FAST TISSUE CLEARING

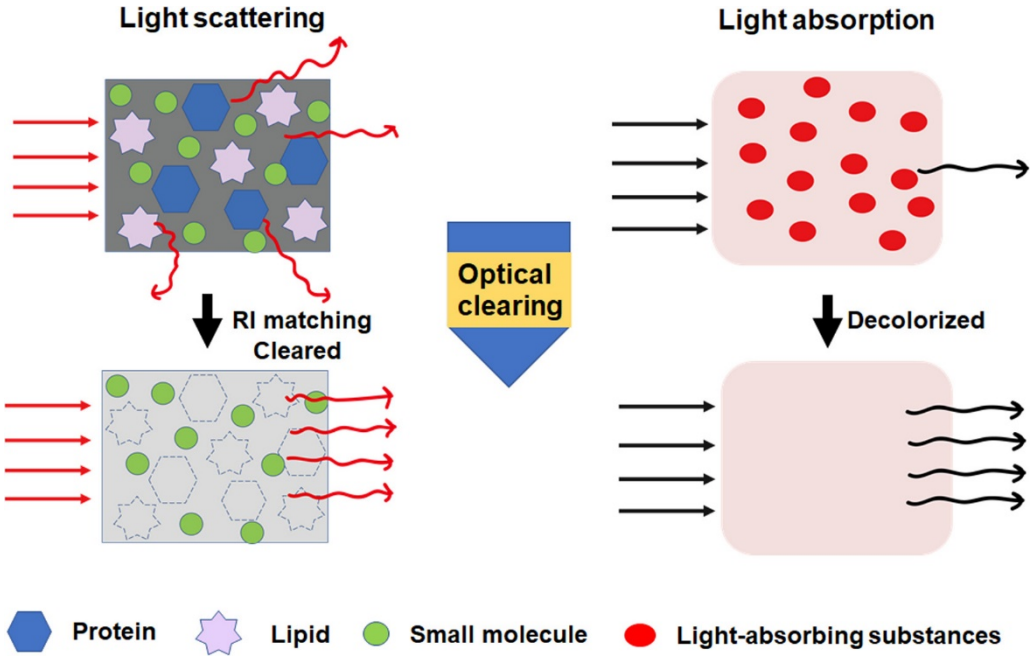
An overview of state-of-the-art methods

Technical Journal Club

12th April 2022

Dalila Vena

Mechanisms and Principles of tissue clearing



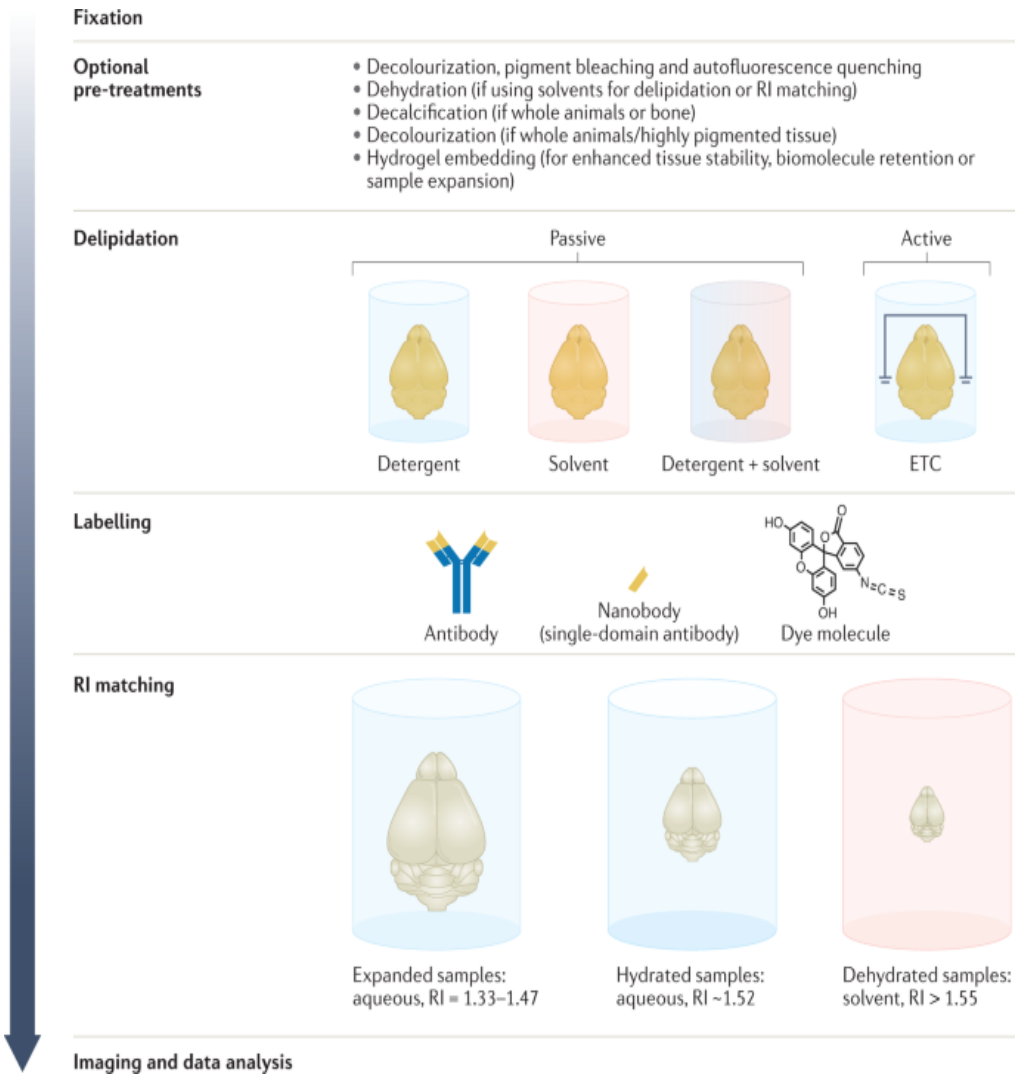
Biological tissues are opaque because of:

- Light scattering (heterogenous components with different refractive index) which cause decay of light intensity
- Light absorption (by endogenous molecules) which further attenuates light transmission

https://doi.org/10.1111/joa.13309

Main common steps in clearing protocols

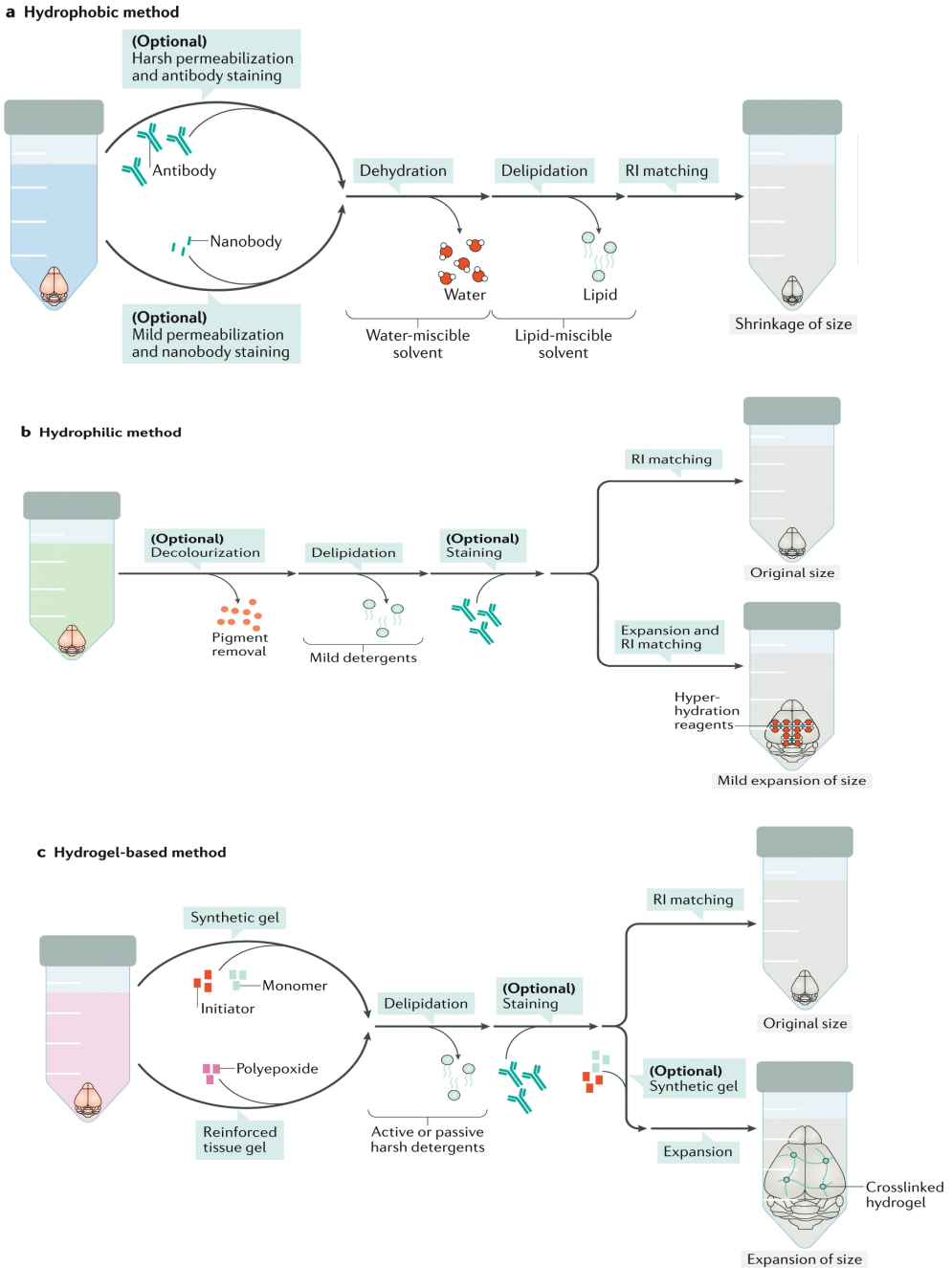
1. Perfusion
2. Fixation
3. Delipidation
   - Passive (simple immersion)
   - Active (electrophoresis)
4. RI matching: tissues are immersed in high-RI agents; thus, the inner RI of tissues tends to be homogeneous, and the scattering of light can be minimized



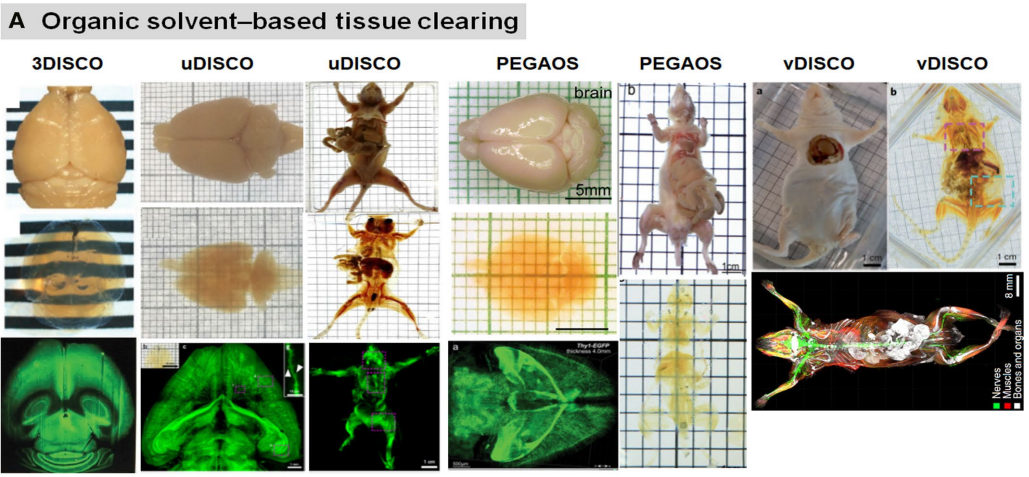
https://doi.org/10.1038/s43586-021-00080-9

Three major tissue clearing approaches

1. Organic-solvent based
2. Aqueous-solution based
3. Hydrogel embedding



https://doi.org/10.1038/s41583-019-0250-1



- Dehydration with lipid solvation (methanol, tetrahydrofurane; etc)
- Additional lipid solvation + clearing (methylsalicilate, dibenzyl ether, etc.)

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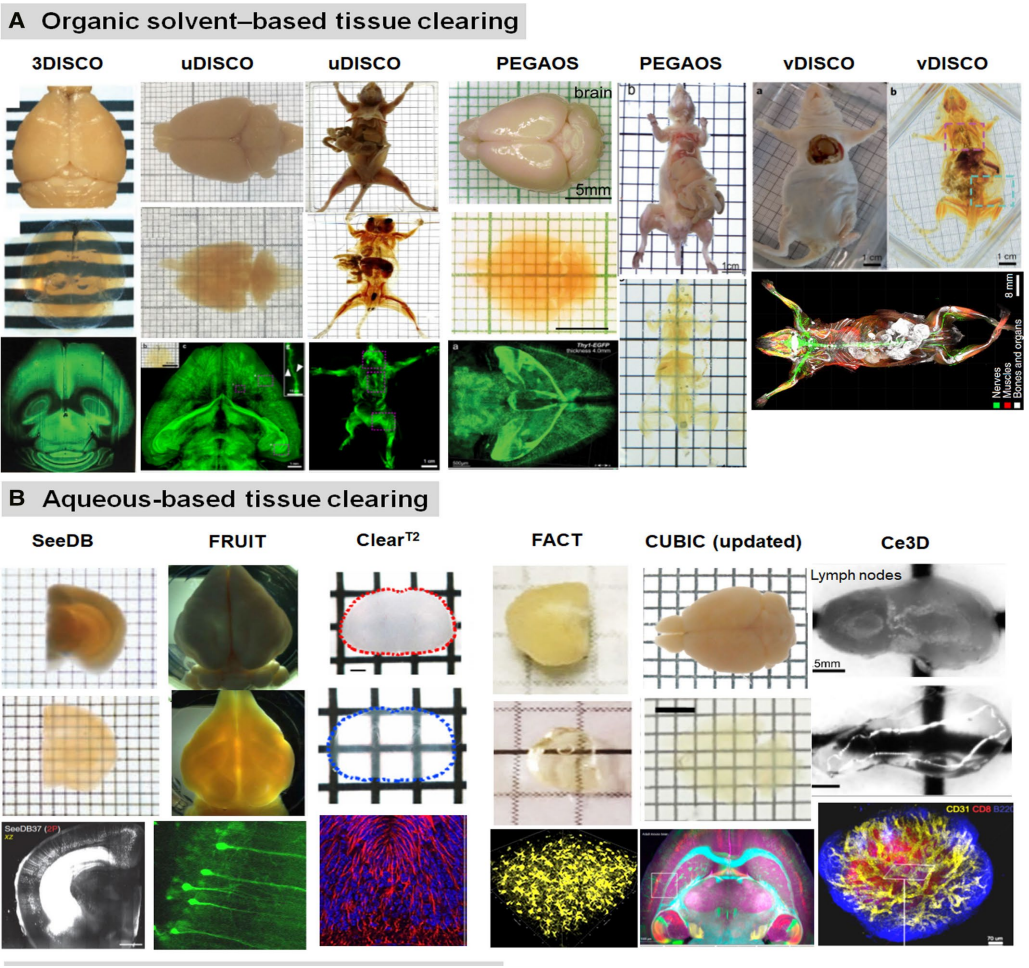

- Dehydration with lipid solvation (methanol, tetrahydrofurane; etc)
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1. Simple immersion in high refractive index solutions (sucrose, fructose, formamide, etc.)
2. Delipidation (detergents)/Hydration (urea)

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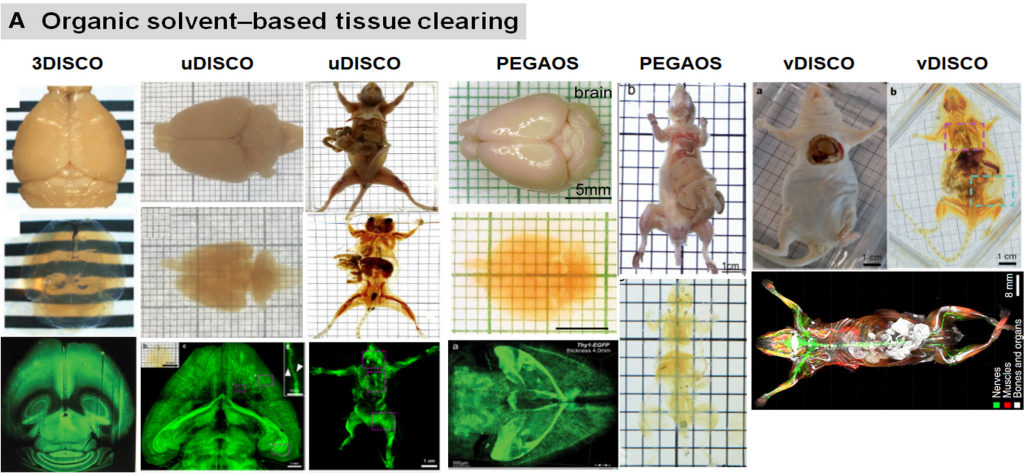

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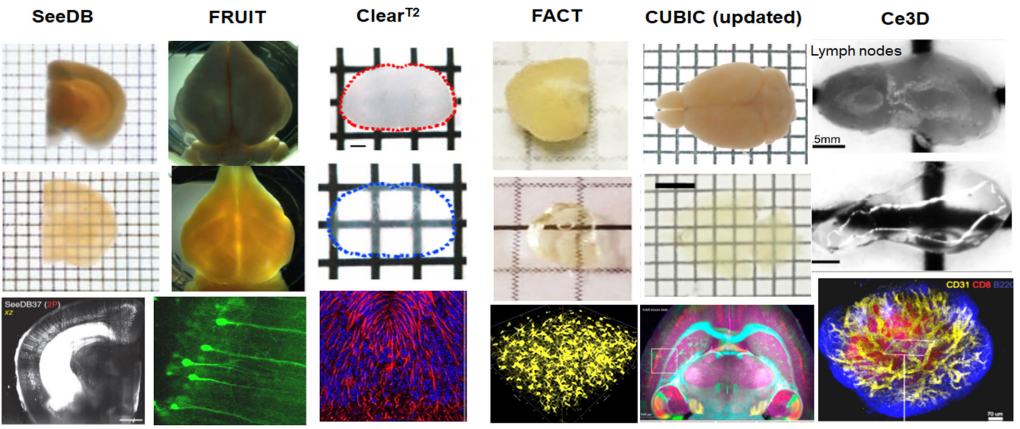

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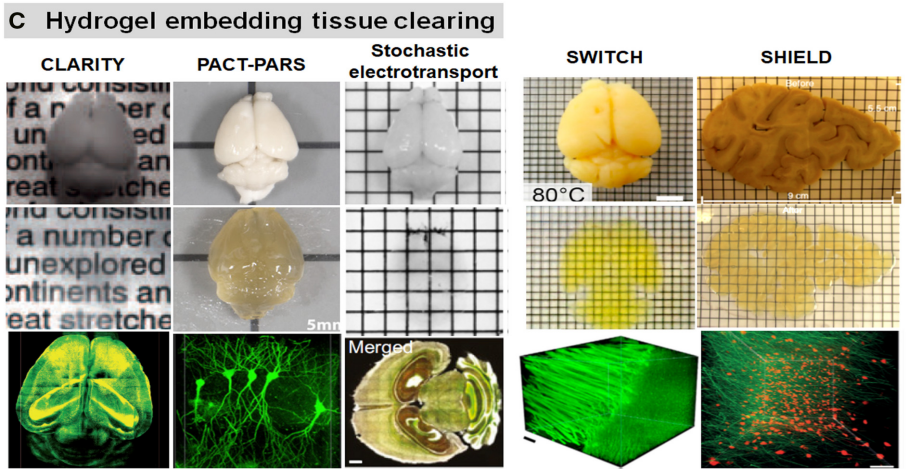

- Hydrogel embedding
- Delipidation
  - passively by incubation in detergent (e.g. SDS)
  - rapidly via electrophoresis
- Immersion in clearing solution (RIMs, glycerol, etc.)

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Organic solvent-based

✓ Excellent tissue transparency

✓ Sub-cellular resolution

✓ Whole-body clearing

✕ Shrinkage of samples

✕ Toxicity of solvents

✕ Quenching of fluorescent protein due to dehydration

Aqueous-based

✓ Simple

✓ Safe

✓ Fluorescence-friendly

✕ Possible loss of native biomolecule due to detergents

✕ Possible damage of tissue architecture

Hydrogel embedding

✓ Uniform transparent tissues

✓ Fluorescence preservation

✓ Minimal structural disturbance

✓ Antigenicity and transcripts preservation

✕ Slow clearing rate

✕ Electrophoretic equipment

Best clearing approach?

Depends on question and research objective

Currently available fast clearing protocols

- **FACT -** Fast Free-of-Acrylamide Clearing Tissue (2017)
  - 1mm thick brain slice
- **FASTClear** (2017)
  - Human tissue
- **RTF** - Rapid clearing method based on Triethanolamine and Formamide (2018)
  - small adult brain blocks
- **Ce3D** - Clearing-Enhanced 3D (2019)
  - Not efficient on brain
- **FOCM** - ultraFast Optical Clearing Method (2019)
  - 300-μm-thick brain slices

PRE-CLARITY - Passive pRe-Electrophroresis CLARITY (2019)
FAST 3D Clear (2022)

SCIENTIFIC REPORTS

OPEN

Modified CLARITY Achieving Faster and Better Intact Mouse Brain Clearing and Immunostaining

Received: 2 October 2018
Accepted: 29 June 2019
Published online: 22 July 2019

Hao Du[1], Peihong Hou[1], Liting Wang[2], Zhongke Wang[3] & Qiyu Li[1]

Cell Reports Methods

CellPress
OPEN ACCESS

Report

A fast, aqueous, reversible three-day tissue clearing method for adult and embryonic mouse brain and whole body

Stylianos Kosmidis,[1,3,4,*] Adrian Negrean,[1,3] Alex Dranovsky,[5] Attila Losonczy,[1,2,3] and Eric R. Kandel[1,2,3,4,6,*]
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https://doi.org/10.1016/j.crmeth.2021.100090

www.nature.com/scientificreports

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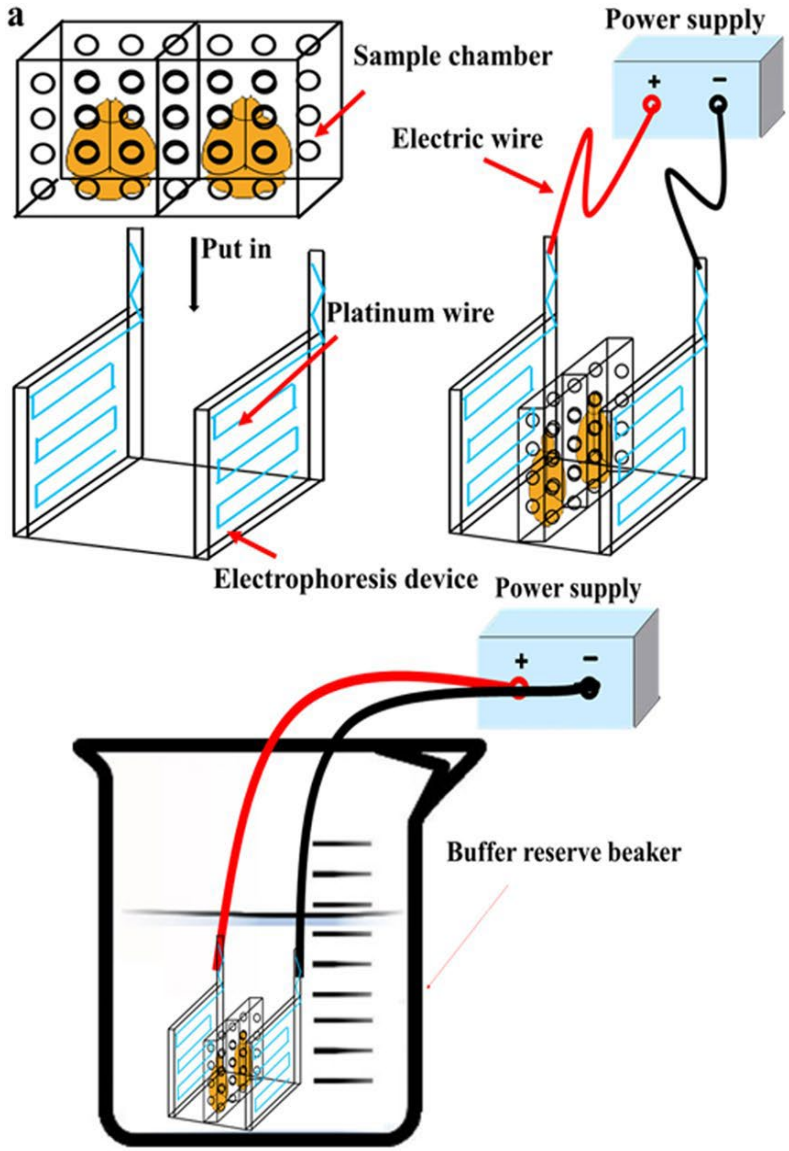
NOVELTIES

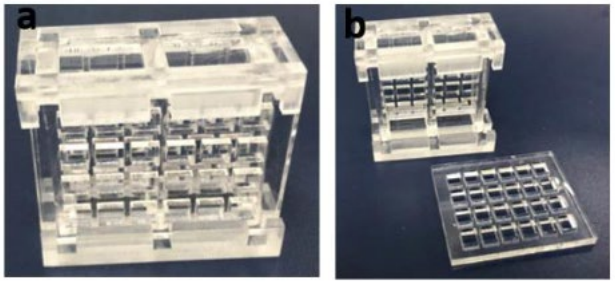
1. NCES (Non-circulation electrophoresis system)
2. PRE-CLARITY (Passive pRe-Electrophroresis CLARITY)
3. CEx staining method

Non-circulation electrophoresis system (NCES)

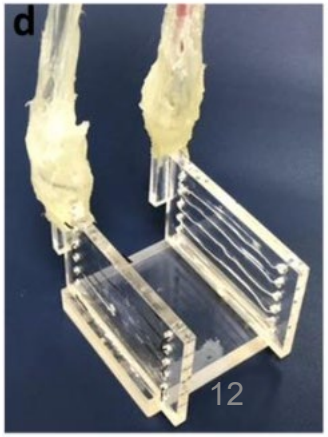


- SDS clearing buffer and electrophoresis in a beaker
- Mobile electrophoresis device
- Cheap plastic-made electrophoresis device and sample chamber
- Possibility to design different size/structure
- Several samples simultaneously

Sample chamber



Electrophoresis device



Modified CLARITY promotes brain clearing speed and improves transparency

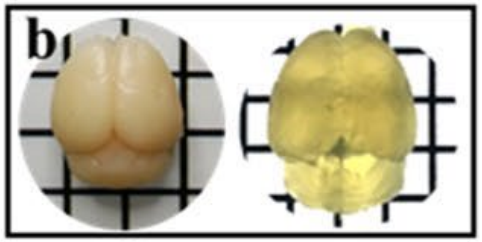
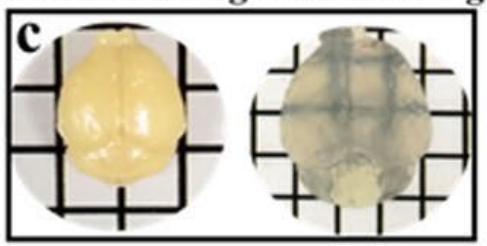
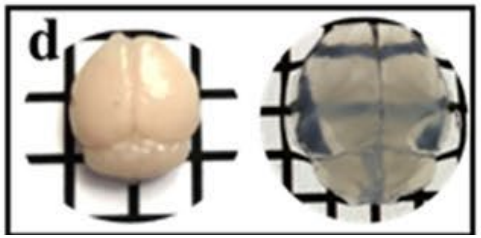
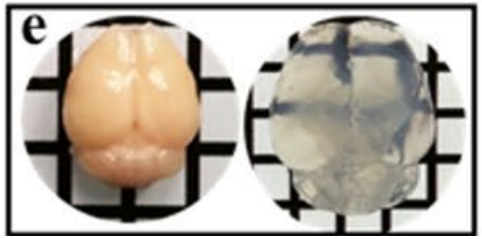
CONTROL GROUP

- Bl6 mice transcardially perfused with **A4P4B0.05 solution** (4% acrylamide, 0.05% bis-acrylamide, 4% PFA and 0.25% VA-044 initiator in PBS)
- Incubated in A4P4B0.05 solution for 24 hours
- The brain was polymerized and extracted from solidified hydrogel.
- Passive/NCES clearing

EXPERIMENTAL GROUP

- Bl6 mice transcardially perfused with PBS followed by 4% PFA.
- Brains were excised and then post-fixed in 4% PFA at 4 °C.
- Fixed brains were incubated in **A4P0B0 hydrogel monomer solution** (4% acrylamide in PBS) supplemented with 0.25% VA-044 initiator for 24 hours at 4 °C.
- A4P0B0-infused samples were polymerized for 6 hours at 37 °C
- Passive/NCES clearing

Modified CLARITY promotes brain clearing speed and improves transparency

	CONTROL GROUP	EXPERIMENTAL GROUP
	A4P4B0.05-processed brain Before clearing After clearing	A4P0B0-processed brain Before clearing After clearing
NCES		
Passive clearing		
	B 5 days electrophoresis clearing at 25 V/37 °C **D** 1 month passive clearing at 50 °C	**C** 60 hours electrophoresis clearing at 25 V/37 °C, with adding 1% α-thioglycerol in clearing buffer **E** 14 days passive clearing at 37 °C, with adding 5% α-thioglycerol in clearing buffer

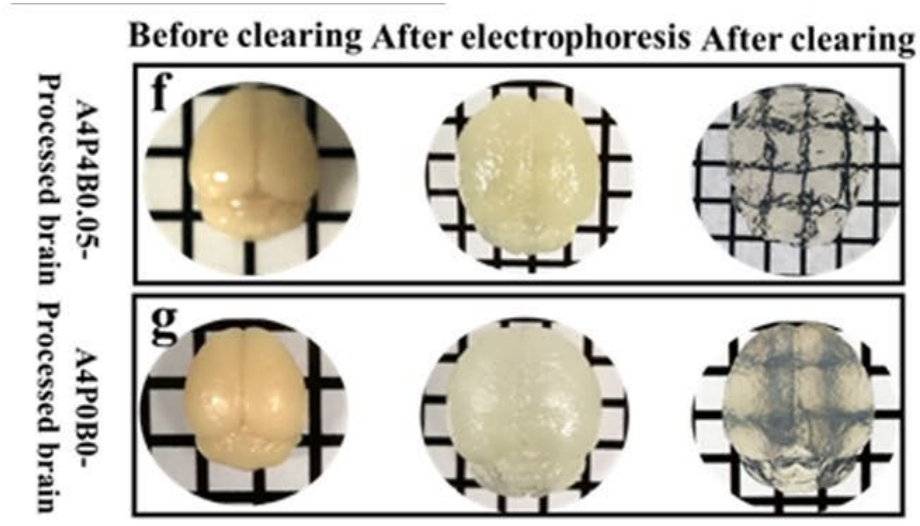
A4P0B0 EMBEDDING METHOD + THIOGLYCEROL + NCES

Faster clearing, higher transparency, mild temperature, prevent brain coloration

PRE-CLARITY achieves higher transparency

CONTROL GROUP

EXPERIMENTAL GROUP



To try to achieve better clarification in the central area of the brain
PRE-CLARITY (Passive pRe-Electrophroresis CLARITY)

- 1-day NCES electrophoresis clearing
- "n days" passive clearing
  - Control group: 15 days (1+15)
  - Experimental group: 5 days (1+5)

PRE-CLARITY
Faster clearing, higher transparency, higher transmittance compared to individual NCES or passive clearing

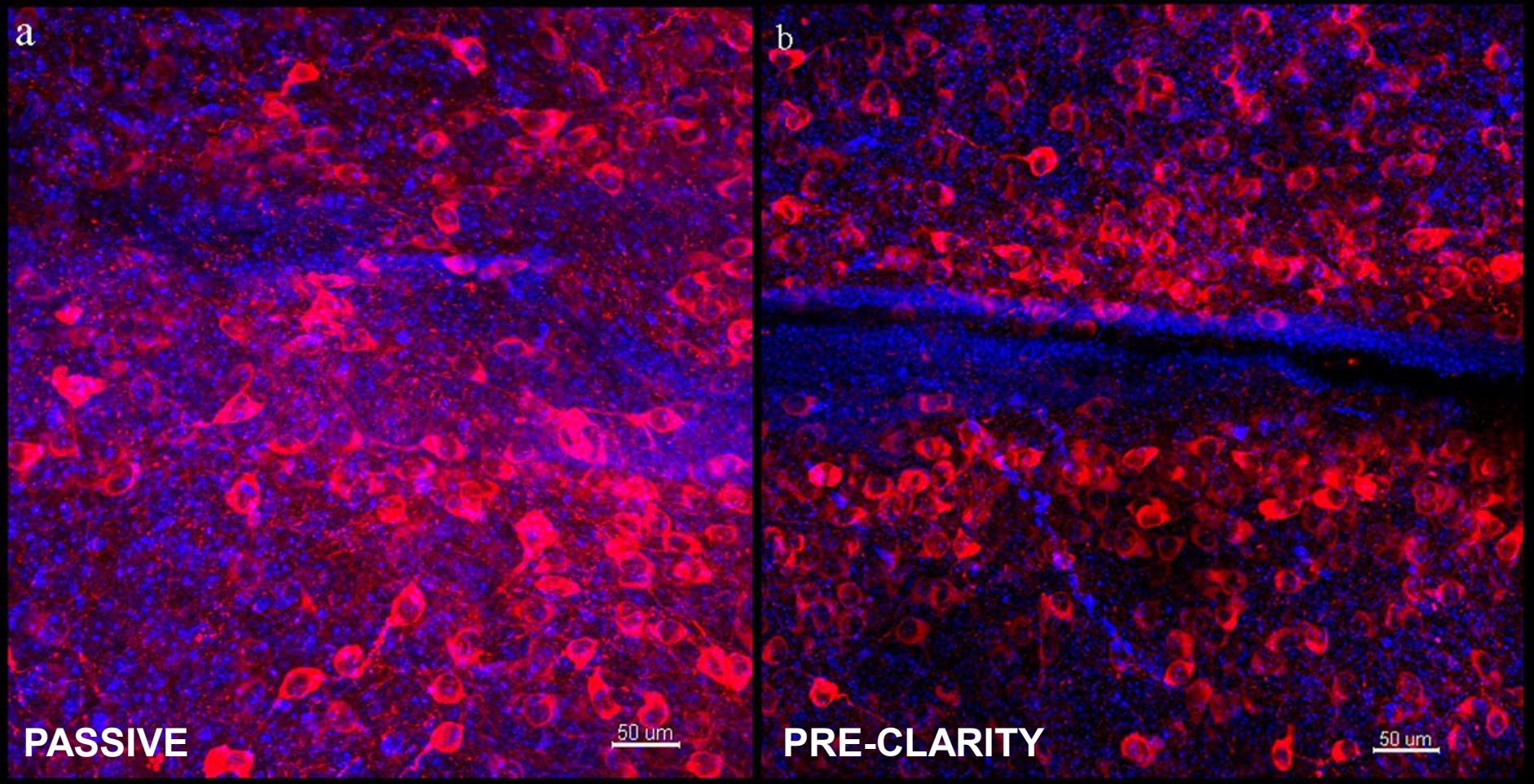


Figure 4. Images of passive and PRE-CLARITY cleared A4P4B0.05-processed brain. (**a**) The image was acquired by confocal microscopy (20x/0.75 dry objective). It is a projection of image stacks and shows stained dopaminergic neurons in the passive cleared A4P4B0.05-processed brain (Red: anti-TH; blue: DAPI; Scale bar: 50 μm; z stack: 516.62 μm). (**b**) The image was acquired by confocal microscopy (20x/0.75 dry objective). It is a projection of image stacks and shows stained dopaminergic neurons in the PRE-CLARITY cleared A4P4B0.05-processed brain (Red: anti-TH; blue: DAPI; Scale bar: 50 μm; z stack: 698.5 μm).

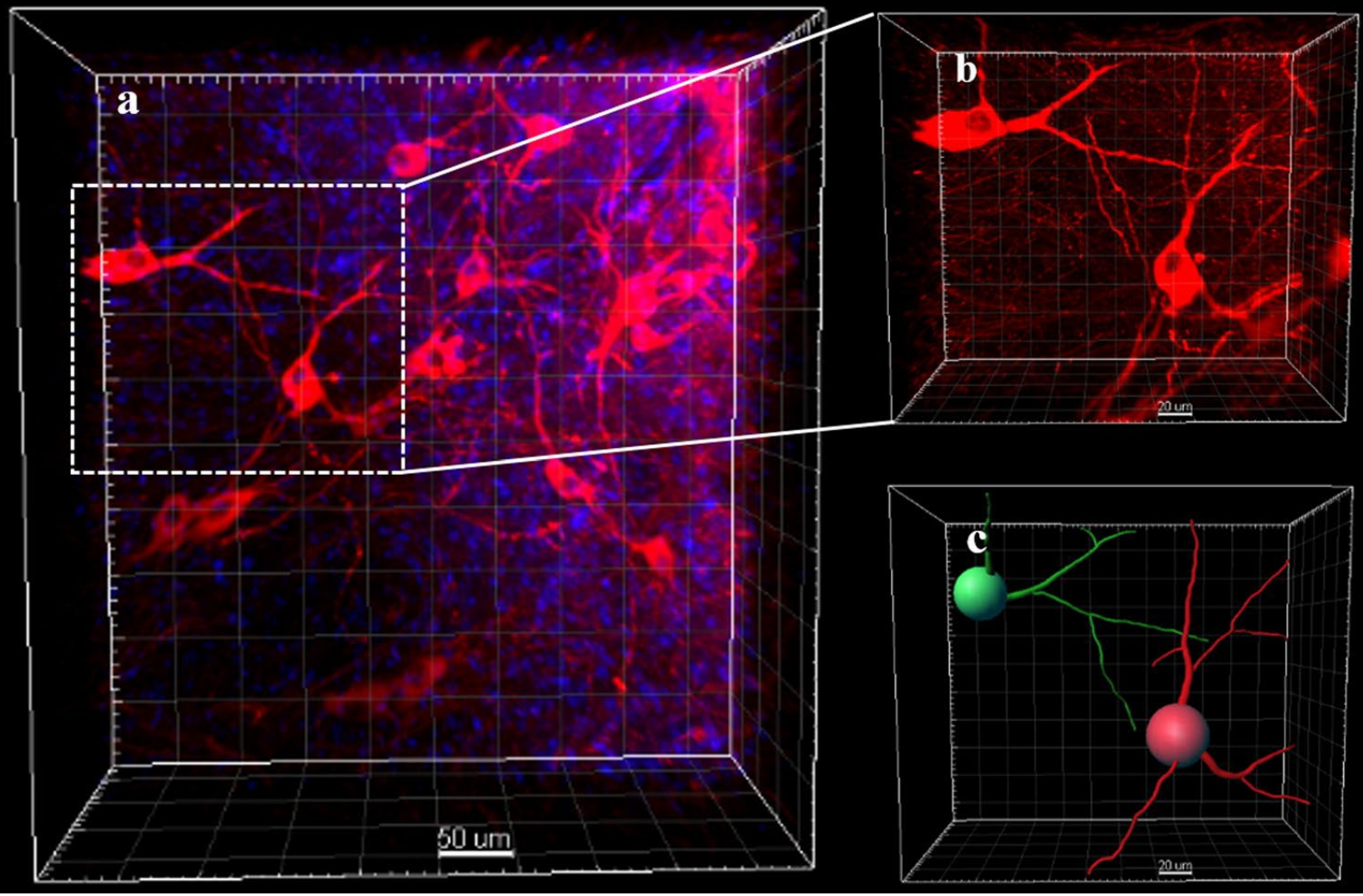


Figure 6. Images of PRE-CLARITY cleared A4P0B0-processed brain. (**a**) The image was acquired by confocal microscopy (20x/0.75 dry objective). It shows dopaminergic neurons stained with Anti-Tyrosine Hydroxylase antibody. (Red: anti-TH; blue: DAPI; Scale bar: 50 μm; square width: 50 μm). (**b**) The amplificatory 3D view of neurons in the white boxed region of figure a. (Scale bar: 20 μm; square width: 20 μm). (**c**) The image shows neuronal dendrites in figure b. It is reconstructed with the filament auto-path toolkit in Imaris 9.0.1 software (Bitplane) (Scale bar: 20 μm; square width: 20 μm).

Faster labeling with CEx staining

Boric acid solution expanded the cleared brain → it might increase the hydrogel network pore size potentially, and then antibodies penetrate into deep tissue easier.

PBST in the washing procedure and PBS in the staining procedure were substituted with **0.2 M boric acid buffer** (pH 7.2)
24h antibody incubation, DAPI added in the last 12 hours

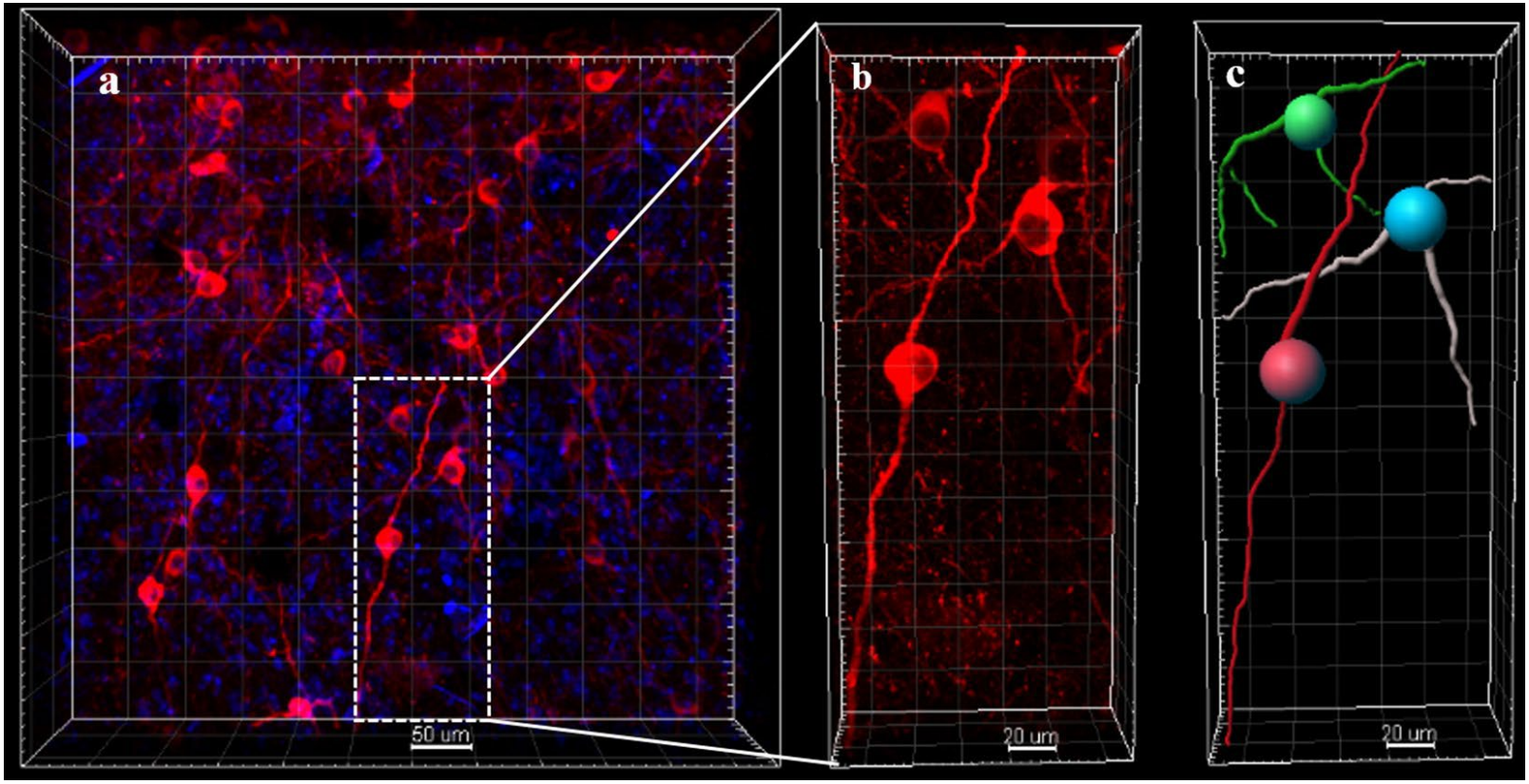
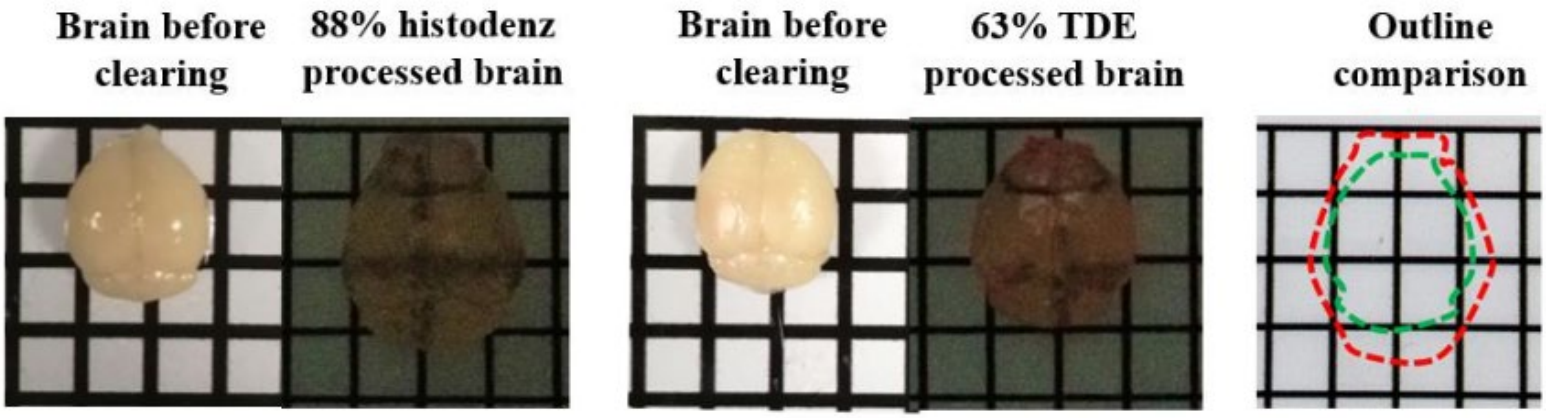


Figure 3. Images of electrophoresis cleared A4P0B0-processed brain. (**a**) The image was acquired by confocal microscopy (20x/0.75 dry objective). It shows dopaminergic neurons (stained with Anti-Tyrosine Hydroxylase antibody) at a part of the midbrain. (Red: anti-TH; blue: DAPI; Scale bar: 50 μm; square width: 50 μm). (**b**) The image shows neurons in the white boxed region of figure a. (Scale bar: 20 μm; square width: 20 μm). (**c**) The image shows dendrites in figure b. It was reconstructed with the filament auto-path toolkit in Imaris 9.0.1 software (Bitplane) (Scale bar: 20 μm; square width: 20 μm).

RI matching solution



TDE has anti-swelling properties

Supplementary figure S7. Comparison of brain after 88% histodenz or 63% TDE processing. The left pair of pictures shows the brain before clearing and after 88% histodenz processing. The middle pair of pictures shows the brain before clearing and after 63% TDE processing. The right picture shows the outline of brain after histodenz (red line) or TDE (green line) processing.

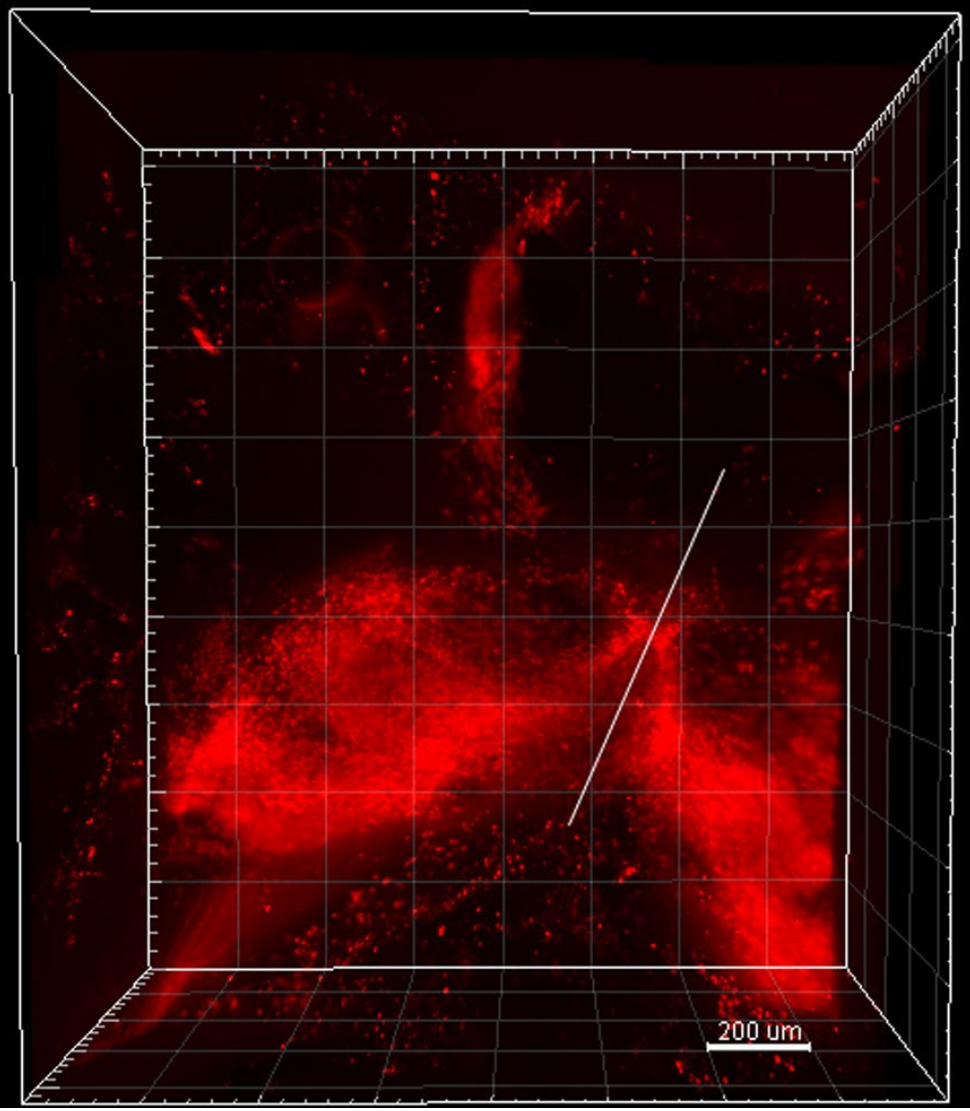


Figure 7. 3D rendering of PRE-CLARITY cleared A4P0B0-processed brain. The image was acquired by lightsheet microscopy (5x/0.16 dry objective). The brain was stained with anti-TH antibody by CEx method. It shows the basal ganglia area has been labeled almost entirely. For some reasons the sample is not at the central position and the white line shows the midline of brain (Scale bar: 200 μm; square width: 200 μm).

Conclusions

ADVANTAGES

- Easy
- Cheap
- Quick
- No electrophoretic equipment needed (peristaltic pump, filter, refrigerated circulator)

LIMITATIONS

- NCES not compatible with A4P4B0.05 solution

Cell Reports Methods

CellPress
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Report

A fast, aqueous, reversible three-day tissue clearing method for adult and embryonic mouse brain and whole body

Stylianos Kosmidis,[1,3,4,*] Adrian Negrean,[1,3] Alex Dranovsky,[5] Attila Losonczy,[1,2,3] and Eric R. Kandel[1,2,3,4,6,*]
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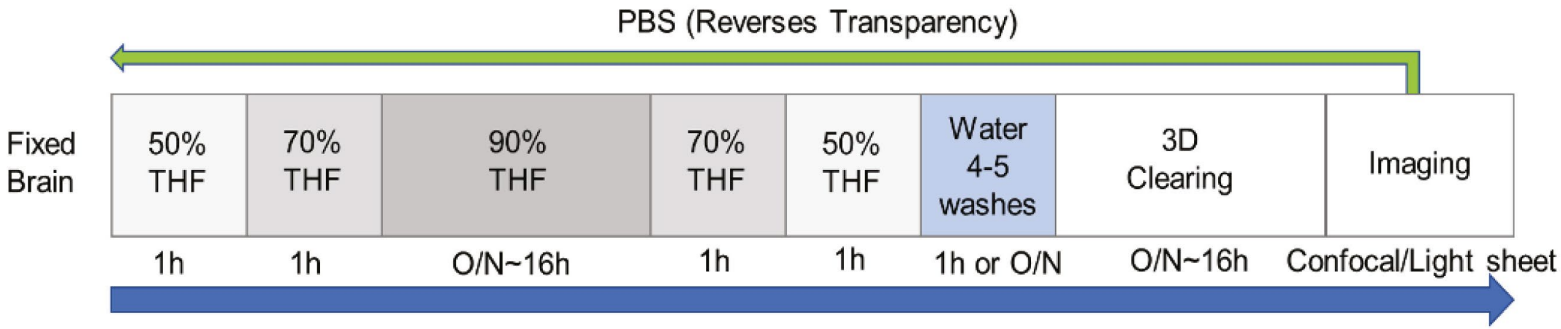
https://doi.org/10.1016/j.crmeth.2021.100090

Fast 3D Clear

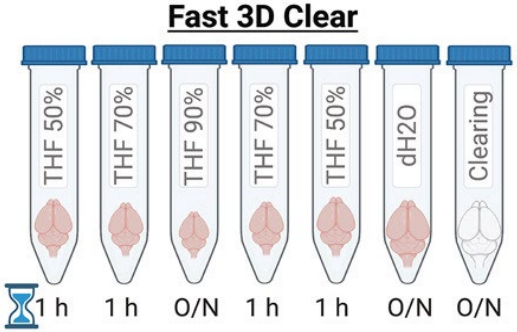
- Perfusion with PBS
- Post-fixation in 4% PFA o/n
- Washes in PBS, 4-5x 10 minutes
- Washes in dH_2O at RT, 2-3 times

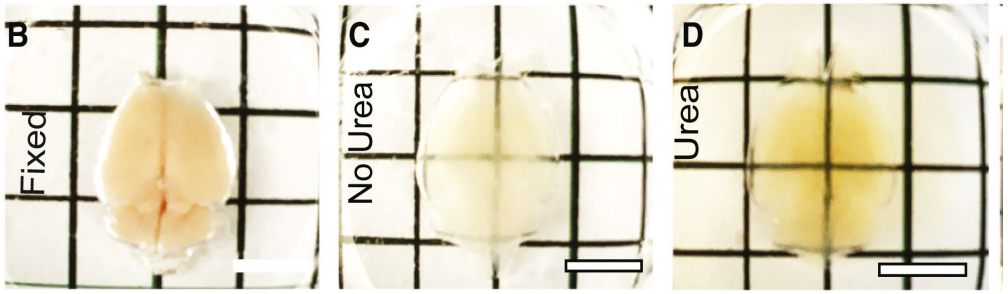


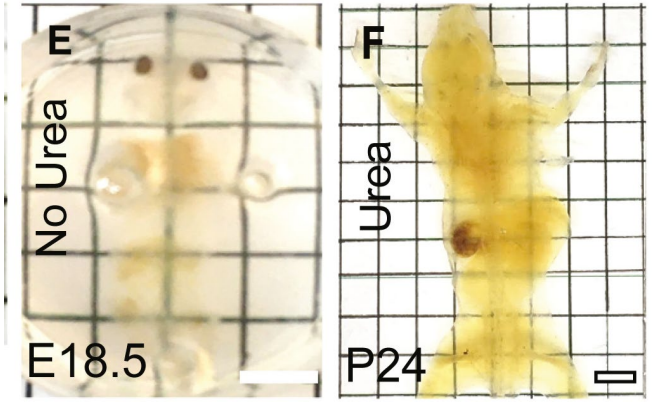
- *Delipidation/Dehydration*: increasing concentration of THF (tetrahydrofuran) pH 9.0
- *Rehydration*: decreasing concentration of THF to reverse shrinkage and preserve fluorescence
- Washes in dH_2O at RT, 4-5 times
- *Clearing*: transfer into aqueous clearing solution (Histodenz, Diatrizoic Acid, N-Methyl-D-Glucamine, Ultrapure Urea. RI 1.512-1.515)

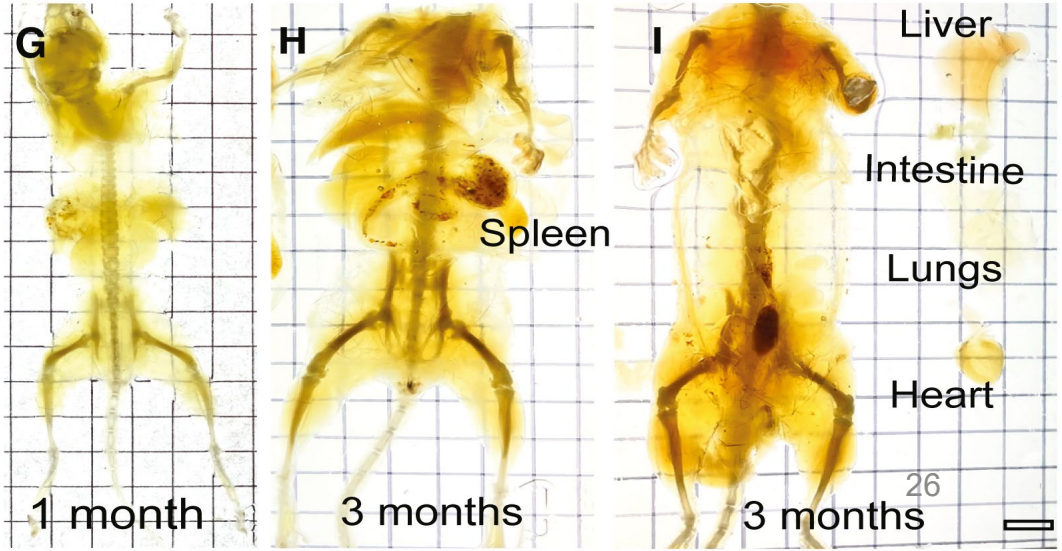
- *Reverse clearing*: PBS washes for 16 hours at 4°C (4-5 washes)

Fast 3D Clear achieves high tissue transparency in brains, whole adult mice and embryos

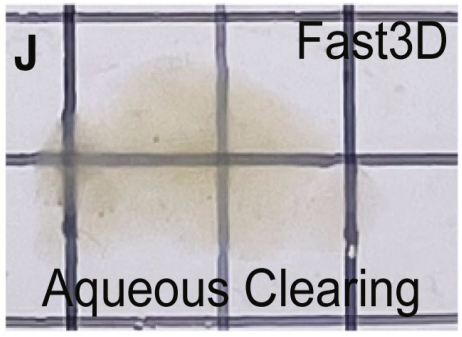


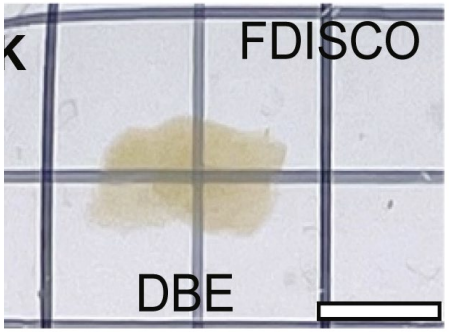


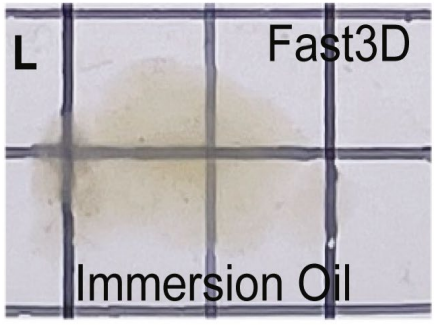


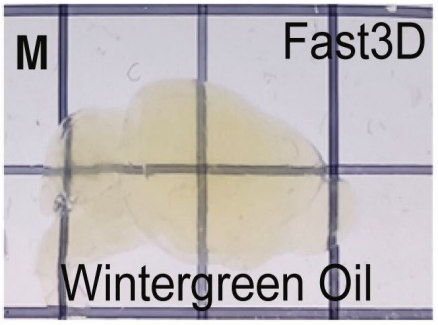


Comparison of Fast 3D Clear with other clearing methods



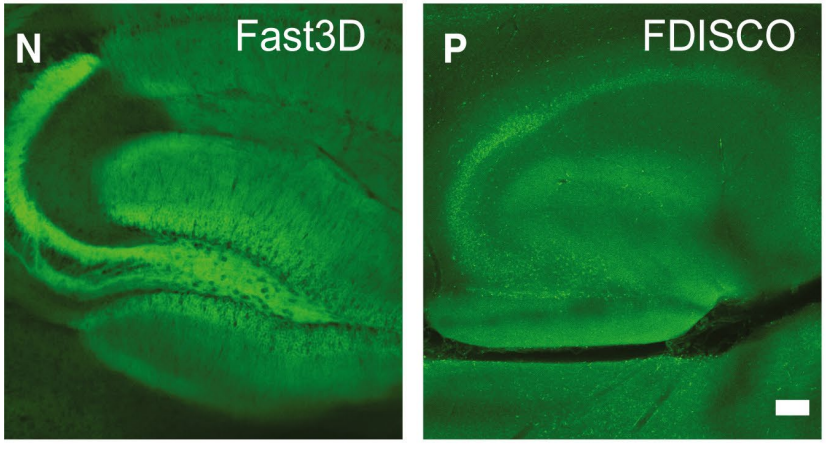


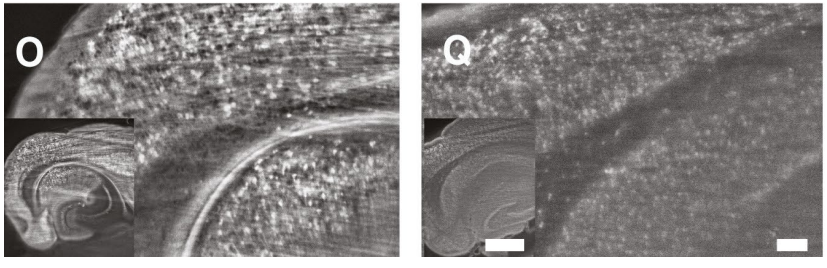


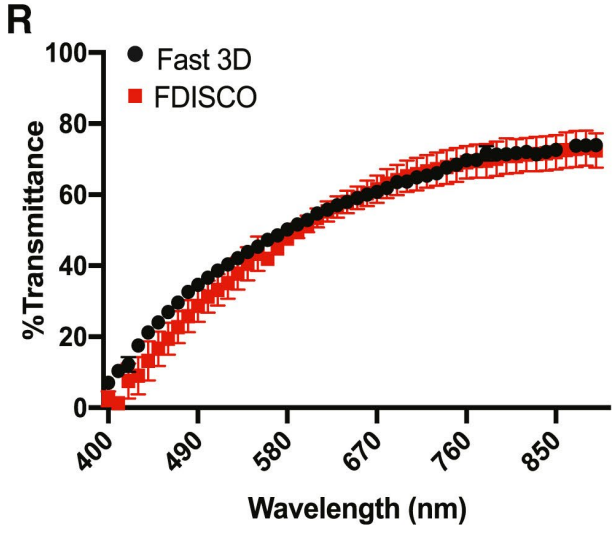


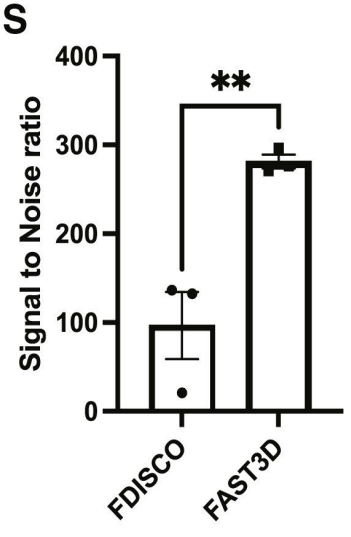
Cargille oil

GCaMP3-
CaMK2-Cre

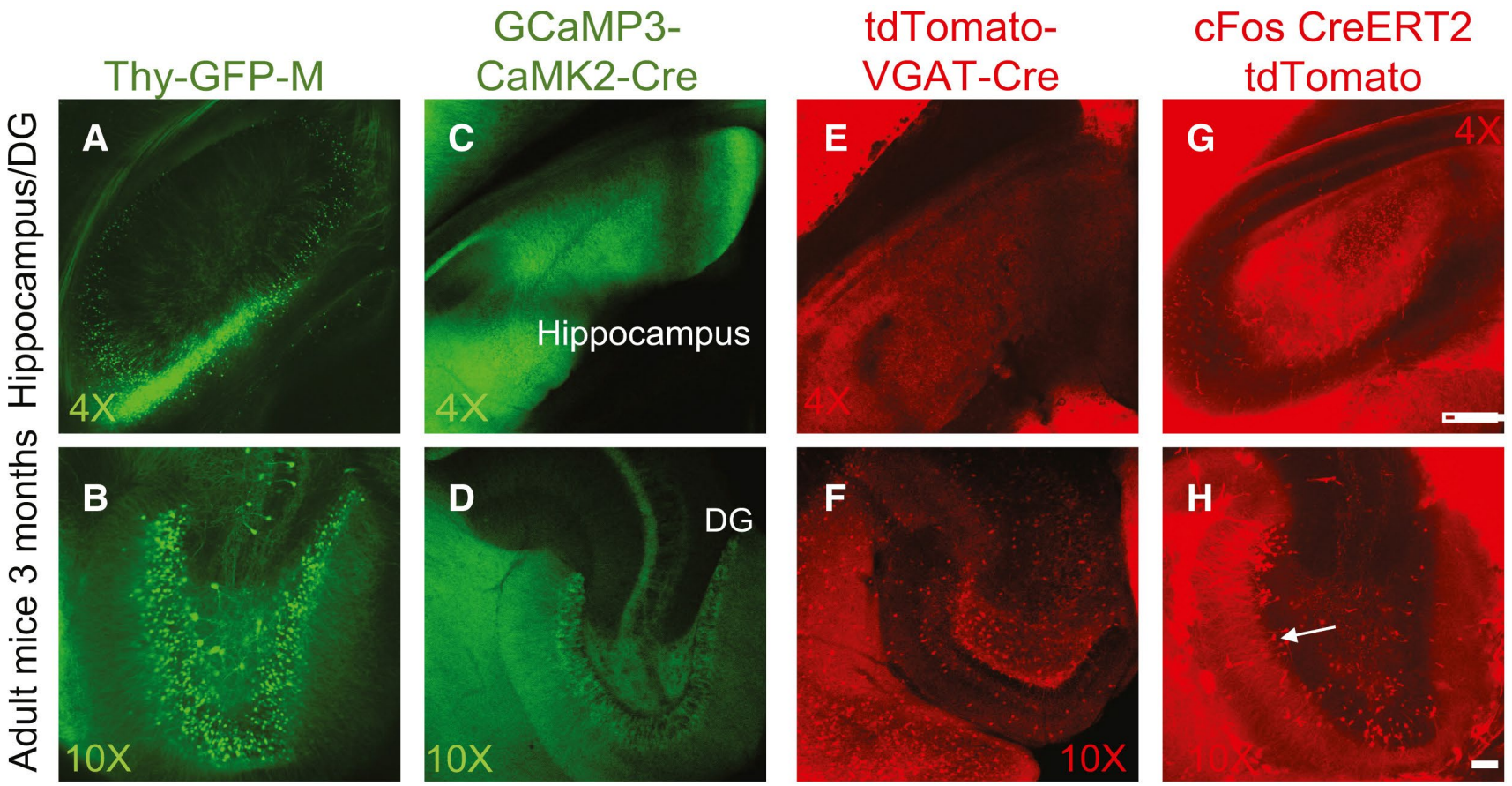






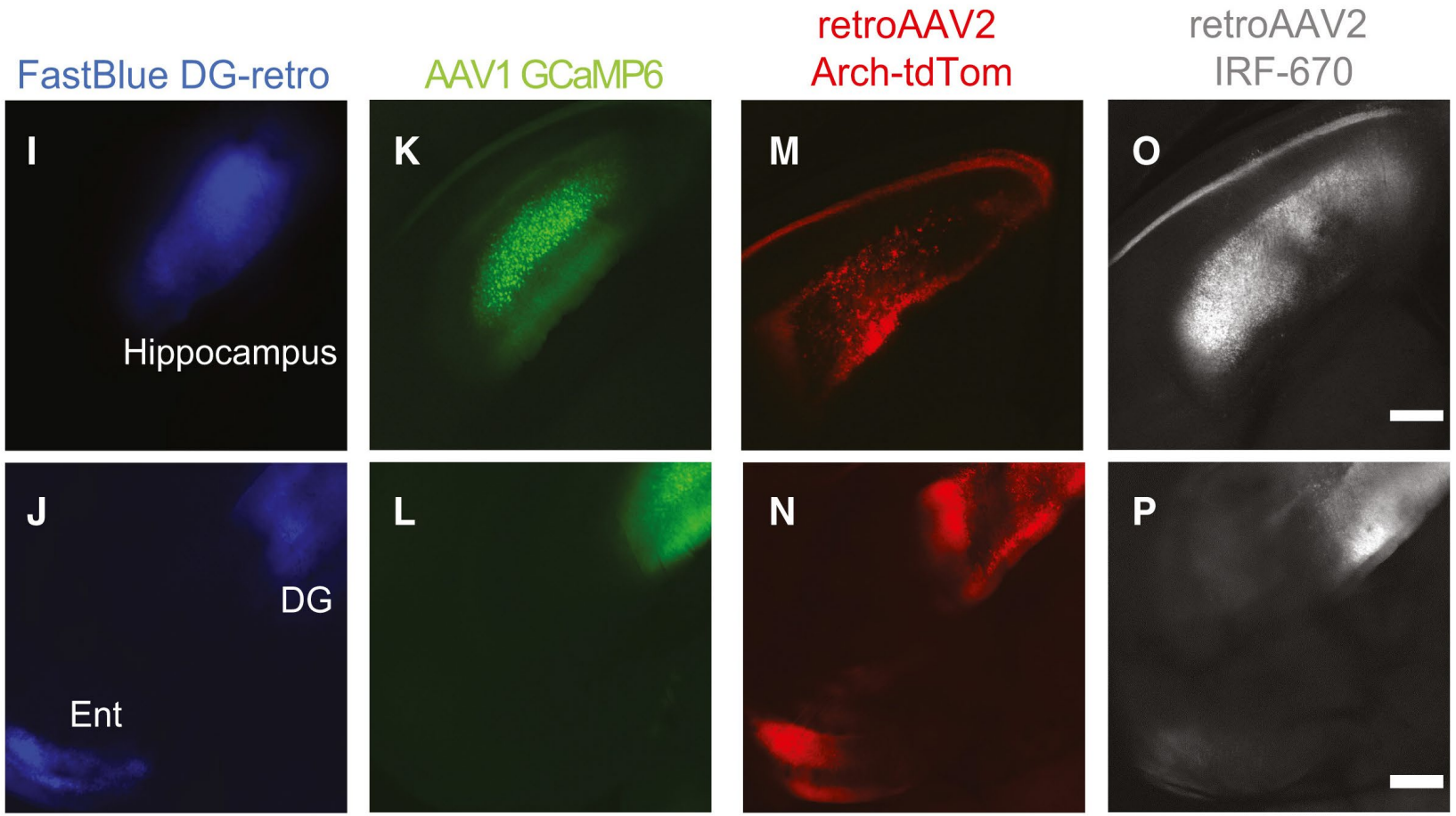


Fast 3D Clear preserves endogenous fluorescence in adult mouse brains, whole adult mice, and embryos (confocal)



(1) Thy1-GFP-M, characterized by high levels of GFP expression in sparse neuronal populations
(2) GCaMP3-CaMK2-Cre in which GCaMP3 calcium-sensitive fluorescent protein is expressed in CaMK2+ neurons
(3) tdTomato-VGAT-Cre, in which tdTomato is expressed in inhibitory neurons
(4) cFos-CreERT2-tdTomato, in which tamoxifen administration results in tdTomato labeling of neurons active during a behavioral task.

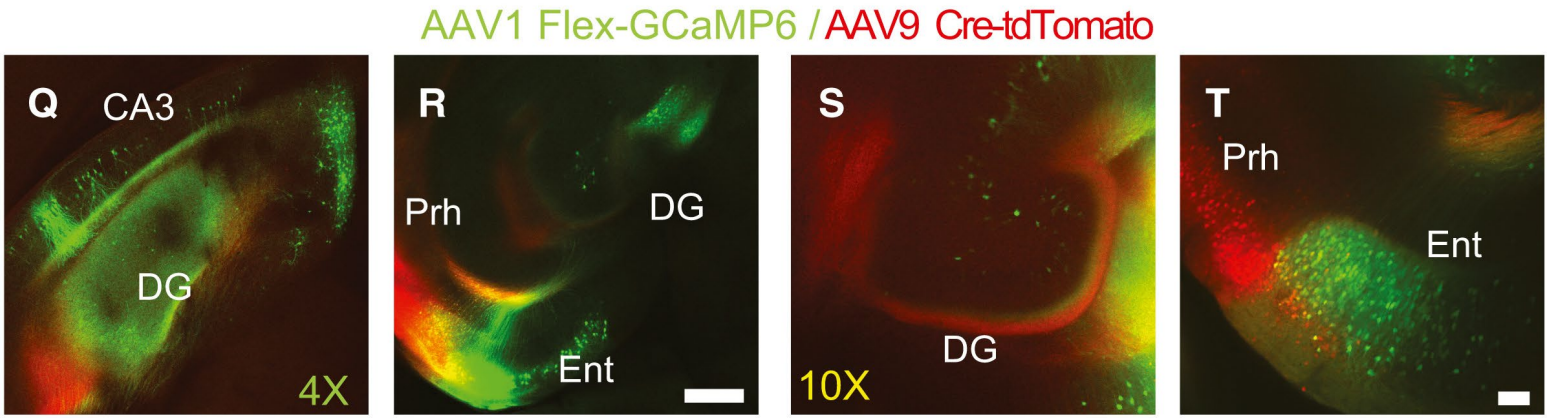
Fast 3D Clear preserves the fluorescence of synthetic and genetically encoded labels at multiple emission wavelengths (confocal)



Intracranial administration of:

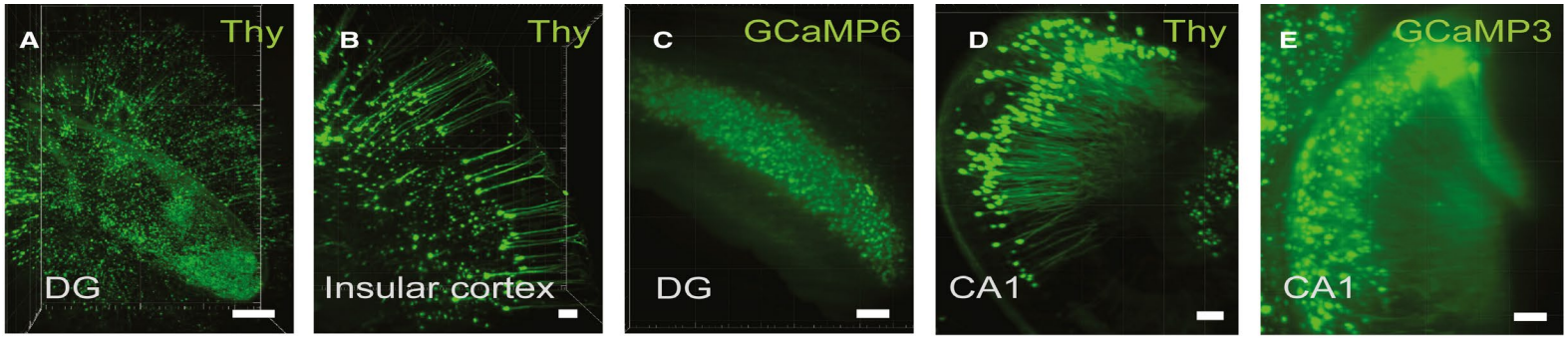
- retrograde neuronal tracer FastBlue (DG injection)
- AAV1 CaMK2-GCaMP6 (DG injection)
- Retro-AAV2 Arch-tdTomato (DG injection)
- Retro-AAV2 IRF670

Fast 3D Clear preserves the fluorescence of synthetic and genetically encoded labels at multiple emission wavelengths (confocal)

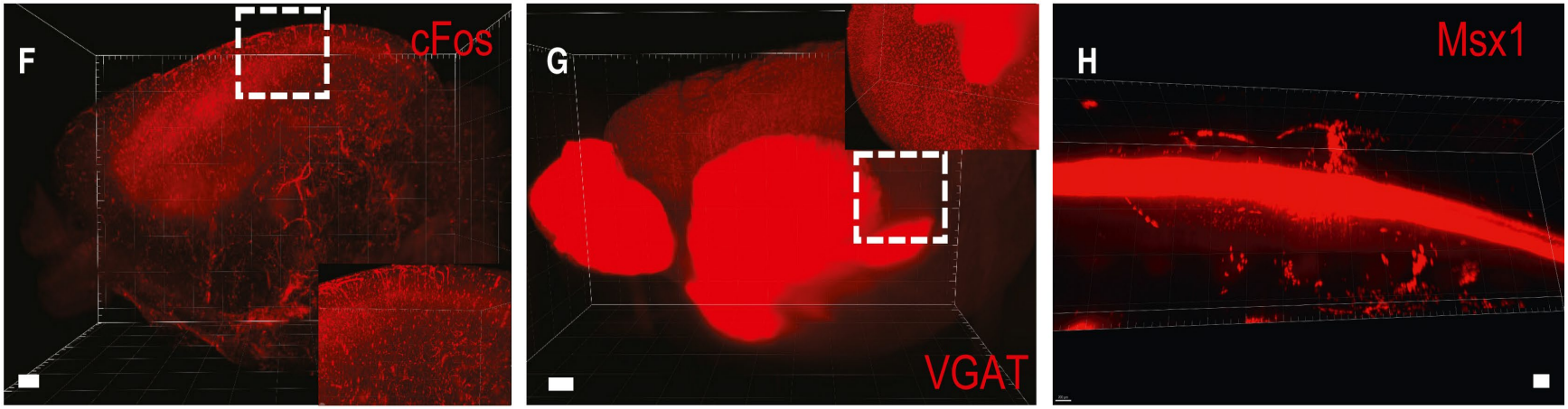


Injection of a Cre-dependent AAV1-GCaMP6 virus into the dorsal hippocampus and a tdTomato-Cre-virus into the ventral Ent/perirhinal (Prh) cortex to assess whether an anterograde transfer of AAV will activate the Cre-dependent expression of GCaMP6 in the dorsal hippocampus.

Fast 3D Clear is compatible with light-sheet (and confocal) **microscopy**

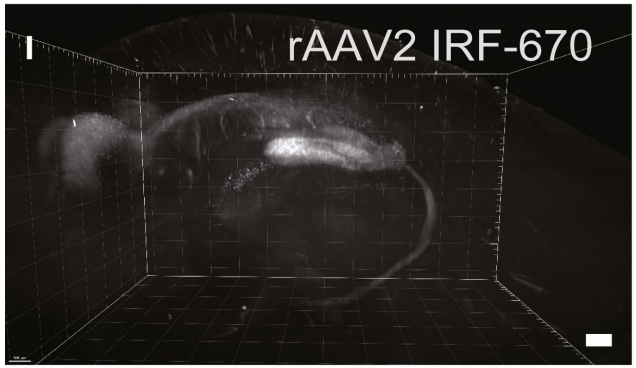


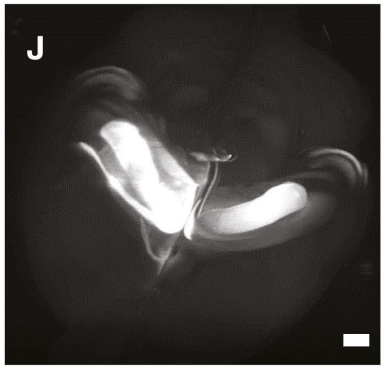
Fast 3D Clear is compatible with light-sheet (and confocal) **microscopy**

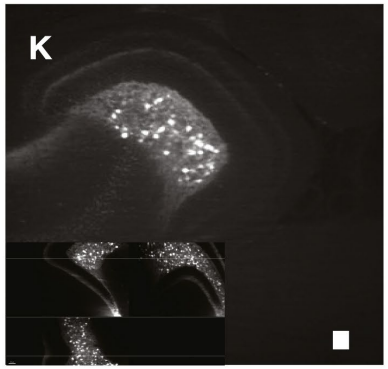


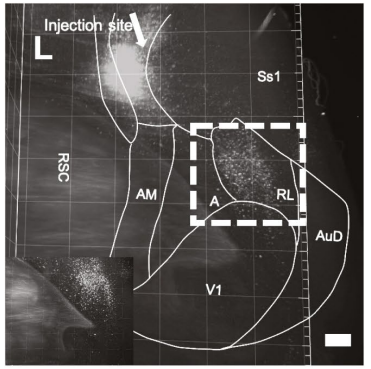
(F–H) 3D visualization of cleared (F) cFos-Cre^{ERT2}-tdTomato brain, (G) VGAT-Cre-tdTomato, and (H) spinal cord from Msx1-Cre^{ERT2}-Tdtomato E18.5 mouse embryos.

Fast 3D Clear is compatible with light-sheet (and confocal) **microscopy**





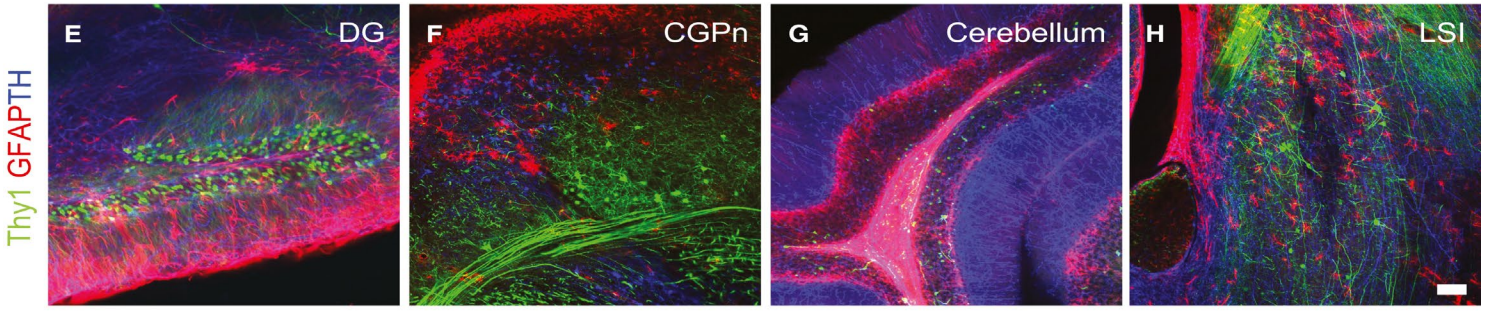




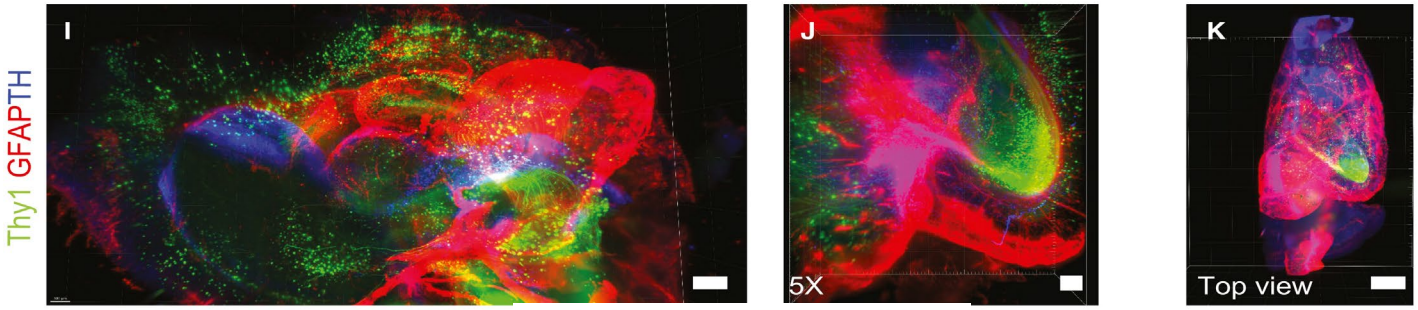
Fast 3D Clear is compatible with fluorescent antibody staining

Thy1-GFP-M mice

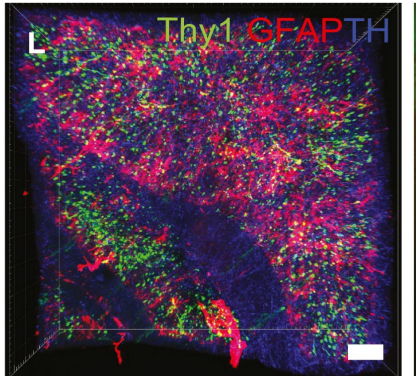
1. Reverse transparency (PBS washes 16 hours at 4°C)
2. iDISCO staining protocol
3. Clearing



Confocal



Light sheet



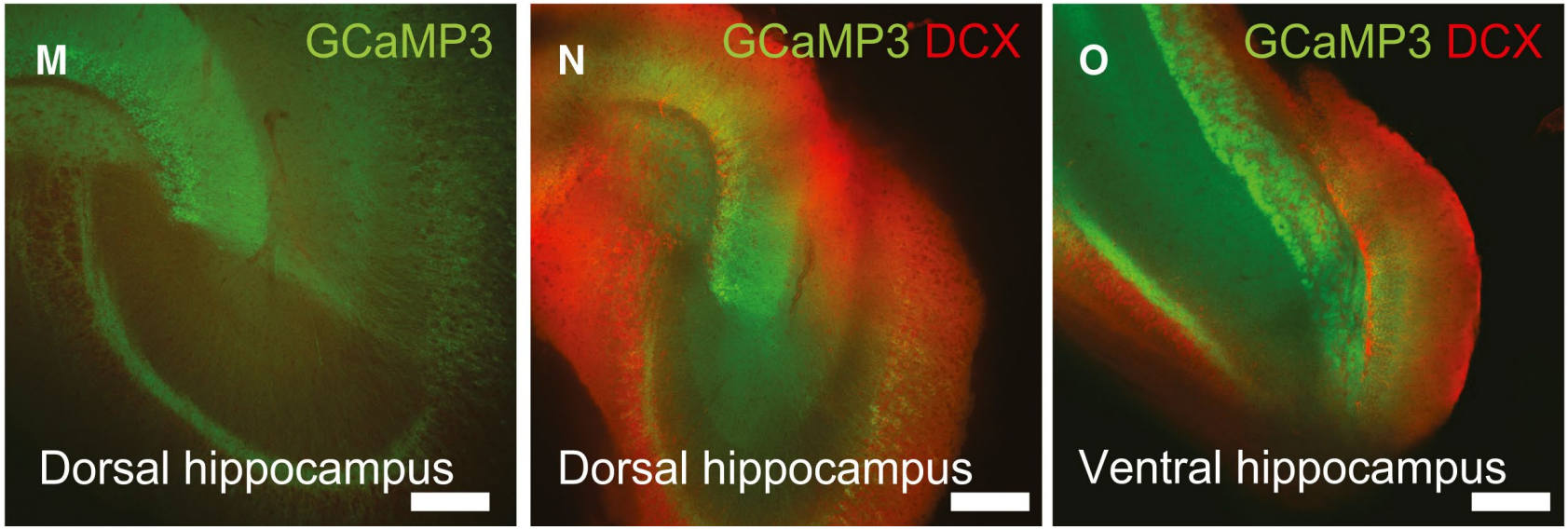
Confocal – 3D visualization cerebellum

Fast 3D Clear is compatible with fluorescent antibody staining

Fast 3D Clear is compatible with fluorescent antibody staining

GCaMP3-CaMK2 hippocampi

1. Reverse transparency (PBS washes 16 hours at 4°C)
2. iDISCO staining protocol
3. Clearing



(M–O) Whole adult mouse hippocampus from GCaMP3-CaMK2-Cre animals cleared with Fast 3D Clear. (M) GCaMP3 fluorescence before staining, (N) fluorescence at 500/30 and 650LP nm for GCaMP3 and DCX in dorsal hippocampus, and (O) ventral hippocampus merged channels. Scale bars, (I, K) 500 μm, (J and L) 200 μm, (E, F, G, H, M, N, O) 80 μm, (A, B, D) 20 μm, (C) 5 μm.

Conclusions

ADVANTAGES

- Speed
- Cost-effective
- Simple
- No tissue shrinkage
- Fluorescence preservation (endogenous and not)

LIMITATIONS

- Not been tested in other organisms other than mice
- Modest clearing of hard tissue (e.g bones)
- Antibody fluorescence stability not validated
- Compatibility with cell registration software not yet known

References

- **REVIEWS**
  - https://doi.org/10.1111/joa.13309
  - https://doi.org/10.1038/s43586-021-00080-9
  - https://doi.org/10.1038/s41583-019-0250-1
- **FACT -** Fast Free-of-Acrylamide Clearing Tissue (2017)
  - 10.1038/s41598-017-10204-5
- **FASTClear** (2017)
  - 10.1111/nan.12361
- **RTF** - Rapid clearing method based on Triethanolamine and Formamide (2018)
  - 10.1038/s41598-018-20306-3
- **Ce3D** - Clearing-Enhanced 3D (2019)
  - 10.1038/s41596-019-0156-4
- **FOCM** - ultraFast Optical Clearing Method (2019)
  - 10.1073/pnas.1819583116

Thank you!