Whole hippocampus high-resolution optical imaging

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Advanced microscopy methods for neuroscience

Biomedical label-free imaging

Single-molecule biophysics

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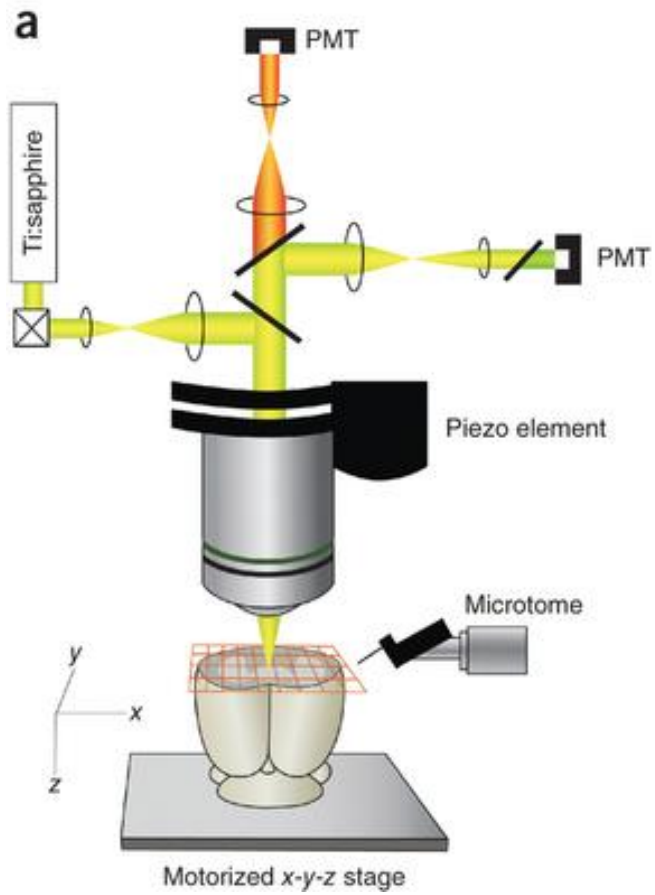
Single-molecule biophysics

Optical techniques: serial two-photon microscopy (STP)



Osten and Margrie, Nat Meth 2013

In serial two-photon imaging the brain is imaged with a scanning two-photon microscope up to a depth of several hundredths of microns, and then sliced away.

Pros

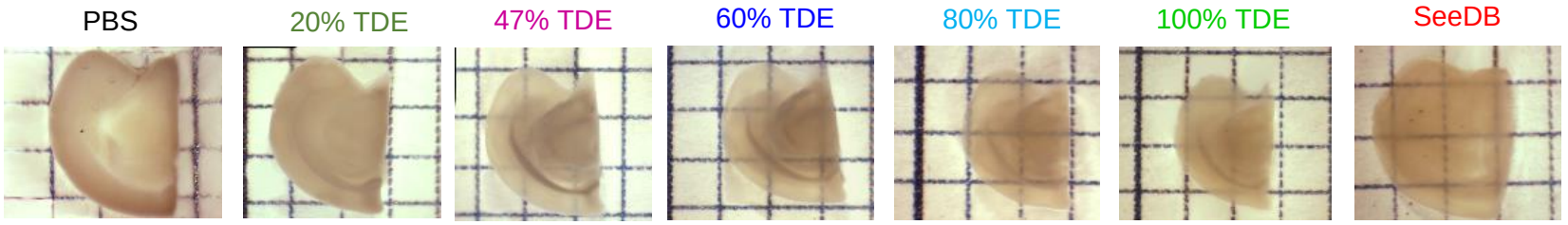
- **High resolution**
- **High sensitivity**

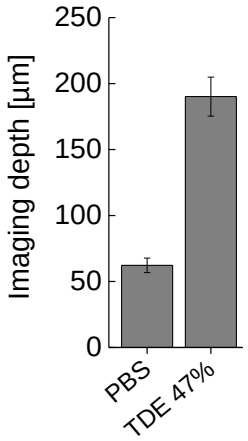
Cons

- **Limited penetration depth in fixed tissue (about 50-100 µm)**
- **Sparse axial sampling (1 µm every 50): in fact the initial layers are damaged by the cut, and the deep ones are not imaged clearly.**

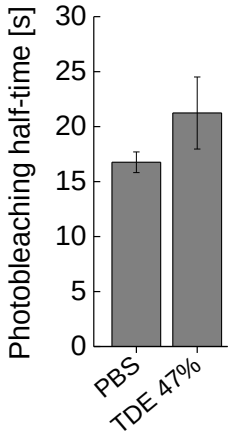
A new versatile clearing method: 2,2’ Thiodiethanol (TDE)

1. Direct clearing of small regions

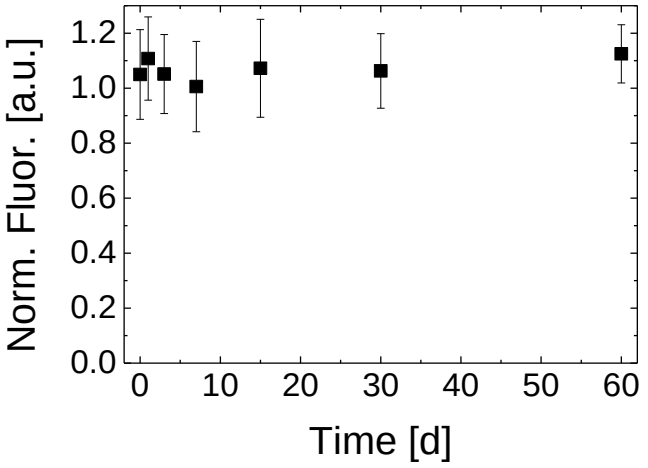




Imaging 4 times deeper than in fixed tissue



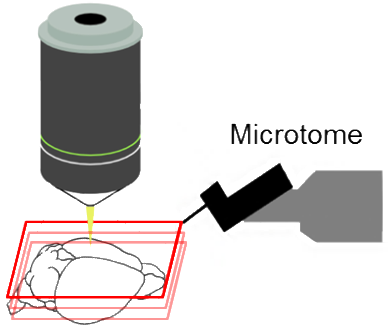
No increase in photobleaching



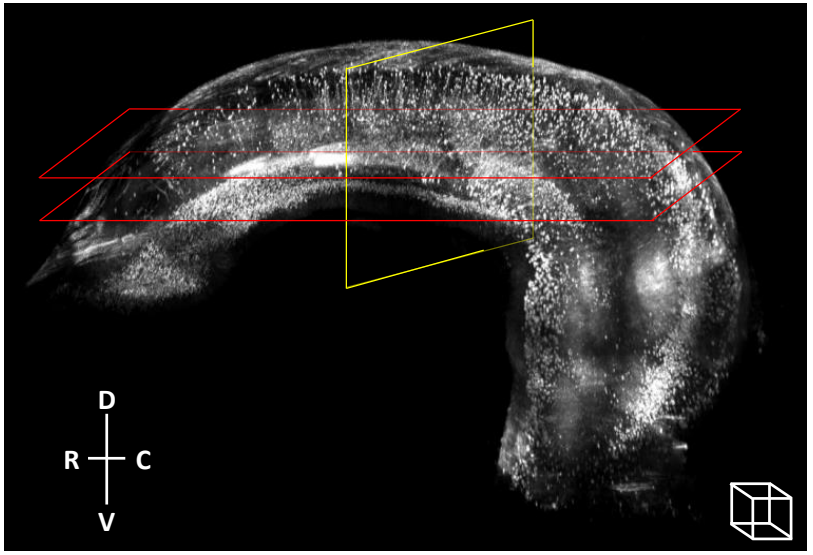
Fluorescence stable for months

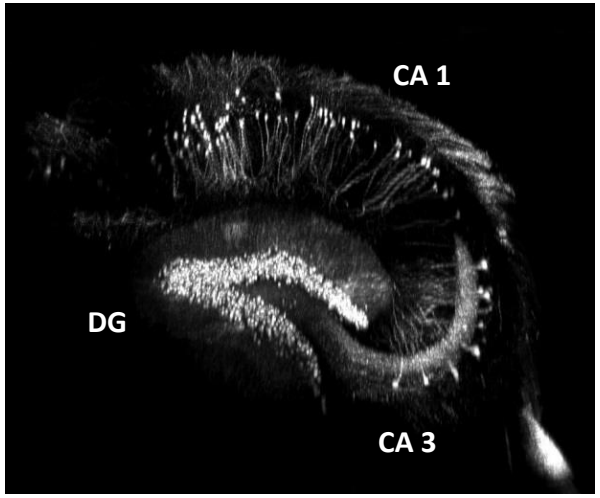
Costantini et al., Sci. Rep., *in press*

3D reconstruction of TDE cleared hippocampus with two–photon serial sectioning

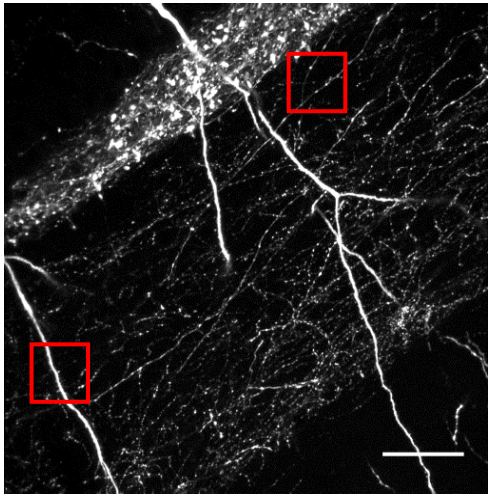


- **Micrometric resolution**
- **NO loss of information**

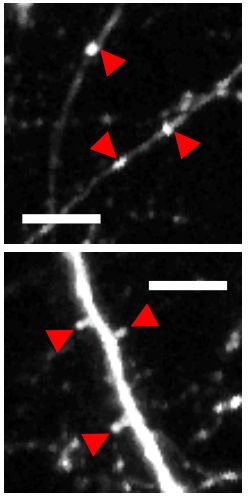




Thy1-GFP-M transgenic mouse



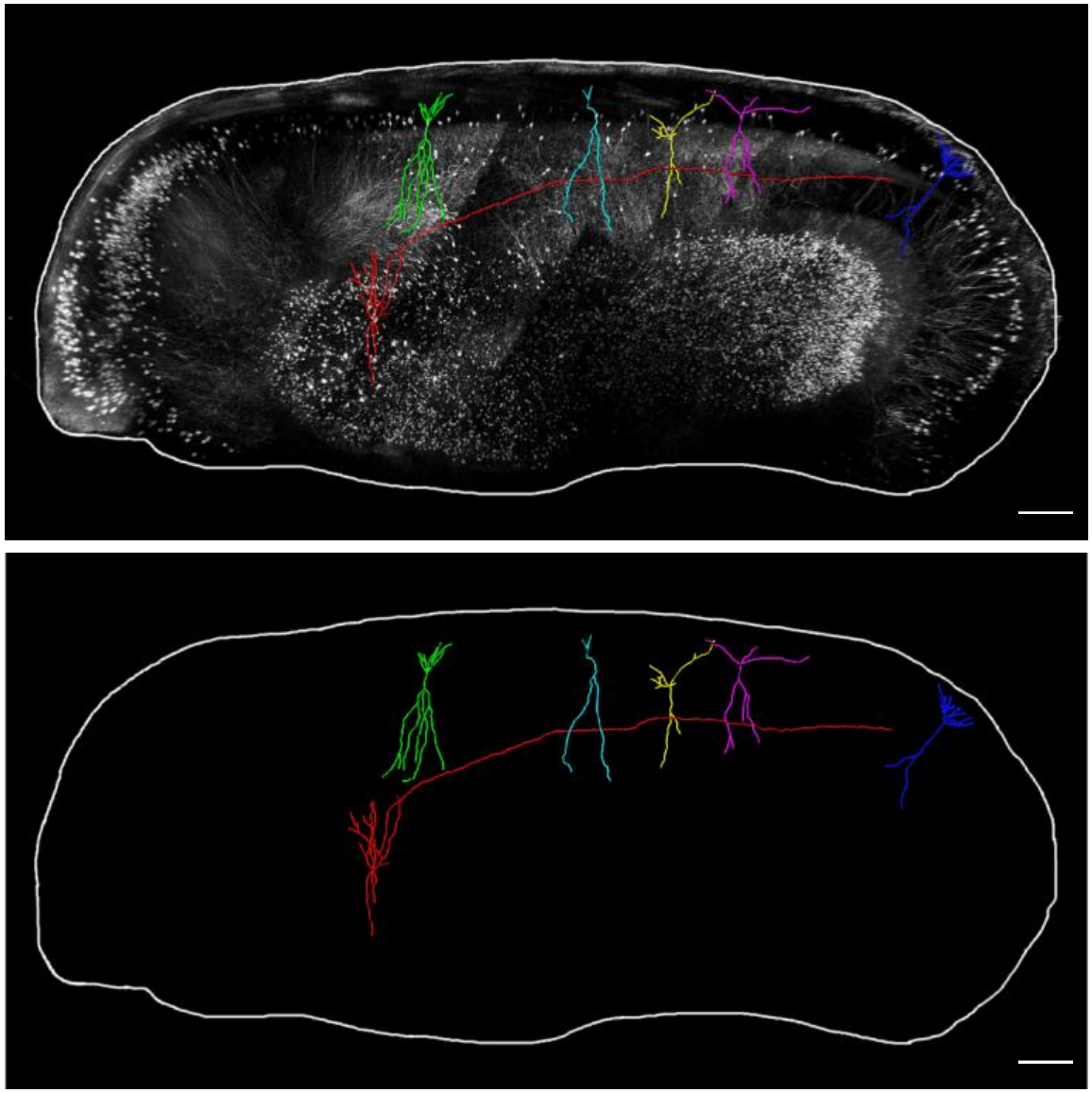
Scale bar 50 µm



Scale bar 10 µm

3D reconstruction of TDE cleared hippocampus with two–photon serial sectioning

Tracing of single neurons elongating through the entire hippocampus

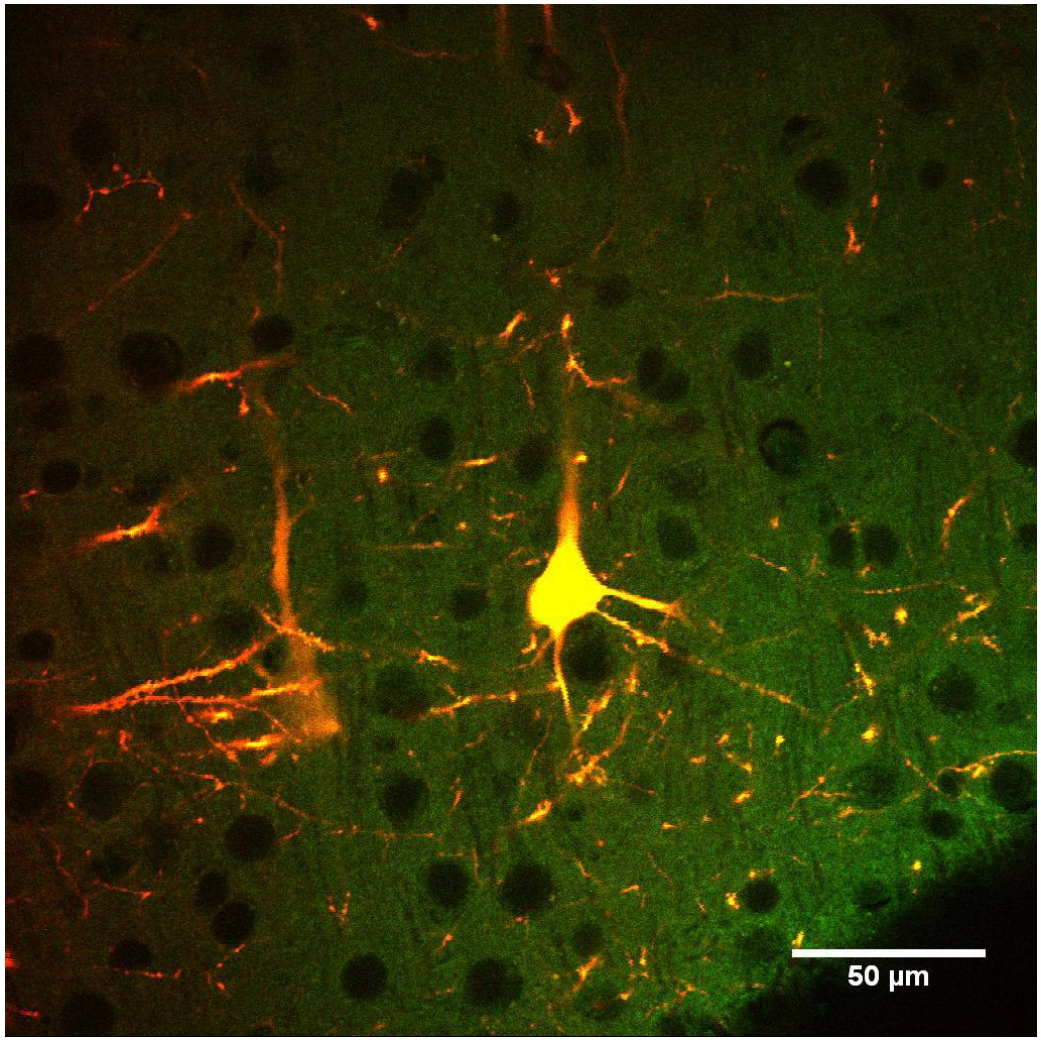


Scale bar 300 µm

IHC labeling + STP

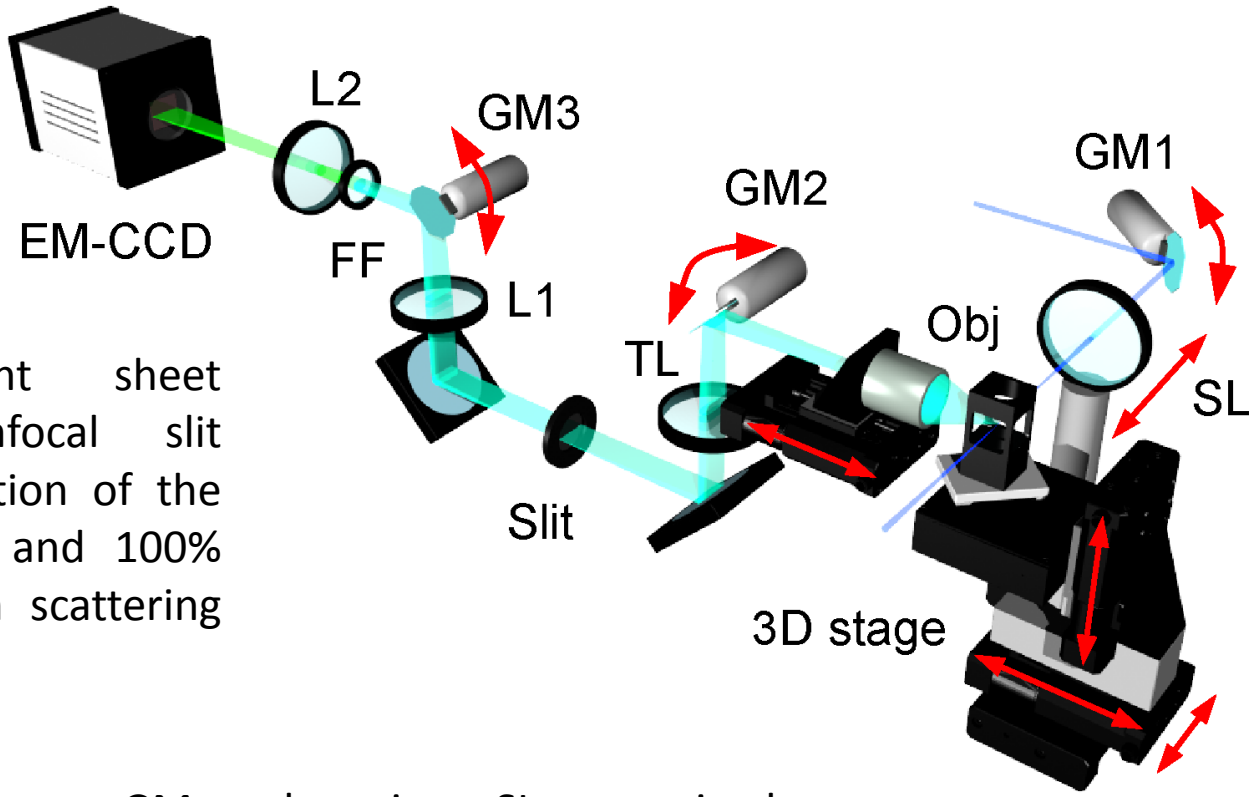
1 mm GFP-M mouse brain slice processed with CLARITY, immersed in TDE, and imaged with STP.
The sample was immunostained with an anti-GFP IgG alexa fluor 594 conjugate
(FOV=266 x 266 µm, z-step=5 µm, depth=400 µm, λ= 820nm)
Acquisition time: 6 minutes

Green channel: GFP
Red channel: anti-GFP antibody



Optical techniques: confocal light sheet microscopy (CLSM)

CLSM combines light sheet illumination with confocal slit detection, allowing rejection of the out-of-focus background and 100% contrast enhancement in scattering samples.

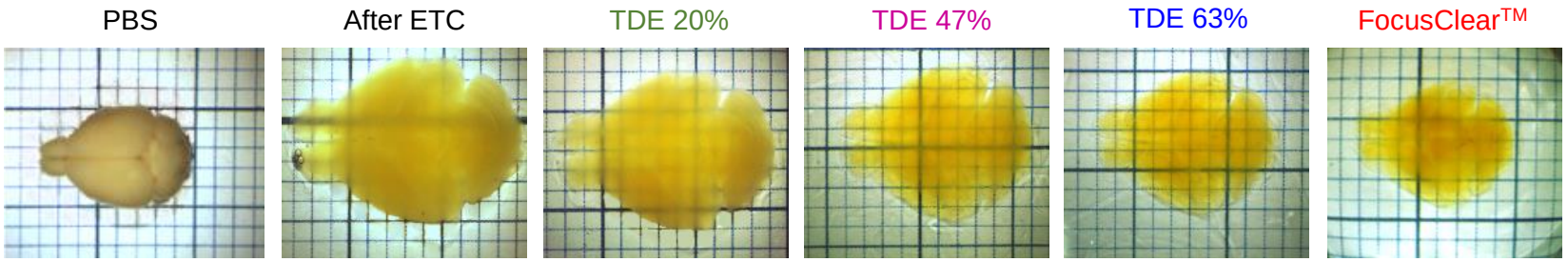


GM = galvo mirror, SL = scanning lens,
TL = tube lens, L = lens, FF = fluorescence filter

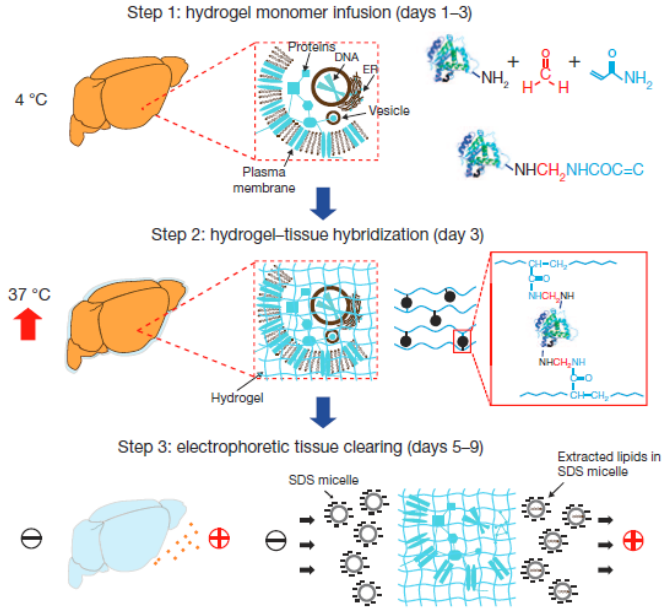
Silvestri et al., Opt. Exp. 2012

A new versatile clearing method: 2,2’ Thiodiethanol (TDE)

2. Whole-brain clearing in combination with CLARITY



Costantini et al., Sci. Rep., *in press*

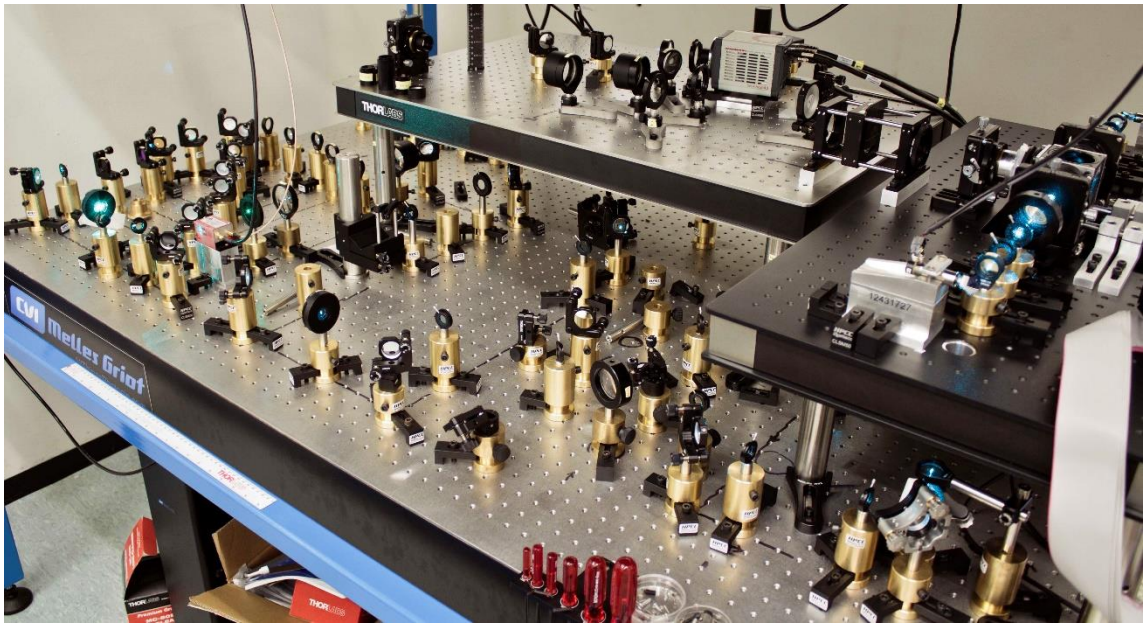


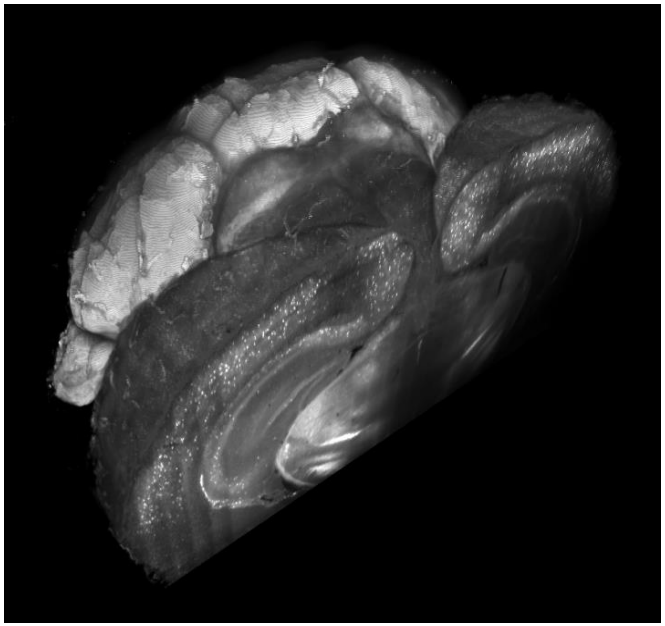
TDE is a valid alternative to FocusClear for refractive index matching in the CLARITY method.

Focus clear	**20$/ml**	**2-3000$/sample**
TDE	**0.2$/ml**	**20-30$/sample**

Chung et al., Nature 2013

Whole-brain imaging with light sheet microscopy





A 2nd generation light sheet microscope has been built

S/N improved by a factor 20

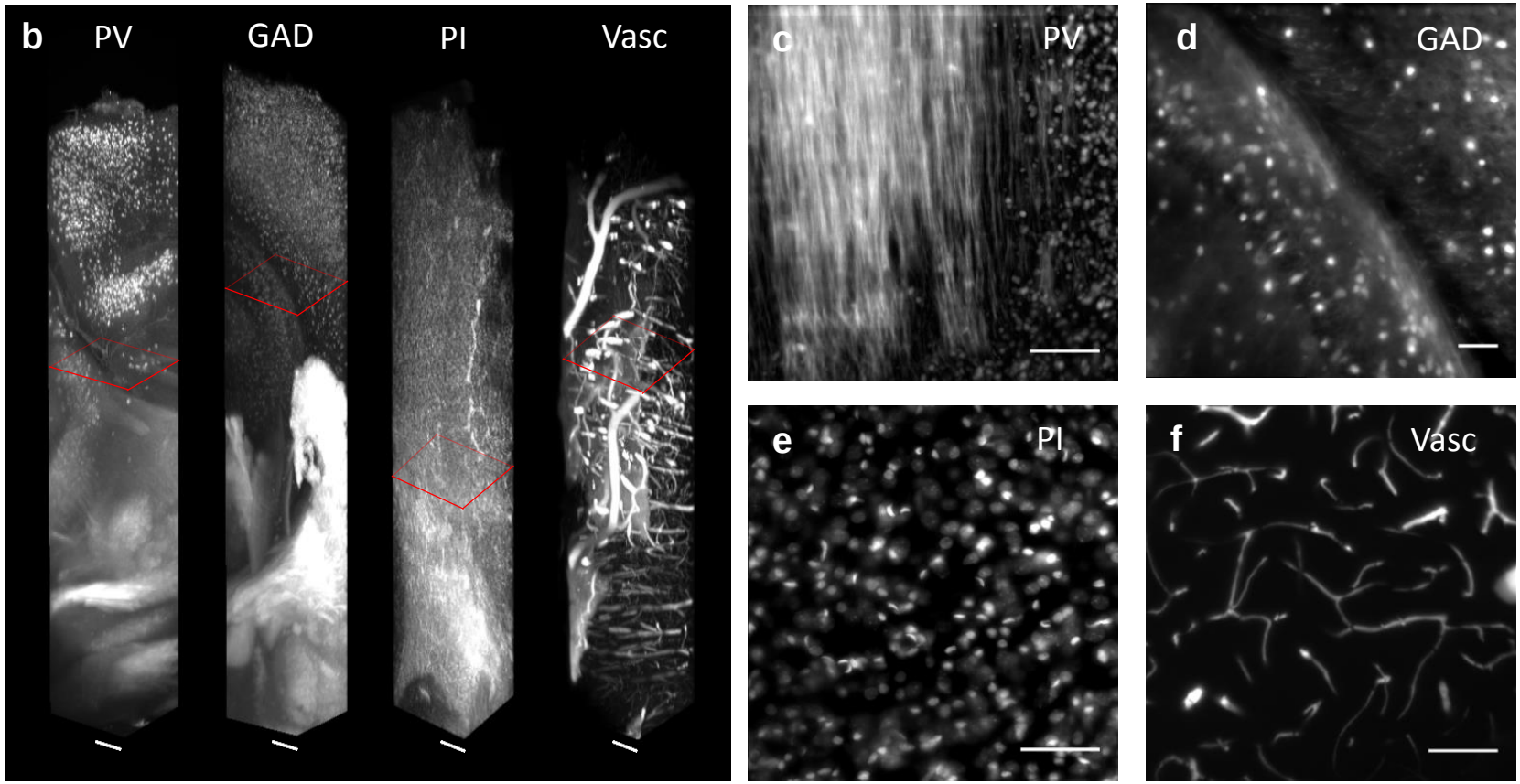
3D rendering from a PV-dTomato mouse brain (parvalbuminergic neurons labeled)

Main features:

- **Double-side illumination**
- **Optimized optics for CLARITY solution**
- **Confocal slit detection**
- **Multi-color imaging**

Costantini et al., Sci. Rep., *in press*

Whole-brain imaging with light sheet microscopy



PV: PV-dTomato mouse (parvalbuminergic neurons labeled)
GAD: GAD-dTomato mouse (GABAergic neurons labeled)
PI: propidium iodide staining (all nuclei labeled)
Vasc: vasculature filling with FITC-albumin

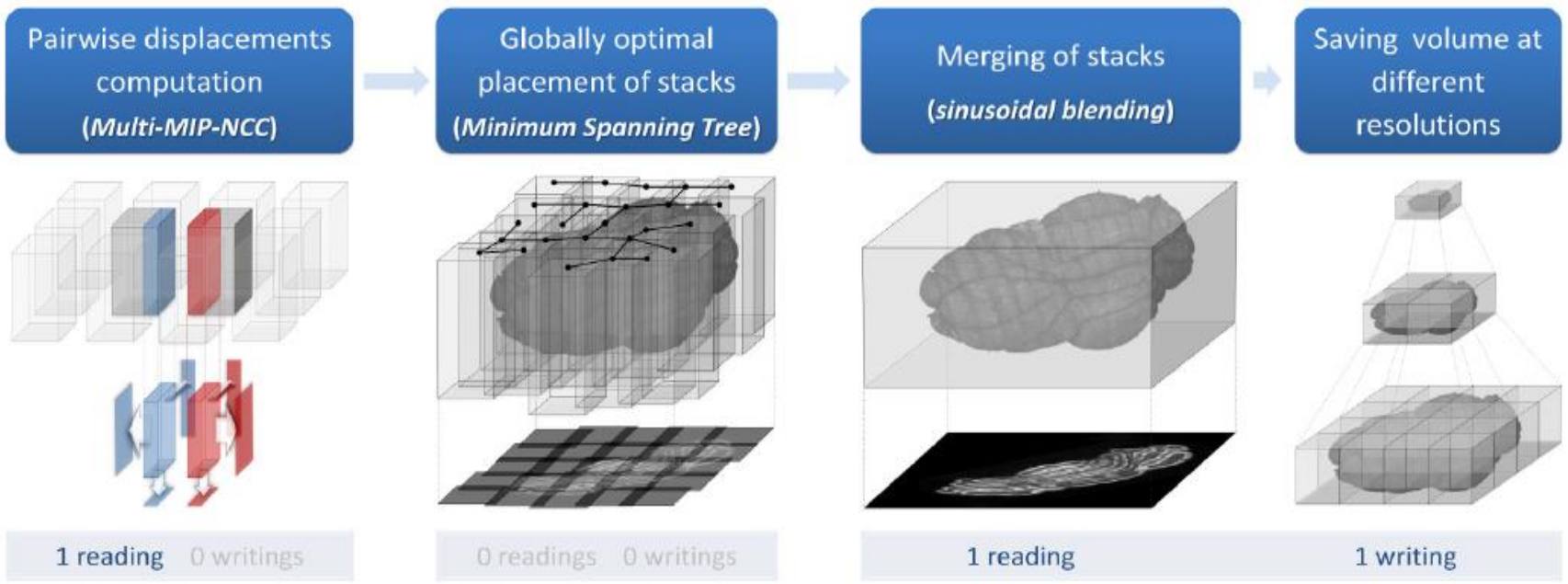
Scale bar 100 µm

Costantini et al., Sci. Rep., *in press*

Image management and processing

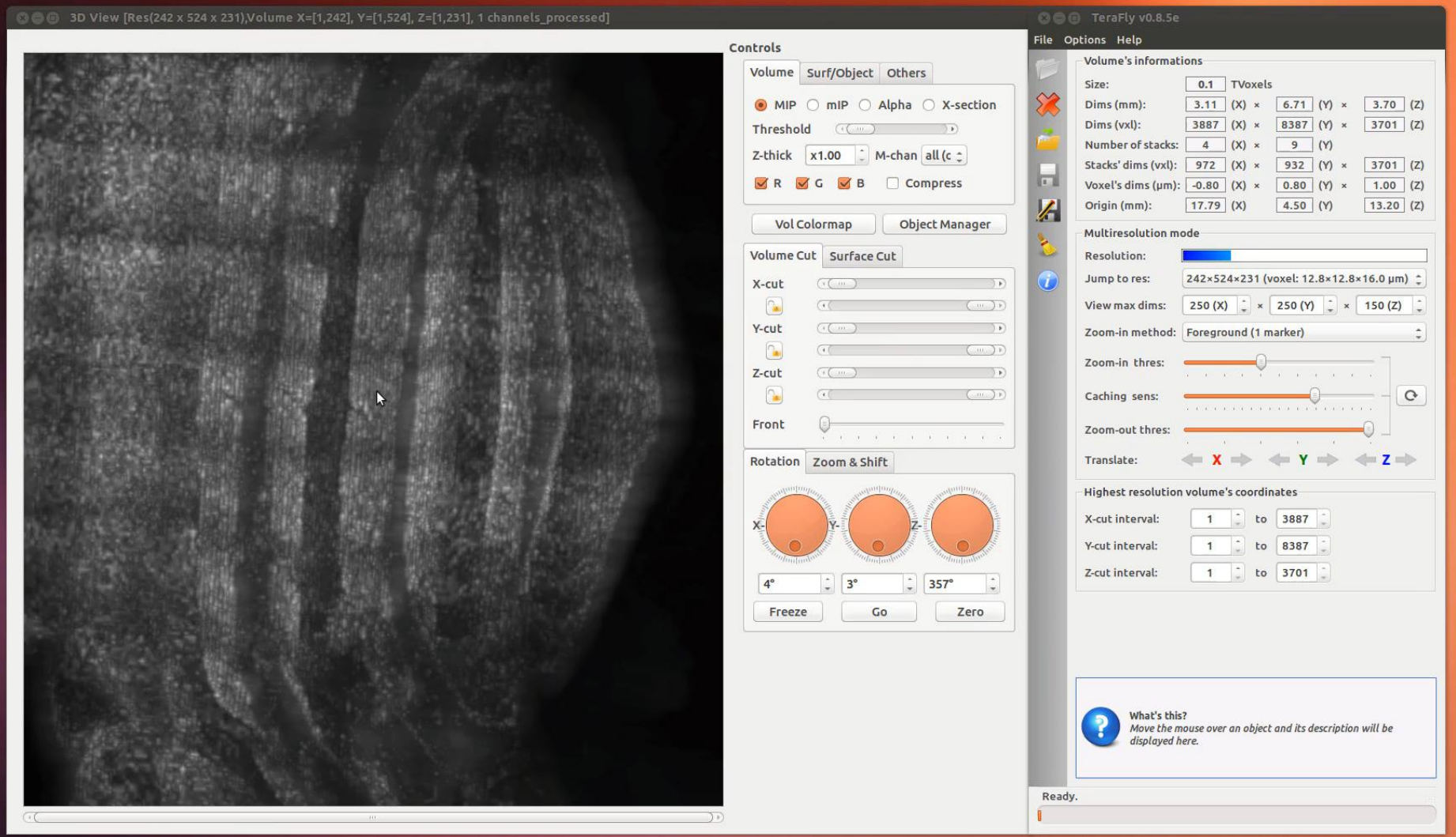
- **10 Gb/s** dedicated connection from **LENS** to **CINECA**
- Connection from **LENS** to **Juelich** via CINECA (using **PRACE** infrastructure)
- Data production now: about 2-3 TB per week
- Data production forecast (M18): 20 TB per week

Stitching Teravoxel-sized images: **TeraStitcher**



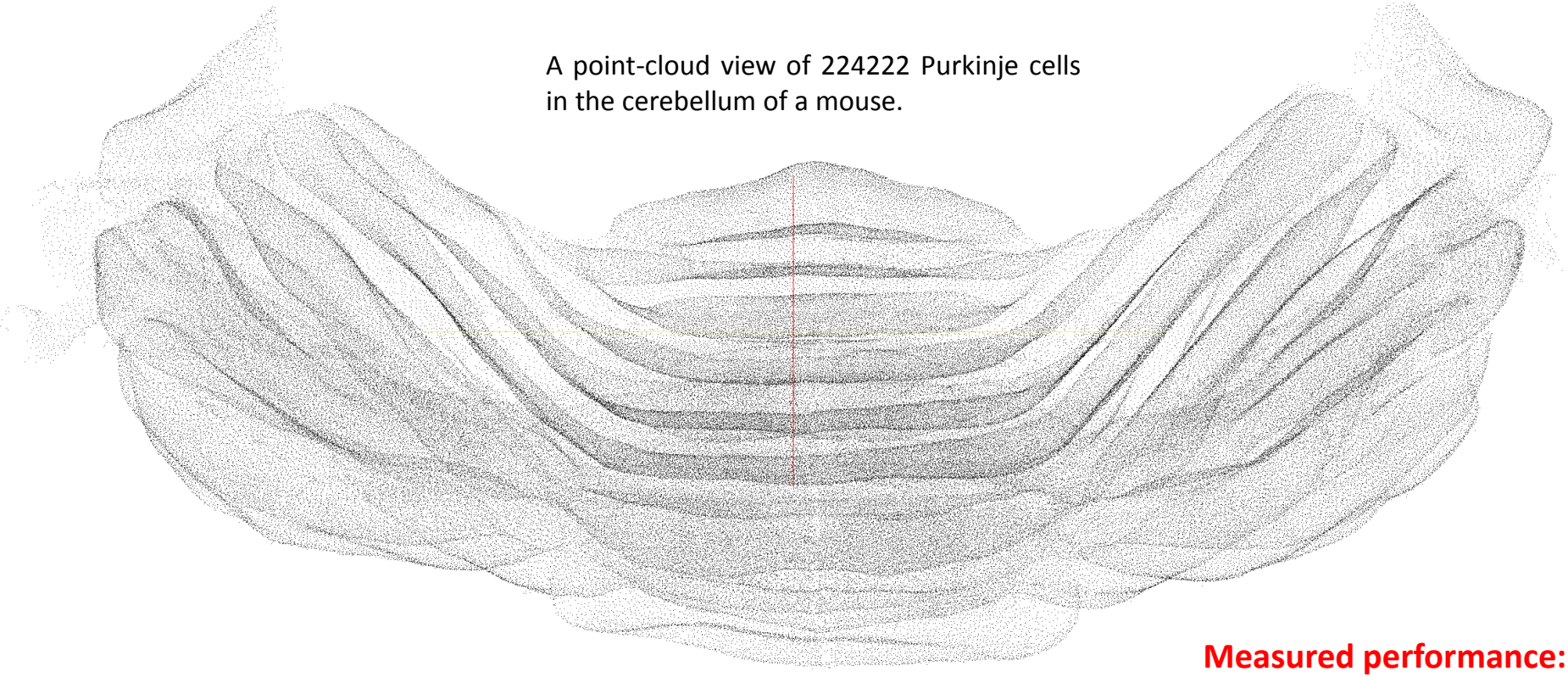
Bria et al., BMC Bioinformatics (2012)
http://github.com/abria/TeraStitcher

TeraFly



Peng et al., Nat. Prot. (2014) - a google-maps inspired brain navigation tool
Available as plugin of Vaa3D **http://www.vaa3d.org/**

Automatic cell localization



A point-cloud view of 224222 Purkinje cells in the cerebellum of a mouse.

The software performs a **"semantic deconvolution"** of the images through a supervised neuronal network to enhance features of interest (cell bodies) and weaken other structures. After this step a **k-means algorithm** is used to localize soma center. The limited memory usage of the software (compared to standard segmentation approaches) makes it highly scalable to large datasets.

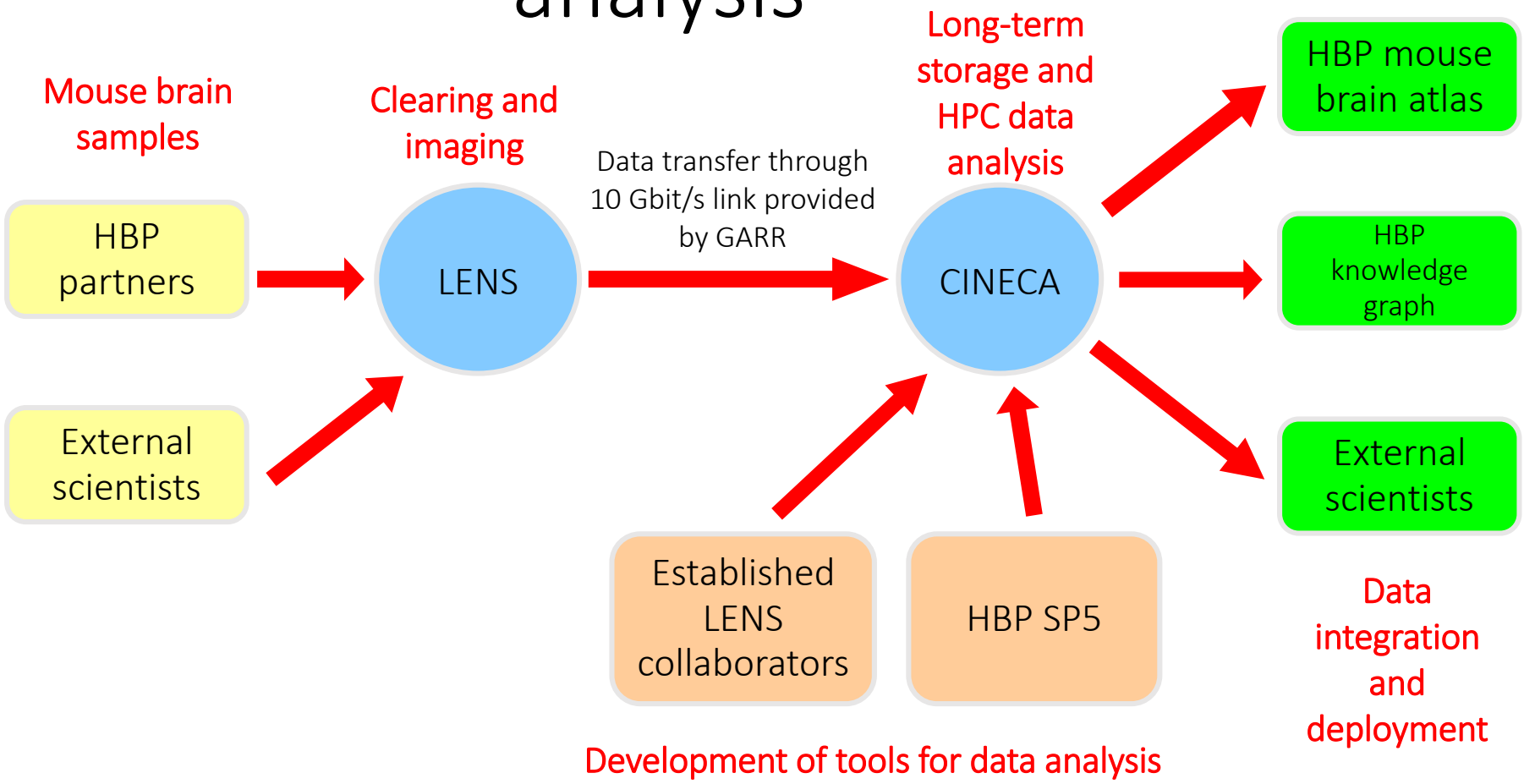
This dataset is being integrated into the HBP mouse brain atlas

Measured performance:
Precision [TP/(TP+FP)] 95%
Recall [TP/(TP+FN)] 97%

TP = True Positives
FP = False Positives
FN = False Negatives

Frasconi et al., Bioinformatics (2014)

An integrated pipeline for Big Data analysis

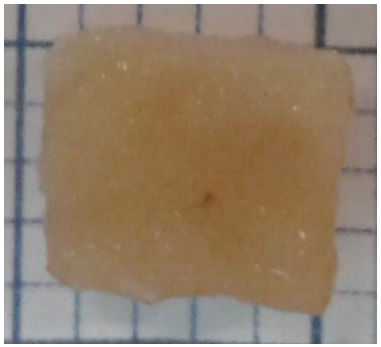


Data (2-3 TB per single imaging dataset) are physically stored @ CINECA. Software tools for data processing, information extraction and atlasing are deployed there (a new HPC machine dedicated to Big Data analytics – PICO – has just been set up). Data will be accessible outside through the HBP portal.

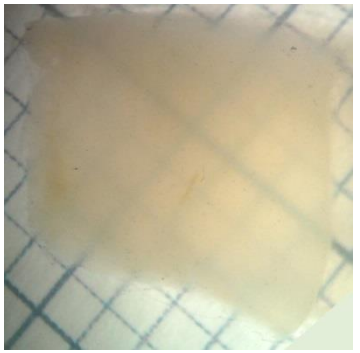
Human brain tissue preparation

Uncleared brain

After polymerization



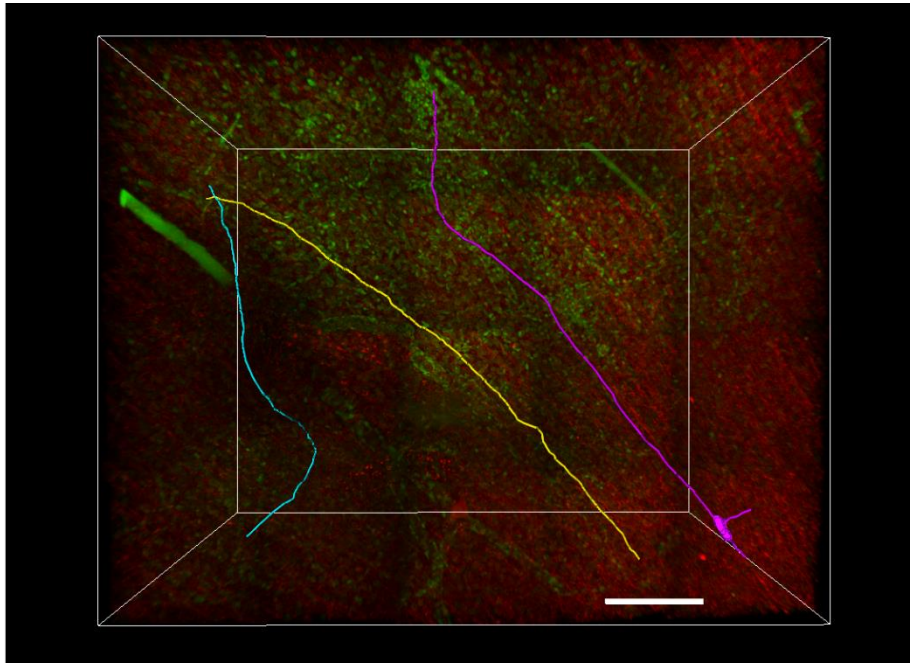
After passive clearing

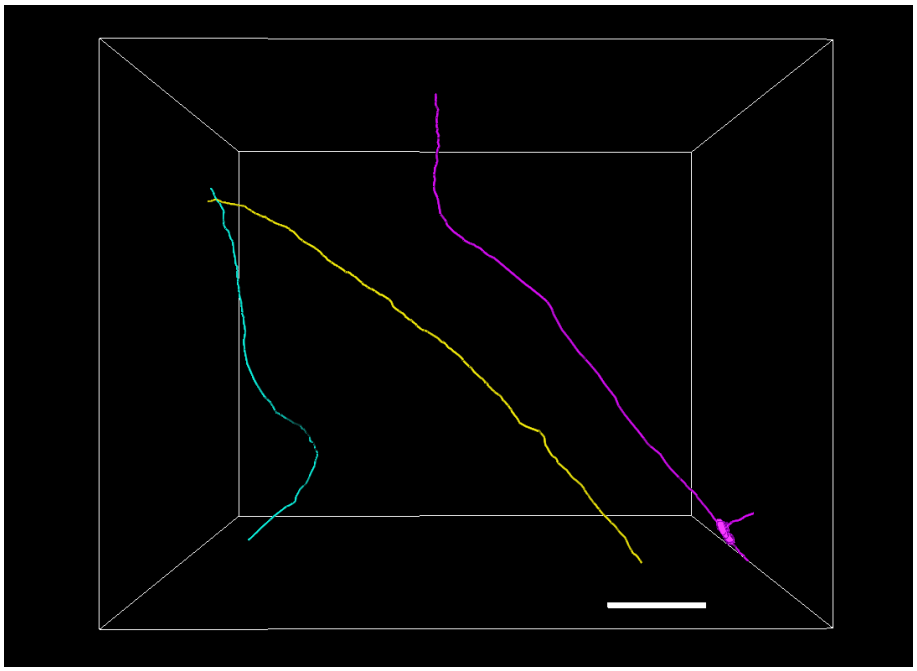


- Passive **CLARITY** protocol treating (hydrogel incubation, degassing and passive clearing) of a human brain block of a patient with hemimegalencephaly (HME) (~ 0,8 x 0,8 x 0,4 cm)
- Performing immunostaining protocol with different antibodies
- Clearing the sample with TDE 47%
- Imaging with two-photon fluorescence microscope

3D reconstruction of neurofilaments in human brain

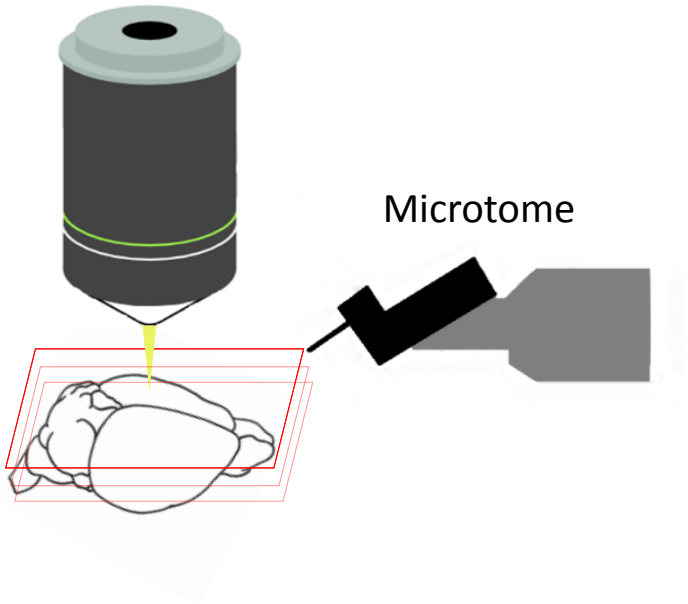
Tracing of fibers, immunostained with anti-PV antibody, elongating through a volume of 1 mm^3





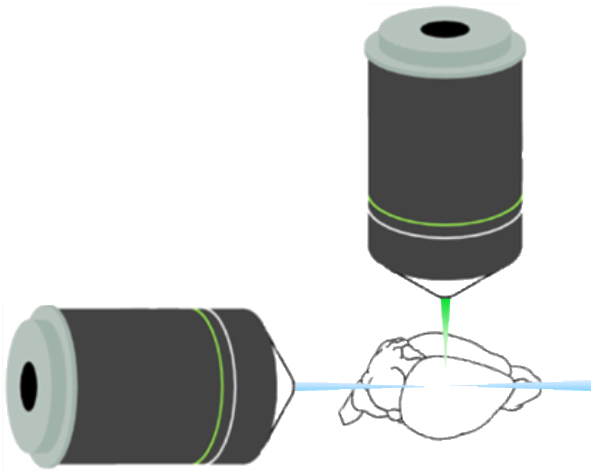
Scale bar 300 µm

STP + optical clearing



- ✓ **Imaging of moderately large areas (imaging the whole hippocampus takes about 2 weeks)**
- ✓ **Molecular specificity (transgenic animal or IHC)**
- ✓ **Manual morphology discrimination**
- ✓ **Manual long-tract axonal tracing (not for all axons)**
- ✓ **Automatic cell counting**
- ✗ **Morphology reconstruction**
- ✗ **Non-fluorescence labeling**

Light sheet microscopy



- ✓ **Imaging of whole mouse brains (about 2 days per samples)**
- ✓ **Molecular specificity (transgenic animal) – ICH over whole mouse brains requires months**
- ✓ **Manual morphology discrimination**
- ✓ **Manual bundle tracing**
- ✓ **Automatic cell counting**
- ✗ **Morphology reconstruction**
- ✗ **Non-fluorescence labeling**

People involved and collaborations

Florence: LENS and University
Francesco Saverio Pavone (Principal Investigator)
Leonardo Sacconi (light sheet microscopy and serial two-photon)
Anna Letizia Allegra Mascaro (serial two-photon)
Marie Caroline Muellenbroich (light sheet microscopy)
Irene Costantini (clearing methods)
Antonino Paolo di Giovanna (serial two-photon)
Paolo Frasconi (automatic cell localization)

Rome: University Campus Bio-medico
Giulio Iannello (image stitching)
Alessandro Bria (image visualization)

École Polytechnique Fédérale de Lausanne
Jean-Pierre Ghobril (vasculature and brain mapping)
Henry Markram (brain mapping)

University of Zurich
Bruno Weber (vasculature mapping)
Matthias Schneider (vessel segmentation)

University of Edinburgh
Fei Zhu (synaptic puncta mapping)
Seth Grant (synaptyic puncta mapping)

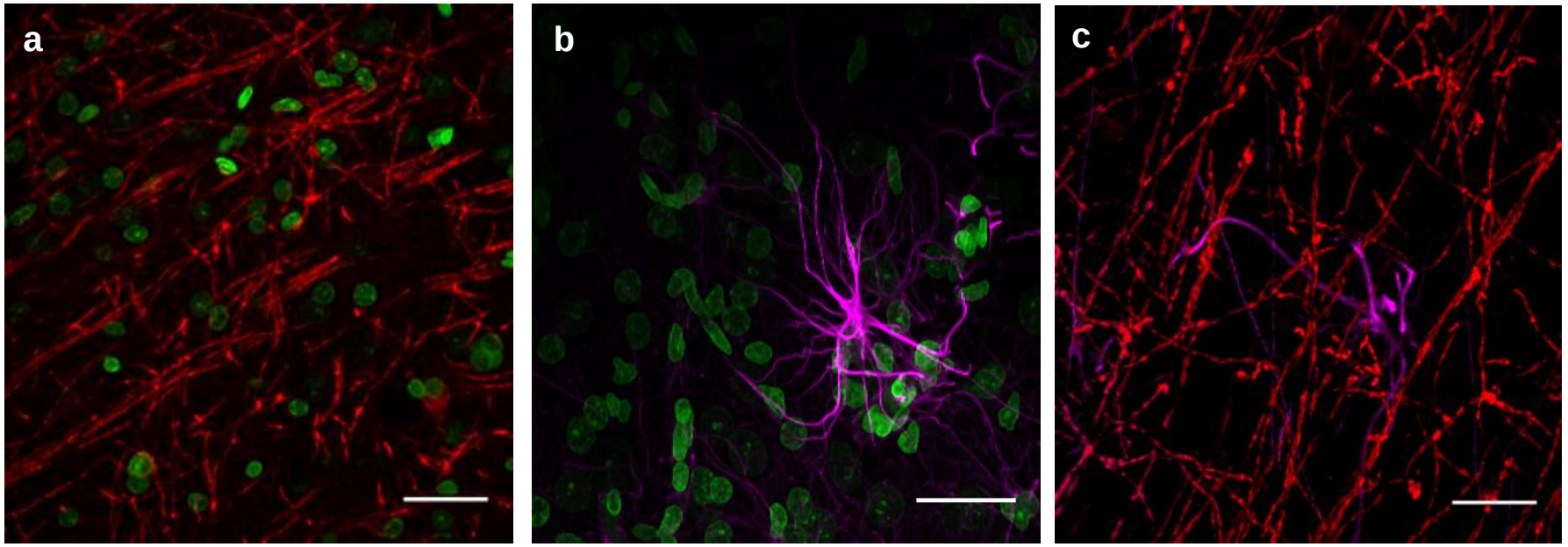
Seattle: Allen Institute for Brain Sciences
Hanchuan Peng (image visualization)

Florence: Meyer Paediatric Hospital
Renzo Guerrini (human brain samples)
Valerio Conti (human brain samples)

Juelich: Forschungszentrum
Katrin Amunts (human brain mapping)
Karl Zilles (human brain mapping)

Human brain imaging

Immunostaining with antibodies against parvalbumin (**PV**) and glial fibrillary acidic protein (**GFAP**) and **DAPI**. Double labelling with the combination of them

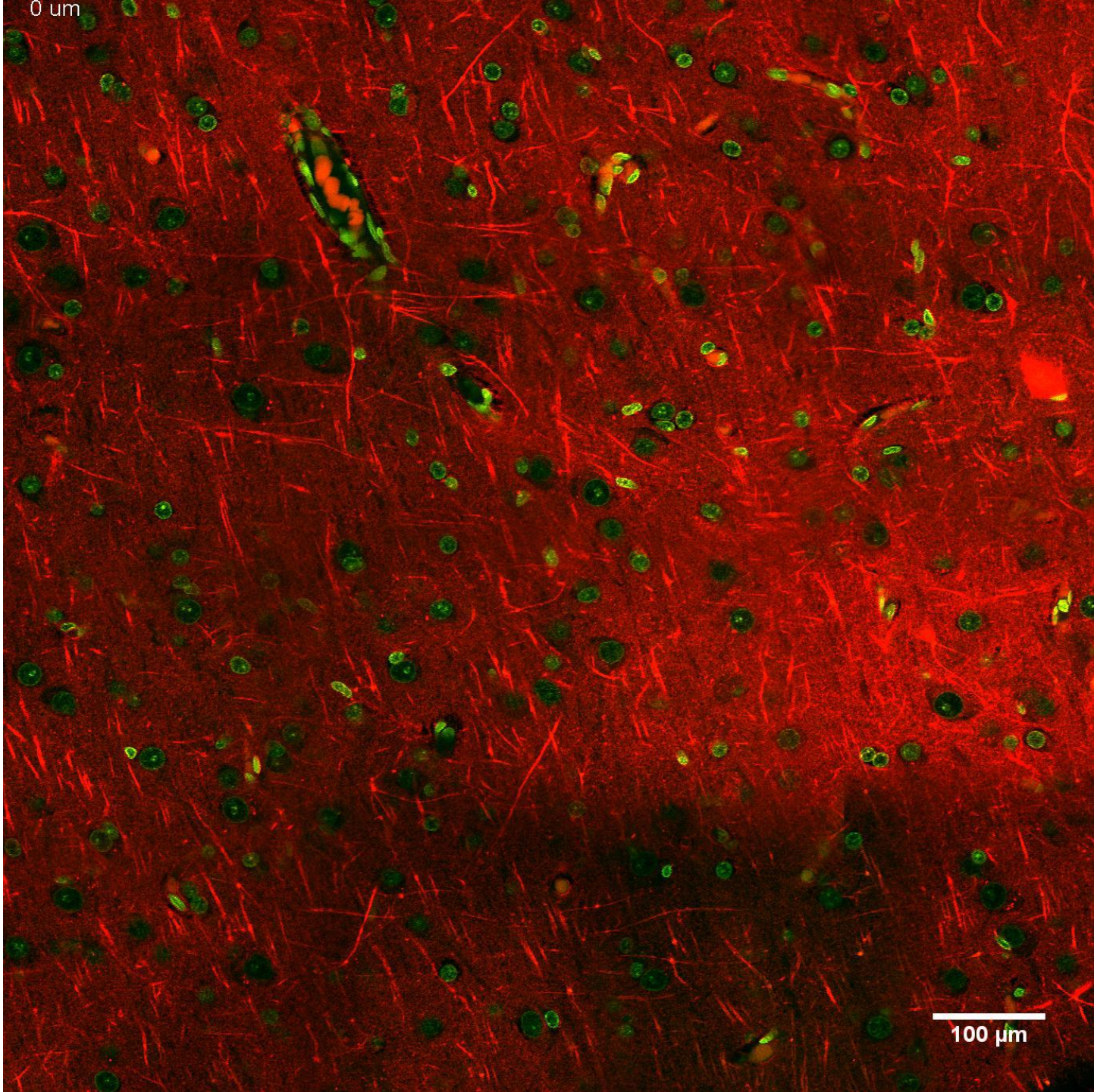


PV in red; GFAP in magenta; DAPI in green . Scale bar = 50 µm

Human brain imaging

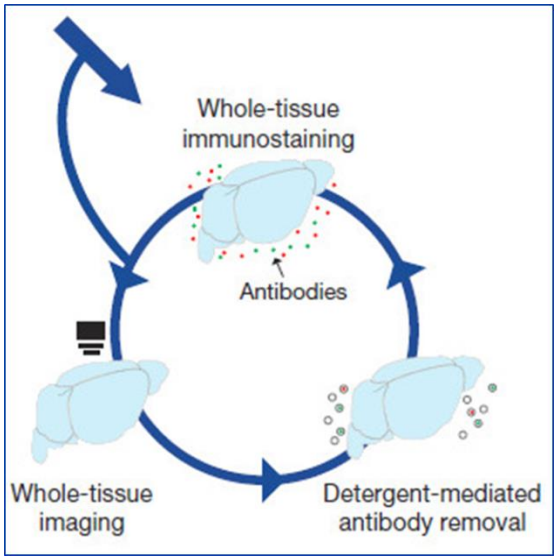
Human brain sample: nuclei in green (DAPI), neurofilament in red (anti-PV/Alexa 568)

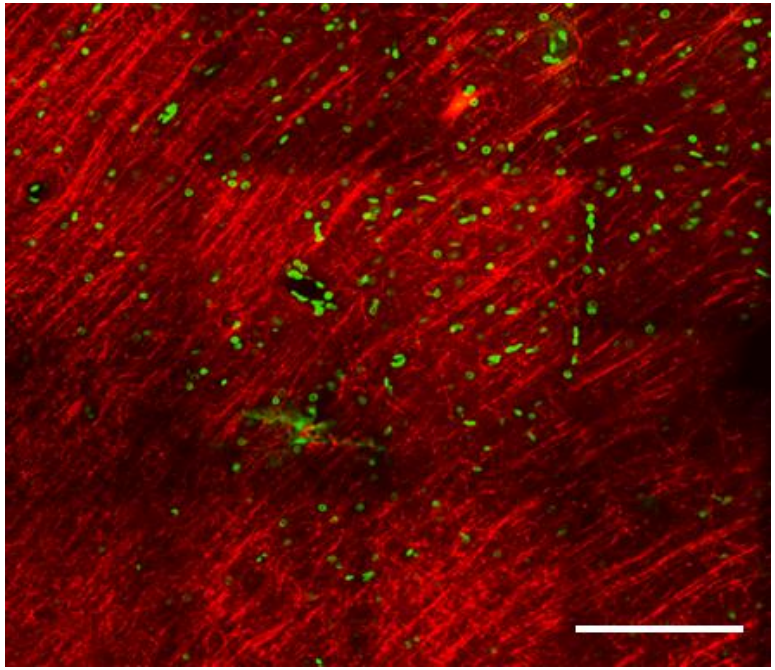
(FOV=1 x 1 mm, z-step=2 µm, depth=400 µm, λ= 800nm)

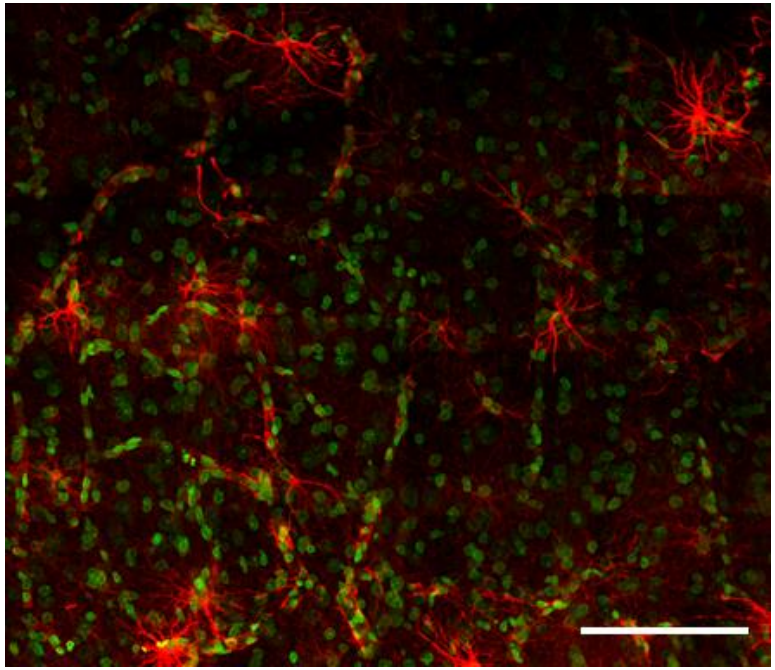


Multi round immunostaining

PV and DAPI
GFAP and DAPI
Scale bar = 300 µm

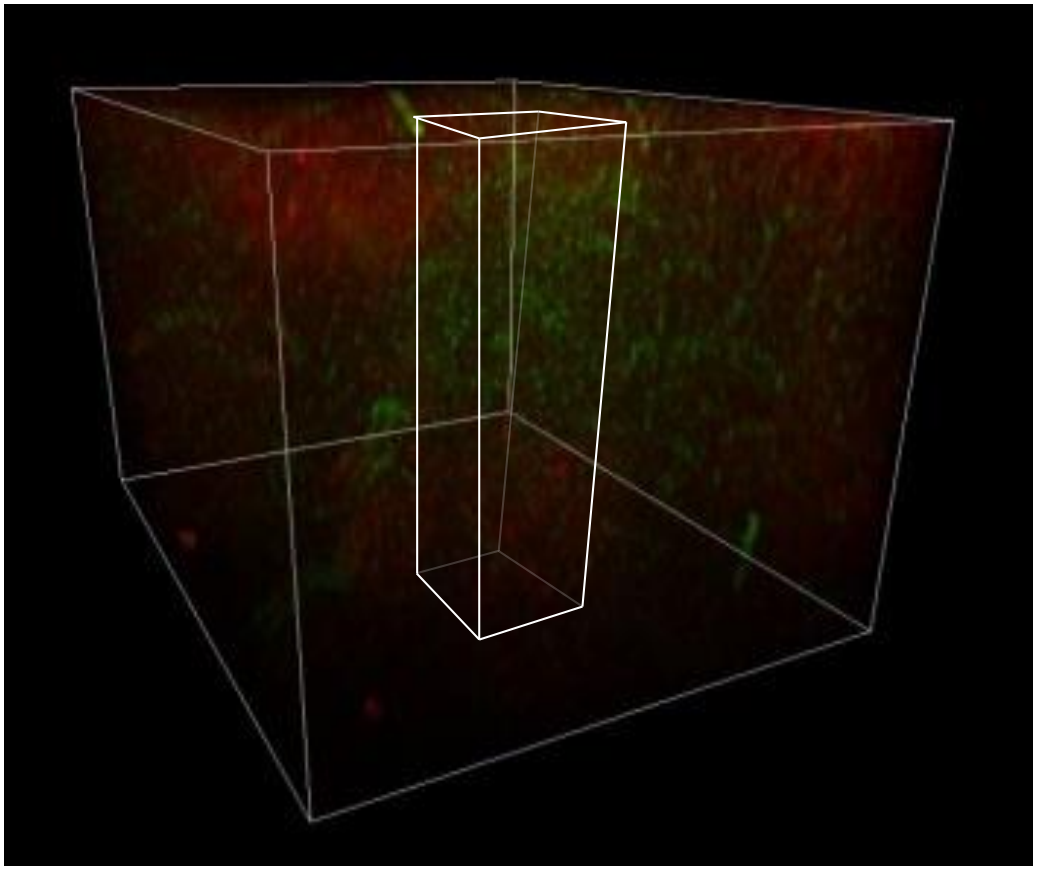


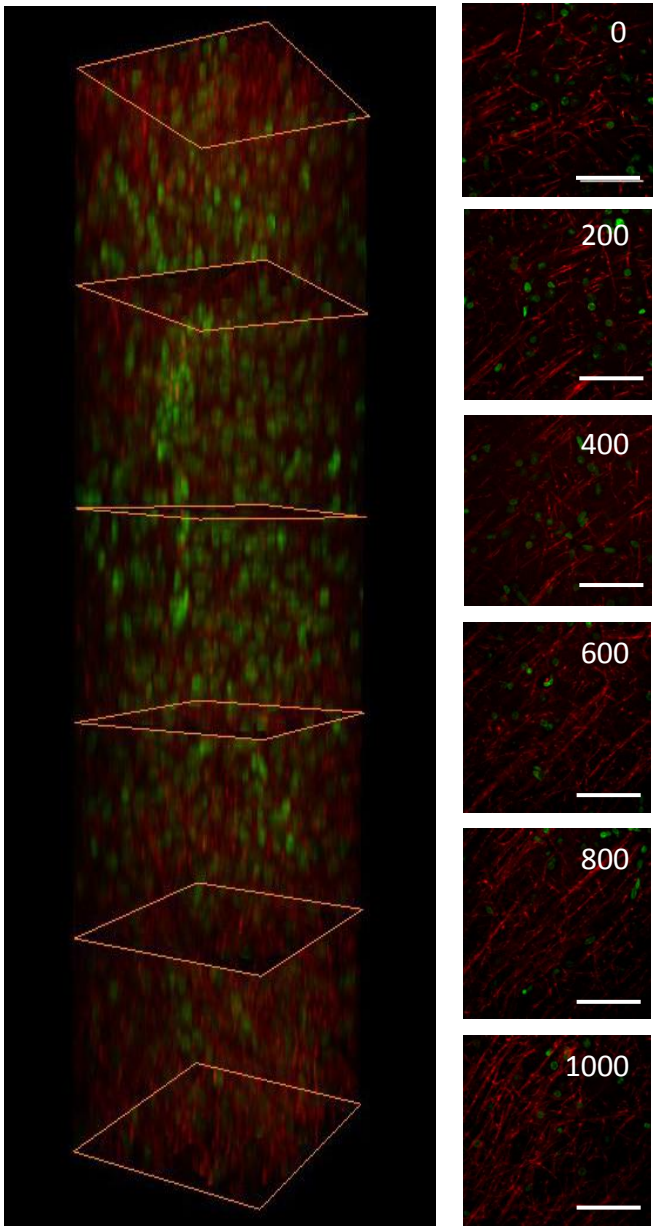




Human brain imaging

1 mm^3 thick block of a formalin-fixed tissue of a patient with hemimegalencephaly (HME), treated with passive CLARITY protocol, PV immunostained and cleared with TDE 47% (20X Sca*l*e objective).





Scale bar = 50 µm

Synaptic puncta density measurement with STP

Mouse brain tissue cleared with TDE and imaged with STP. This is a transgenic mouse in which PSD95 is labeled with GFP, so synaptic puncta becomes visible.

Voxel size 0.26x0.26x1 µm^3

Possible 3D density map reconstruction over large volumes (whole hippocampus)

Data obtained in collaboration with Fei Zhu and Seth Grant, Univ. of Edinburgh