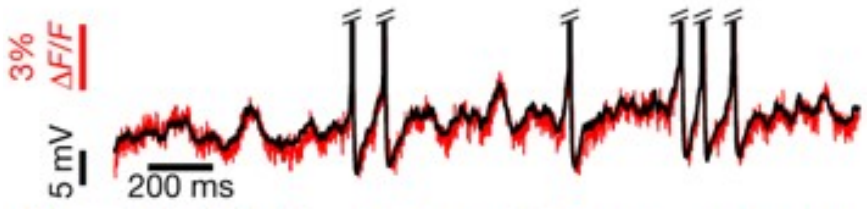
Calcium imaging

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

Voltage imaging

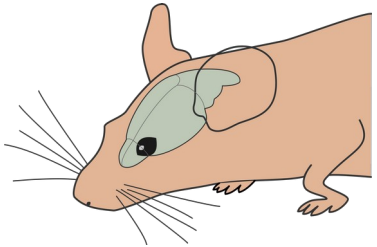
- genetically encoded voltage indicators (GEVIs, e.g. QuasAr, ASAP) report directly transmembrane voltage
- located in cell membrane

Challenges of voltage imaging



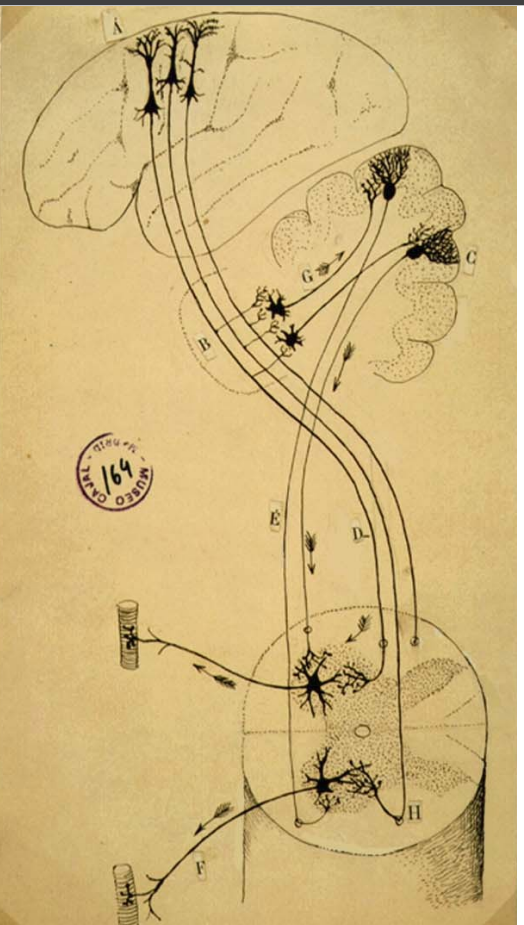
- Requires high-speed microscopes due to short duration of action potentials (~2 ms)
- Photobleaching due to constant illumination
- Requires good membrane trafficking of fluorophores
- Requires exceptionally bright fluorescence due to fewer fluorescent proteins in field of view (volumne vs. surface)

Outline of the talk



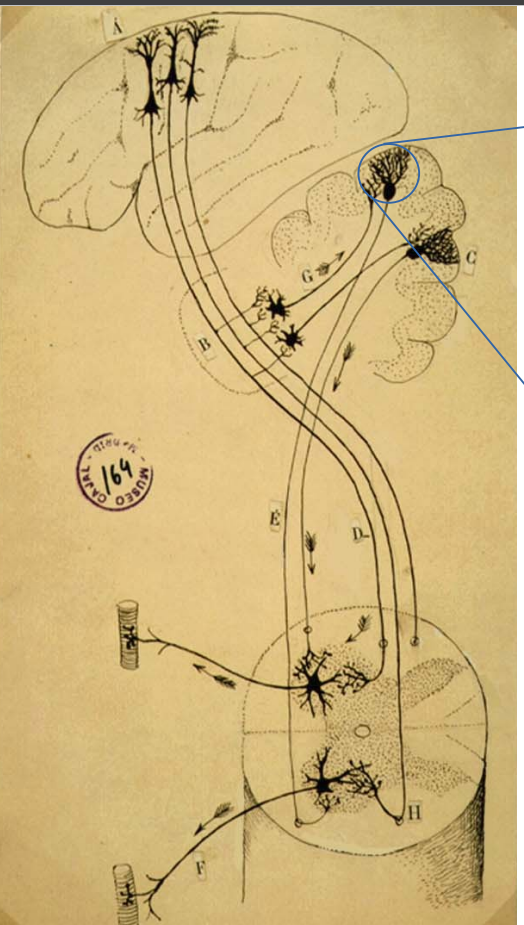
1. Basics of *in vivo* imaging
   - parts list for imaging experiment
   - challenges of deep tissue imaging
   - 1- vs. 2-photon imaging
2. Considerations of *in vivo* imaging in awake animals
   - sensory modalities studied
   - practical implementation : optical access, head-fixed vs. 'freely' moving
   - virtual reality systems
   - movement artifacts
   - calcium vs. voltage imaging
3. Cerebellum and motor control

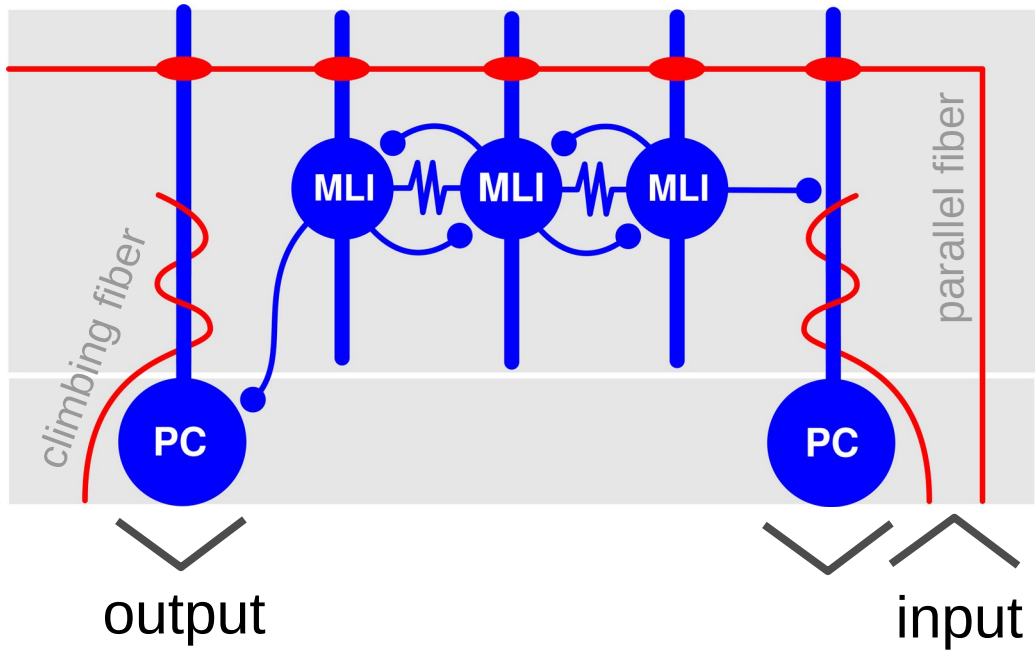
Ongoing project in the lab : Cerebellum and locomotion



- 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*



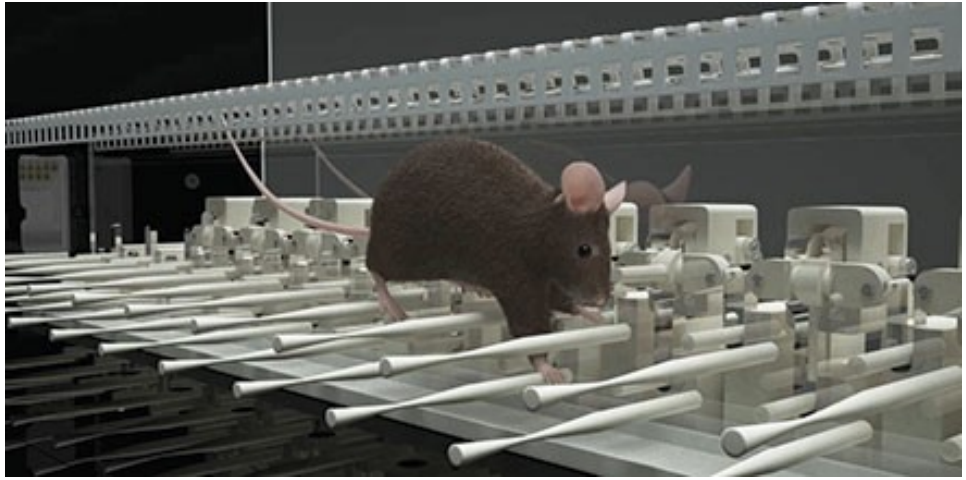


MLI ... molecular layer interneuron

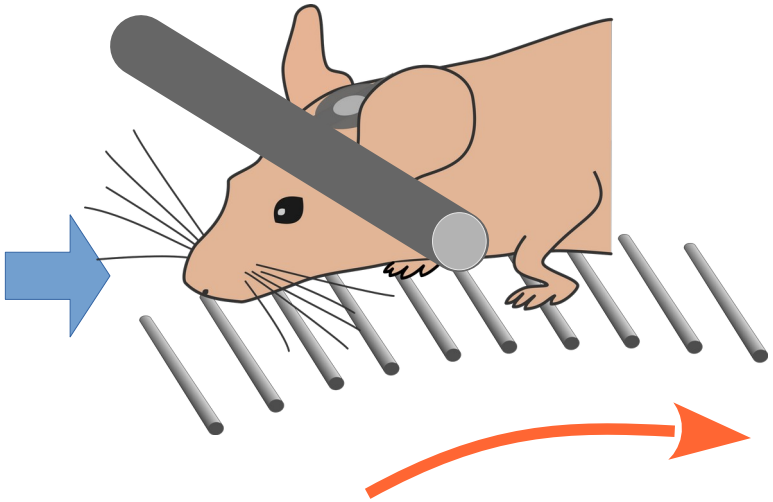
PC ... Purkinje cell

Task to study motor coordination on cellular level

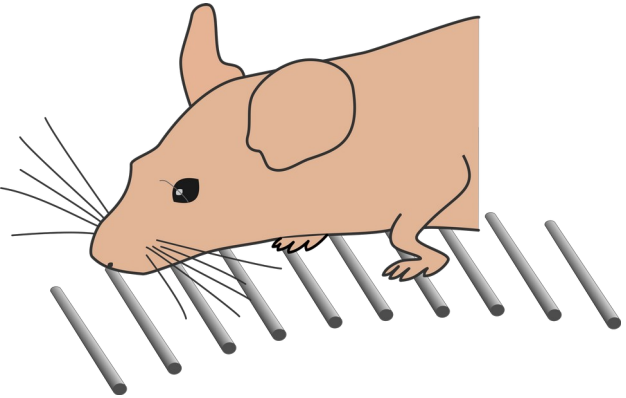
Erasmus Ladder | Noldus



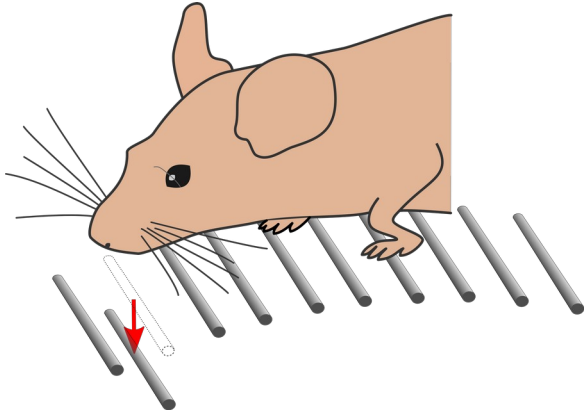
Acquisition of a
complex motor task
in head-fixed animal



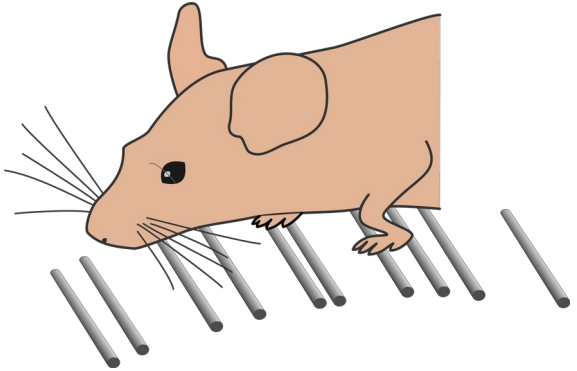
Task to study motor coordination on cellular level



1) acquisition of a complex motor task

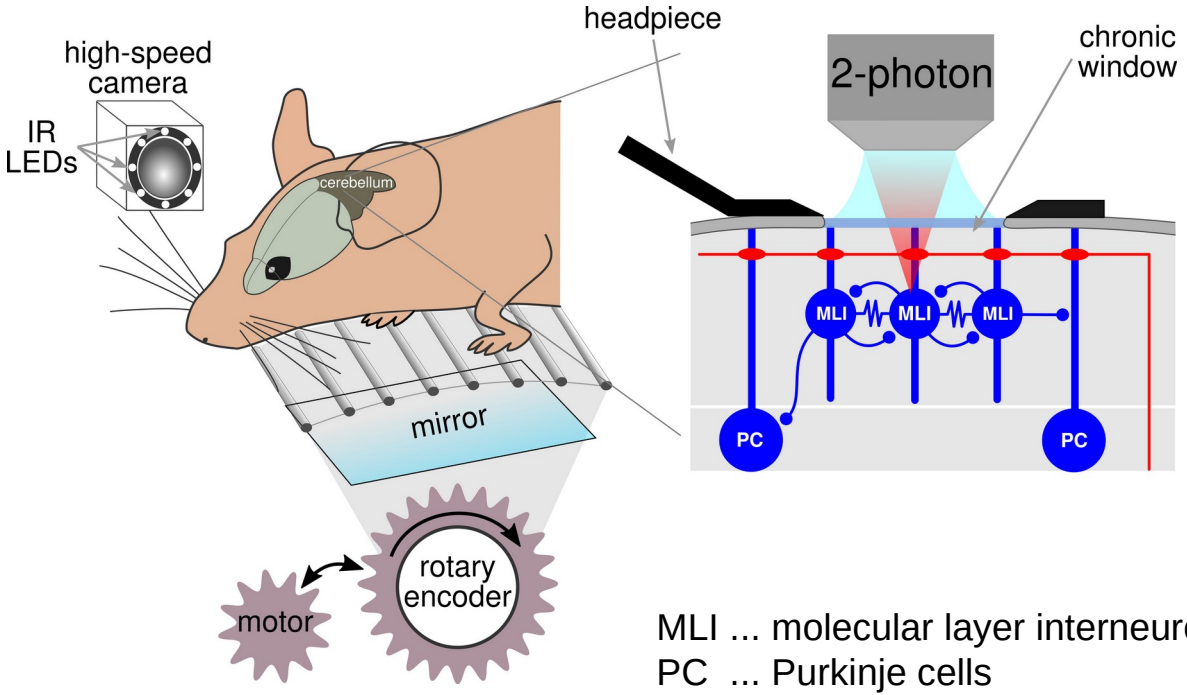


2) adaptation of the motor plan to a sudden environmental change



3) permanent changes of the motor plan in an irregular environment

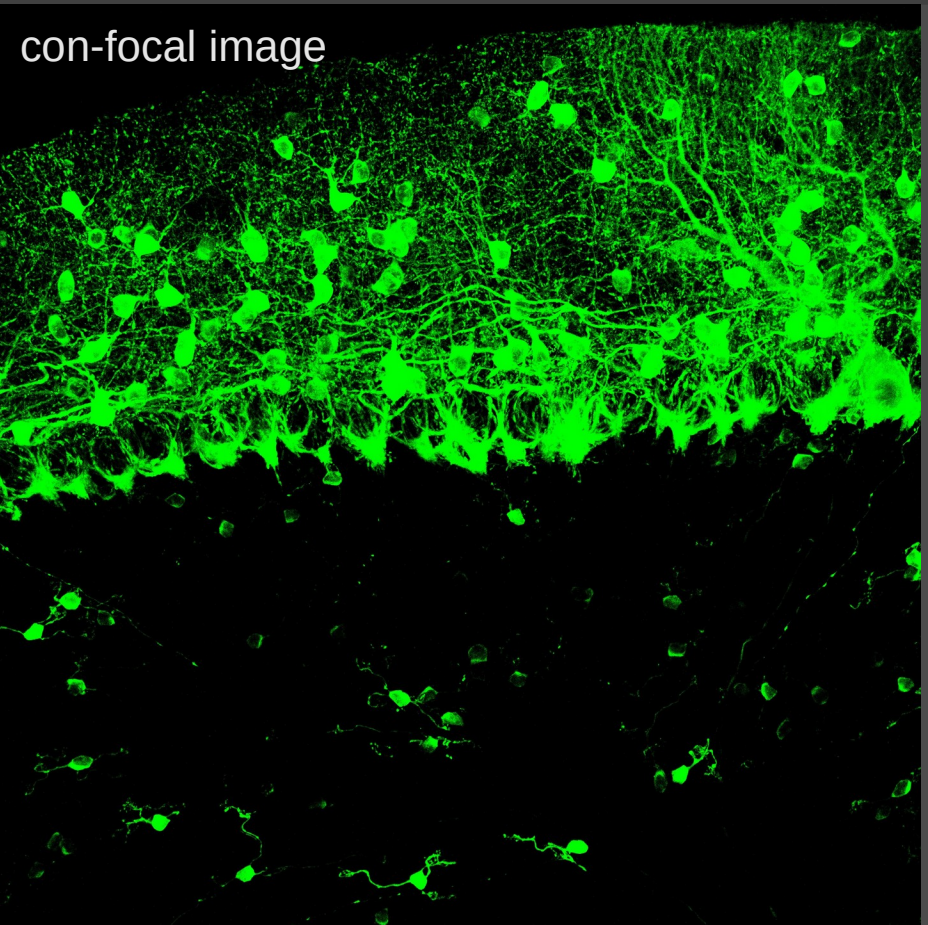
Experimental methods and setup

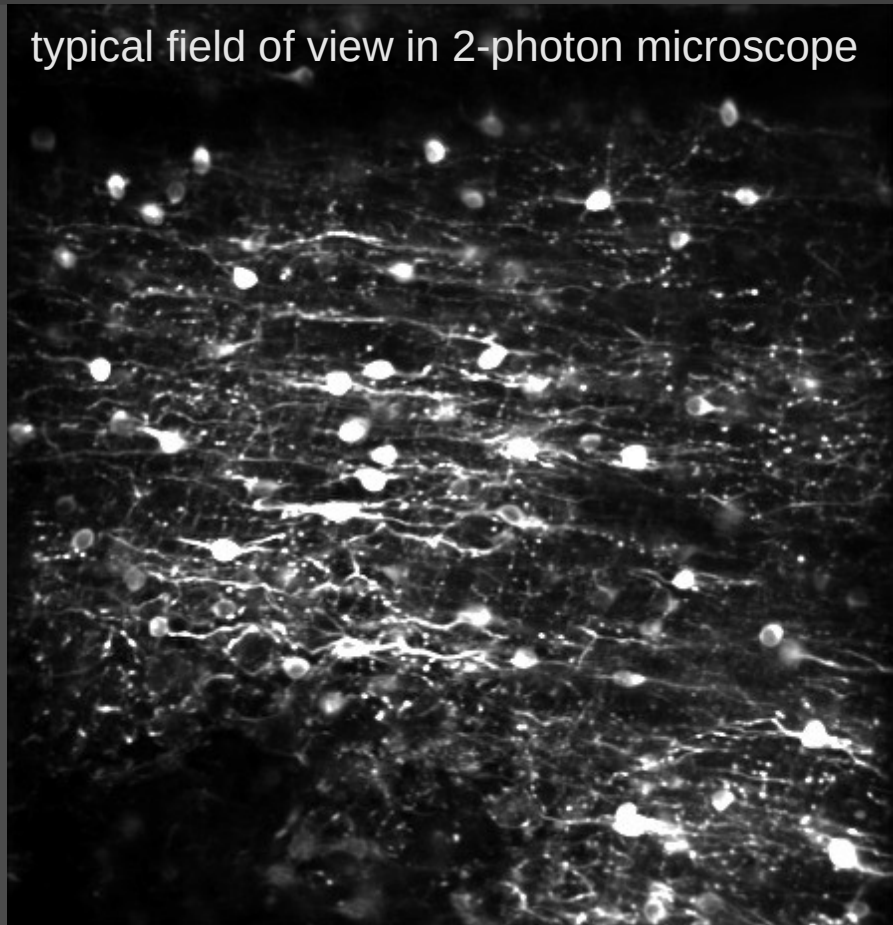


MLI ... molecular layer interneurons
PC ... Purkinje cells

- 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

GCaMP6f expressed in molecular layer interneurons

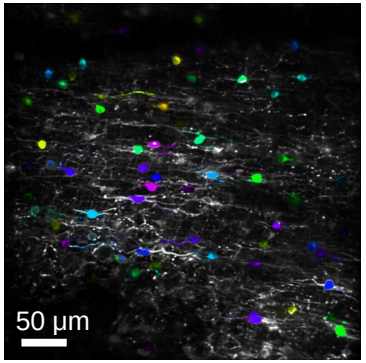




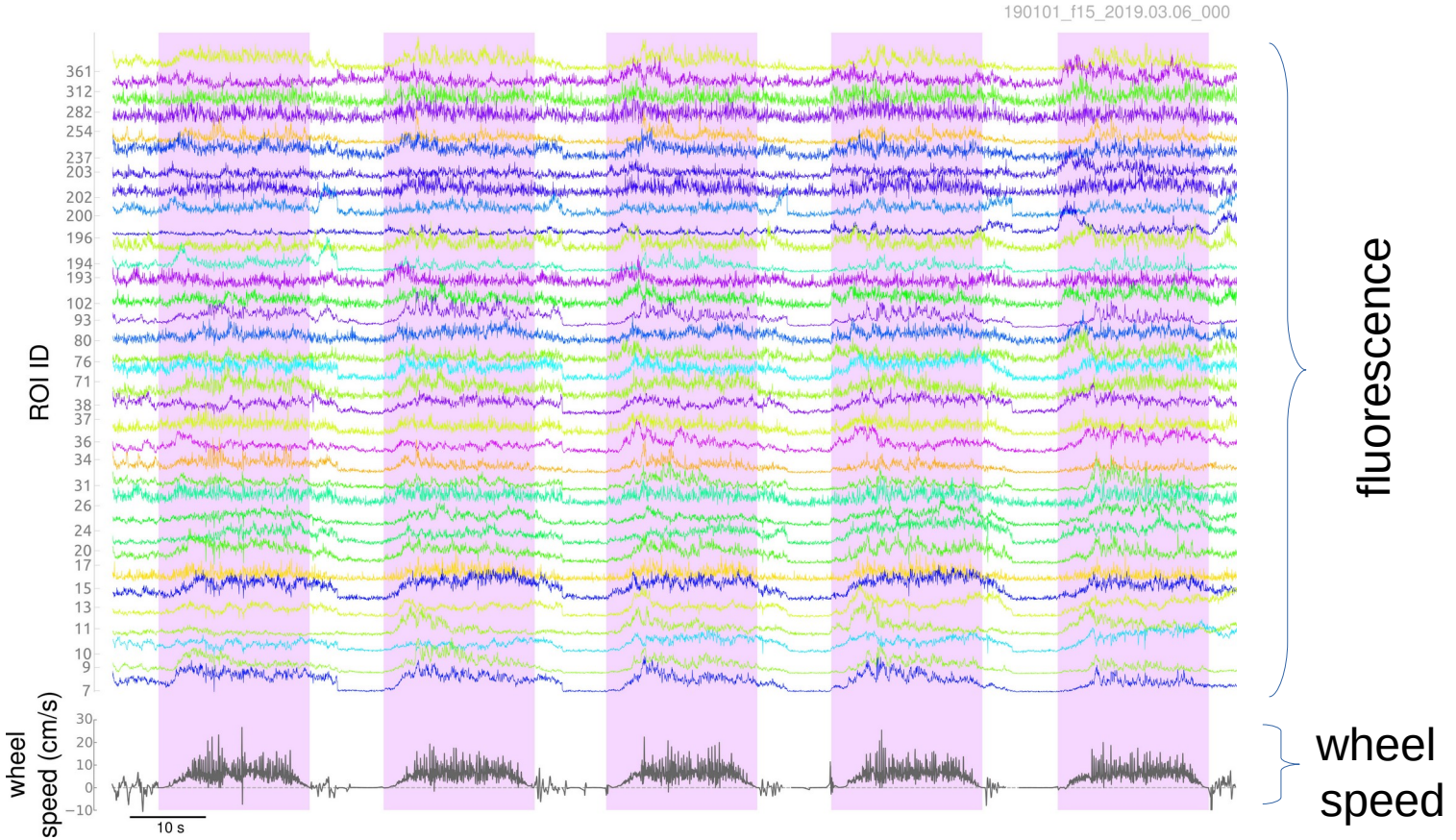
Mouse walking on treadmill with bars (rungs)

Video

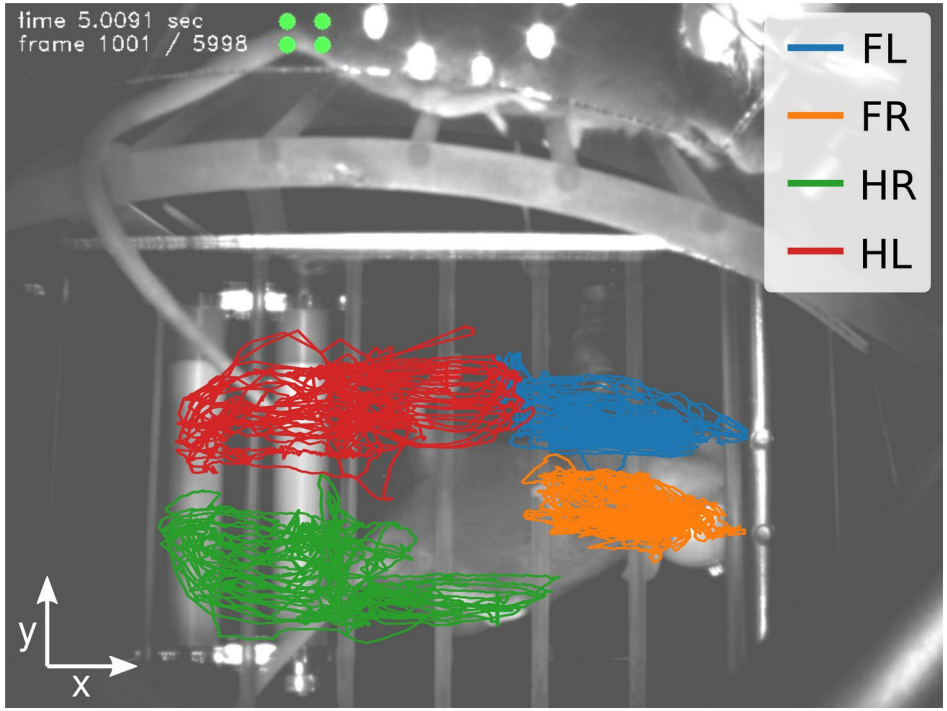
Interneurons exhibit locomotion related activity

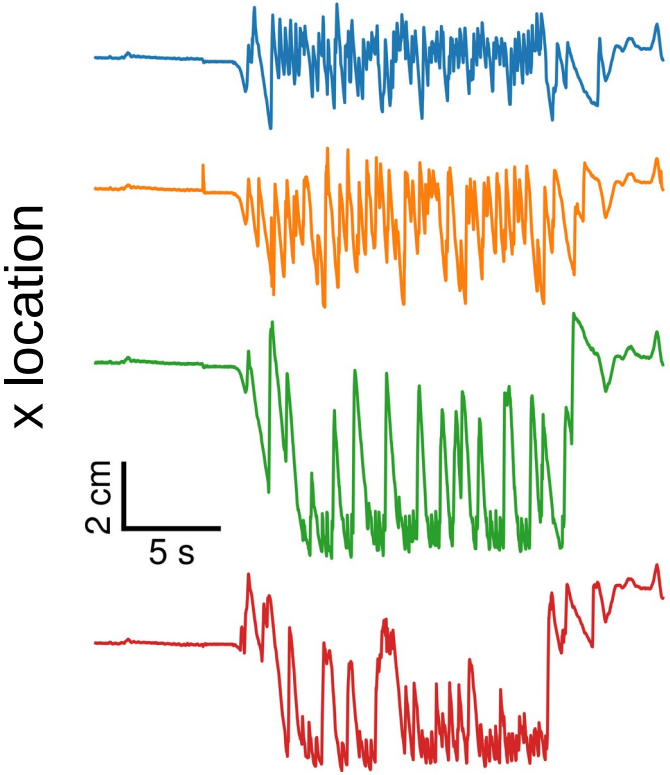


67 ROIs



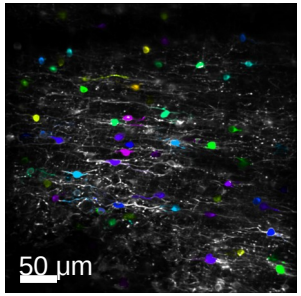
Extraction of paw trajectories with DeepLabCut

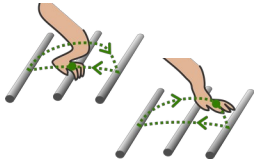


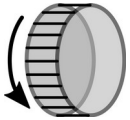


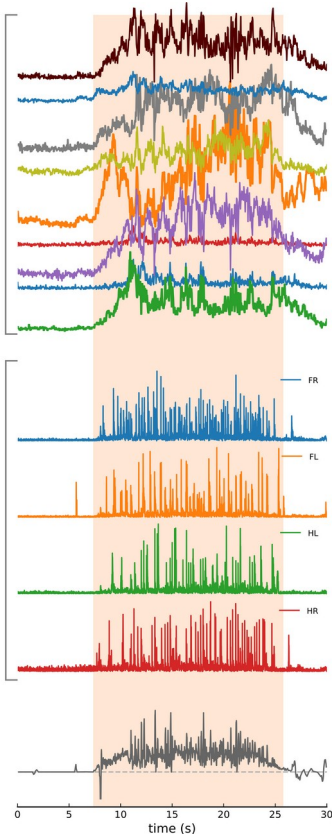
[Mathis *et al. Nat Neurosci* 2018]

Question: Link btw. calcium activity and locomotion?









Calcium imaging data:

- reflecting activity of a local MLI network

Paw trajectories → speed:

- reflecting activity of multiple muscle groups of different angles linked to specific joint

Wheel speed:

- reflecting overall locomotion state involving multiple limbs

In vivo imaging as tool to study sensorimotor integration