application note

Preserving endogenous fluorescent protein signals for volumetric imaging using the X-CLARITY™ systems and reagents for tissue clearing

INTRODUCTION

Tissue clearing techniques have allowed biologists to acquire high-resolution volumetric images without the need to reduce samples to thin serial sections. One major limitation to some techniques is preserving the signal from endogenous fluorescent proteins (FPs). The required dehydration step of solvent-based tissue clearing methods removes water molecules from tissues, which are required to preserve FP signal. Although recent solvent-based techniques 3DISCO and iDISCO have attempted to address this issue, these methods still can only maintain FP emission for a few days. Although other well-known techniques such as CUBIC, Sca*l*e, and PACT preserve FP emission, they are a significant time investment and are limited to small tissue samples. The SWITCH technique utilizes strong fixatives and high temperatures, resulting in a loss of FP signal.

In principle, the CLARITY method is the most compatible for FP imaging, but having to construct one's own electrophoresis chamber with a makeshift cooling system to prevent overheating is a challenge to most.

The X-CLARITY™ Systems

X-CLARITY™ systems and reagents for tissue clearing

The X-CLARITY™ systems and reagents for tissue clearing are based on the CLARITY principle and have been developed to standardize, simplify, and accelerate each step of the tissue clearing process. One of the components of the X-CLARITY™ Tissue Clearing System is the electrophoretic tissue clearing (ETC) chamber with platinum-plated electrodes and built-in cooling system for efficient tissue clearing. A whole mouse brain takes just 6 hours to clear and endogenous FP signals are preserved. In this report, we present a protocol for sample processing for volumetric imaging using the X-CLARITY™ systems and reagents for tissue clearing with a focus on the preservation of FP signals.

Sample preparation

All animal experimental procedures were conducted in accordance with KBRI IACUC guidelines. Adult Thy1-YFP mice (20 weeks old) were anaesthetized with Avertin (250 mg/kg, Sigma) and perfused transcardially with 30 mL PBS, followed by 20 mL fresh 4% PFA (10 mL/min). Brains were extracted and incubated in 4% PFA for 24 hours at 4 °C. Brains were then washed and stored in PBS for 24 hours at 4 °C.

Technical Tips:

- Post-fixation is an important step in the X-CLARITY™ protocol. A whole mouse brain should be post-fixed for at least 24 hours. Excessive fixation can lead to increased clearing time.
- Post-fixed tissues should be washed for at least 24 hours as residual PFA can react with acrylamide during hydrogel polymerization and lead to increased clearing time.

X-CLARITY™ Hydrogel Solution Kit
C1310X

X-CLARITY™ Polymerization System
C20001

Electrophoretic Tissue Clearing Solution
C13001

X-CLARITY™ Tissue Clearing System
C10001

X-CLARITY™ Mounting Solution
C13101

Hydrogel infusion and polymerization

A hydrogel solution was prepared using the X-CLARITY™ Hydrogel Solution Kit (Logos Biosystems, C1310X). Brains were incubated in hydrogel solution (5 mL/brain) for 24 hours at 4°C. Brains in solution were placed in the X-CLARITY™ Polymerization System (Logos Biosystems, C20001) for 2 hours at 37°C and -90 kPa to initiate polymerization.

Technical Tips:

- The main difference of the original CLARITY and X-CLARITY™ chemistry is the use of *bis*-acrylamide and PFA in hydrogel solution. The use of *bis*-acrylamide and PFA leads to higher cross-liking density, but this also leads to increased clearing time.
- Since *bis*-acrylamide is not used in X-CLARITY™ chemistry, gelation is not observed after hydrogel polymerization. If the liquid hydrogel solution has become a sticky, more viscous solution, acrylamide polymerization has been successful.

Electrophoretic Tissue Clearing

The hydrogel-embedded brains were rinsed with Electrophoretic Tissue Clearing Solution (Logos Biosystems, C13001) and then placed in the ETC Chamber of the X-CLARITY™ Tissue Clearing System (Logos Biosystems, C10001). Electrophoretic Tissue Clearing Solution was circulated through the chamber and 0.7 A was applied across the brains for 6-12 hours at 35°C. After clearing, brains were washed with PBS overnight at room temperature to remove residual SDS.

Technical Tips:

- Endogenous FP signals can degrade at higher temperatures. To prevent excessive Joule heating, optimize the electric current applied during electrophoresis. Use lower currents to minimize high temperature increases. This may also affect clearing time.

Immunostaining

Clarified brains were cut into 1 mm slices or hemispheres for immunostaining. Brain samples were incubated in anti-Collagen IV (1:100, Abcam) in 6% BSA, 0.2% Triton X-100, 0.01% sodium azide in 0.1 M PBS for 24 hours at 37°C with gentle shaking. After 24 hours, samples were washed with PBST (0.2% Triton X-100, 0.01% sodium azide in 1X PBS) overnight at 37°C. Samples were then incubated with donkey anti-rabbit Cy3 Fab fragment (1:250, Jackson ImmunoResearch) and TO-PRO-3 Iodide (1:1000, ThermoFisher Scientific) in 6% BSA, 0.2% Triton X-100, 0.01% sodium azide in 0.1 M PBS for 24 hours at 37°C with gentle shaking. Samples were washed with PBST overnight at 37°C.

Refractive index matching and imaging

Stained samples were rinsed with distilled water for 5 minutes and immersed in X-CLARITY™ Mounting Solution (Logos Biosystems, C13101) for 1 hour at room temperature with gentle shaking. The solution was replaced and samples were incubated for another 1-2 hours.

For confocal imaging, brain slices were placed in a 35 mm glass-bottomed dish (SPL Life Sciences) with fresh mounting solution and imaged with a LSM 710 (Carl Zeiss) using the EC Plan-Neofluar 10x/0.3 objective. ZEN software (Carl Zeiss) was used to process the images. For light sheet imaging, brain hemispheres were placed in an imaging chamber with fresh mounting solution and imaged with a Lightsheet Z.1 (Carl Zeiss) using a EC Plan-Neofluar 5x/0.16 objective lens.

ZEN (Carl Zeiss) software was used to process confocal images. Amira 3D software (FEI) was used to process light sheet images and render a 3D video.

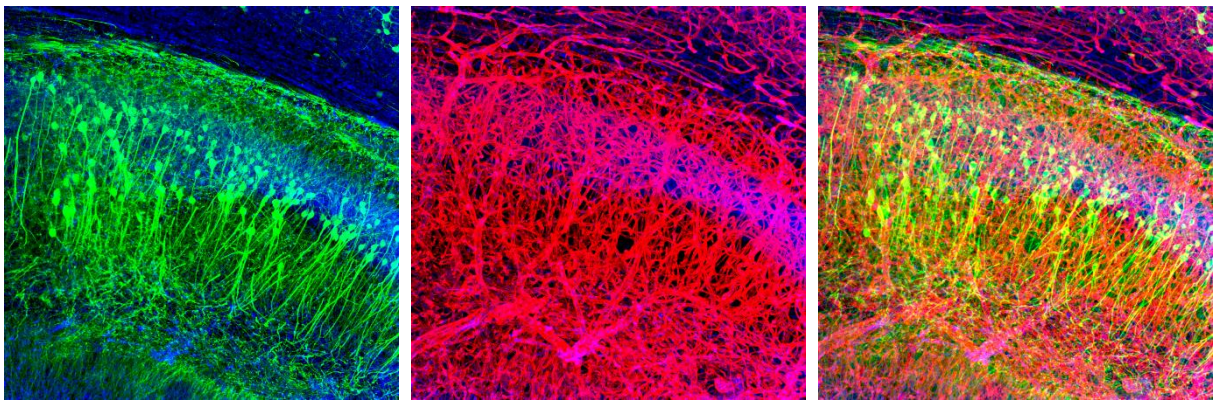
Technical Tips:

- Fluorescence imaging should be done immediately after RI matching to minimize the loss of fluorescence signal. Stained samples can be stored in the mounting solution for up to 3 days without significant signal loss, but for long term storage, place samples in PBS at 4°C.
- For more information, read "*The X-CLARITY™ Mounting Solution: an improved RI matching solution for tissues cleared by the X-CLARITY™ Tissue Clearing System*".

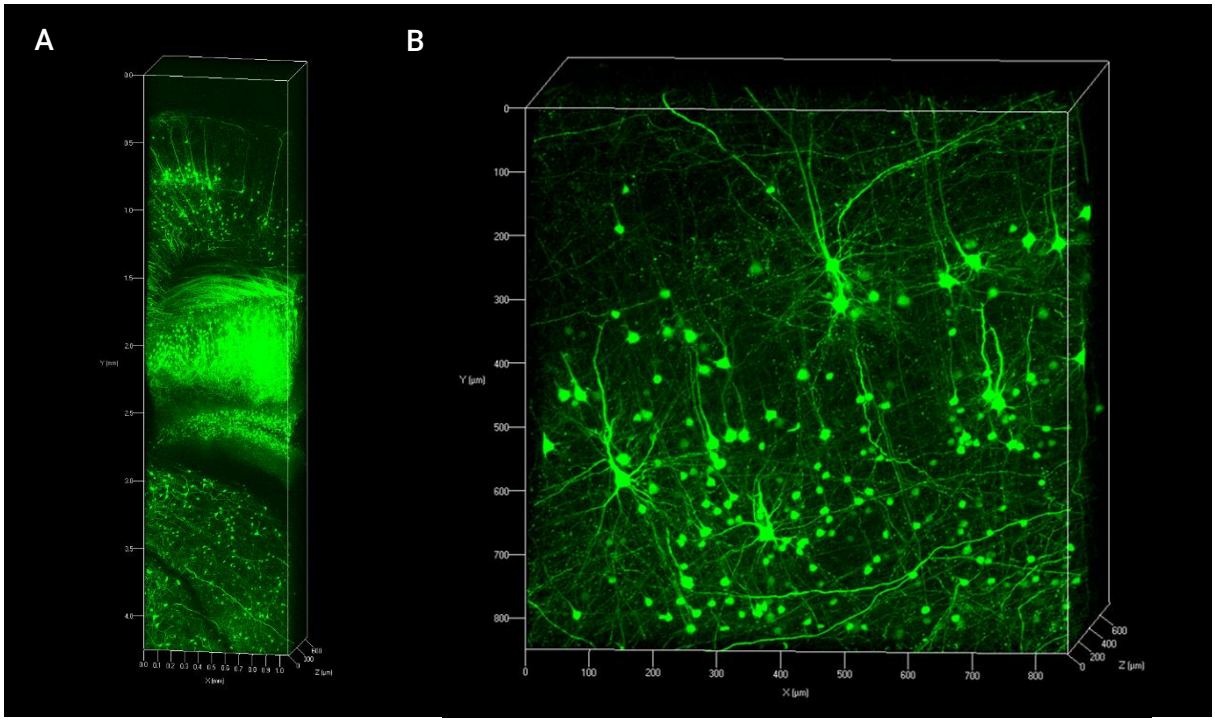
Results

Endogenous Thy1-YFP was stable and well-preserved upon clearing with the X-CLARITY™ systems and reagents. Anti-Collagen IV antibody and TO-PRO-3 Iodide penetrated the sample efficiently.

Light sheet images were rendered into a high resolution 3D video and can be viewed here:
A journey through a Thy1-YFP mouse brain.



Thy1-YFP mouse brain slices cleared with the X-CLARITY™ systems and reagents.
Thy1-YFP (green), Anti-Collagen IV (red), TO-PRO-3 (blue)



Long-term preservation of the Thy1-YFP signal after clearing with the X-CLARITY™ systems and reagents.
(A) Thy1-YFP signal immediately after clearing. (B) Thy1-YFP signal one month after clearing.

Conclusion

The procedure described here is a step-by-step protocol to obtain images of endogenous FP signals from transparent mouse brains. As demonstrated here, the Thy1-YFP signal is well-preserved after clearing with the X-CLARITY™ systems and reagents for tissue clearing. The signal was found to be stable even a month after the sample was cleared. The X-CLARITY™ Tissue Clearing System has an ETC chamber with platinum-plated electrodes that generate a dense and stable electric current, resulting in remarkably fast clearing times and consistent clearing. This combined with the integrated cooling system effectively preserves the fluorescence signal of endogenous FPs, allowing for volumetric imaging of large samples at single-cell resolution for improved data quality.

A whole adult mouse brain before (L) and after (R) clearing with the X-CLARITY™ systems and reagents

References

1) Lee, E. et al. ACT-PRESTO: Rapid and consistent tissue clearing and labeling method for 3 dimensional (3D) imaging. Scientific Reports 6, 18631 (2016).

2) [Application Note] An automated and high-throughput polymerization solution for downstream tissue clearing: the X-CLARITY Polymerization System, Logos Biosystems (2016).

3) Richardson, D. & Lichtman, J. 2015. Clarifying tissue clearing. Cell 162, 246-257 (2015).

4) Jun, Li et al. Fast immune-labeling by electrophoretically driven infiltration for intact tissue imaging. Scientific Reports 5, 10640 (2015).

5) Chung, K. & Deisseroth, K. CLARITY for mapping the nervous system. Nature Methods 10, 508–513 (2013).

Ordering information

Hydrogel Infusion & Polymerization		
C1310X	X-CLARITY™ Hydrogel Solution Kit X-CLARITY™ Hydrogel Solution X-CLARITY™ Polymerization Initiator	1 kit
C20001	X-CLARITY™ Polymerization System	1 unit
C20002	X-CLARITY™ Heat Block for 6 x 50 mL tubes	1 unit
C20003	X-CLARITY™ Heat Block for flat-bottom plates	1 unit

Tissue Clearing		
C10001	X-CLARITY™ Tissue Clearing System Starter Kit X-CLARITY™ ETC Chamber X-CLARITY™ ETC Controller X-CLARITY™ Pump X-CLARITY™ Reservoir Tissue Containers Container Holder for 1 Tissue Container Electrophoretic Tissue Clearing Solution	1 set
C12001	Tissue Containers	1 box
C12002	Container Holder for 1 Tissue Container	1 unit
C12004	Mouse Brain Slice Holder	1 unit
C12007	Whole Rat Brain Holder	1 unit
C12010	1.5 Φ Holder for 36 Mouse Brain Slices	1 set
C12011	1.5 Φ Holder for 6 Slices	1 set
C12012	1.5 Φ Holder for 1 Sample	1 set
C12013	1.5 Φ Holder for 6 Mouse Brains	1 set
C12014	1.5 Φ Holder for 48 Samples	1 set
C12015	1.5 Φ Holder for 192 Samples	1 set
C12020	0.6 Φ Holder for 36 Mouse Brain Slices	1 set
C12021	0.6 Φ Holder for 6 Slices	1 set
C12022	0.6 Φ Holder for 1 Sample	1 set
C12023	0.6 Φ Holder for 6 Mouse Brains	1 set
C12024	0.6 Φ Holder for 48 Samples	1 set
C12025	0.6 Φ Holder for 192 Samples	1 set
C13001	Electrophoretic Tissue Clearing Solution	12 x 1 L

Labeling and Imaging		
C13101	X-CLARITY™ Mounting Solution	1 x 25 mL
C13102	X-CLARITY™ Mounting Solution Value Pack	10 x 25 mL
C13107	X-CLARITY™ Mounting Solution Bulk Pack	20 x 25 mL

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