CLARITY

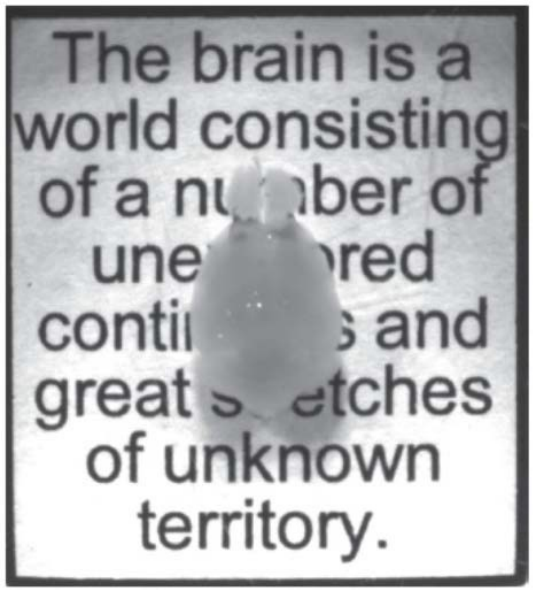
structural and molecular interrogation
of intact biological systems

Journal Club

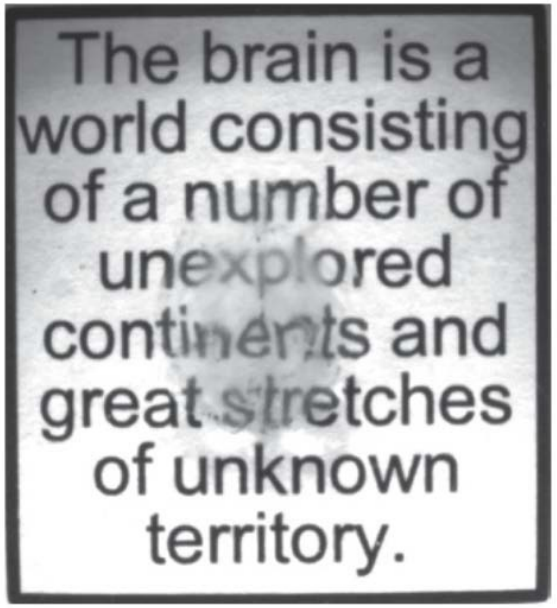
23rd April 2013

Despina Goniotaki

Imaging of intact tissue



Clearing



- Diffraction limit of light
- Opaque specimens - light scattering
- Sectioning - fragmented tissues

Chung K *et al.*, *Nature* (2013)

How to introduce chemicals to the tissue

1. Perfusion - is the process of delivery of fluid (such as blood) to a specific organ or area of the body

2. Immersion - relies on diffusion to transport molecules from the surface to the interior

Chemical Clearing: Samples are immerged in a medium/solution having a refractive index similar to proteins

Keller P. & Dodt HU, *Current opinion in neurobiology* (2012)

Chemical Clearing

Background

1900 ⟶ Blood vessels

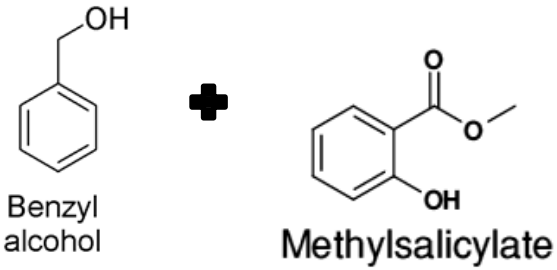
Werner Spalteholz

Spalteholz, W. (S. Hierzel, 1914)
Über das Durchsichtigmachen von menschlichen und tierischen Präparaten

Lunvall H.

?

Lundvall H Anatomischer Anzeiger 1905, 27:520-523.
Weiteres über Demonstration embryonaler Skelette.



Keller P. & Dodt HU, *Current opinion in neurobiology* (2012)

Background

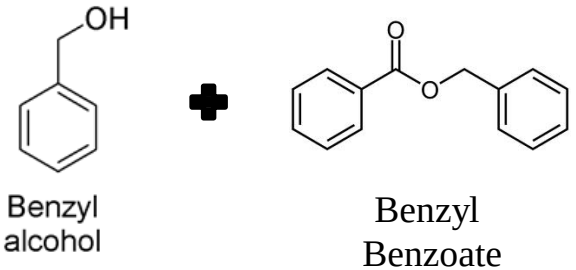
1990 ⟶ Xenopus embryogenesis

Klymkowsky MW

Dent JA, Polson AG, Klymkowsky MW (Development 1989, 105:61-74)
A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in Xenopus.

Hanken J

Klymkowsky MW, Hanken J (Methods in Cell Biology, Volume 36, 1991:413-435)
Whole-mount staining of Xenopus and other vertebrates. In Xenopus laevis: Practical Uses in Cell and Molecular Biology - Edited by Kay BK, Peng HB. Academic Press



Benzyl alcohol + Benzyl Benzoate ⟶ **Murray's clear/ BABB**

Keller P. & Dodt HU, *Current opinion in neurobiology* (2012)

Experimental design

I. Choice of Tissue

Tissue should be labelled with a strong fluorophore

1. expression of fluorescent proteins (transfection, transduction)

2. labeling of cells/structures with synthetic dyes

3. transgenic animals (e.g. Thy1-GFP mice)

II. Chemical Clearing

1. Tissue Dehydration

2. Dehydrated Tissue is impregnated with an optical clearing agent

3.Clear Tissue hardens & becomes transparent

III. Imaging

Ertürk A. *et al*, *Nature Protocols* (2012)

Chemical Clearing

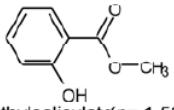
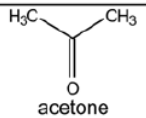
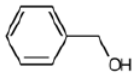
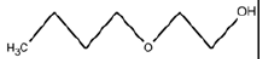
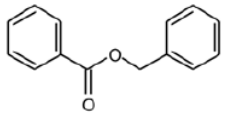
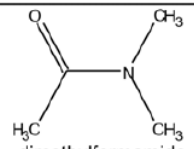
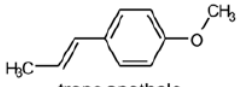
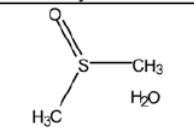
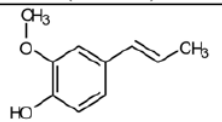
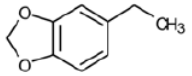
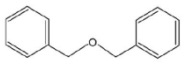
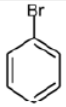
involves 2 main steps:

- Tissue Dehydration

- Tissue Clearing

Common dehydration agent: ethanol
Common clearing solution: BABB

Chemical Clearing

dehydration		clearing (after ethanol dehydration)	
dehydration medium	**GFP fluorescence preservation after clearing with BABB**	**clearing medium**	**clearing result after dehydration alcohol dehydration**
 methanol	Severe reduction of fluorescence.	 methylsalicylate (n= 1.536)	Specimens poorly cleared, fluorescence strongly reduced.
 acetone	No fluorescence detectable	 benzyl alcohole (n= 1.539)	Specimens poorly cleared, fluorescence reduced.
 2-butoxyethanol	Severe reduction of fluorescence.	 benzybenzoate (n= 1.566)	Specimens poorly cleared, fluorescence reduced.
 dimethylformamide	Severe reduction of fluorescence.	 trans-anethole (n= 1.560)	Good clearing result, with brownish dis colorations. Good preservation of GFP fluorescence.
 dimethylsulfoxid (DMSO)	Severe reduction of fluorescence.	 isoeugenole (n= 1.577)	Good clearing result, but strong brownish discolorations. Good preservation of GFP fluorescence.
 dioxane	Severe reduction of fluorescence.	 isosafrole (n= 1.573)	Specimens poorly cleared, fluorescence strongly reduced.
THF  tetrahydrofurane	Good preservation of fluorescence	 1,5-bromopentane (n= 1.513)	Specimens not cleared at all.
DBE  Dibenzyl ether (DBE)	Good preservation of fluorescence	 bromobenzene (n = 1.560)	Good clearing result. Good preservation of GFP fluorescence, but significantly toxic and highly volatile.

2 step procedure:

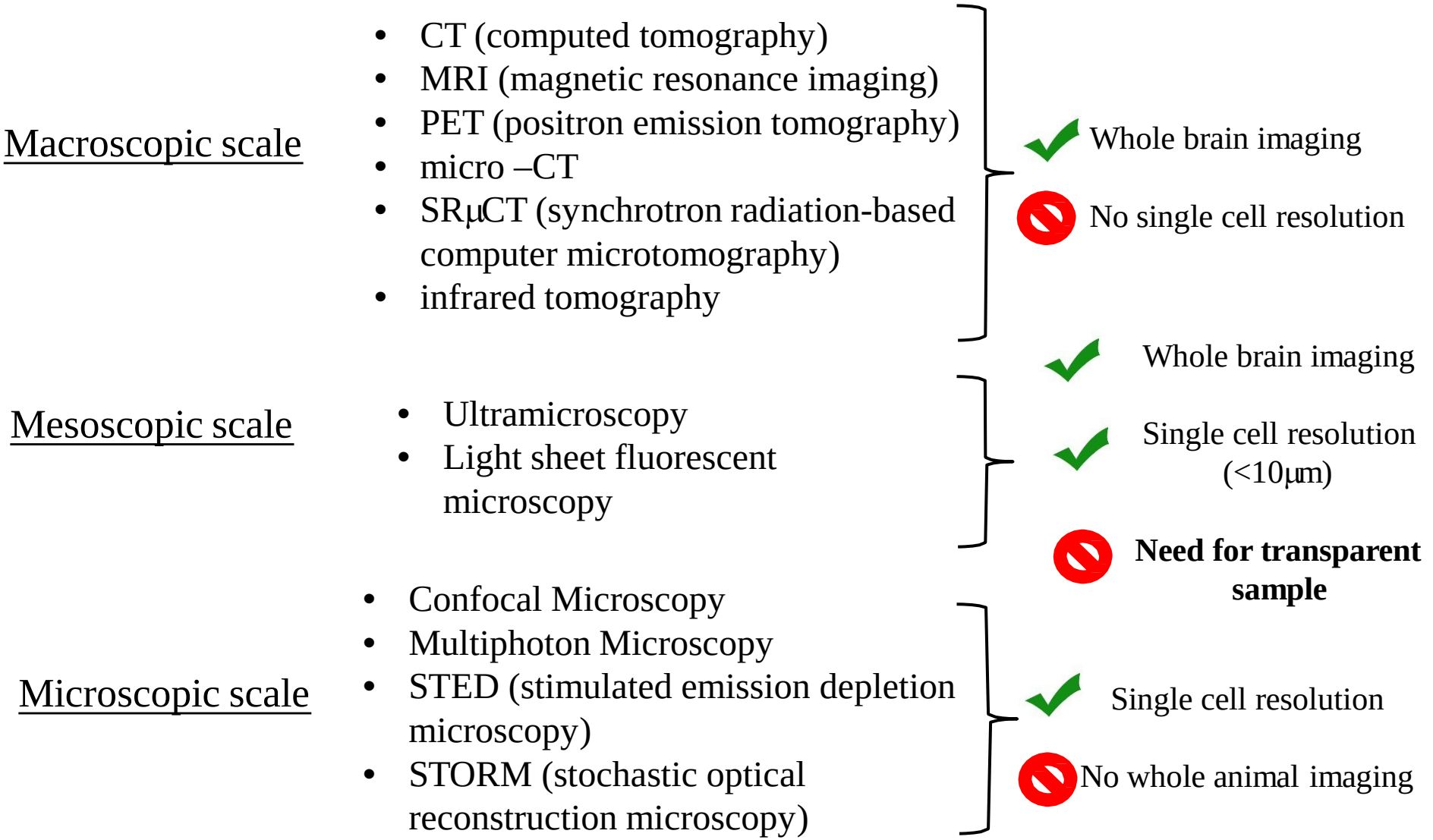
- Tissue Dehydration
- Tissue Clearing

Interestingly, both efficient dehydration compounds are ethers without further functional groups

Becker K. *et al*, *PLOS one* (2012)

Imaging Techniques

Available Imaging Techniques



Paper 1

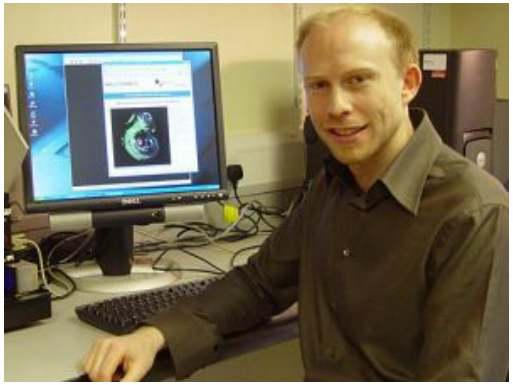
Optical Projection Tomography as a Tool for 3D Microscopy and Gene Expression Studies

James Sharpe *et al.*

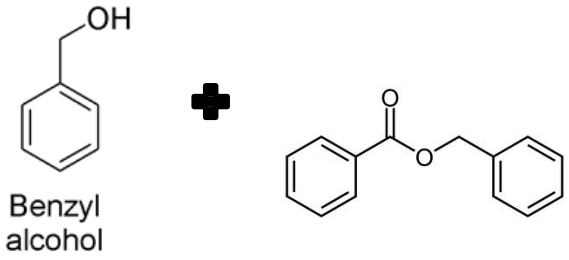
Science **296**, 541 (2002);
DOI: 10.1126/science.1068206

2002 ⟶ Optical projection tomography (OPT) + Chemical Clearing

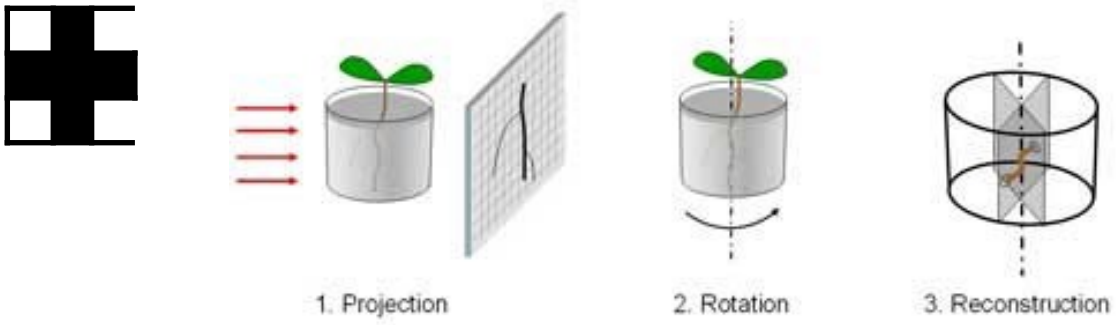
James Sharpe



Murray's clear / BABB



Optical Projection Tomography (OPT)



Science
AAAS

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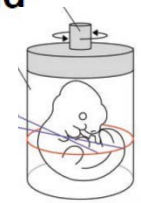
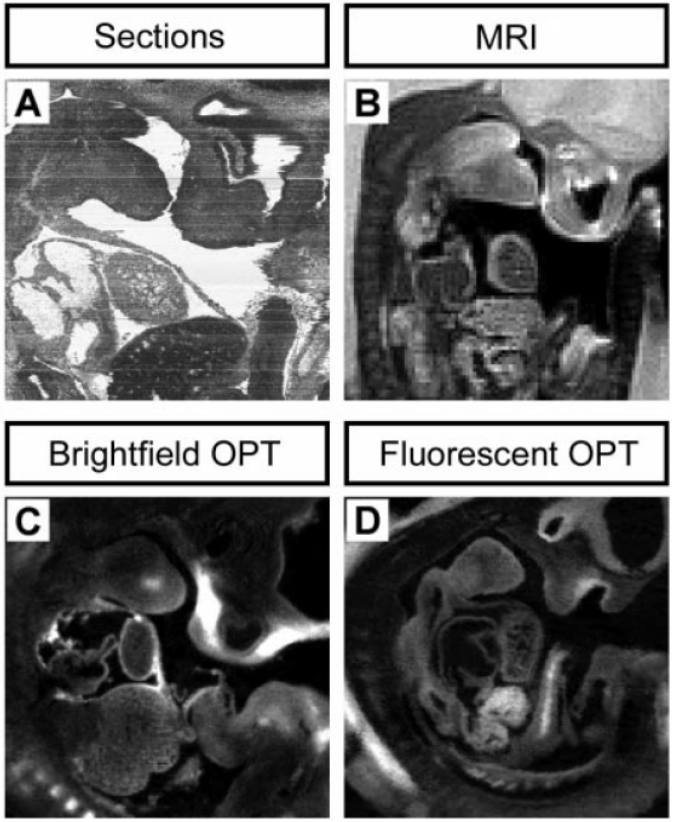
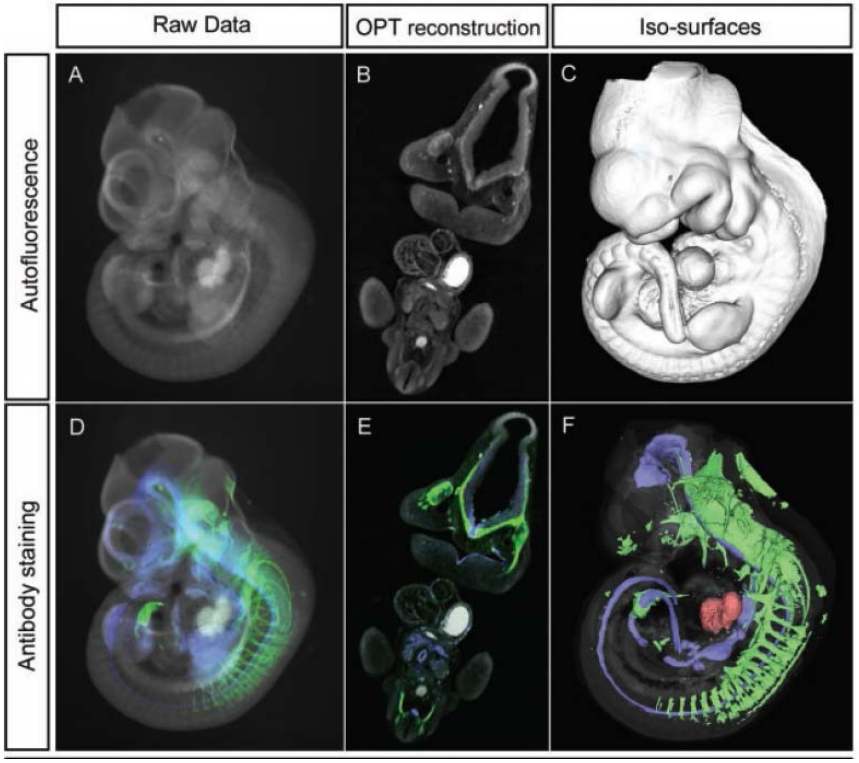




Comparison of OPT microscopy with other 3D reconstruction techniques.
Sagital sections of E10.5-12.5 mouse embryos



Fluorescence OPT imaging of multiple signals within an E10.5 mouse embryo with HNF3b antibodies (labeling the floorplate of the neural tube) and the endoderm and neurofilament antibodies (labeling the developing nerve tracts).

Science
AAAS

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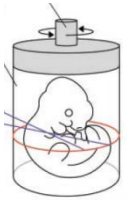


Advancements

- ✓ ***OPT enables quick data acquisition from large specimens***
- ✓ ***Method is compatible with autofluorescence & immunocytochemistry***
- ✓ ***1st time to image whole mouse embryos***

Limitations

- ✓ ***Specimen should be transparent for OPT to be performed***
- ✓ ***Resolution limited to 15-20 μm***
- ✓ ***Clearing can only be used in fixed tissues***

Paper 2

NATURE METHODS

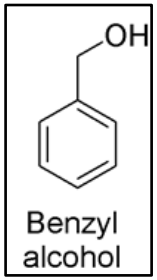
Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain

Hans-Ulrich Dodt[1,3], Ulrich Leischner[1], Anja Schierloh[1], Nina Jährling[1,3], Christoph Peter Mauch[1], Katrin Deininger[2], Jan Michael Deussing[1], Matthias Eder[1], Walter Zieglgänsberger[1] & Klaus Becker[1,3]

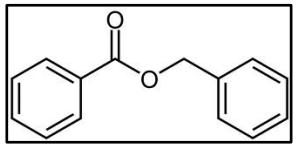
2007 ⟶ Ultramicroscopy + Chemical Clearing

Dodt HU

Murray's clear / BABB

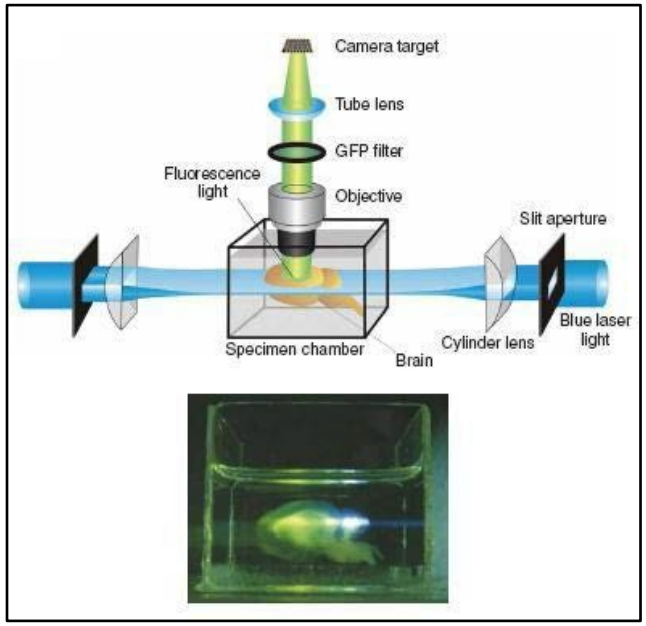


+



Benzyl Benzoate

Ultramicroscopy principle



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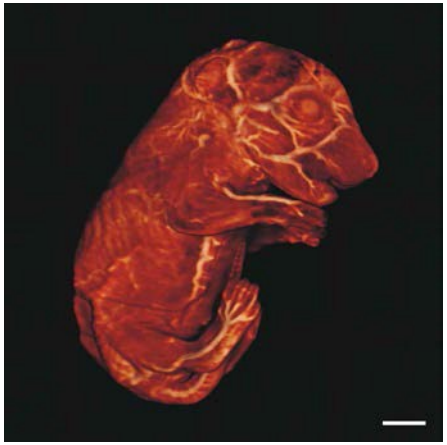
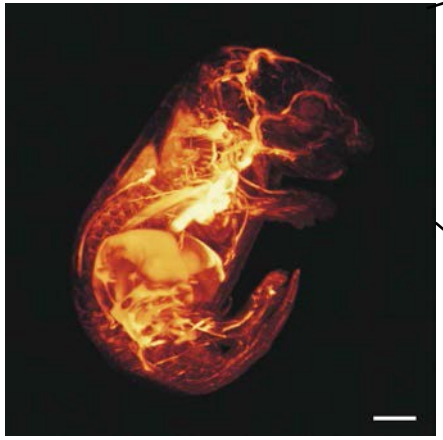
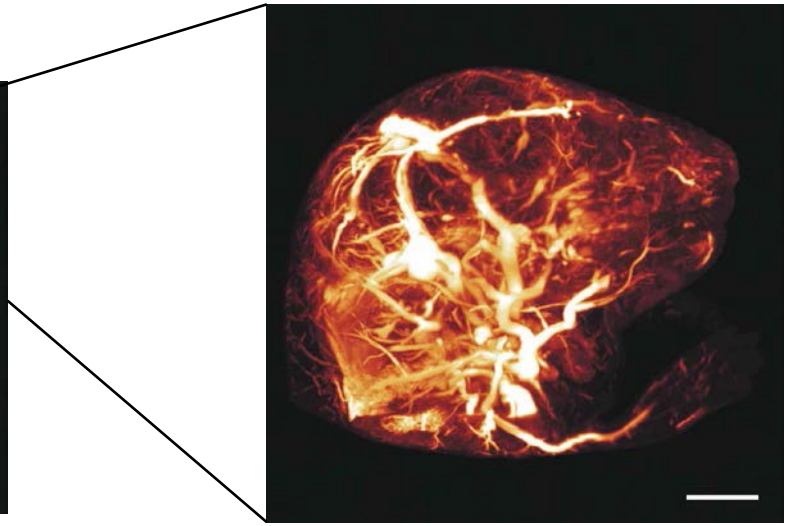
Imaging of mouse embryos



Surface of the mouse embryo (scale bar 2mm)



Blood vessel system of the mouse embryo (scale bar 2mm)



Blood vessel system of the head of the mouse embryo (scale bar 1mm)

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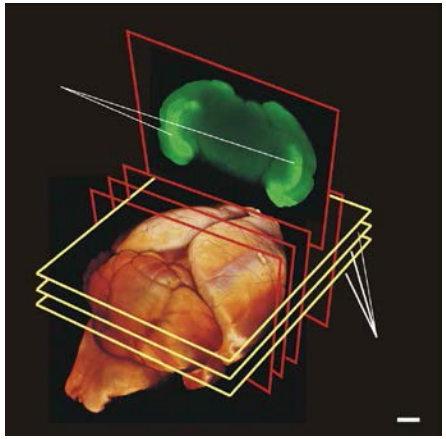
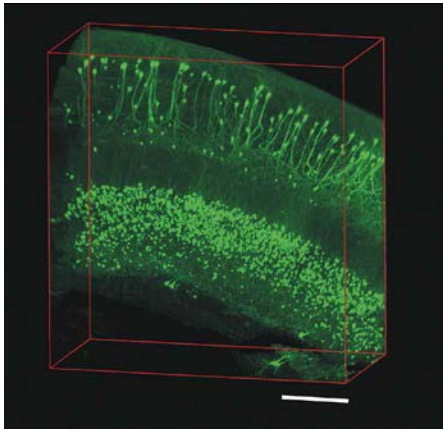
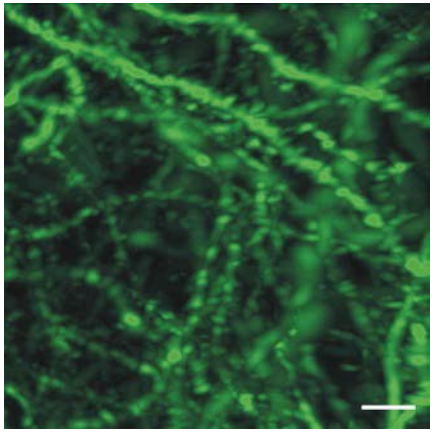
Thy1-GFP-M transgenic mice



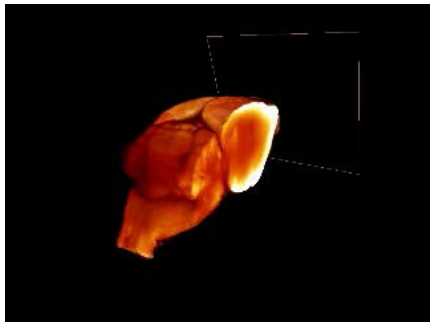
Whole mouse brain reconstructed from 550 optical sections (scale bar 1mm)

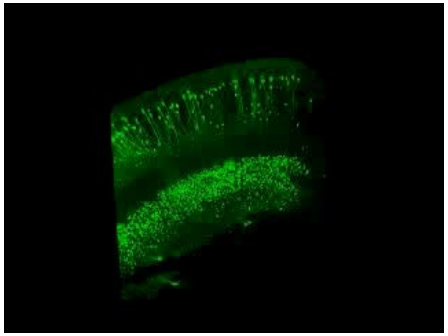


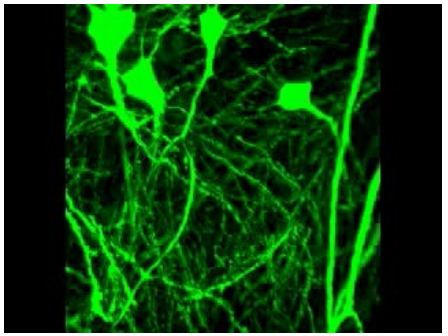
3D reconstruction of part of a whole hippocampus using 132 optical sections (scale bar 200μm)



3D reconstruction of dendritic spines of CA1 pyramidal neurons (scale bar 5μm)







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Advancements

- ✓ *Clearing of whole brains of 3-4week old mice*
- ✓ *Method is compatible with GFP labeling, autofluorescence & immunocytochemistry*
- ✓ *Allows resolution of 5-10μm*

✓ *1st time to preserve GFP fluorescence after tissue clearing (images from dendrites & spines)*

Limitations

- ✓ *The working distance of the objectives*
- ✓ *The moderate apertures of the objectives (0.7 for the 10x)*
- ✓ *Need for transparency (mainly myelinated areas)*
- ✓ *Clearing can only be used in fixed tissues*
- ✓ *Dehydration process (non isotropic shrinking effects)*

Paper 3

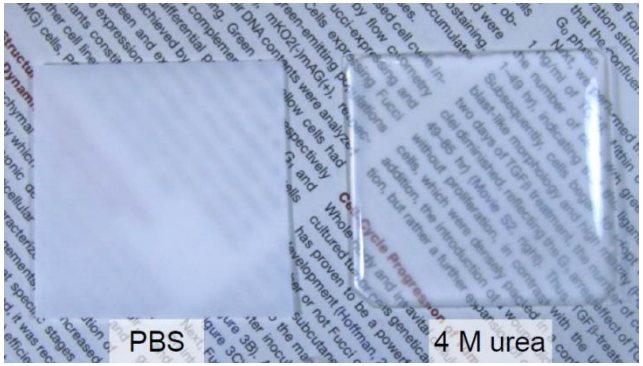
nature neuroscience

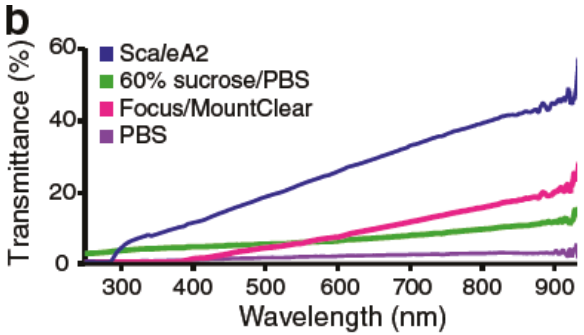
TECHNICAL REPORTS

Sca*l*e: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain

Hiroshi Hama[1], Hiroshi Kurokawa[1,2], Hiroyuki Kawano[1,3], Ryoko Ando[1], Tomomi Shimogori[1], Hisayori Noda[1,4], Kiyoko Fukami[2], Asako Sakaue-Sawano[1,3] & Atsushi Miyawaki[1,3]

2011 ⟶ Novel Chemical Clearing Compound **(SCALE)**

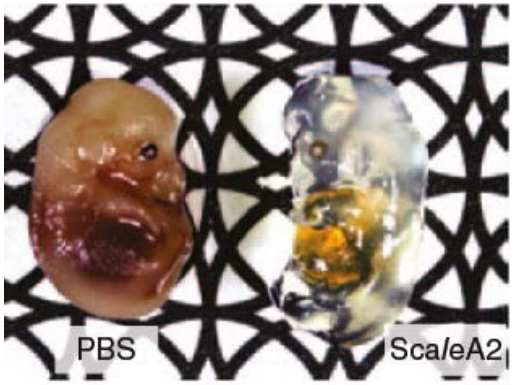




Scale: aqueous reagent that renders biological samples optically transparent (colorless & alkalic - pH7.7)

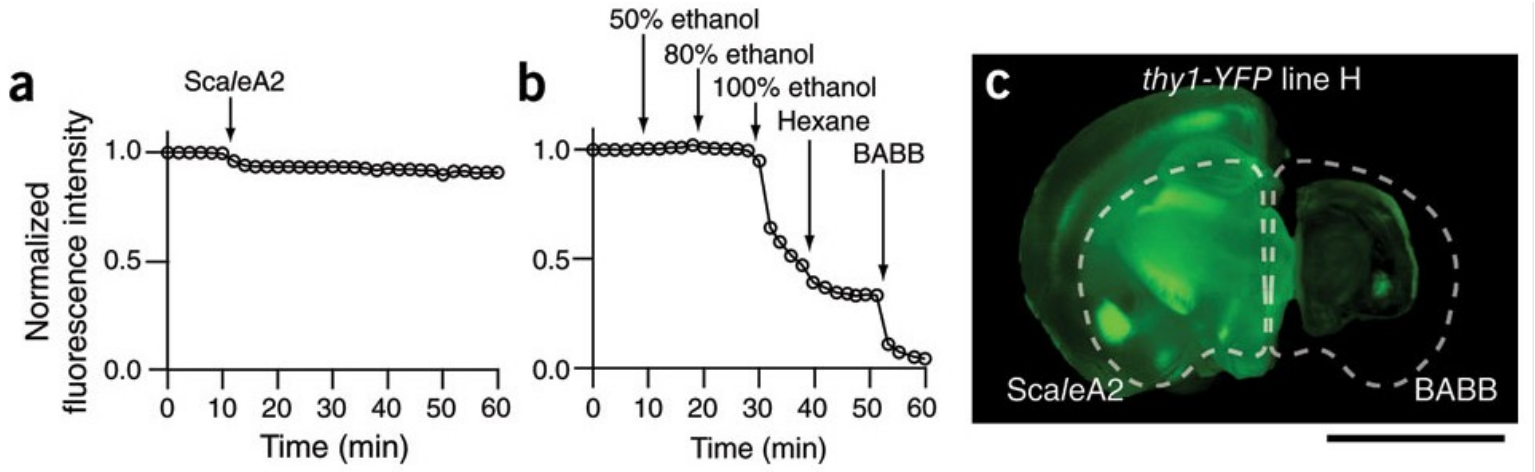
Composition: of 4-8 M urea, 10-60% (wt/vol) glycerol and 0.1% (wt/vol) Triton X-100.

Variants: ScaleA2, ScaleB2, ScaleU2



In vivo stability of EGFP fluorescence upon ScaleA2 treatment

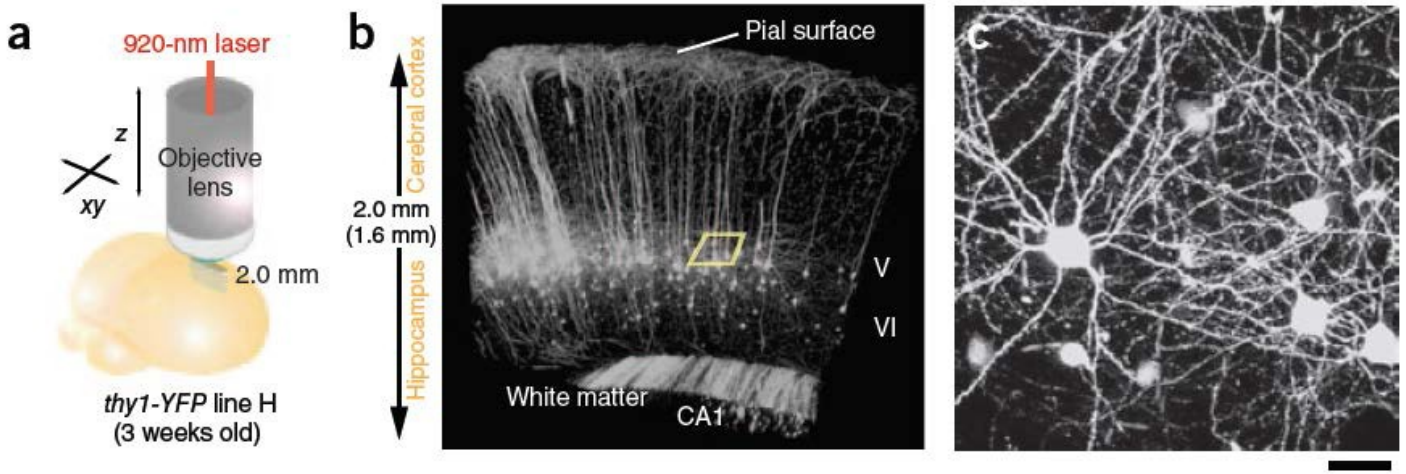
HELA cells

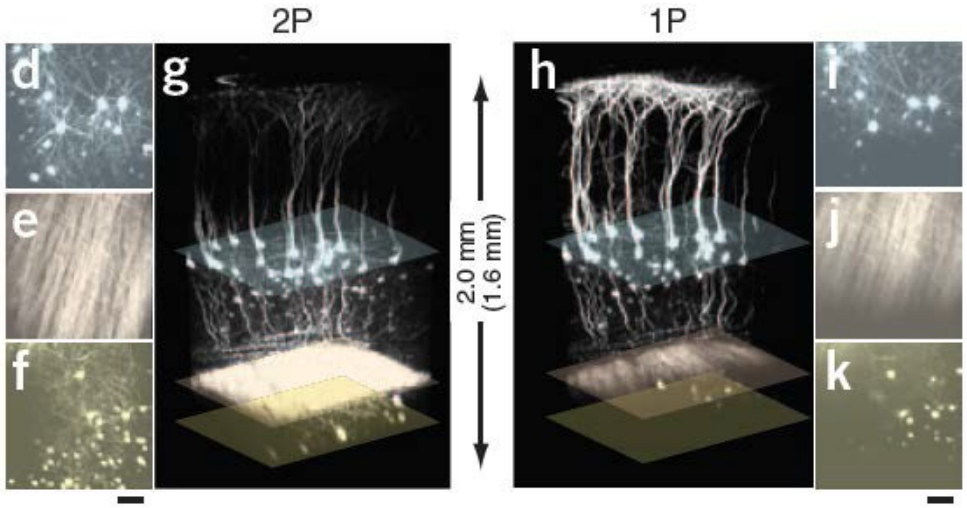


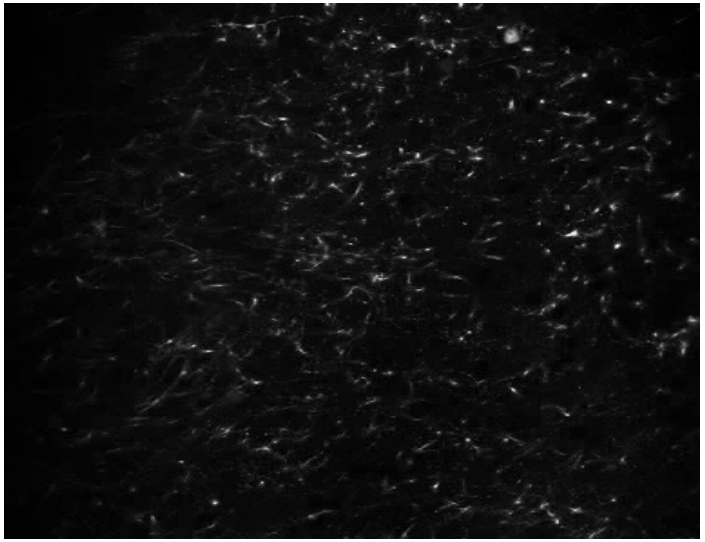
3D reconstruction of neuronal structures

YFP-H mice

Individual spines were discernable at a depth of 0.9mm

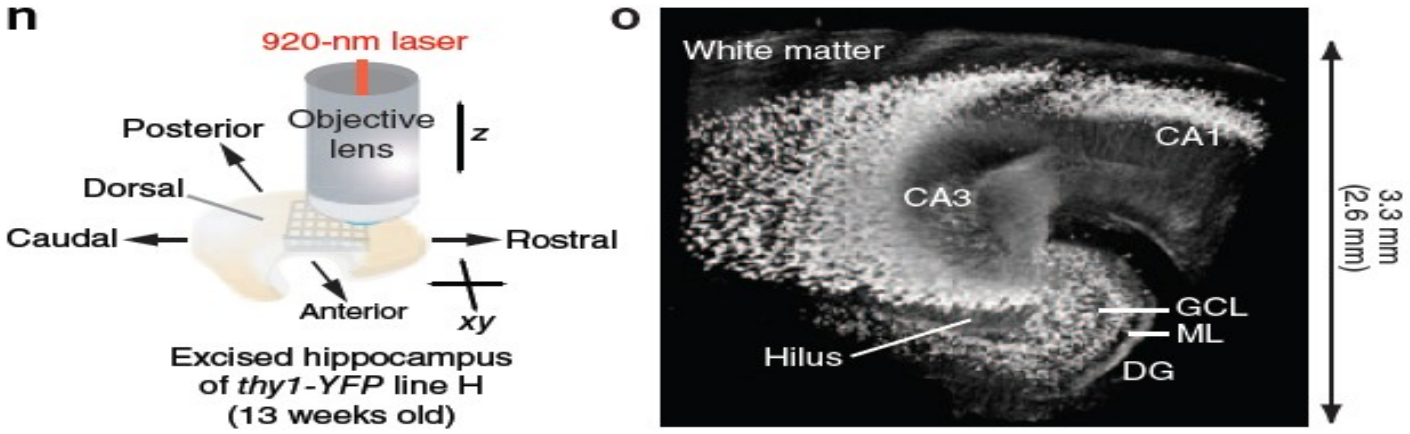






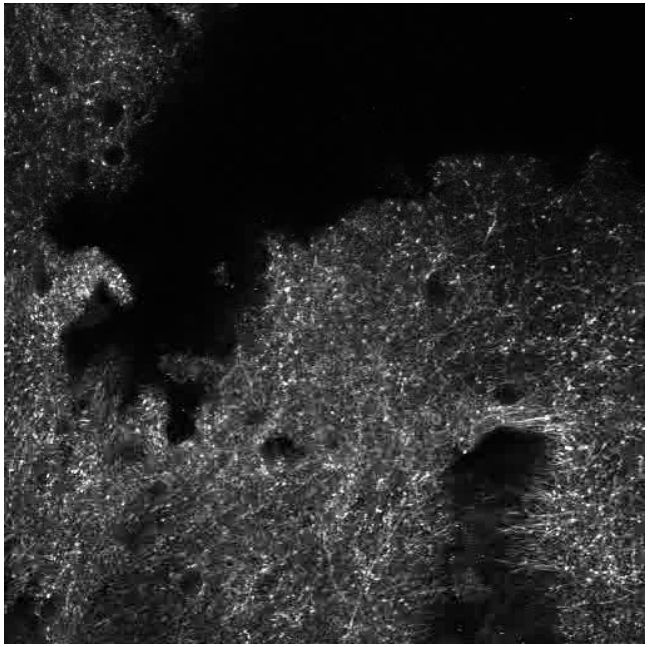
Beyond the current imaging depth limit

YFP-H mice

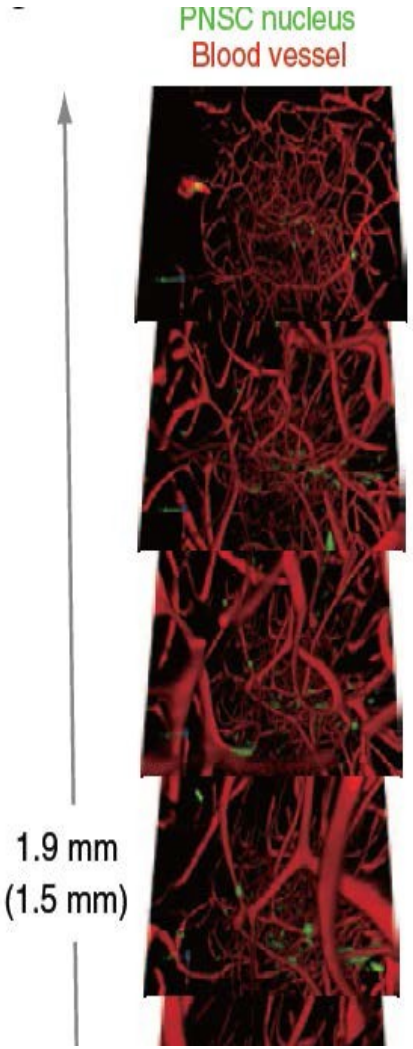


Customized 25x objective (25mm working distance, NA=1.0)

- Generation of long quadratic prisms
- Reconstruction extended from brain surface to DG

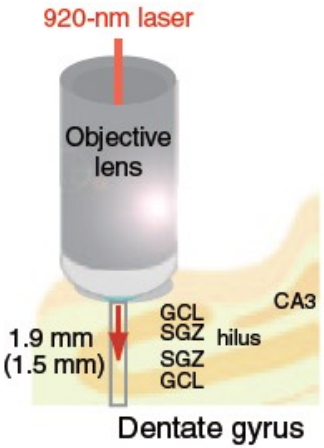


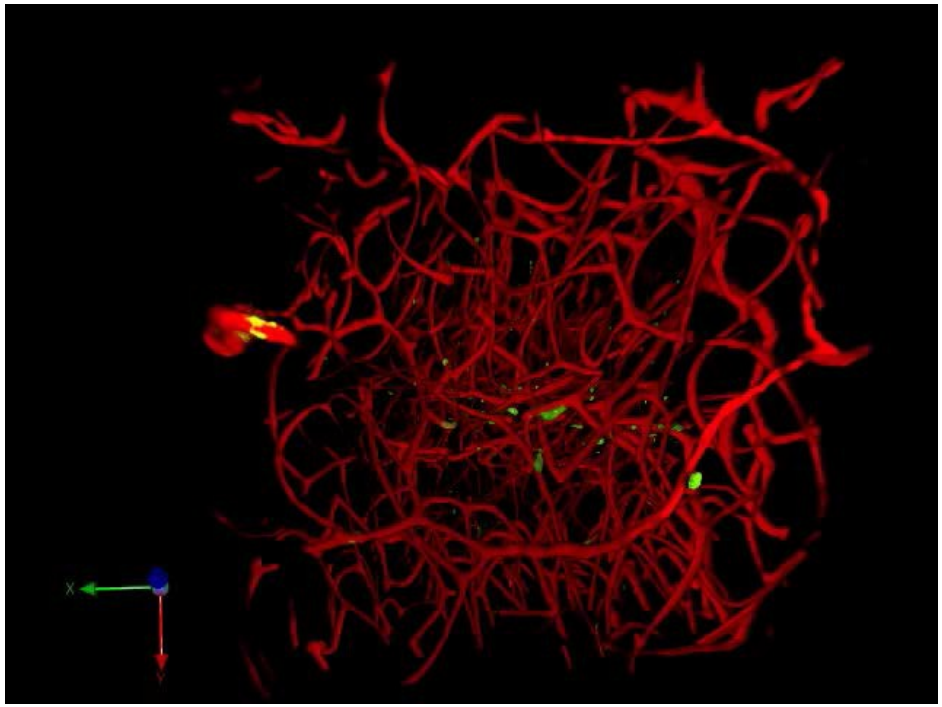
Neural Stem Cell (NSC) association with blood vessels in the subgranular zone (SGZ) of DG



- Scale optical clearing of SVZ

AIM: understand how NSCs associate with blood vessels





nature neuroscience

TECHNICAL REPORTS

Sca/e: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain

Hiroshi Hama[1], Hiroshi Kurokawa[1,2], Hiroyuki Kawano[1,3], Ryoko Ando[1], Tomomi Shimogori[1], Hisayori Noda[1,4], Kiyoko Fukami[2], Asako Sakaue-Sawano[1,3] & Atsushi Miyawaki[1,3]

Advancements

- ✓ ***Reconstruction of neuronal population & projections***
- ✓***Unlike organic solvent-based compounds, SCALE allows signal preservation of fluorescent proteins***
- ✓ ***Inexpensive and simple formula →might allow clearing of primate/human samples***
- ✓ ***Compatibility with most light microscopy systems***

Limitations

- ***Clearing can only be used in fixed tissues***
- ✓***Long incubation periods to achieve adequate transparency***
- ✓ ***Tissue fragility***

Paper 4

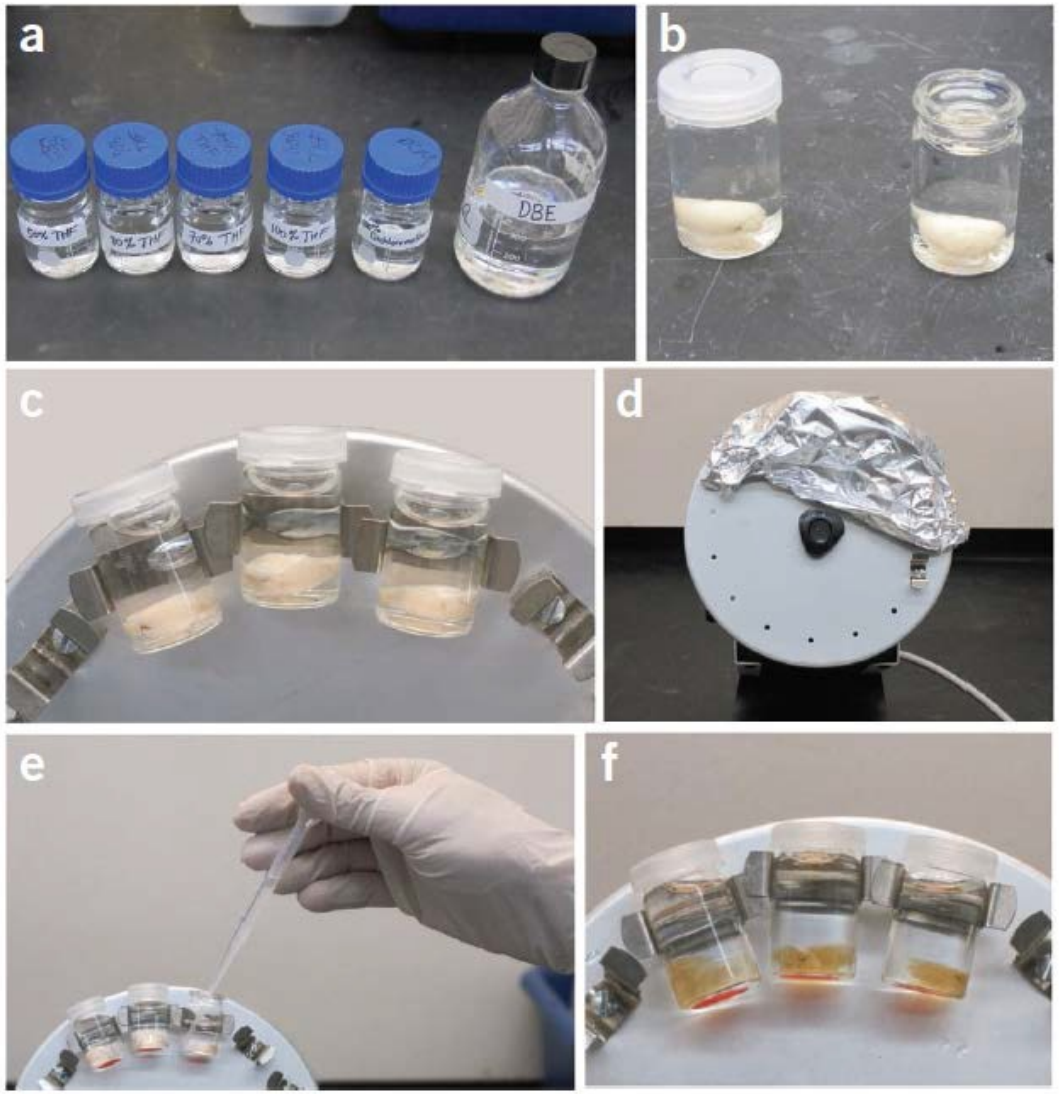
NATURE PROTOCOLS

Three-dimensional imaging of solvent-cleared organs using 3DISCO

Ali Ertürk[1], Klaus Becker[2,3], Nina Jährling[2–4], Christoph P Mauch[5], Caroline D Hojer[6], Jackson G Egen[6], Farida Hellal[7], Frank Bradke[7], Morgan Sheng[1] & Hans-Ulrich Dodt[2,3]

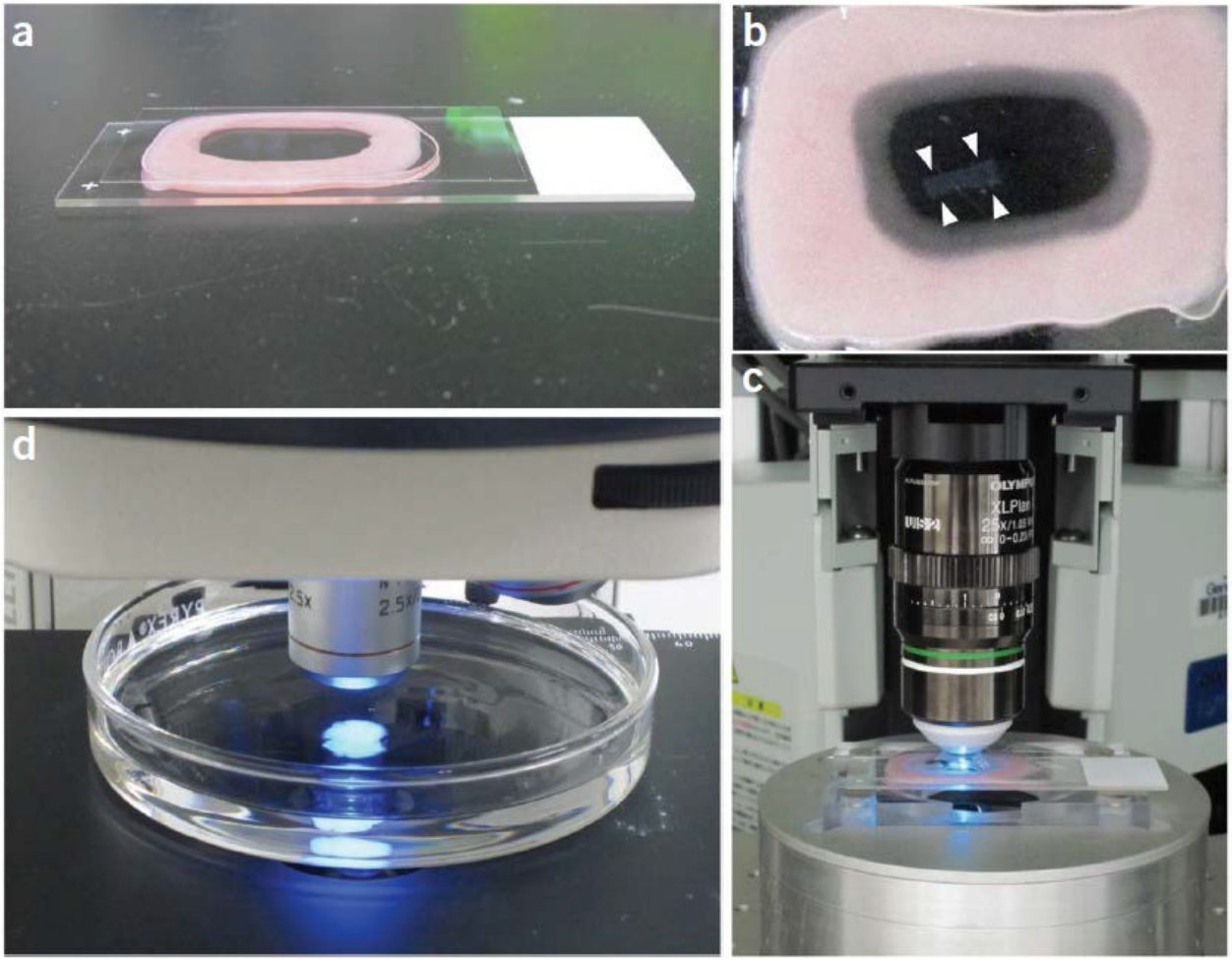
Clearing Protocol

Combination of THF & DBE for tissue dehydration-clearing



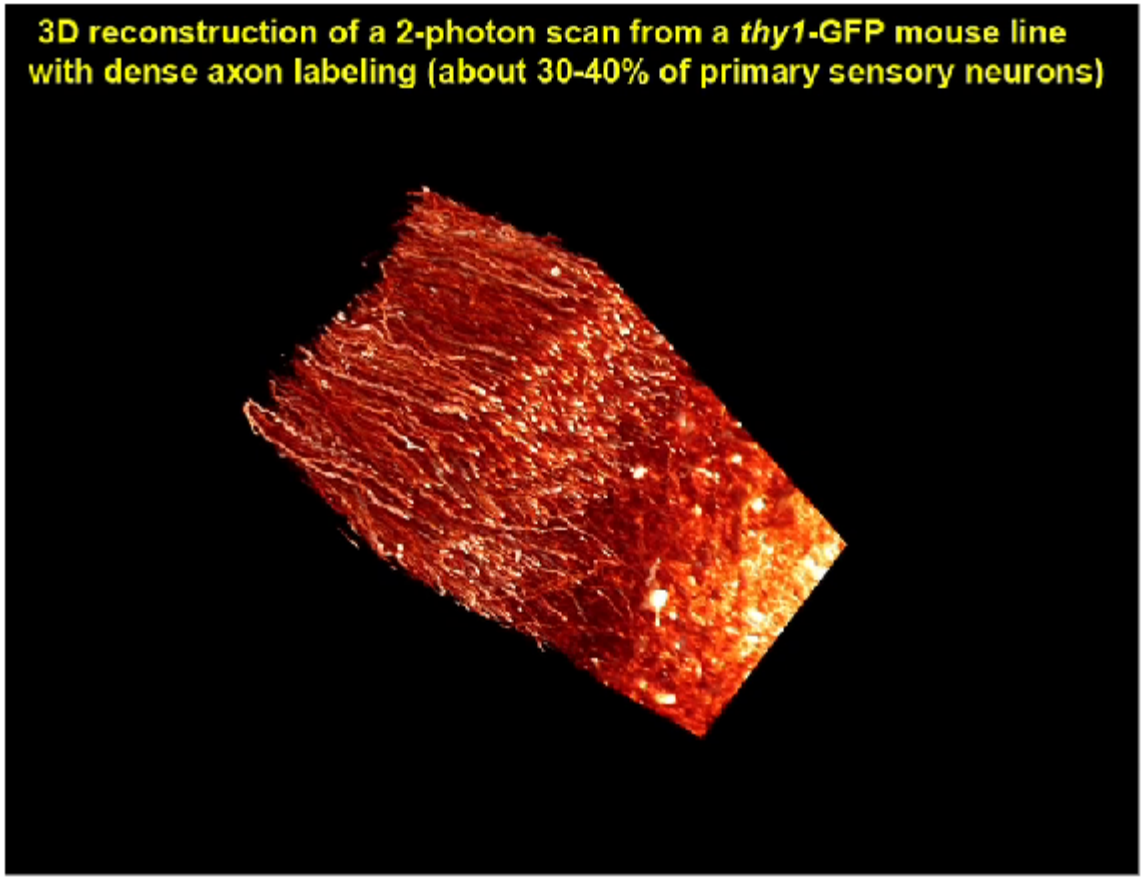
Imaging

Confocal or Two-photon microscopy of the cleared tissue to obtain high resolution images



Study neuronal degeneration-regeneration

Axon tracing in the spinal cord

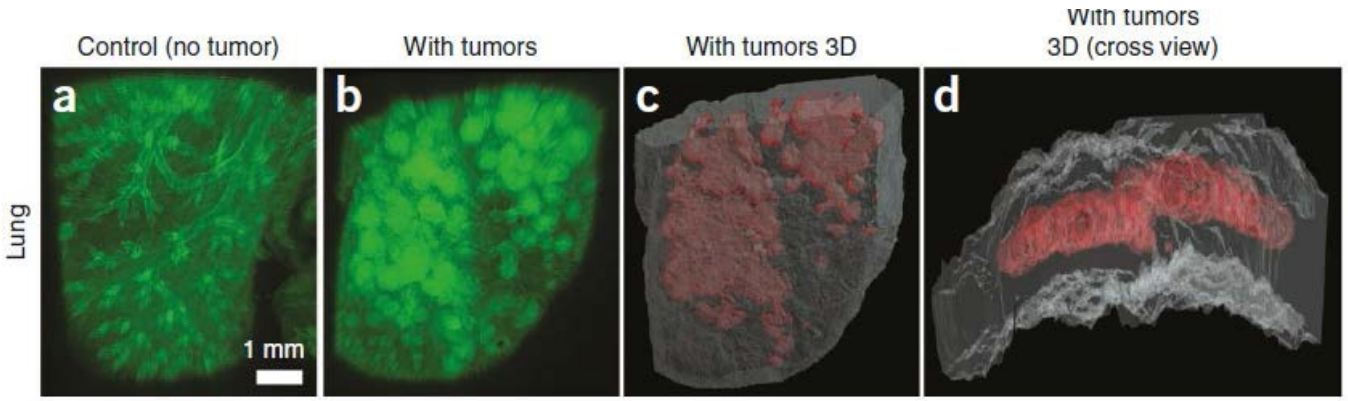


Assessment of tissue integration

Induced pluripotent stem cell graphs integration or distribution & volume of tumors in large organs

Kras ^{LSLG12D} mice

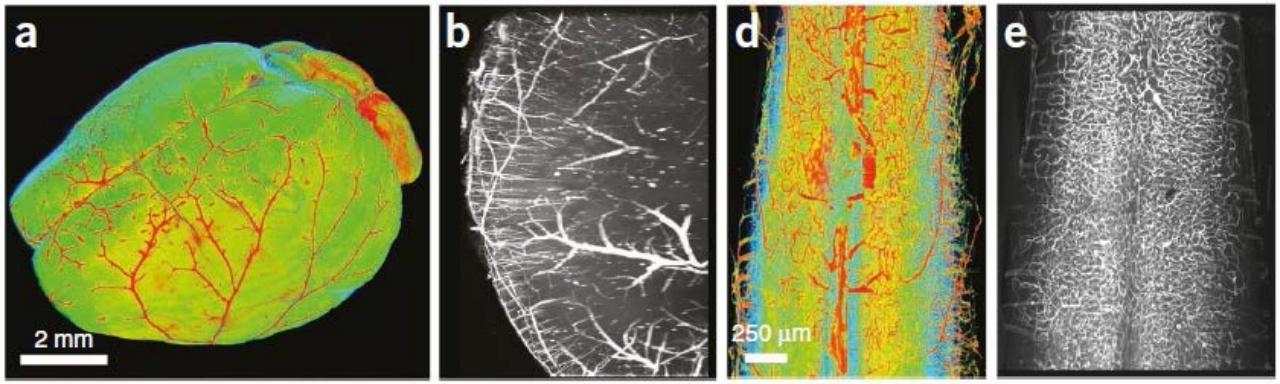
(Initiation of Lung tumors with LV-cre vector expressing doxycycline-inducible GFP)

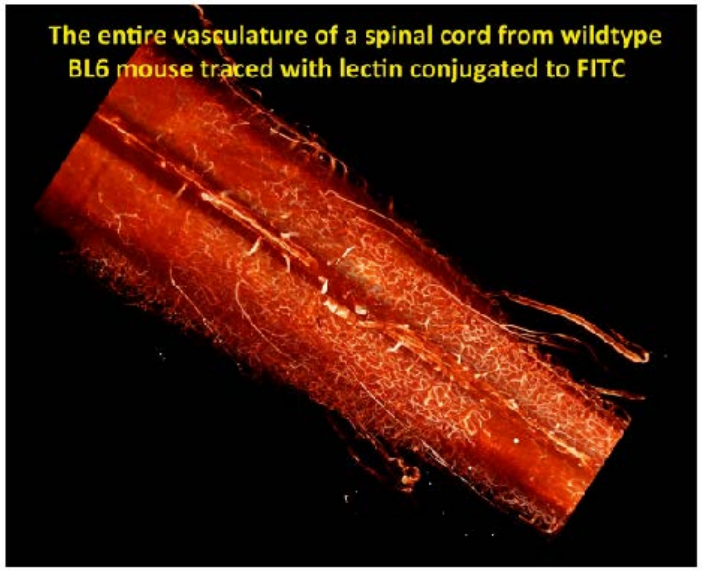


Visualization of blood capillaries in entire organs

Details of vasculature in the brain & spinal cord

Labeling of vasculatire with lectin-FITC & imaging with Ultramicroscopy





NATURE PROTOCOLS

Three-dimensional imaging of solvent-cleared organs using 3DISCO

Ali Ertürk[1], Klaus Becker[2,3], Nina Jährling[2–4], Christoph P Mauch[5], Caroline D Hojer[6], Jackson G Egen[6], Farida Hellal[7], Frank Bradke[7], Morgan Sheng[1] & Hans-Ulrich Dodt[2,3]

Advancements

- ✓ ***Very fast to perform***
- ✓ ***Combination of THF & DBE as clearing compounds → increased transparency of adult brain***
- ✓ ***Method can be used for clearing and imaging of several organs***
- ✓ ***Compatibility with diverse labeling methods***
- ✓ ***Compatibility with various microscopy techniques***

Limitations

- ✓ ***Clearing can only be used in fixed tissues***
- ✓ ***Clearing dissolves lipid structures (samples cannot be used for electron microscopy or be amenable to lipophilic dye staining-tracing)***
- ✓ ***Cleared tissues cannot be stored for long time (degraded fluorescence)***
- ✓ ***Immunolabeling is a challenge (limited antibody penetration)***

Paper 5

NATURE |

ARTICLE

doi:10.1038/nature12107

Structural and molecular interrogation of intact biological systems

Kwanghun Chung[1,2], Jenelle Wallace[1], Sung-Yon Kim[1], Sandhiya Kalyanasundaram[2], Aaron S. Andalman[1,2], Thomas J. Davidson[1,2], Julie J. Mirzabekov[1], Kelly A. Zalocusky[1,2], Joanna Mattis[1], Aleksandra K. Denisin[1], Sally Pak[1], Hannah Bernstein[1], Charu Ramakrishnan[1], Logan Grosenick[1], Viviana Gradinaru[2] & Karl Deisseroth[1,2,3,4]

C: clear

L: lipid-exchanged

A: anatomically

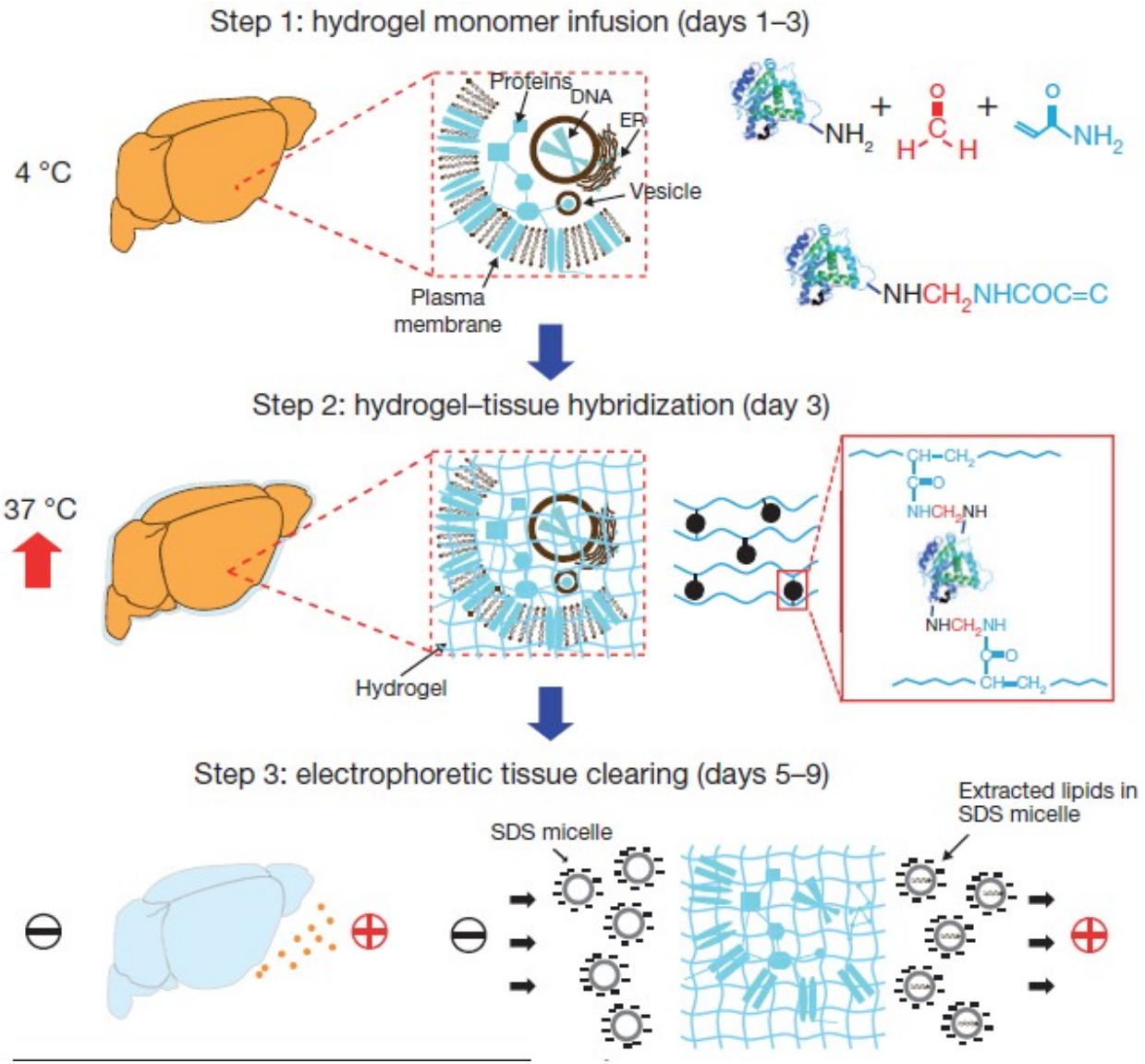
R: rigid

I: imaging/immunostaining-compatible

T: tissue

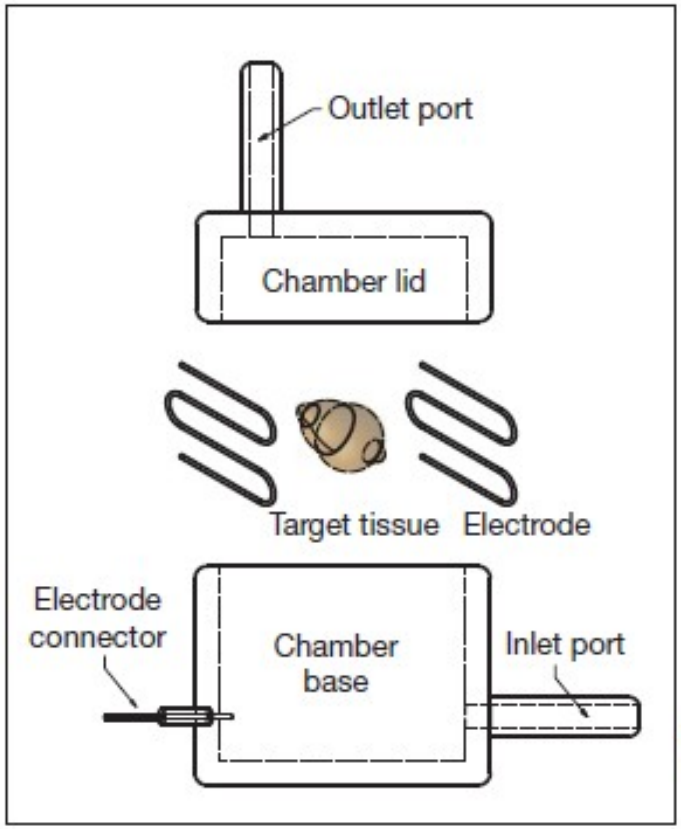
Y: hYdrogel

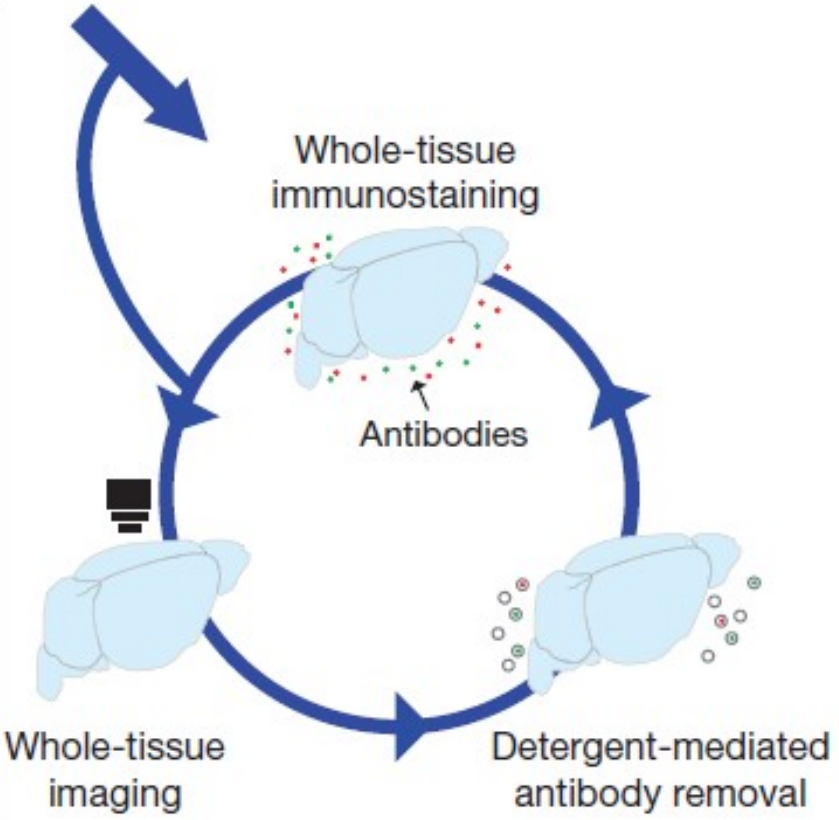
Clearing Protocol



Clearing Protocol

Electrophoretic Tissue Clearing (ETC)





C

L

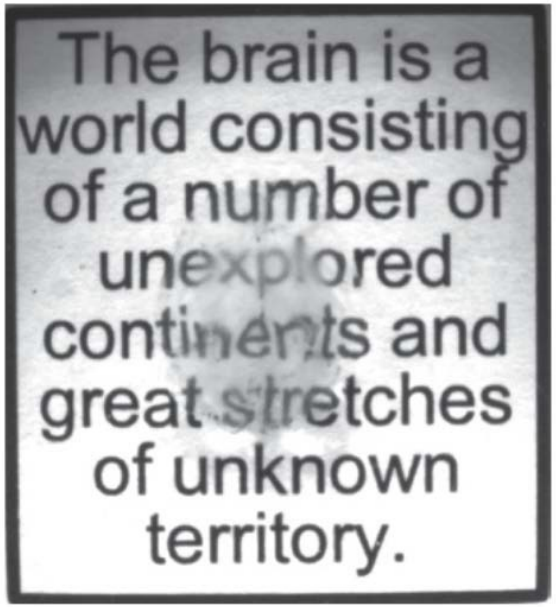
A

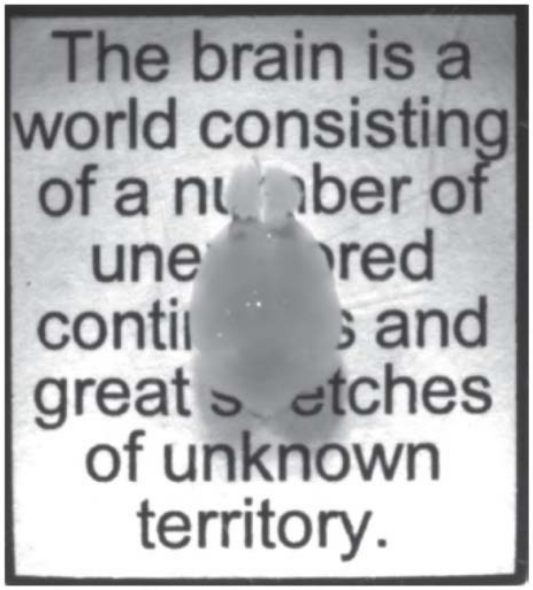
R

I

T

Y


The brain is a
world consisting
of a number of
unexplored
continents and
great stretches
of unknown
territory.


The brain is a
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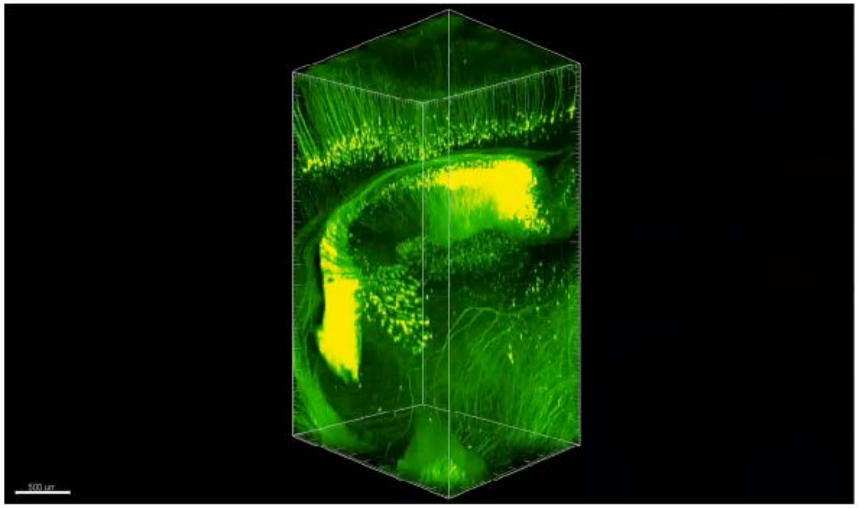
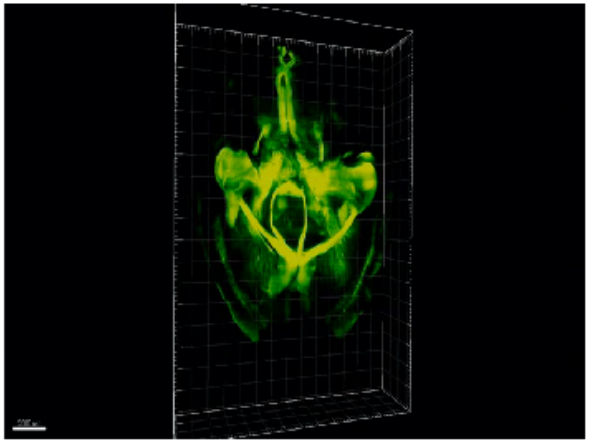
Adult mouse Brain Imaging

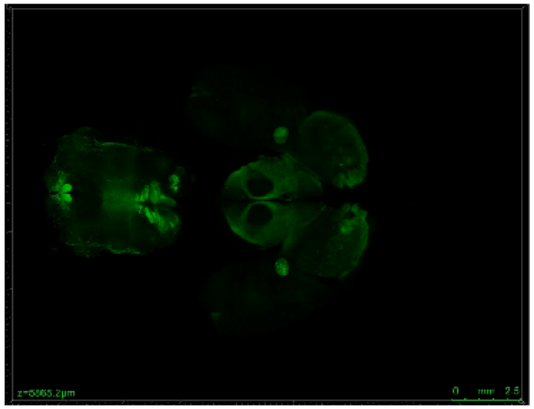
3 month old Thy1-eYFP line-H mice



Hippocampus_ Thy1 eGFP mice



Pia surface → thalamus



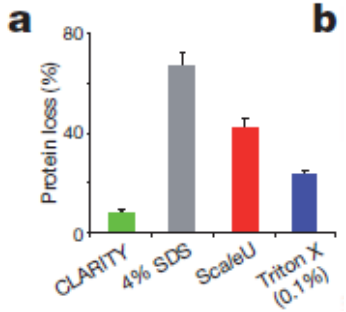
Neuronal networks in all regions
of the brain

TEM image
(myelinated axons in the hippocampus)

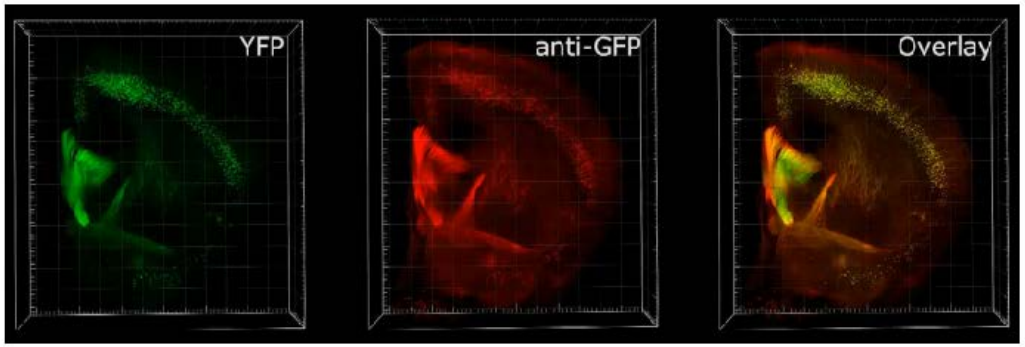
Molecular Phenotyping of Intact Tissue

3 month old Thy1-eYFP line-H mice

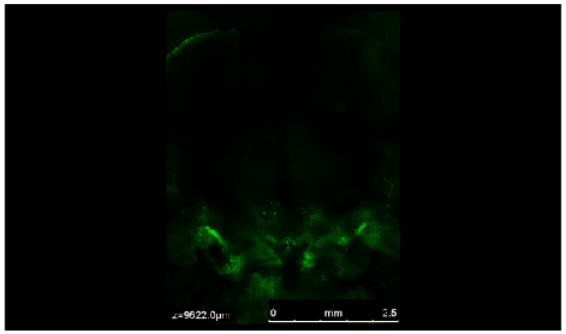
Protein Loss
upon different clearing compounds

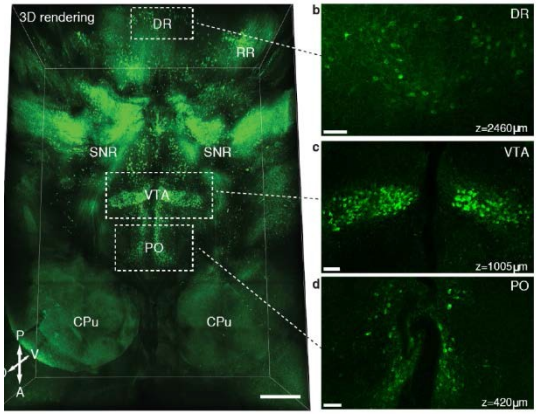


3D visualization of 1mm thick coronal block of Thy1-eGFP mouse (12w old) immunostained for GFP

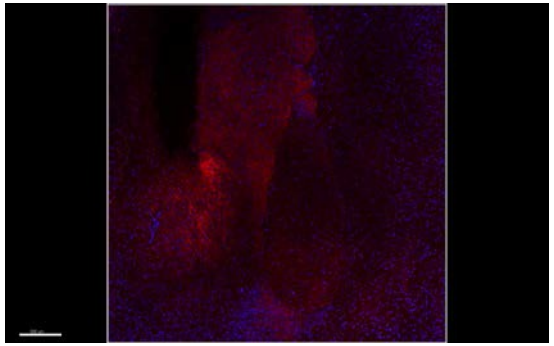


<u>Tyrosine Hydroxylase (TH) staining</u>
(5mm thick adult mouse brains)





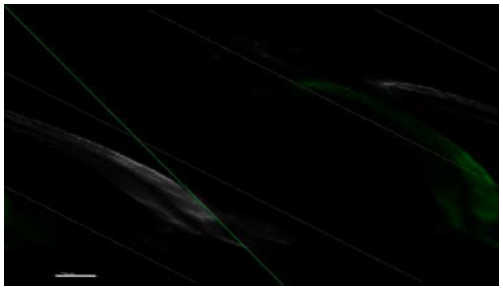
<u>TH positive fibers in the amygdala</u>



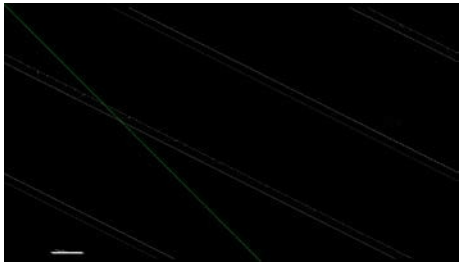
Multi-round Molecular Phenotyping

3 month old Thy1-eYFP line-H mice

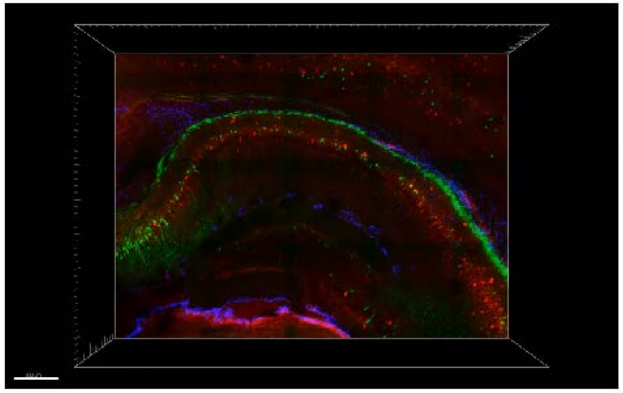
3D visualization of 1mm thick coronal block of Thy1-eGFP mouse (12w old) immunostained for TH (red:TH, green:eGFP)



3D visualization of 1mm thick coronal block of Thy1-eGFP mouse (12w old) immunostained for TH (red:TH, green:eGFP) after elution



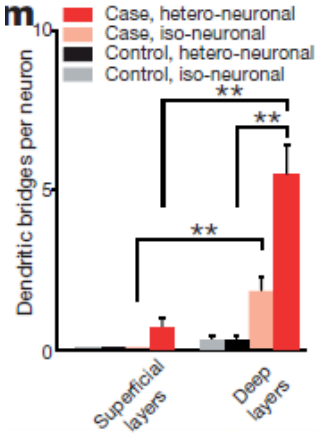
3D visualization of 1mm thick coronal block of Thy1-eGFP mouse (12w old) immunostained for PV (red), GFAP (blue), DAPI (white)



Three-dimensional view of hippocampus showing eYFP expressing neurons (green), parvalbumin-positive neurons (red) and GFAP (blue).

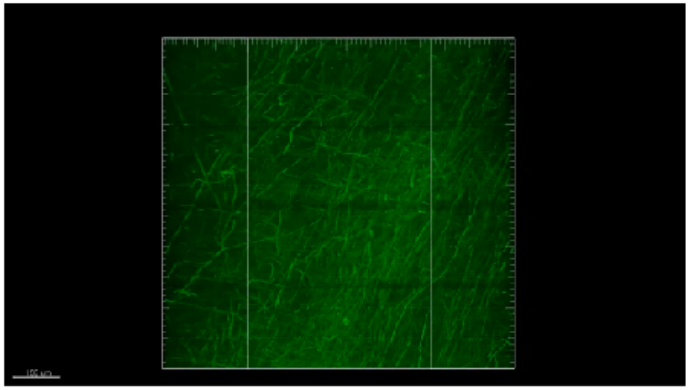
Human Brain structural/molecular phenotyping

Human frontal lobe (BA10), 500μm thick, intact blocks

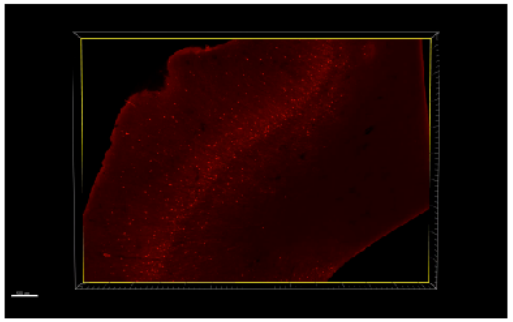


Counting dendritic bridges

Single axonal tracing –rendering of neurofilament positive axonal fibres



Visualization of parvalvumin-positive neurons in the neocortex of an autism case



NATURE PROTOCOLS

Three-dimensional imaging of solvent-cleared organs using 3DISCO

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Advancements

- ✓ *Applicable to different fixed tissues (mouse, zebrafish, post-mortem human brains)*
- ✓ *Enables multi-round molecular phenotyping*
- ✓ *Removal of lipid bilayers*
- ✓ *Compatibility with diverse labeling methods*
- ✓ *Clarified tissue potentially compatible with electron microscopy*
- ✓ *Tissue stability*

Limitations

- ✓ *Clearing can only be used in fixed tissues*
- ✓ *Need for Clarity-optimized long working-distance objectives*
- ✓ *Data processing & storage*

Summary

- ✓ Tissue Clearing
- ✓ Tissue Stability
- ✓ Compatibility with ultramicroscopy, 1- & 2- photon and confocal microscopy
- ✓ Multiple Applications (mouse brain, human samples, zebrafish, vasculature, all organs)

Outlook

- ✓ Tissue Clearing → stability / transparency / tissue damage / time
- ✓ Improved Resolution
- ✓ Compatibility with electron microscopy
- ✓ Tissue Fixation
- ✓ Huge amount of data

Thank you!