Confidential

Clearing a Mouse Bone with the X-CLARITY™

The following is an optimized protocol of Logos Biosystems for clearing a mouse bone 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.*

Mouse femur isolation, perfusion, fixation and decalcification

Materials: Mouse (12-week old ICR female mouse was used in this protocol.)
4% PFA (cold), freshly prepared
Formic acid (Duksan, cat. 724)
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, remove a hind limb, and isolate femur by dissection.
4. Incubate the femur in 10 mL 4% PFA for 24 hours at 4°C (do not exceed 24 hours).
5. Rinse the femur with 1X PBS for 24 hours at 4°C.
6. Incubate in 1X PBS for 24 hours at 4°C.
7. Incubate the femur in 100% formic acid for 6~10 hours at room temperature.
8. Rinse the femur with 1X PBS for overnight at 4°C.

Note: *For femur, 10 mL of each solution volume is enough.*
Note: *The sample may be stored for a long time at step 6, 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.*
Note: *You can use another chemical for decalcification instead of formic acid (step 7).*

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 8~10 hours:

	Recommended
Current	1.0 ~ 1.5 A
Temperature	37°C
Pump speed	30 rpm

Note: *Check the sample every 2 hours.*
Note: *If environmental temperature is high or if you want to keep endogenous fluorescent proteins, lower the current to 0.8~1.2 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.

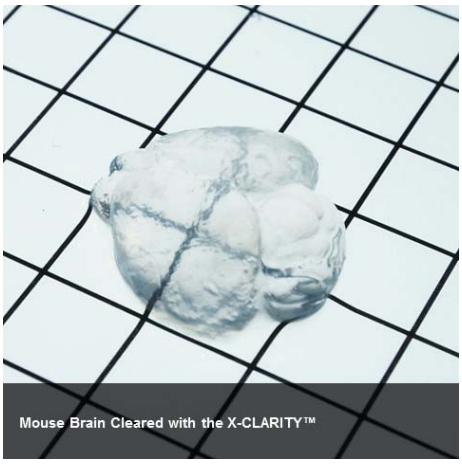
Note: *The sample may be stored in 1x PBS at 4℃ for up to 1 week until immunolabeling.*

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.*



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).