Confidential

# Clearing an adult mouse brain with the X-CLARITY™

The following is an optimized protocol of Logos Biosystems for clearing an adult mouse brain with the X-CLARITY™ Tissue Clearing System. We recommend that you read the available online references on the CLARITY method, the X-CLARITY™ Polymerization System user manual, and the X-CLARITY™ Tissue Clearing System user manual before starting this procedure.

⚠ *Read the MSDS for all reagents. Wear appropriate personal protective equipment. Work under a chemical fume hood when necessary.*

## Brain extraction, perfusion, and fixation

**Materials:** 4% PFA (cold), freshly prepared
1X PBS (cold)

1. Anesthetize the mouse ethically and responsibly.
2. Perfuse the mouse transcardially with at least 100 mL 1X PBS and 30 mL fresh 4% PFA.
3. Rapidly decapitate the mouse and remove the brain.
4. Incubate the brain in 15 mL of 4% PFA for 24 hours at 4°C (do not exceed 24 hours).
5. Rinse the brain with 1X PBS several times.
6. (optional) Cut into 1~2 mm slice section.
7. Incubate in 1X PBS for 24 hours at 4°C.

**Note:** *For a mouse brain, 15 mL of each solution volume is enough.*
**Note:** *The sample may be stored for a long time at step 7, but we recommend storing it up to a maximum of 3 months to preserve protein information from biological tissue.*
**Note:** *The quality of 4% PFA is very important. We recommend dissolving the powder (SIGMA Aldrich Cat# 158127) every time to ensure fresh quality. It should be well-dissolved and well-adjusted with the pH.*

## Hydrogel mixture incubation

**Materials:** 1X PBS (cold)
X-CLARITY™ Hydrogel Solution Kit (Cat# C1310X); the kit contains X-CLARITY™ Hydrogel Solution (Cat# 13103) and X-CLARITY™ Polymerization Initiator (Cat# 13104).

1. Dissolve 2.5 g of X-CLARITY™ Polymerization Initiator in 10 mL 1X PBS to make a 25% (w/v) stock solution. Aliquot (e.g. 0.5 mL each) and store at -20℃ for up to 6 months. Thaw at 4℃ or on ice before use.
2. Mix one part of the aliquoted X-CLARITY™ Polymerization Initiator to 100 parts of the X-CLARITY™ Hydrogel Solution. For example, to make ~50 mL of the hydrogel mixture, mix 0.5 mL of the aliquoted X-CLARITY™ Polymerization Initiator to the 50 mL of X-CLARITY™ Hydrogel Solution.
3. Incubate the sample in the hydrogel mixture at 4℃ for 24 hours. Please keep the sample in the hydrogel mixture on ice until transferring it to 4℃ refrigerator. The sample should be fully submerged in the hydrogel mixture. If you use a 96-well plate, we recommend using 0.3 mL per each well. In the case of a 384-well plate, you may use up to 0.1 mL per each well.

**Note:** *We don't recommend a long time incubation exceeding 48 hours.*
**Note:** *The volume of hydrogel may be enough if the sample is fully submerged. But we recommend preparing the 15 mL of hydrogel mixture at minimum.*

## Hydrogel polymerization

Read the X-CLARITY™ Polymerization System user manual carefully in its entirety prior to polymerizing the sample. Use the system as specified.

1. Run with the following settings:

| | Recommended |
|---|---|
| Vacuum (kPa) | -90 |
| Temperature (℃) | 37.0 |
| Timer (hh:mm) | 03:00 |
| Vessel type | Well plate / tube |

**Note:** *When using conical tubes, do not screw the cap onto the tube. Simply place the cap on the tube.*

2. After polymerization, gently shake the sample on a shaker for 1 minute. If you used a conical tube, invert the sample gently for 1 minute.
3. Rinse the sample with 1X PBS.

   **Note:** *The polymerized sample may be stored for long time at step 3 with 1x PBS at 4℃, but we recommend storing it up to 1 week to preserve protein information from biological tissue.*

## Electrophoretic tissue clearing

Read the X-CLARITY™ Tissue Clearing System user manual carefully in its entirety prior to clearing the tissues. Use the system as specified.

**Materials:** Electrophoretic Tissue Clearing Solution (Cat# C13001)
1X PBS

1. Run with the following settings for 6~8 hours:

| | Recommended |
|---|---|
| Current | 1.0 ~ 1.2 A |
| Temperature | 37°C |
| Pump speed | 30 rpm |

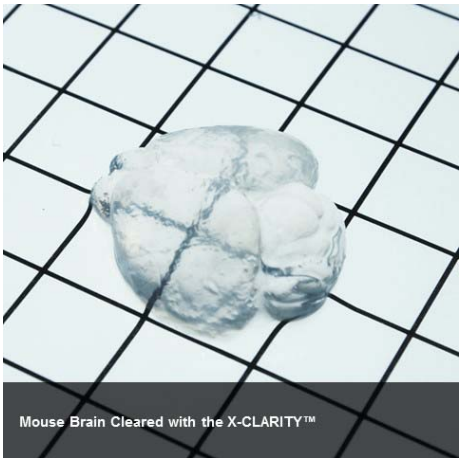
**Note:** *Check the sample every 2-3 hours.*
**Note:** *If environmental temperature is high or if you want to keep endogenous fluorescent proteins, lower the current to 0.8~1.0 A. This may increase clearing time. Alternatively, you can set the pump speed to maximum. This may reduce life time of the pump tube.*
**Note:** *It is normal for the temperature to exceed the set temperature. It depends on the resistance value of sample. If you don't want to make it at a high temperature, set the lower current.*

2. Wash the sample with 1X PBS overnight at room temperature to remove SDS.
   **Note:** *The sample will become opaque and white in PBS, and it is completely normal for the cleared tissue-gel matrix.*
   **Note:** *The brain may be stored in 1x PBS at 4℃ for up to 1 week until immunolabeling.*

## Antibody Labeling / Imaging

**Materials:** X-CLARITY™ Mounting Solution (Cat# C13101)
6% BSA in 1X PBS with 0.2% Triton X-100, 0.01% sodium azide
Primary antibody and secondary antibody
Cover glass bottom dish

1. Perform the antibody labeling by incubating the sample with the appropriate antibodies followed by proper washing steps.
   **Note:** *It is highly recommended to incubate the sample with the high concentrated antibody for longer period at 37℃ (at least 24 hours per each antibody).*
   **Note:** *We don't recommend using fluorescent direct-tagged antibody. It has too weak signal.*
   **Note:** *The positive control staining protocol with the anti-collagen IV antibody is available upon request.*
2. Rinse the sample with distilled water (5 minutes x 3 times) to remove phosphate. This can prevent precipitation after RI matching.
3. Incubate the sample in an appropriate amount of X-CLARITY™ Mounting Solution for 1 hour at room temperature. Replace with fresh X-CLARITY™ Mounting Solution and incubate for additional 1-2 hours. Mount to a cover glass bottom dish to image the sample.
   **Note:** *If you plan to image the sample later days after antibody labeling, store the sample in 1X PBS at 4°C until imaging. And before 1 day for imaging, replace the PBS with the X-CLARITY™ Mounting Solution. We don't recommend storing samples in X-CLARITY™ Mounting Solution. For optimal fluorescence imaging, take image within 1 week after mounting.*
   **Note:** *In some cases, the sample can become opaque in the mounting solution. The possible reasons are:*
   *1. Insufficient washing: Please wash the sample thoroughly to completely remove SDS and phosphate.*
   *2. Low temperature: The RI matching should be performed at room temperature or higher. Incubating the sample in the mounting solution at 37℃ for 30 minutes or 60℃ for 5 minutes could make the sample clear again.*
   *3. Too much evaporation: Evaporation could affect the RI of mounting solution, so please minimize evaporation during RI matching. Replacing the mounting solution with fresh one could make the sample clear again.*


## References

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