SAINTS-PERES
Paris Institute for
the Neurosciences

In vivo imaging in awake animals

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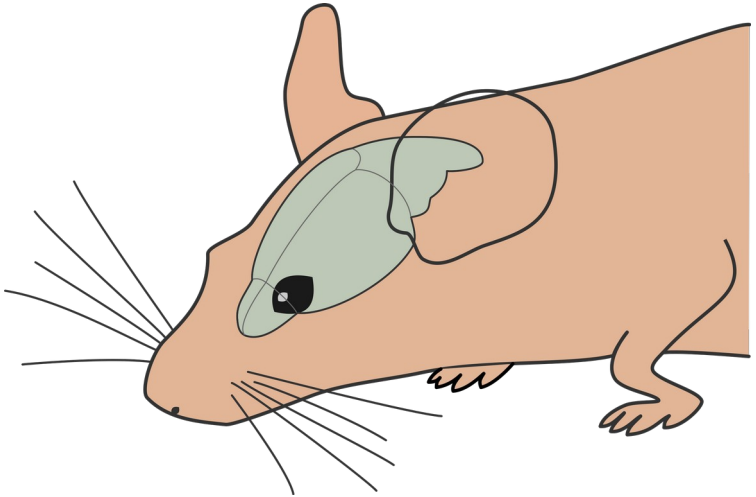
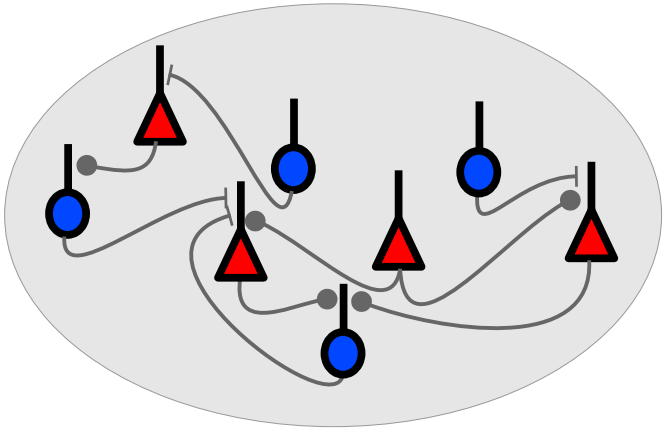
Aim

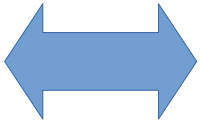
- study brain activity during relevant tasks – tasks which the brain has evolved and optimized to deal with
- explore brain function in its natural environment
- record (neural activity) from the brain of an *alive, awake* animal performing a task



Major challenge in neuroscience

neural circuits



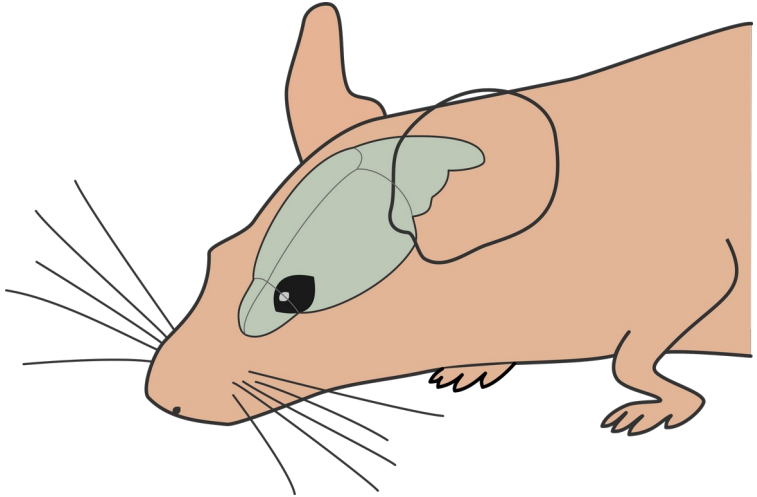


behavior

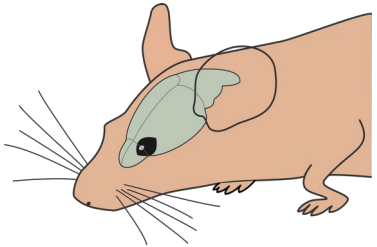
How do neural circuits encode, store, modify and retrieve information?

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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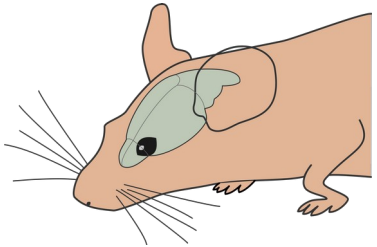

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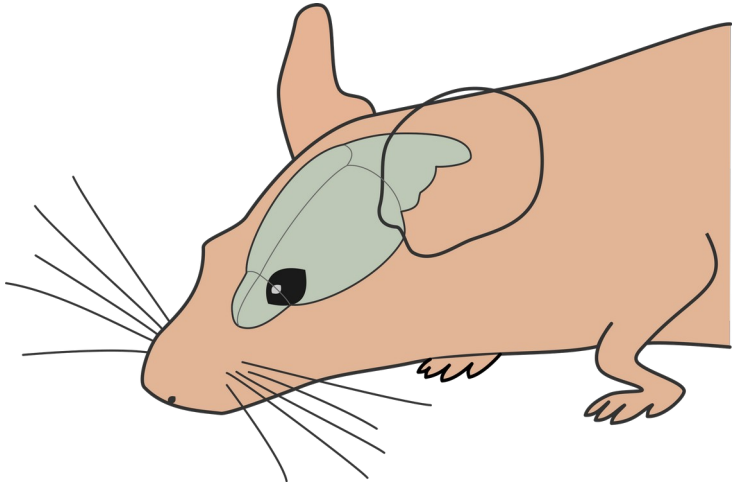
General parts list for *in vivo* imaging

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



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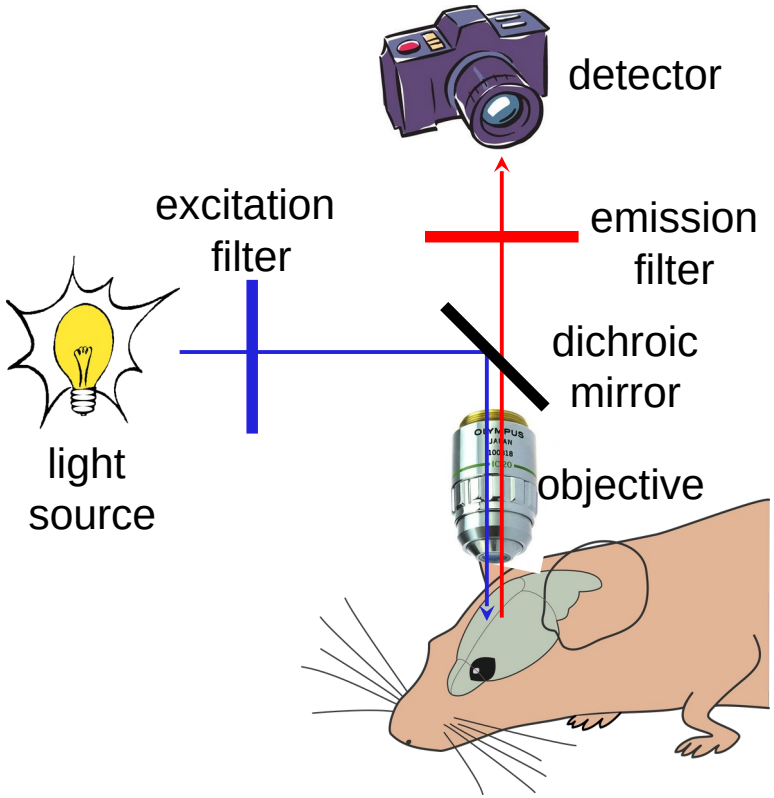
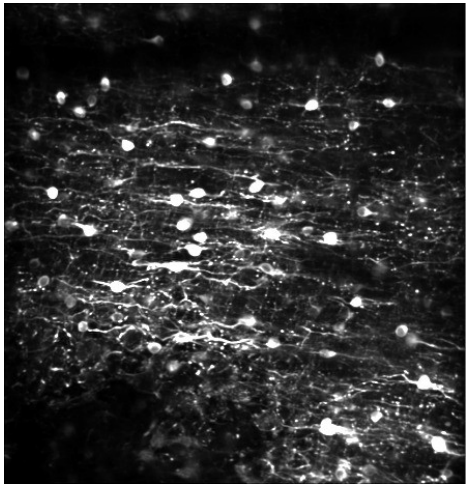
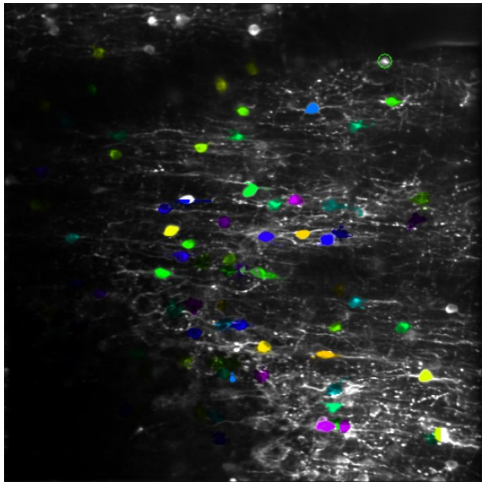
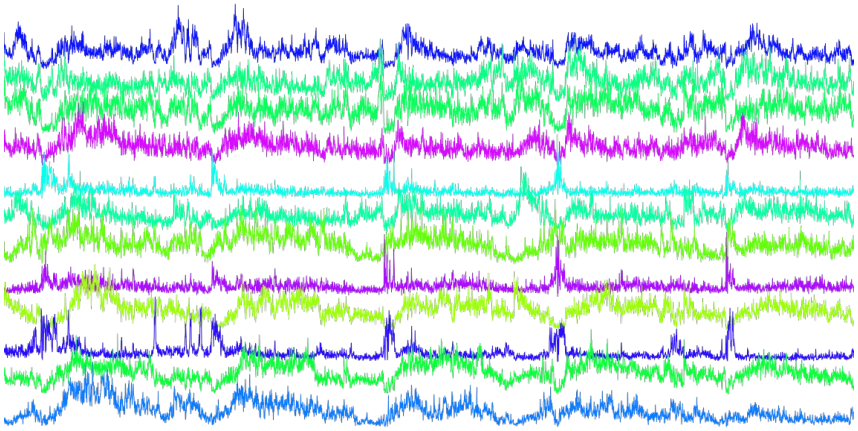


- **Light source**: LED, laser, mercury vapor lamp,...
- **Excitation filter**: enables to select a specific excitation range.
- **Dichroic mirror**: reflects wavelengths that are under/above a cutoff value and transmit wavelengths above this value.
- **Objective** : focuses light on region of interest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

Current method of choice : Calcium imaging using GECIs





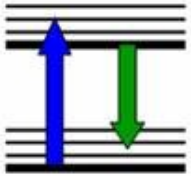


- Genetically encoded calcium indicators (GECIs) can be targeted to specific neuron populations
- Calcium transients serve as proxy readout of neural activity
- Non-invasive and repeatable means to measure neural activity from large populations of neurons

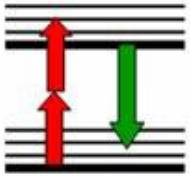
Fluorescence induced by 1- or 2-photons

fluorophore

1-photon excitation

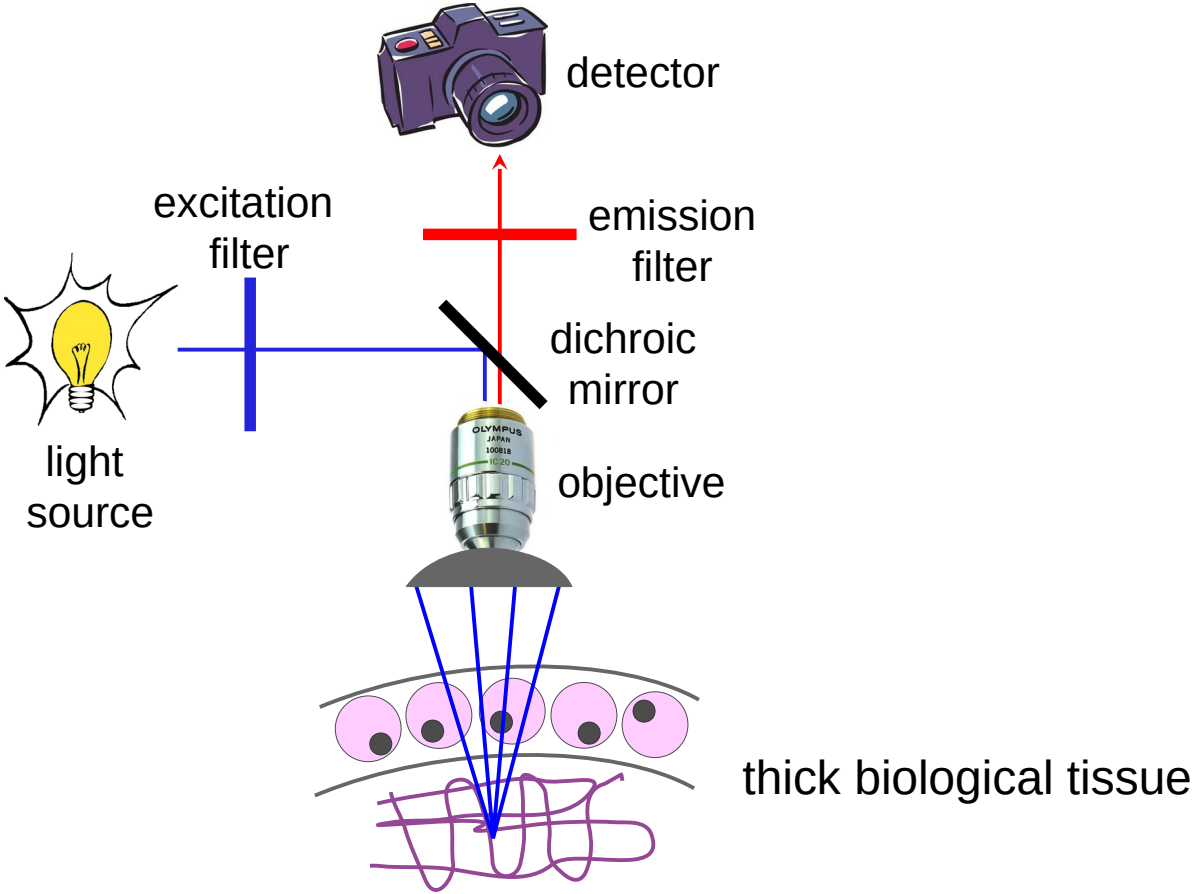


2-photon excitation

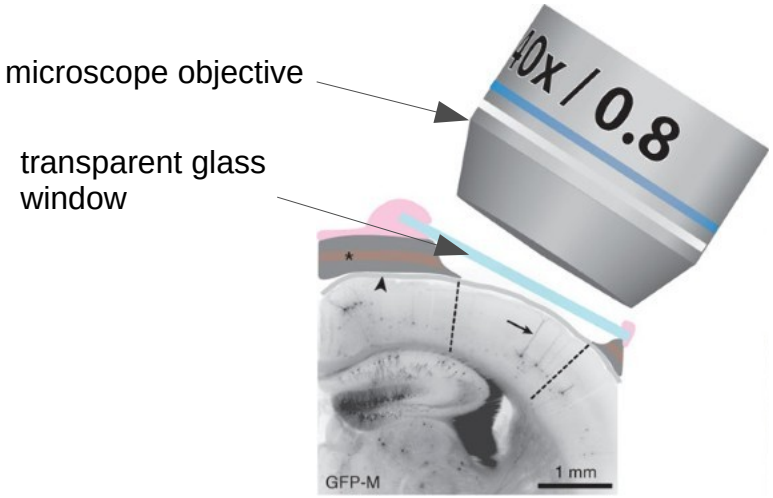


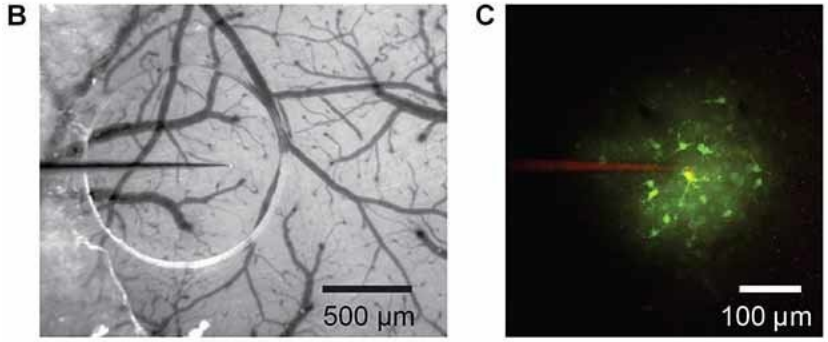
- Fluorescence: emission of light by the fluorophore that has absorbed light; emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation

Challenge: optical access to tissue to be imaged

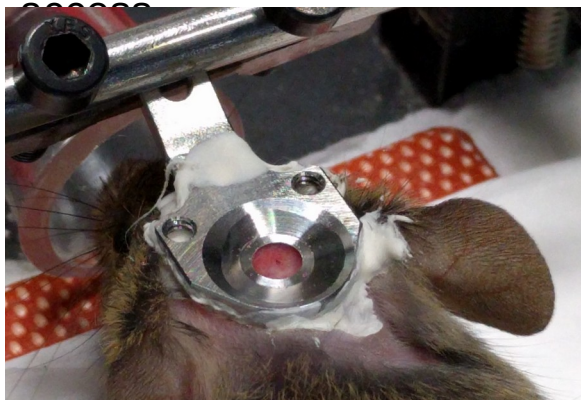


Optical access through chronic window

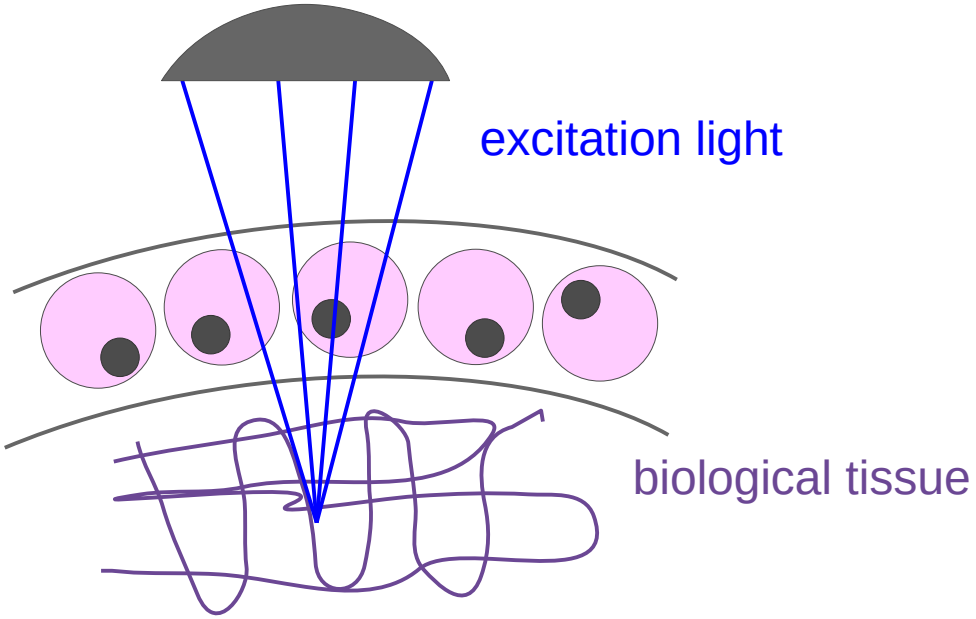




- Transparent window implanted in place of skull over region of interest :
  maximal achievable imaging depth up to 600-800 µm with 2-photon imaging; and 200 µm with 1-photon imaging
- bone thinning can provide sufficient visibility
- access port can allow for additional electrode

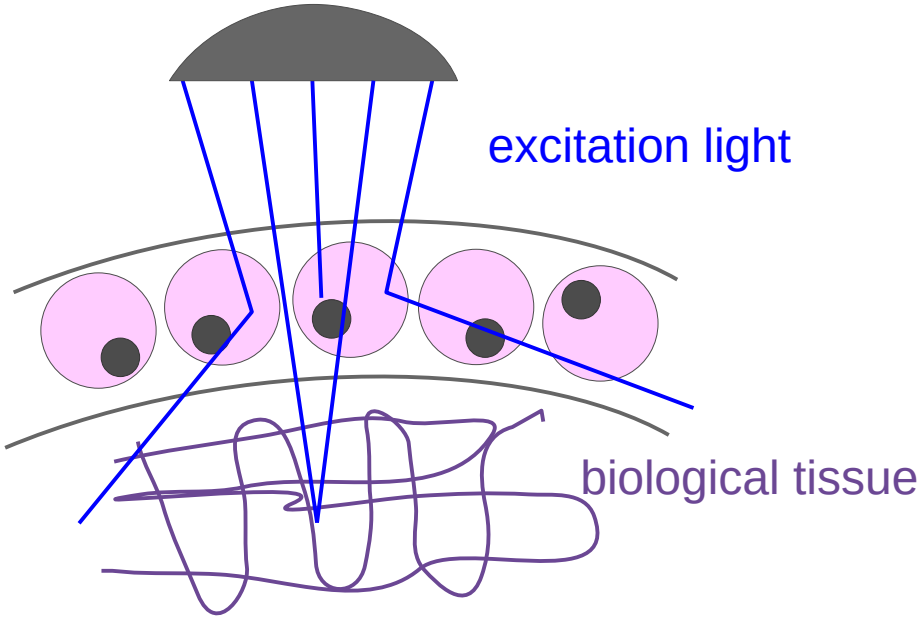


Imaging of thick biological tissue



Ideal case

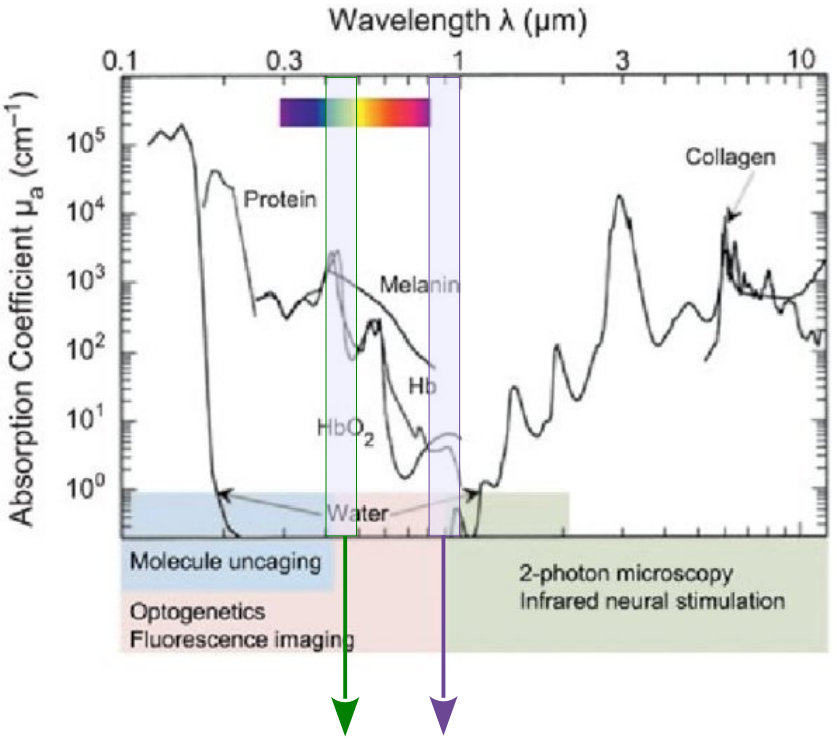
What limits imaging depth ?



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

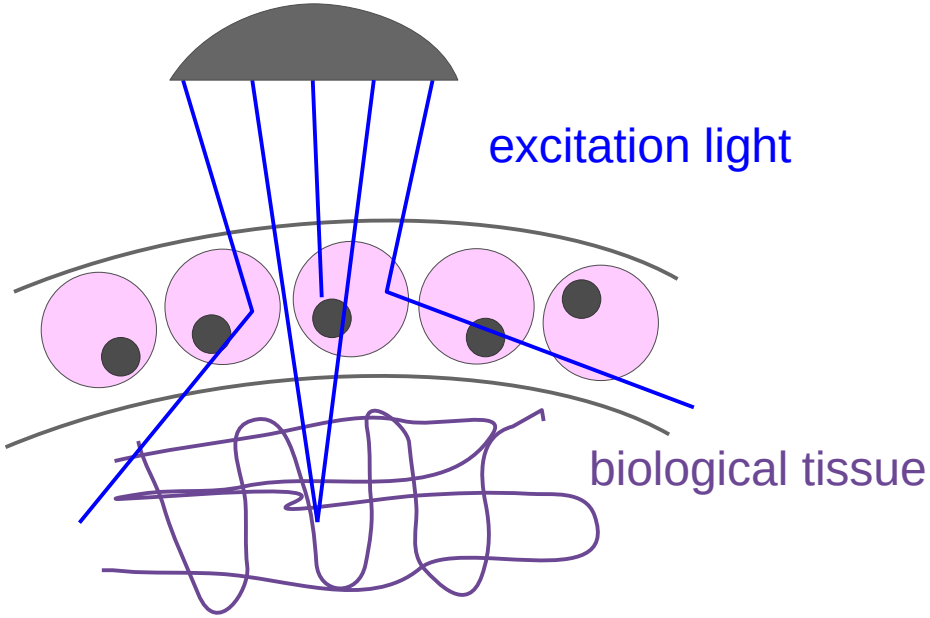


one photon excitation range

two-photon excitation range

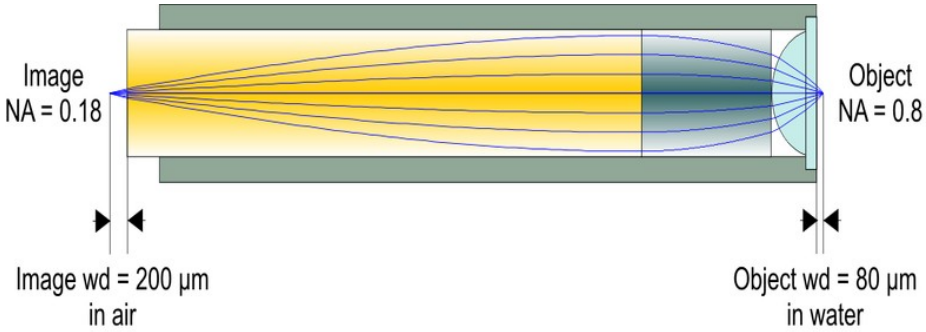
- commonly used fluorescent dyes have excitation spectra in the 400 to 500 nm range → wavelengths used to excite the same dyes with two-photons tend to be between about 800 and 1000 nm
- infrared light can penetrate deeper in biological tissue due to little absorption
- commonly used: titanium-sapphire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

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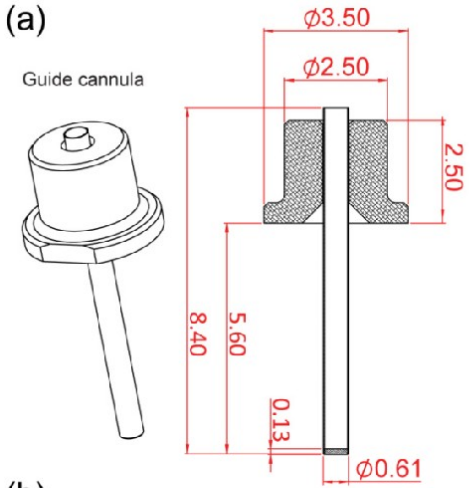

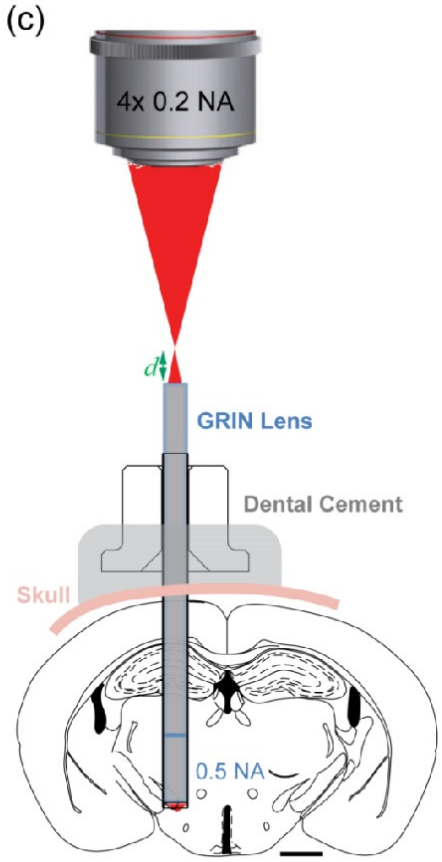

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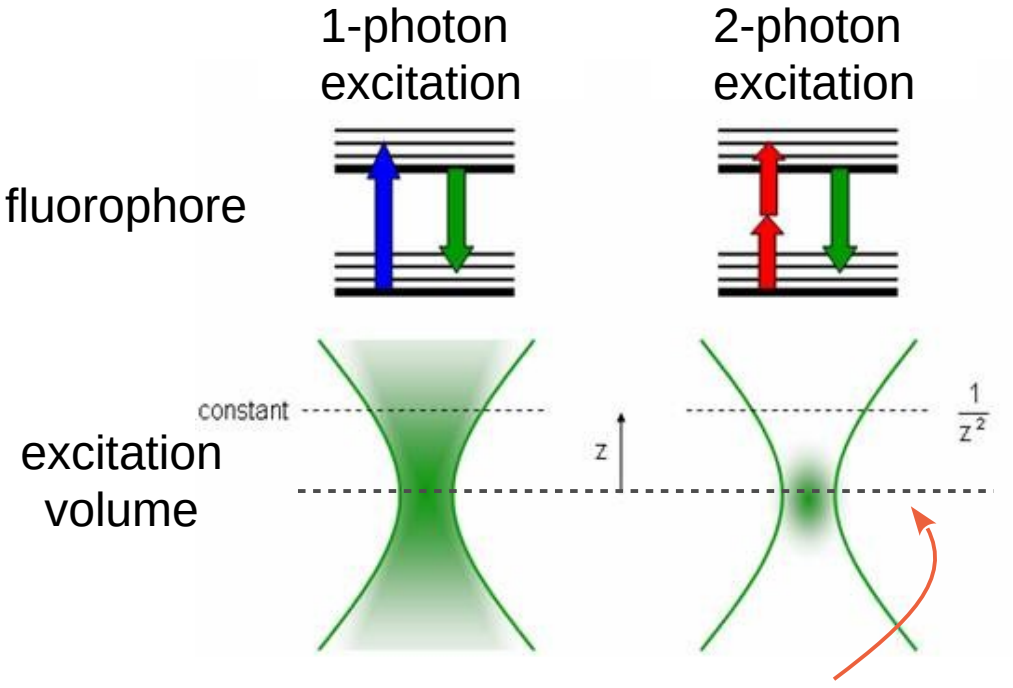
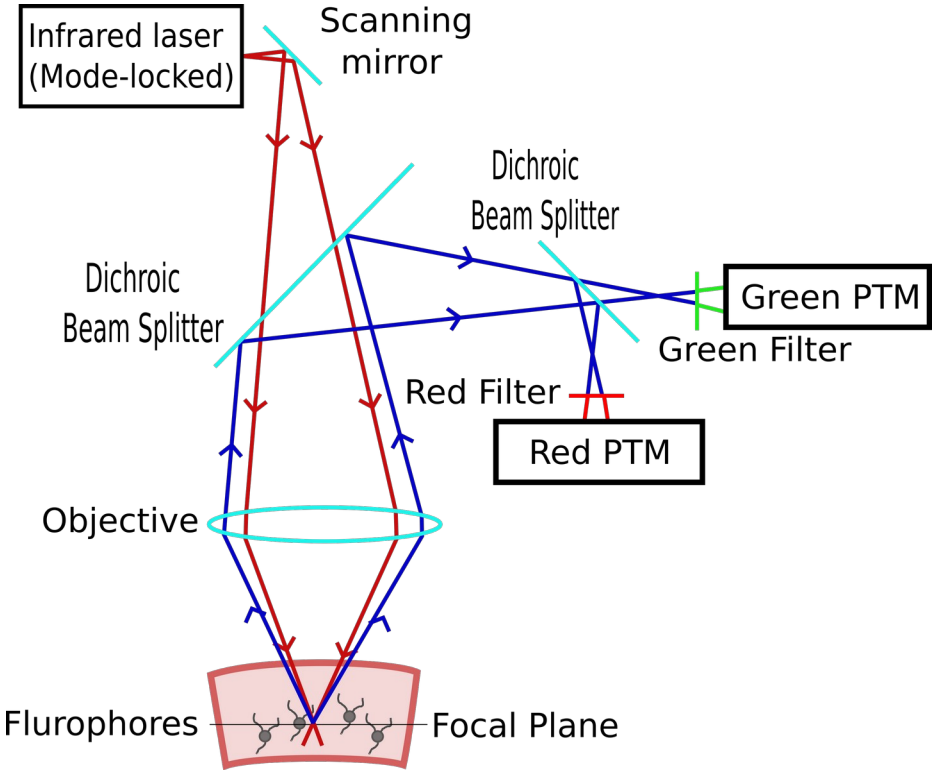
One photon vs. 2-photon fluorescence : resolution



[z is distance from focal plane]

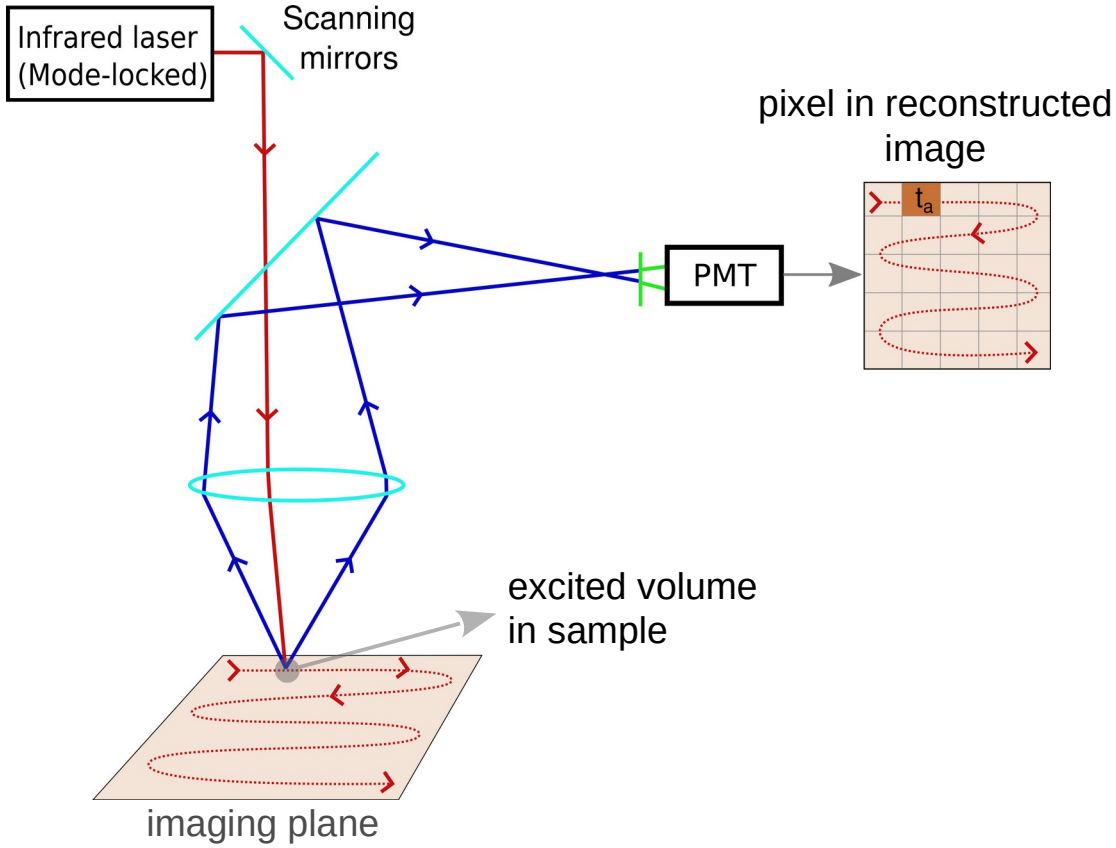
- excitation volume/fluorescence is confined to the focal center of the laser beam
- both photons must arrive nearly simultaneous (< 1 fs)
- fluorescence falls off as ~$1/z^2$, while it falls off as $1/z$ with single photon excitation
  → 3D-imaging with out-of-focus background rejection similar to a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

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



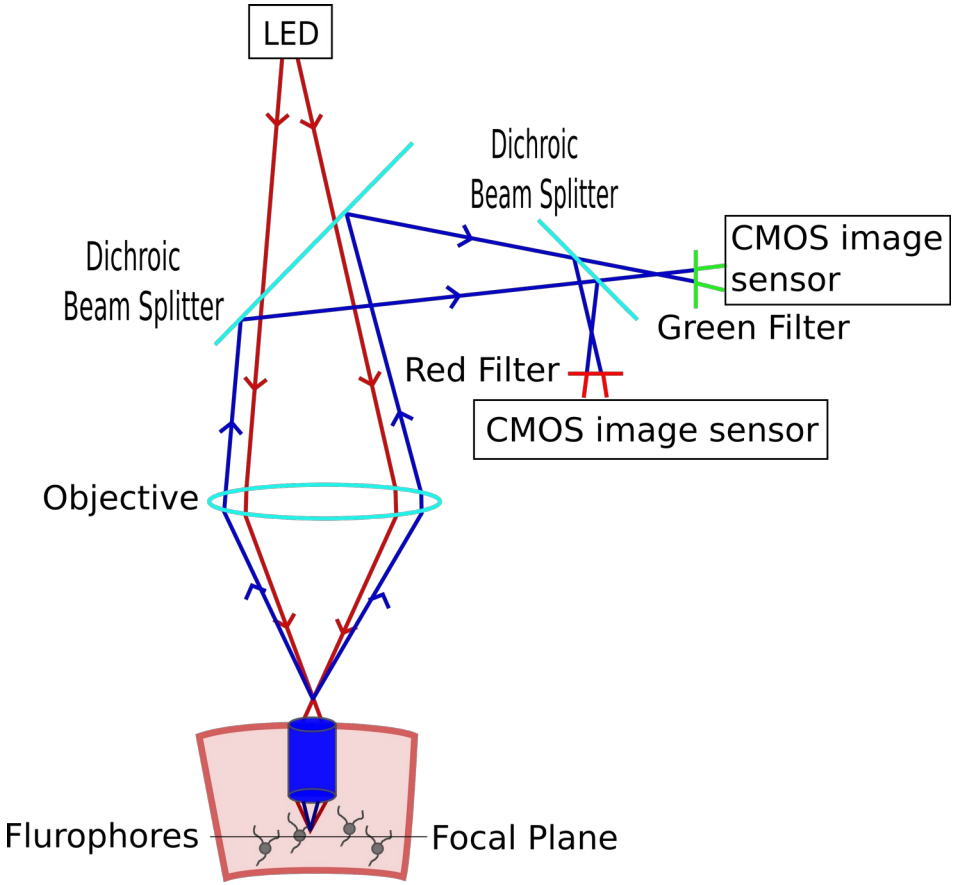
- **Light source**: laser producing light pulses on the order of femtoseconds (10^{-15} s)
- **Excitation filter**: not required since laser produces single wavelength
- **Scanning mirrors:** directs/scans the laser beam over the sample
- **Dichroic mirror**
- **Objective**: focuses light on region of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

2-photon imaging : functioning



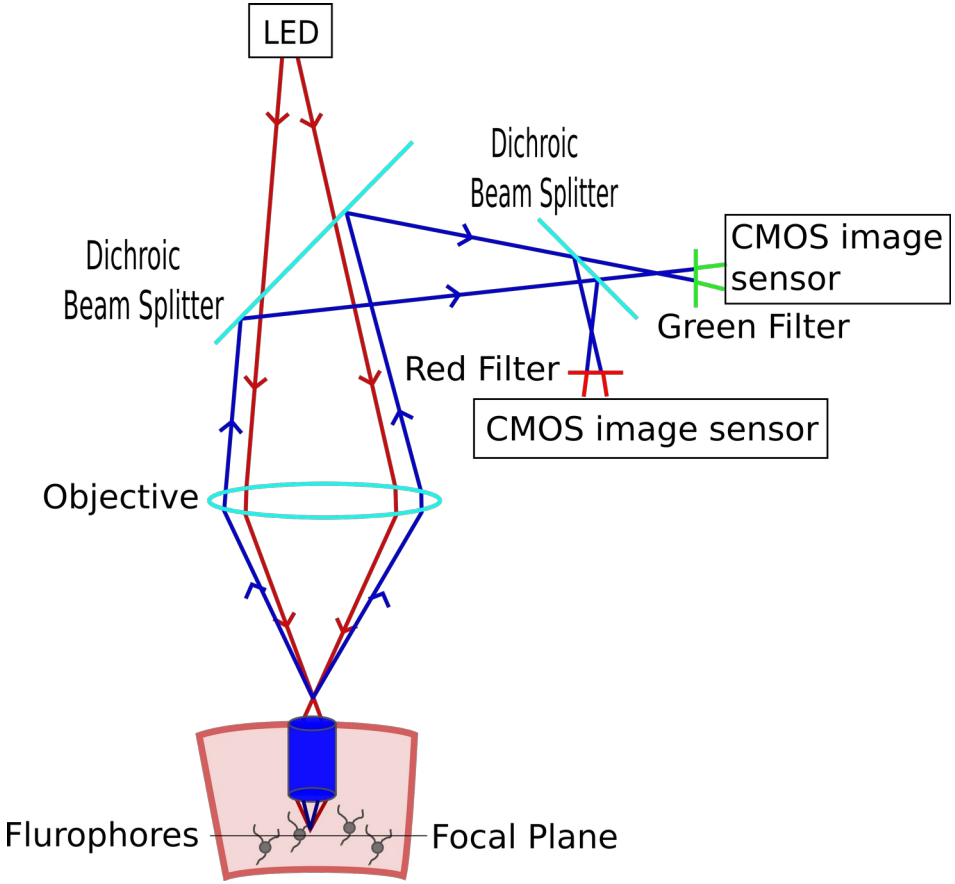
- A small excitation volume is excited by the laser light – defines resolution
- All fluorescent light is collected from the sample (indirect and direct light)
- Scanning mirrors move the laser beam across the imaging region – sequential acquisition of image (typical frame rate 30 Hz)

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 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

1-photon (epifluorescence) imaging

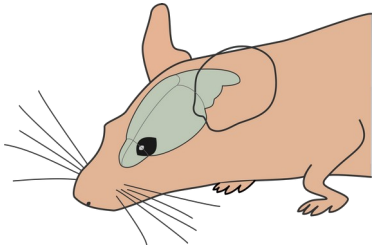
- poor resolution makes it impossible to image neurites or spines
- insertion of GRIN lens destroys neural tissue above the region to be imaged

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)

Outline of the talk



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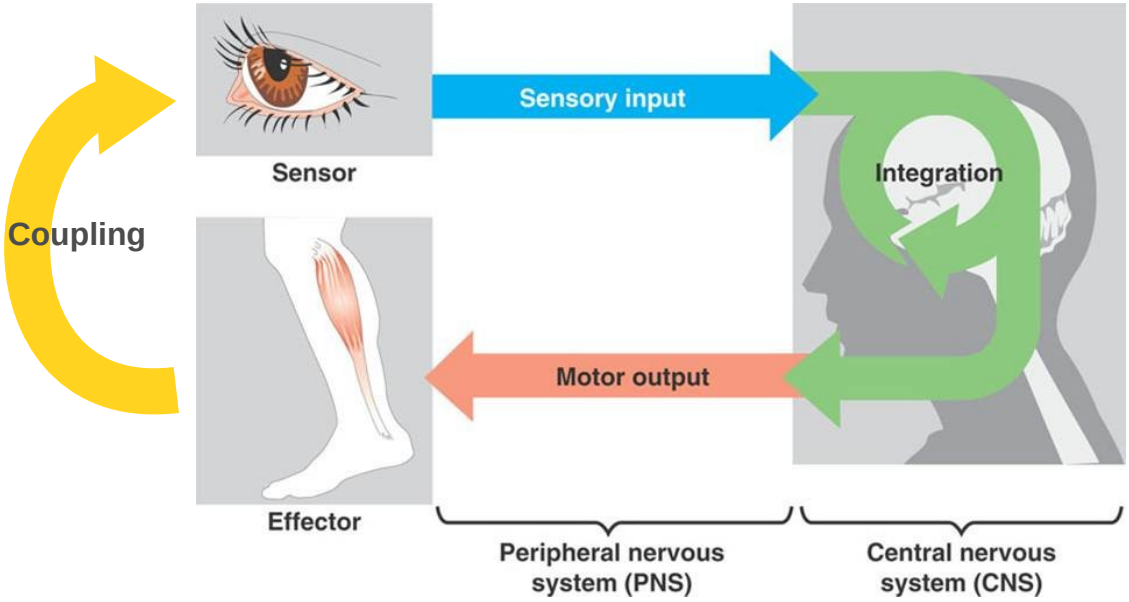
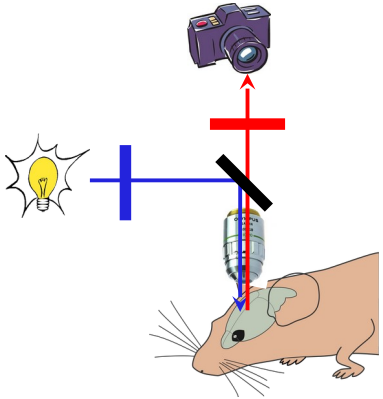
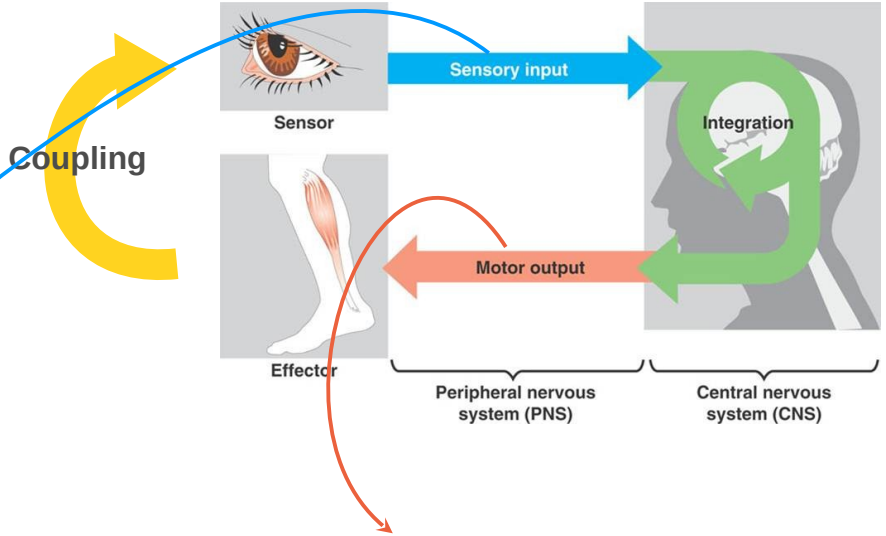
Rational behind *in vivo* experiments



- **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

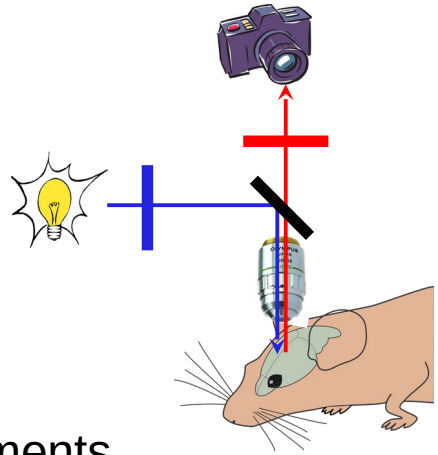




- **sensory stimuli :**
  - easy to implement : touch (whisker), vision (static), smell, taste, sound
  - difficult : vision (dynamic), equilibrium (vestibular)

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

Assure stability btw. imaging system and imaging tissue



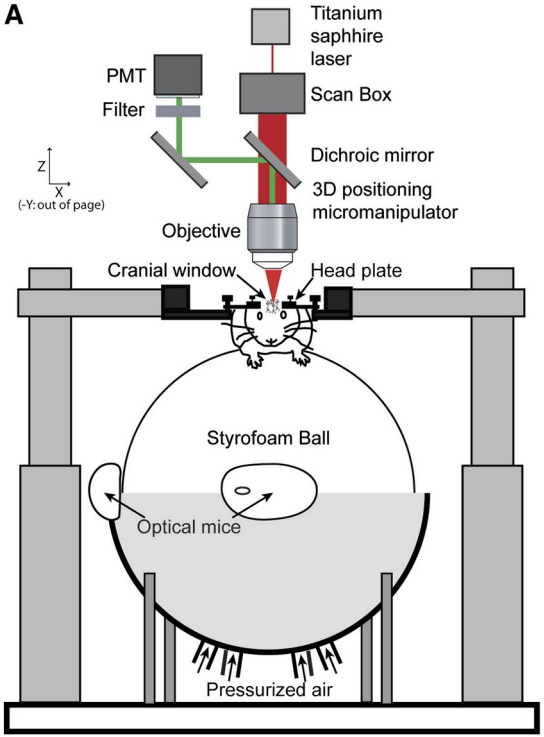
1) Minimize relative movements between animal to be imaged and the microscope

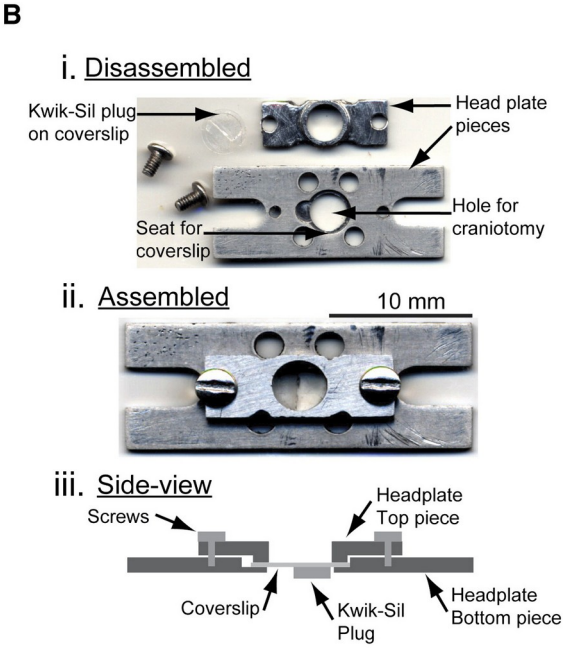
→ fix the animal head under the microscope

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

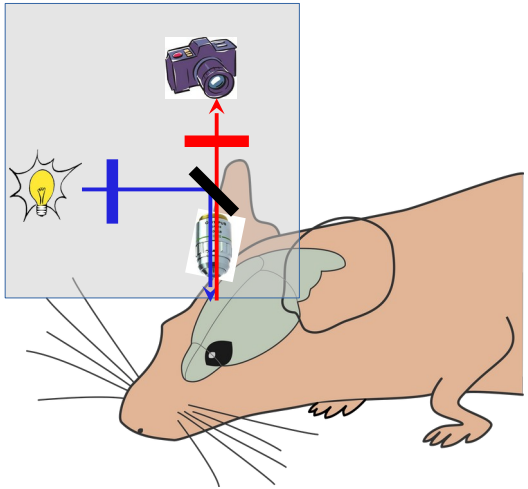




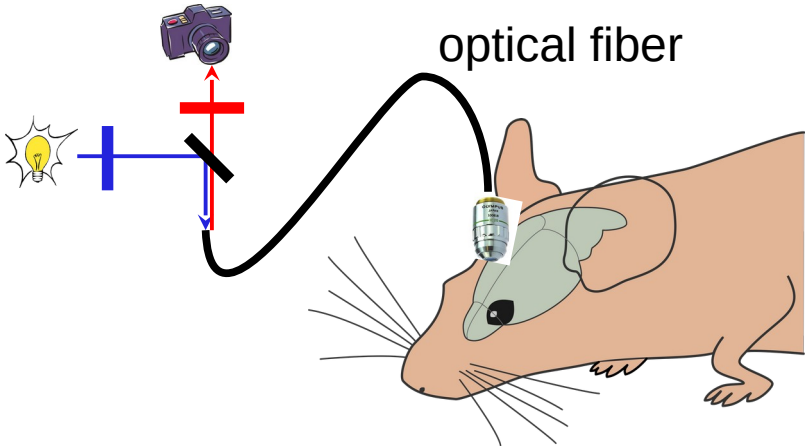
[Dombeck *et al.* Neuron 2007]

- 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

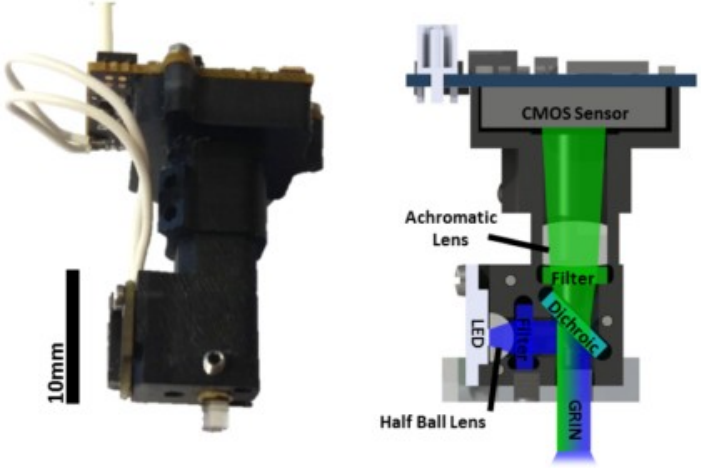


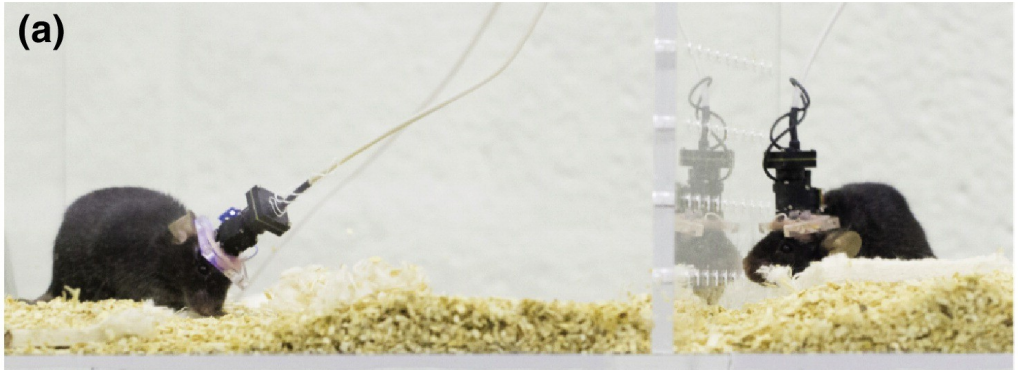
- flexible optical fiber connects static microscope parts (light source/detector) and animal-mounted optics
- allows for 2-photon imaging in 'freely' moving animals

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

miniscope

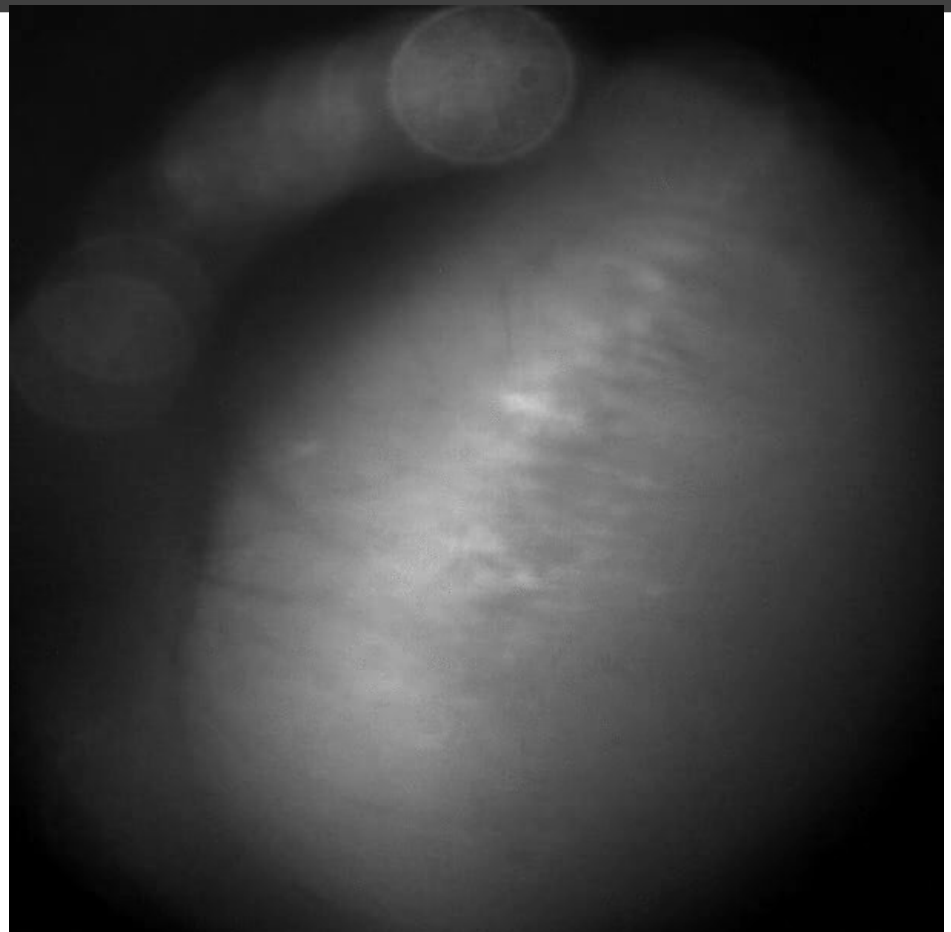
weight ~ 2g



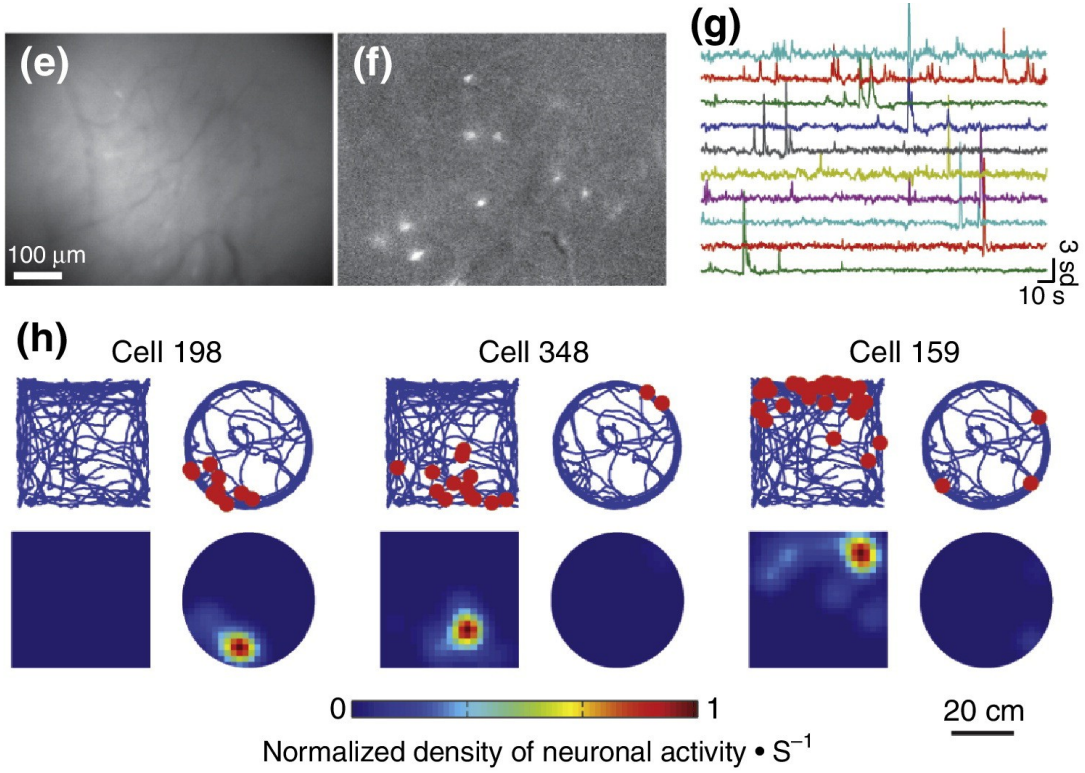


[Ziv & Ghosh, *Current Opinion in Neurobiol* 2015]

Hippocampal Ca dynamcis in behaving mice



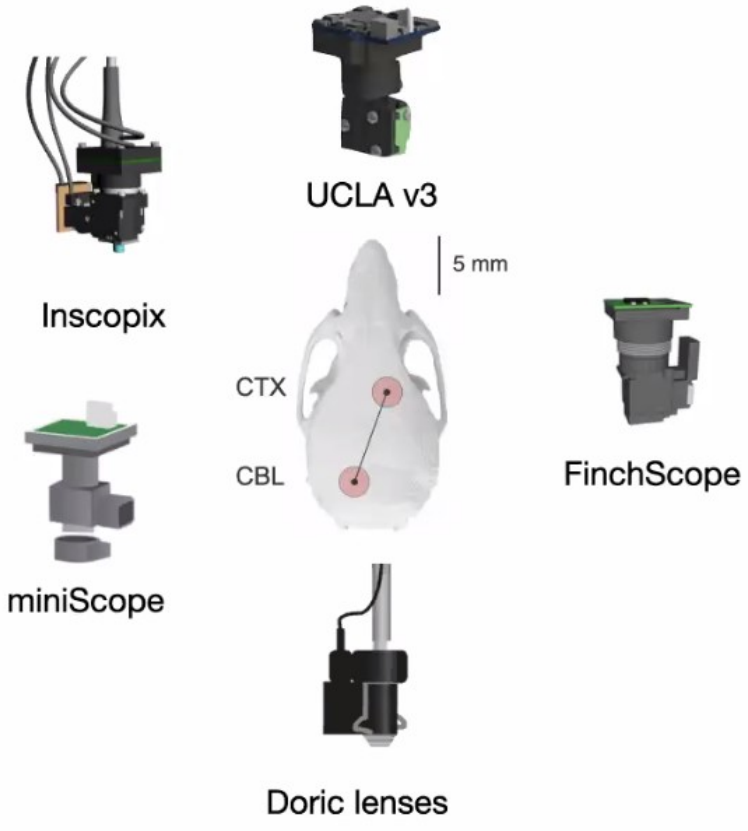
Hippocampal Ca dynamcis in behaving mice



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

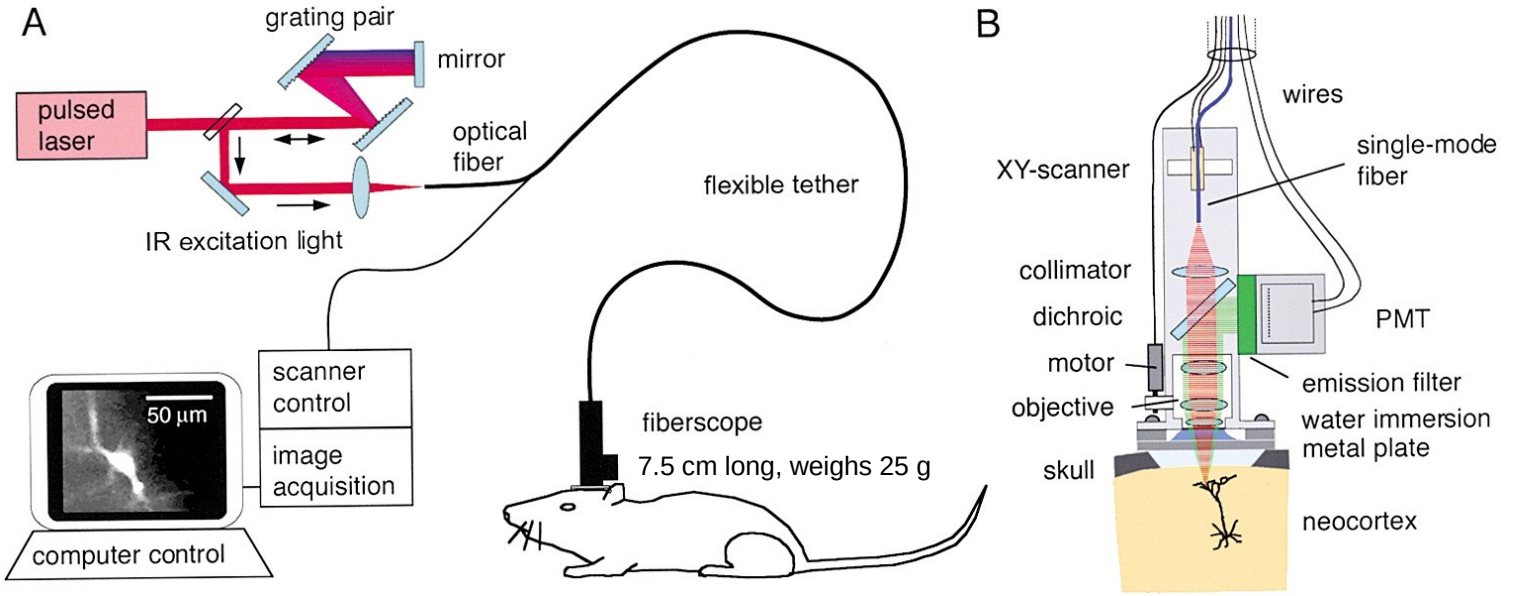
- epifluorescence imaging of pyramidal cells in CA1 region of the hippocampus
- cells in this region feature place-cells : cell which fire when animal enter a particular place in environment

Different wide-field (1p) miniscopes available



[status in 2017]

2p-laser scanning fiber-coupled microscope



[Helmchen et al. *Neuron* 2001]

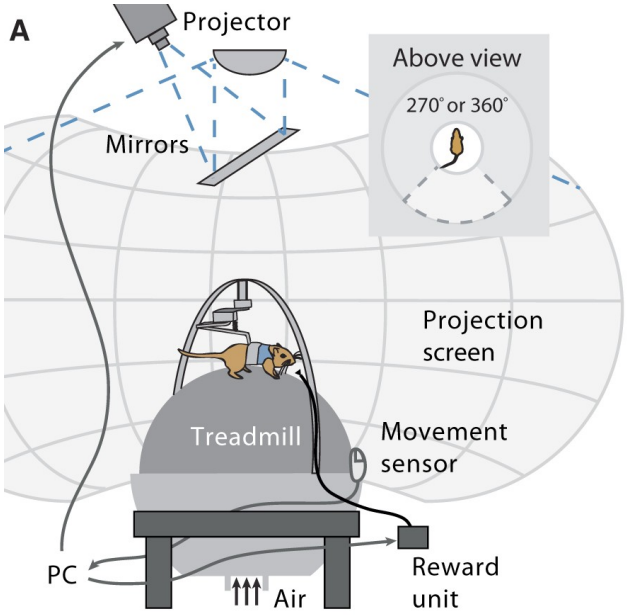
- light source at remote location from the animal
- scanning mirrors and detector in fiberscope on the animal's head
- too heavy and bulky for small animal applications

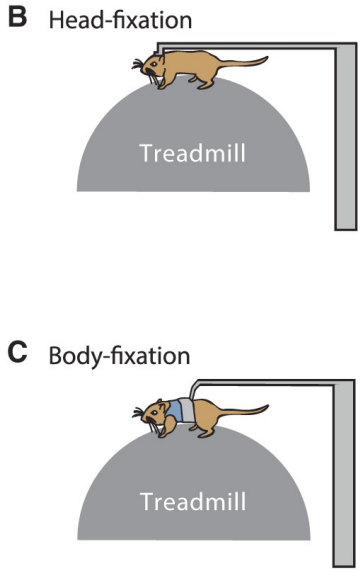
Summary of miniature 2-photon microscopes

	Denk group 2001	Helmchen group 2008	Kerr group 2009	Schnitzer group 2009
Resolution (μm)	unknown Unknown	0.98 7.68	1 unknown	1.3 10.3
Maximum field of view (μm²)	65 x 65	200 x 200	250 x 250	100 x 295
Maximum speed (Hz)	2 (128 x 128)	8 (512 x 512)	8.2 (64 x 64)	4 (400 x 135)
Scanning mechanism	Fiber scan	Fiber scan	Fiber scan	MEMS scan
Weight	25 g	0.6 g	5.5 g	2.9 g
Excitation wavelength	820–850 nm	812 nm	925 nm	790–810 nm
Pulse width after the objective	100 fs (10 mW) 1 ps (180 mW)	100 fs	1.8 ps	~110 fs
Laser power after the objective	Not shown	~150-200 mW	100-150 mW	27 mW
Free-moving experiment	Rat	Not shown	Rat	Not shown
GFP/GCaMP imaging	Not capable	Not capable	Low efficiency	Not capable

as of today, no functioning, commercially-availabe miniature 2-photon microscope exists

Virtual reality (VR) systems : visual VR

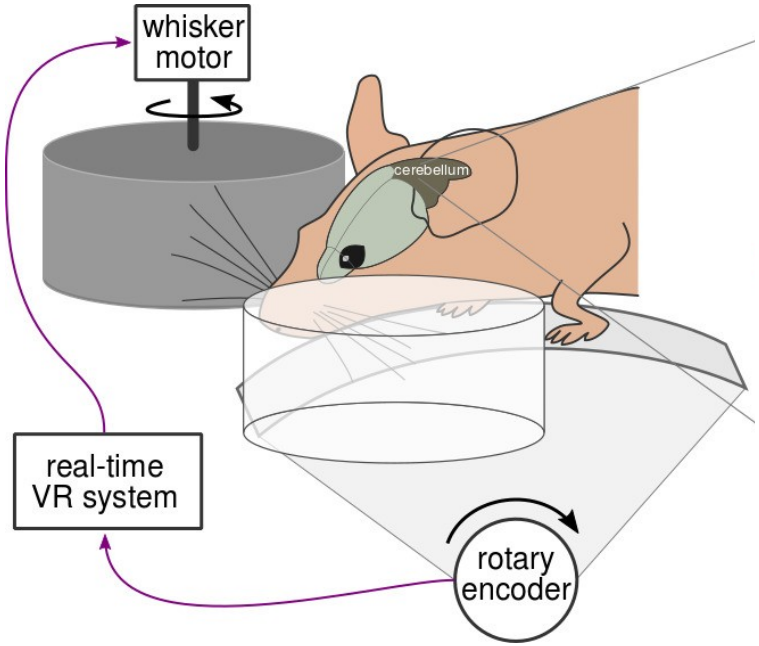




[Thurley & Ayaz, *Current Zoology* 2017]

- 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

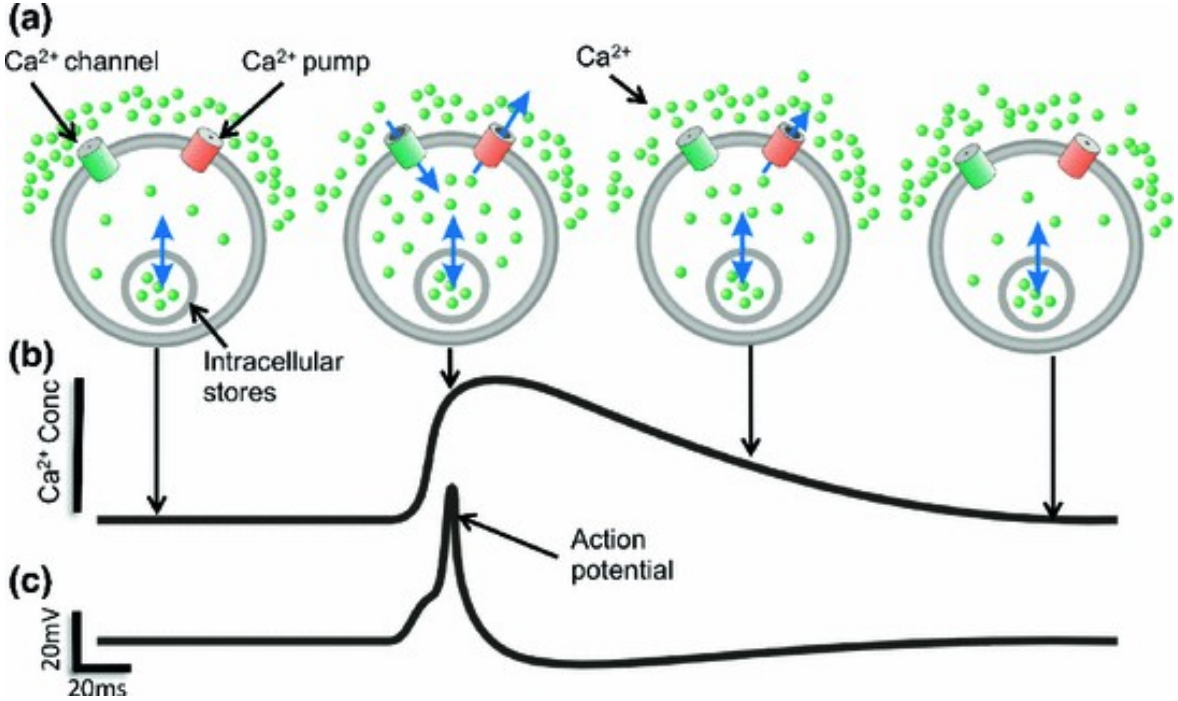
Virtual reality (VR) systems : tactile VR



[Stell *unpublished* 2019]

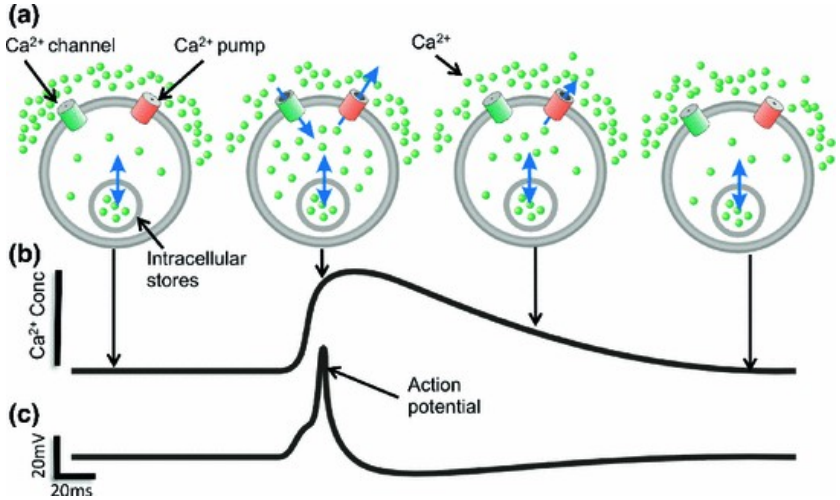
- creating a sensorimotor loop between locomotion and tactile feedback (i.e. mechanic stimulation linked to movement)
- animal is restrained, animals paw movement is recorded and controls rotation of whisker wheels

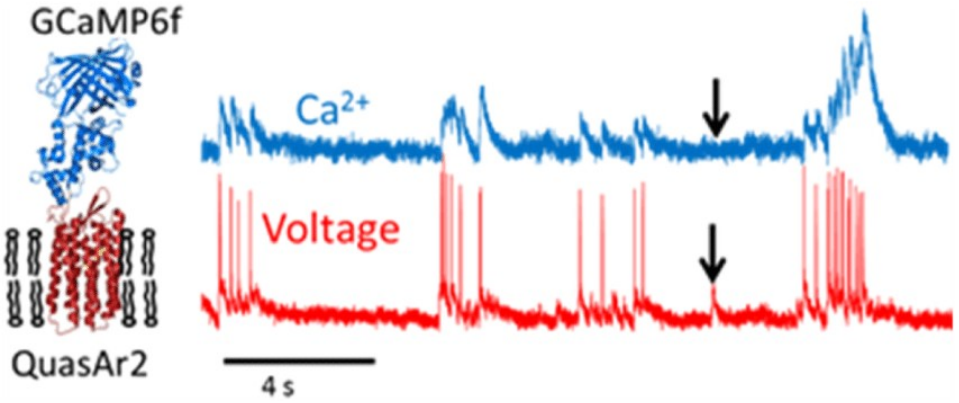
Calcium vs. voltage imaging



- 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





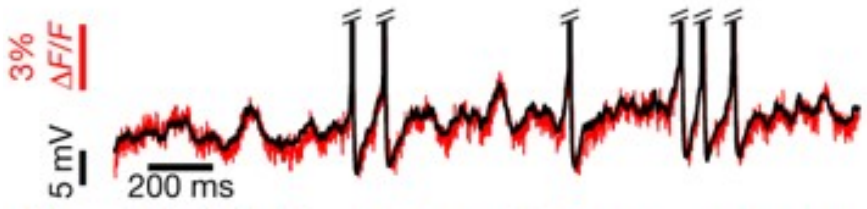
Calcium imaging

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

Voltage imaging

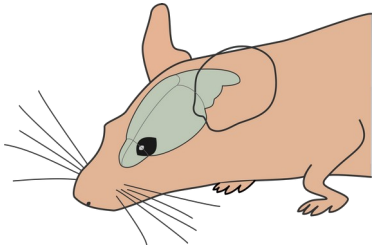
- genetically encoded voltage indicators (GEVIs, e.g. QuasAr, ASAP) report directly transmembrane voltage
- located in cell membrane

Challenges of voltage imaging



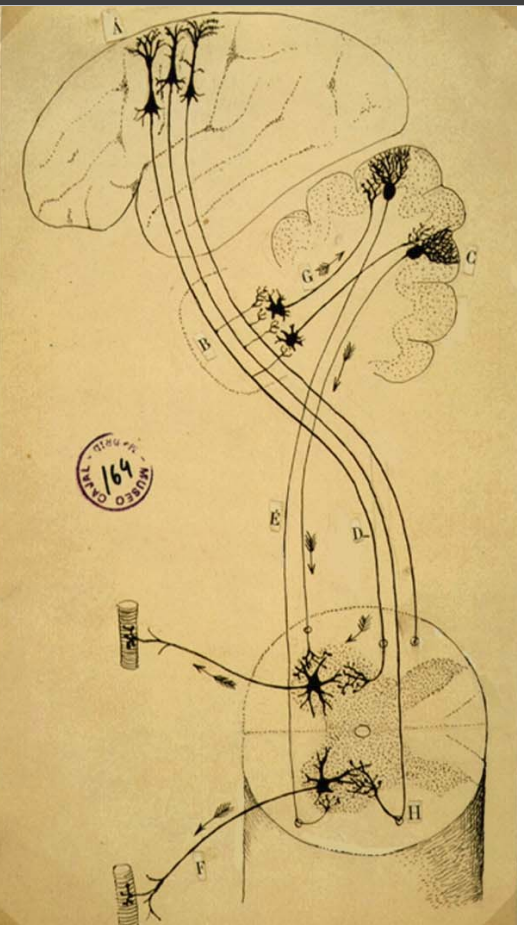
- Requires high-speed microscopes due to short duration of action potentials (~2 ms)
- Photobleaching due to constant illumination
- Requires good membrane trafficking of fluorophores
- Requires exceptionally bright fluorescence due to fewer fluorescent proteins in field of view (volumne vs. surface)

Outline of the talk



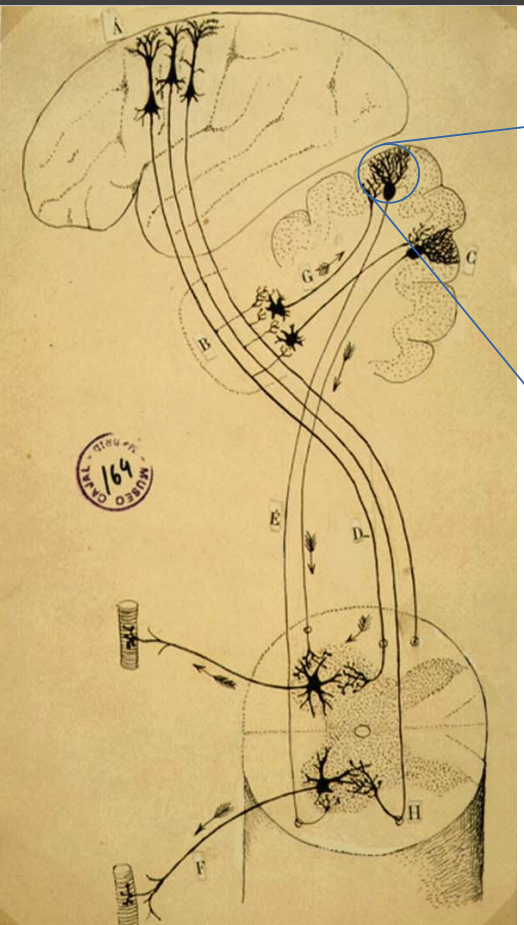
1. Basics of *in vivo* imaging
   - parts list for imaging experiment
   - challenges of deep tissue imaging
   - 1- vs. 2-photon imaging
2. Considerations of *in vivo* imaging in awake animals
   - sensory modalities studied
   - practical implementation : head-fixed vs. 'freely' moving
   - virtual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

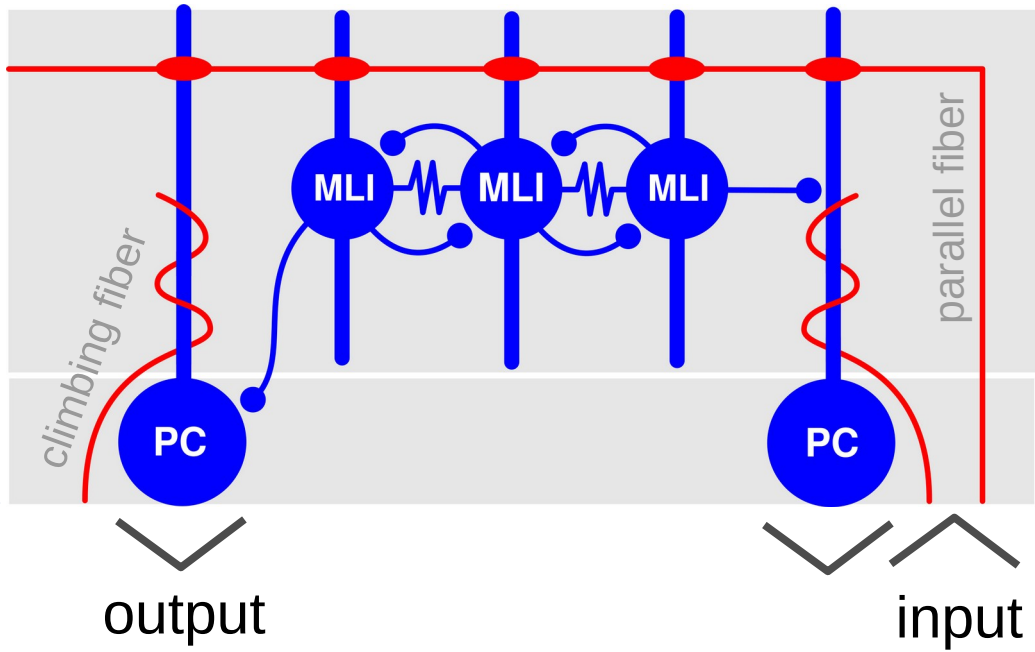
Ongoing project in the lab : Cerebellum and locomotion



- 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*



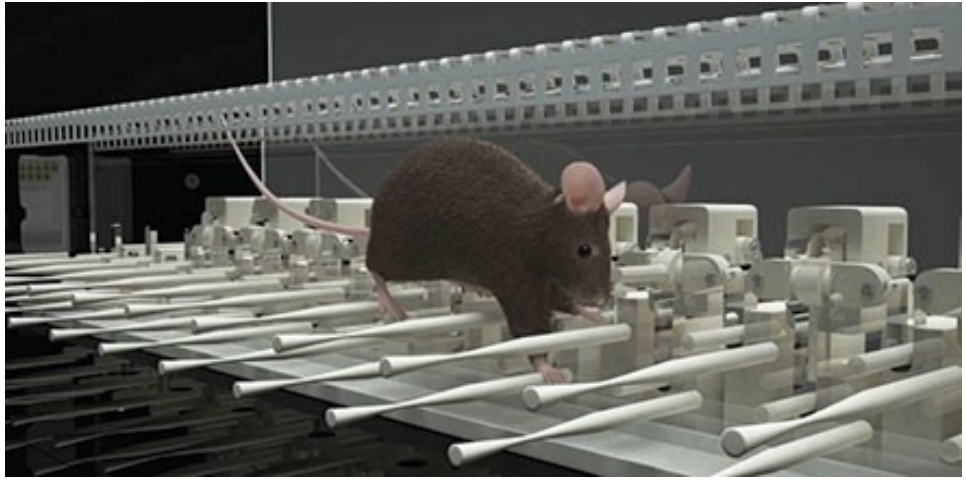


MLI ... molecular layer interneuron

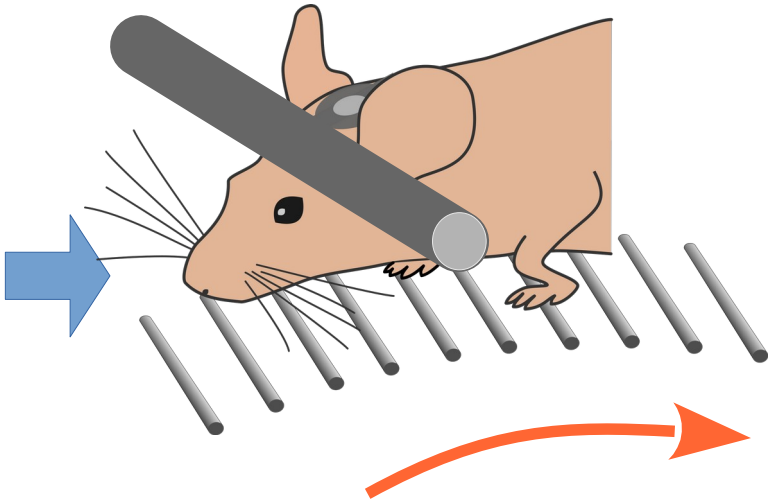
PC ... Purkinje cell

Task to study motor coordination on cellular level

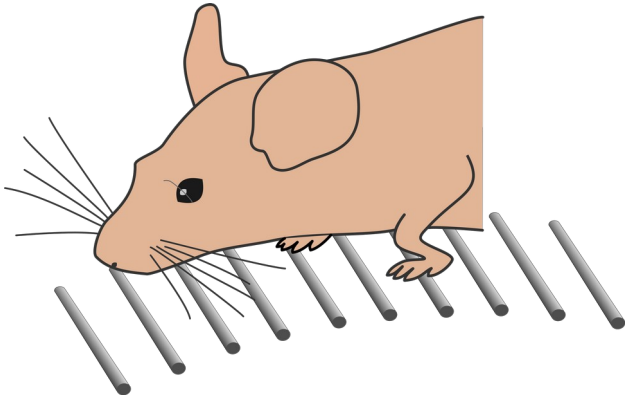
Erasmus Ladder | Noldus



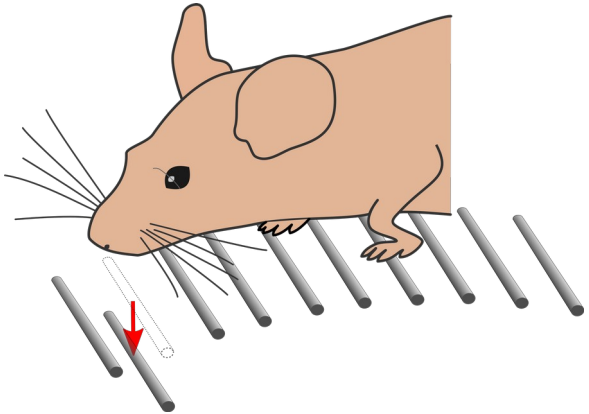
Acquisition of a
complex motor task
in head-fixed animal



Task to study motor coordination on cellular level

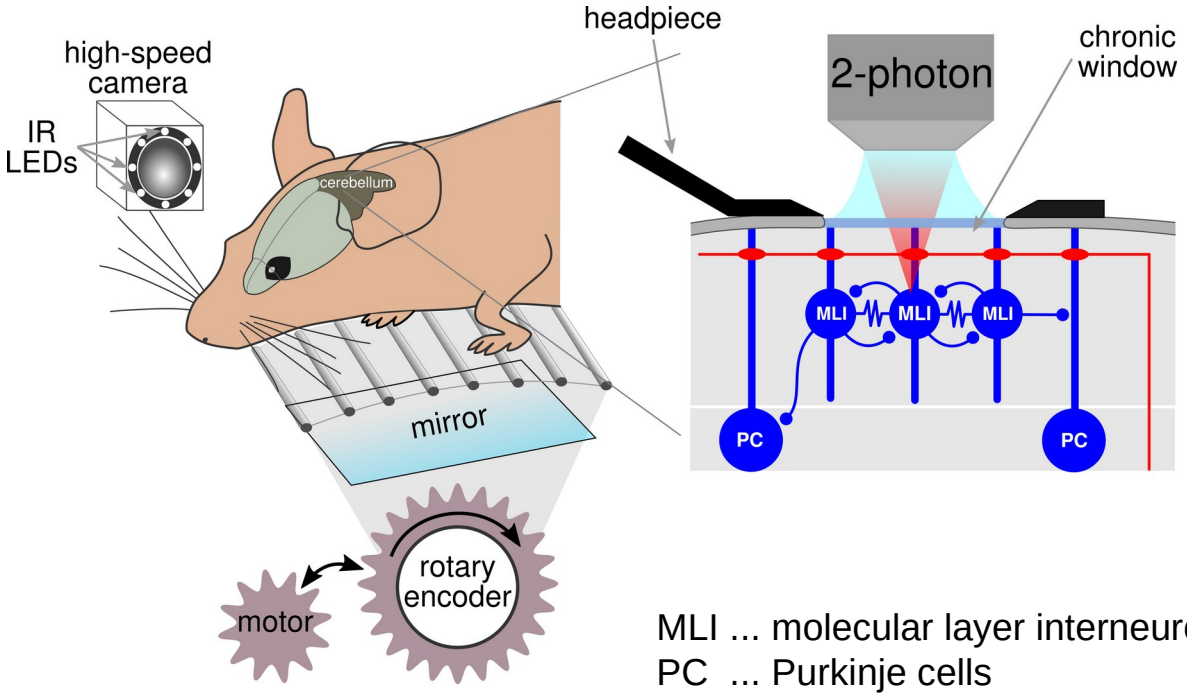


1) acquisition of a complex motor task



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

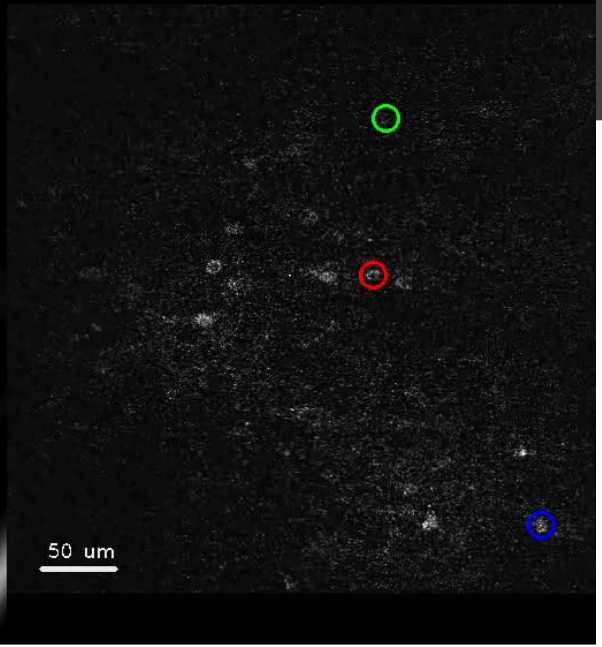
Experimental methods and setup

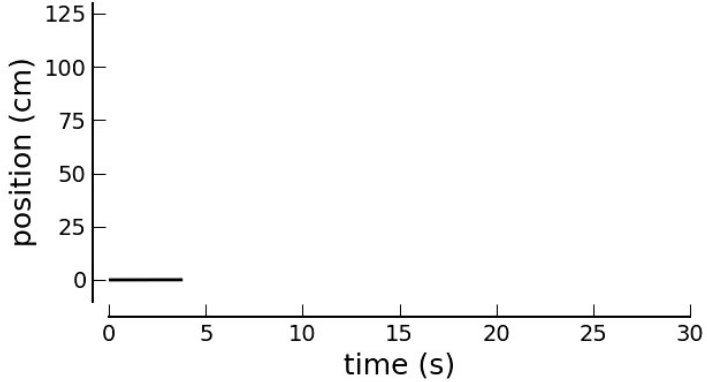


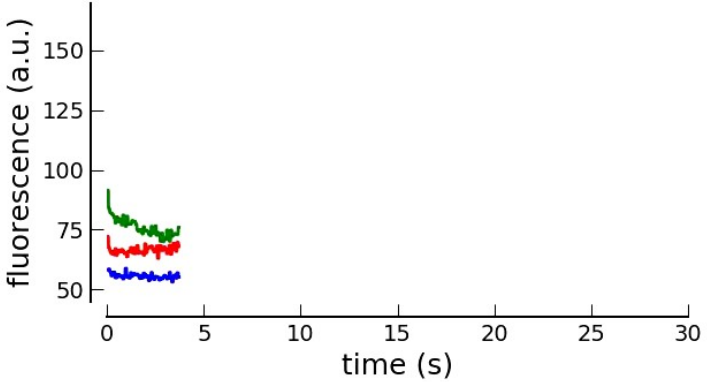
MLI ... molecular layer interneurons
PC ... Purkinje cells

- 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

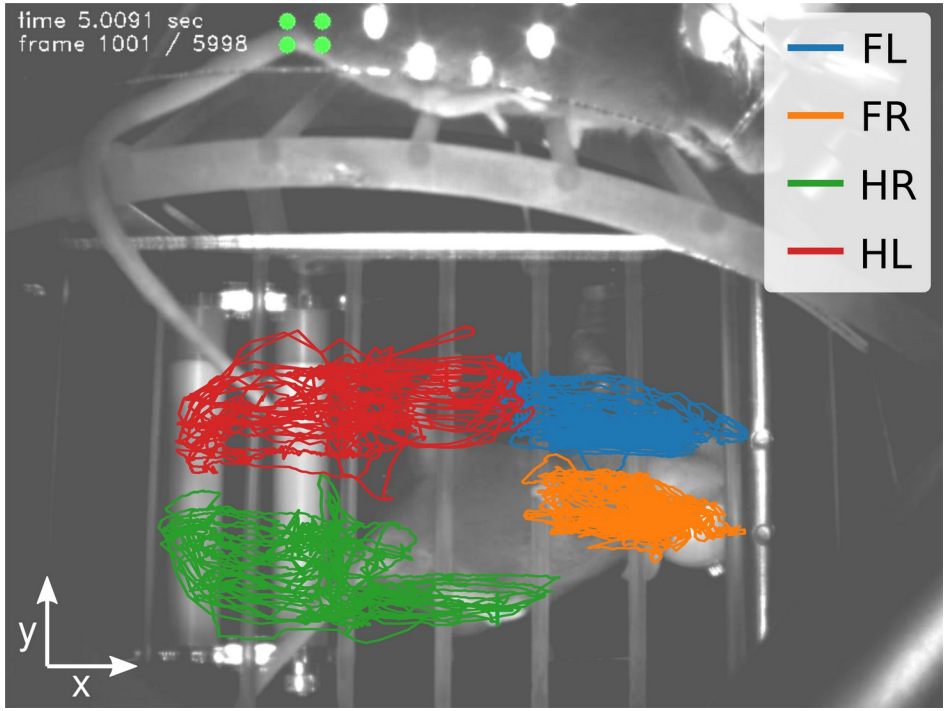

time 3.7301 sec
frame 0745 / 5998

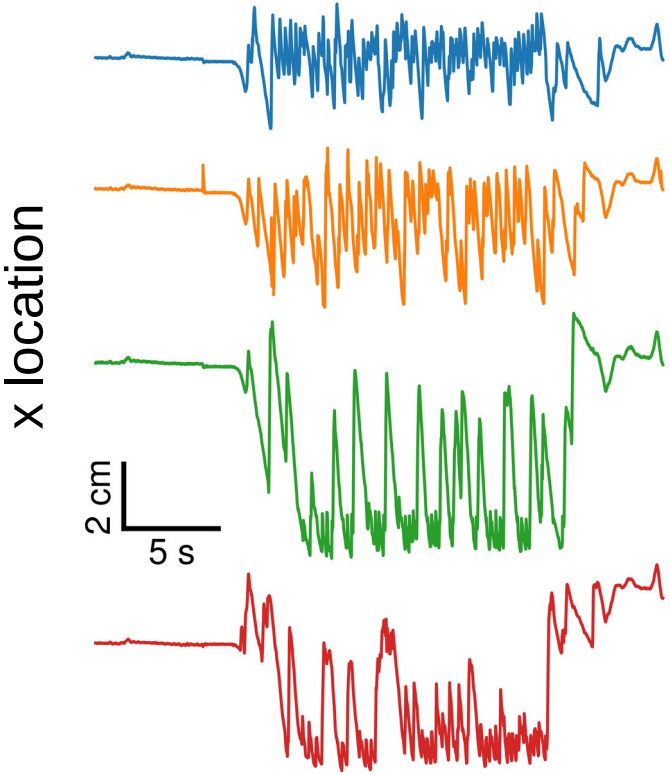

50 um


position (cm)
125
100
75
50
25
0
0
5
10
15
20
25
30
time (s)


fluorescence (a.u.)
150
125
100
75
50
0
5
10
15
20
25
30
time (s)

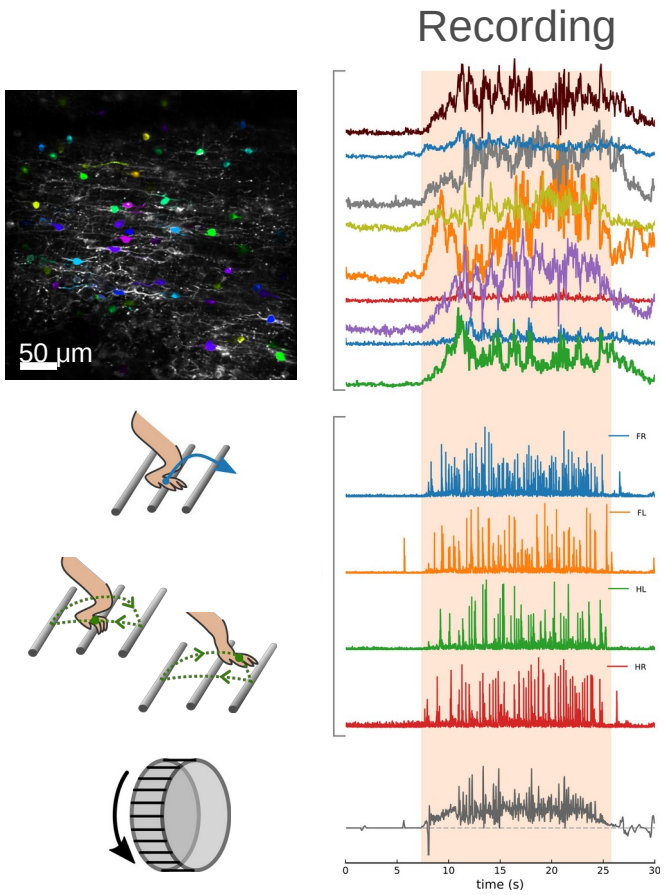
Extraction of paw trajectories with DeepLabCut





[Mathis *et al. Nat Neurosci* 2018]

Question: Link btw. calcium activity and locomotion?



Calcium imaging data:

- reflecting activity of a local MLI network

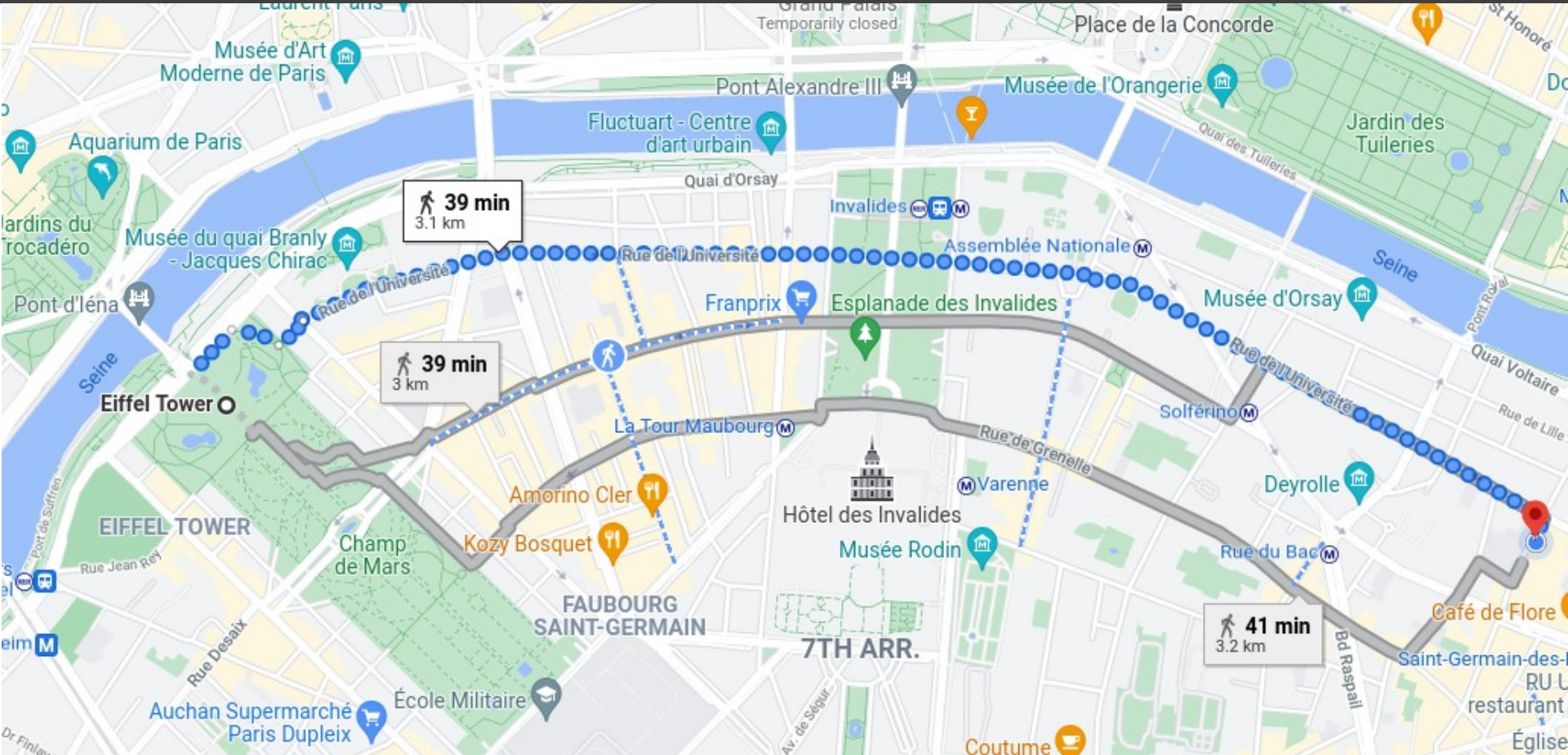
Paw trajectories → speed:

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

Wheel speed:

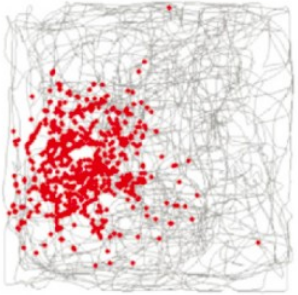
- reflecting overall locomotion state involving multiple limbs

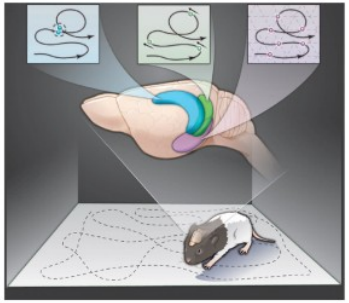
Investigating neural circuits for orientation



Cells and circuits coding for space

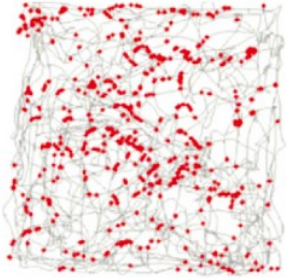
Place cells
hippocampus

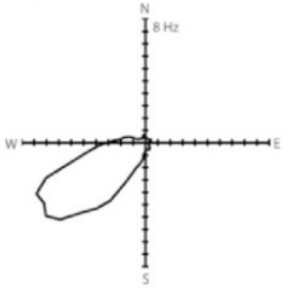




O'Keefe et Nadel 1978

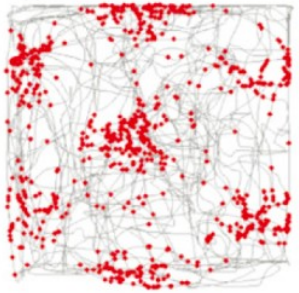
Head direction cells
presubiculum

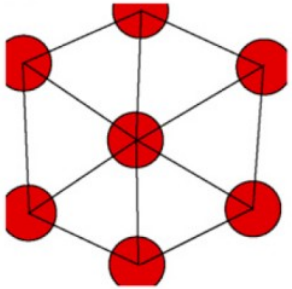




Taube et al. 1990

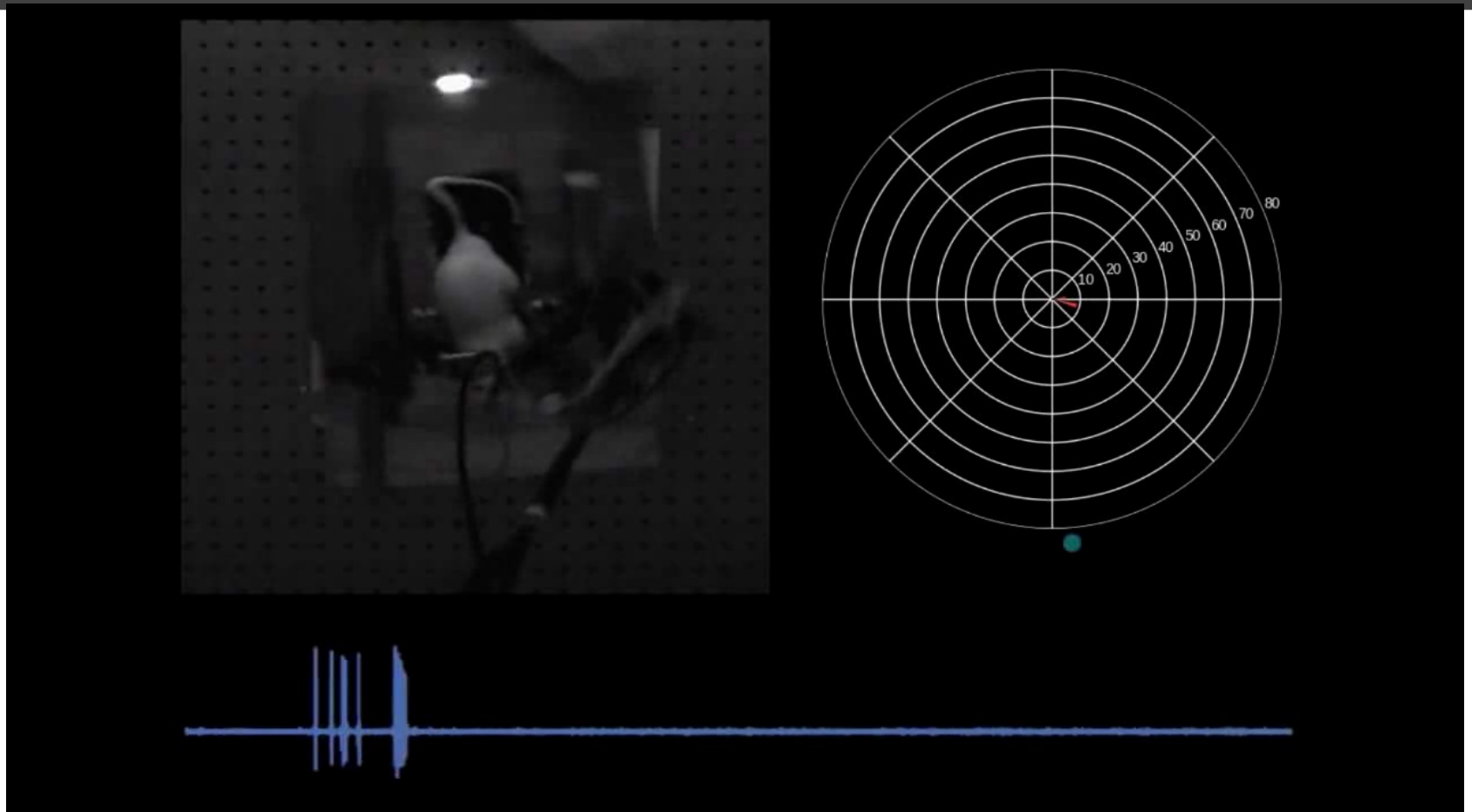
Grid cells
entorhinal cortex



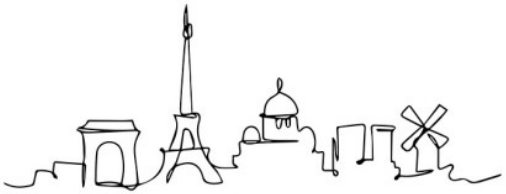


Fyhn et al., 2004

Head-direction neurons in the presubiculum



Presubiculum integrates vestibular and visual inputs



Visual inputs

Visual cortical areas

Thalamic and brain stem areas

Presubiculum

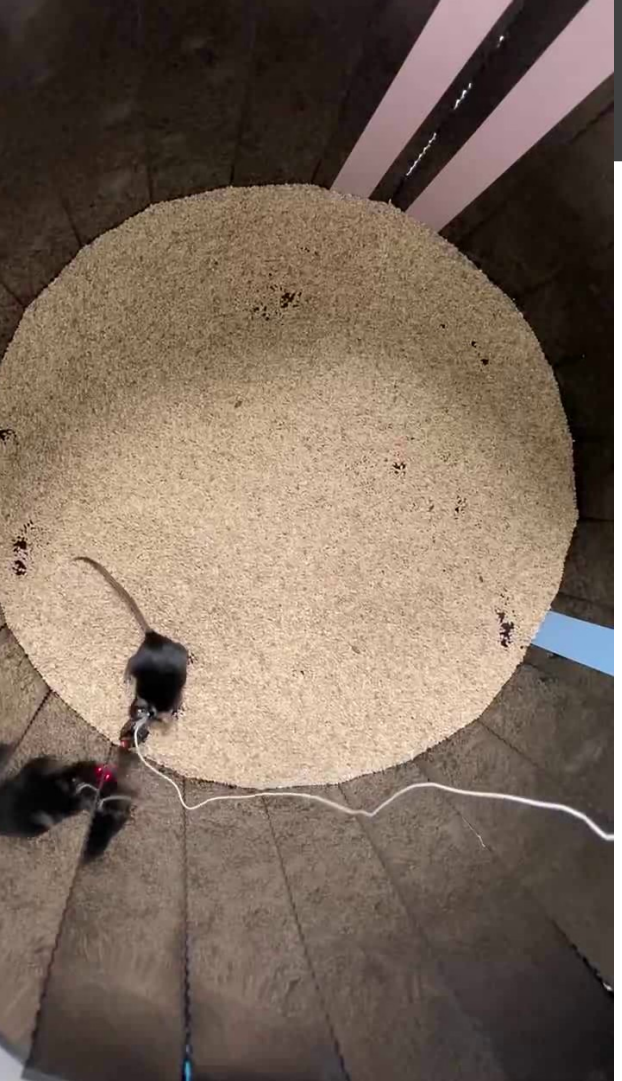
head-direction cells

Vestibular inputs

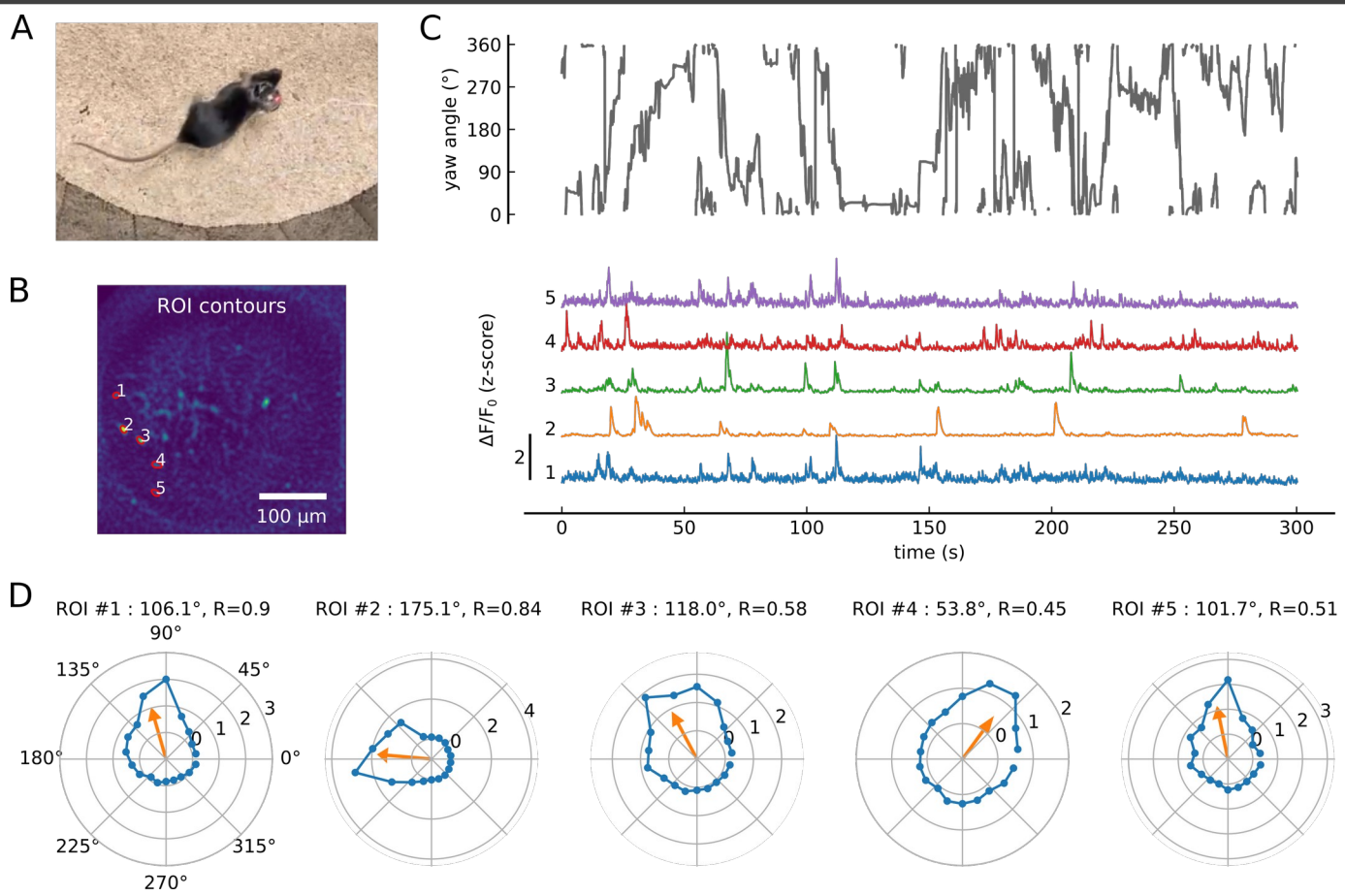
Question :

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

Calcium imaging in presubiculum



Experiments with miniscope : head-direction neurons



In vivo imaging as tool to study sensorimotor integration