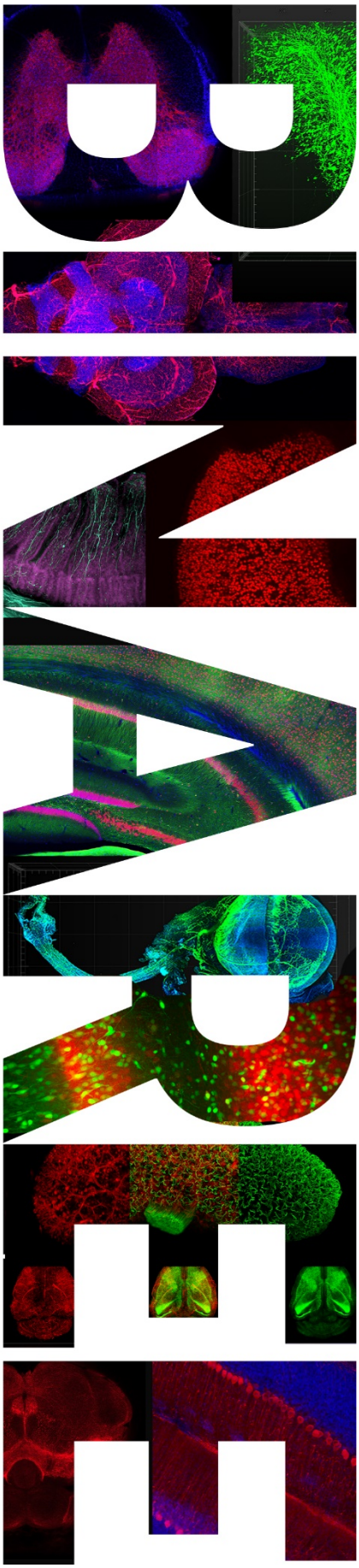

BINAREE

make visible

TISSUE CLEARING

PROTOCOL

HRTC-002

NAME OF PROTOCOL | Binaree Tissue Clearing™ kit (High Resolution) | Cat. No. HRTC-002

CODE OF PROTOCOL: C1001
REVISION OF PROTOCOL: 1.1.8 (2022.08.17)

1. Preparation l Planning your test

(OPTION)

FIXING → CLEARING → STAINING → MOUNTING → IMAGING & STORE

When we designed the protocol, it was considered that clearing effectiveness and working time are the most important factors in the protocol process.

Enjoy the tissue clearing!

2. Preparation l Taking the solutions

2-1. All the solutions should be stored at 4 °C.

2-2. Check Tissue Clearing Solution A & B and Mounting & Storage Solution for crystallization or precipitation before use.

2-3. Contents of the kits

	Binaree Tissue Clearing kit (HRTC-002)	Mounting & Storage Solution (SHMS-060)
① Starting Solution	30 ml	
② Tissue Clearing Solution A	30 ml	
③ Tissue Clearing Solution B	60 ml	
+ Mounting & Storage Solution		60 ml

- The Mounting & Storage Solution (Cat. No. SHMS-060) is not included in Binaree Tissue Clearing Kit (HRTC-002).
- The solutions may become crystallized or precipitated. If this occurs, incubate it at 37 °C for 1-2 h before use.

3. Preparation l Fixing the sample

3-1. The mouse is transcardial perfused with 4% PFA.

3-2. Incubate the sample with 4% PFA at 4 °C for overnight.

3-3. Wash the sample with 1X PBS while shaking at 4 °C for 20 min × 3 times.

Note. Incubate the ②③ Tissue Clearing Solution A & B and + Mounting & Storage Solution at 37 °C for 1-2 h before use.

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4. Protocol l Clearing the fixed sample

Please refer to APPENDIX and adjust the clearing step according to the tissue thickness.

4-1. Incubate the sample with ① **10 ml Starting Solution** at 4 °C until the sample sinks.

4-2. Transfer the sample to ② **10 ml Tissue Clearing Solution A** and agitate at 50 rpm /37 °C for 48 h.

4-3. Wash the sample with distilled water while shaking at 50 rpm /4 °C for 30 min × 4 times.

The sample may become opaque and swell. This does not affect the clearing process; the sample will be cleared again in Mounting & Storage Solution.

4-4. Transfer the sample to ③ **10 ml Tissue Clearing Solution B** and agitate at 50 rpm /37 °C for 48 h.

If the tissue does not enough clear in step 4, washing (4-3) & tissue clearing (4-4) should be repeated until cleared.

4-5. Wash the sample with distilled water while shaking at 50 rpm /4 °C for 30 min × 4 times.

The sample may become opaque and swell. This does not affect the clearing process; the sample will be cleared again in Mounting & Storage Solution.

4-6. (optional) Add nuclear stain solution (e.g. DAPI, 20-40 μg/ml in distilled water) while shaking at 4 °C for overnight.

Wash the sample with distilled water while shaking at 50 rpm /4 °C for 30 min × 3 times.

4-7. Transfer the sample to + **20 ml Mounting & Storage Solution** and agitate at 50 rpm /37 °C for 12-24 h.

5. Clearing Tips

5-1. If the sample contains air bubbles → Centrifuge the sample at 3,000 rpm / 24 °C for 1 min.

5-2. If the sample is not entirely cleared → Repeat from step 4-3 to step 4-4.

5-3. If the rpm is not specified → Operate the shaking incubator gently.

5-4. In steps 4-3 and 4-5, the sample may be washed with 0.1X PBS instead of distilled water.

5-5. It is recommended to use the vial for tissue clearing rather than the chamber slide.

Drying causes crystallization of Tissue Clearing Solution A & B and Mounting & Storage Solution.

6. Storage & Imaging Tips

6-1. After imaging, the samples are stored at room temperature in the Mounting & Storage Solution. Never refrigerate.

6-2. Take images within 7 days after the clearing for the best results.

6-3. Take images on the microscope. We recommend using a Light Sheet Fluorescence Microscope (LSFM) or Confocal Laser Scanning Microscope (CLSM). Analyze and visualize the images with a microscopy image analysis software.

6-4. Mounting & Storage Solution is a solvent-free material that is safe to use in the Light Sheet Fluorescence Microscope (LSFM).

6-5. Refractive Index (RI) of the Mounting & Storage Solution is **1.45.**

6-6. Be careful of making bubbles while filling the microscope chamber with the sample and the Mounting & Storage Solution. The bubbles may disturb the imaging.

6-7. To take images of tissue with less than 1 mm thickness via confocal microscope, use a slide chamber (2 wells or 4 wells) like the image below. Sealing the chamber with label tape reduces drying. Too much of the Mounting & Storage Solution can cause the sample in the chamber to shake. The optimal volume for 1 mm-thick tissue is 200 µl.

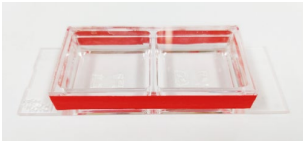

Figure 1. When taking images through confocal microscopy, the image chamber must be seal by label tape.

7. Appendix

Read not only the appendix but also the protocol. The protocol describes the method in detail.

7-1. Mouse ≤ 8-weeks-old

step	Summary	Temp. (°C)	1 mm thickness	3 mm thickness	≤ 7 mm thickness (ex. half brain, spleen, spinal cord)	≥ 7 mm thickness (ex. whole brain, lung, kidney, heart)	Spheroid Organoid
3-2	4% PFA	4 °C	12 - 24 h	12 - 24 h	12 - 24 h	12 - 24 h	15 min - 1 h
3-3	Wash with 1X PBS	4 °C	20 min × 3 times	20 min × 3 times	20 min × 3 times	20 min × 3 times	20 min × 3 times
4-1	Starting Solution*	4 °C	2 - 12 h	12 - 24 h	24 h	24 - 48 h	2 - 12 h
4-2	Tissue Clearing Solution A	37 °C	4 - 6 h	24 - 48 h	48 h	48 - 72 h	
4-3	Wash with distilled water	4 °C	10 min × 3 times	30 min × 4 times	30 min × 4 times	30 min × 4 times	
4-4	Tissue Clearing Solution B	37 °C		24 - 48 h	24 - 48 h	48 - 72h	4 - 24 h
4-5	Wash with distilled water	4 °C		30 min × 4 times	30 min × 4 times	30 min ×4 times	10 min ×4 times
4-6	(option) DAPI staining	4 °C	1 h	6 - 12 h	12 - 24 h	12 - 24 h	1 h
4-7	Mounting & Storage Solution	37 °C	< 1 day	> 1 day	> 1 day	> 2 days	> 6 h

Note. *Samples were incubated in Starting Solution until the sample sanks. However, even if the sample (ex. lung, spinal cord, spheroid, etc.) does not sink in the Starting Solution after 2 days, proceed to the next steps.

Note. If the tissue does not enough clear in step 4-4, washing (step 4-3) & tissue clearing (step 4-4) should be repeated until cleared.

Note. When using tissue from mice older than 8 weeks, repeat the tissue clearing (step 4-4) & washing (step 4-5) until the tissue is clear.

Note. Spheroid and organoid are not incubated with Tissue Clearing Solution A.

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8. Protocol l (Option) Immunostaining for 1 mm-thick tissue

8-1. Tissue Preparation

8-1-1. Perfuse mouse with 4% PFA using transcardial perfusion.
8-1-2. Post-fix sample with 4% PFA overnight (12-16 h) at 4 ℃. Do not over-fix the sample.
8-1-3. Wash the sample with 1X PBS while shaking at 4 ℃ for 20 min × 3 times.
8-1-4. Cut tissues into 1 mm-thick sections using Mouse Brain Slicer.

8-2. Tissue Clearing (1 mm-thick tissue)

8-2-1. Incubate the sample with 6 ml Starting Solution while shaking at 4 ℃ until the sample sinks (at least 24 h or more).
8-2-2. Incubate the sample with 3 ml Tissue Clearing Solution A in a shaking incubator at 37 ℃ for 4-6 h.
8-2-3. Wash the sample with distilled water while shaking at 4 ℃ for 30 min × 4 times.
Note. Tissue may become opaque and swell at step 8-2-3.
If the tissue does not enough clear in step 8-2-2, washing (8-2-3) & tissue clearing solution B should be used to incubate until cleared (at least 12 h or more).

8-3. Permeabilization and Blocking

You can perform standard immunostaining procedures.
8-3-1. Incubate the sample with permeabilization solution in a shaking incubator at 37 °C for 2-3 days.
Note. You can perform standard immunostaining procedures. Permeabilization is a key step for successful antibody labeling. Therefore, the permeabilization condition should be optimized depending on the tissue and antibodies.
Note. Sample may become opaque at permeabilization step. Permeabilization solution (recommendation):
1X PBS or TBS containing 0.2-0.5% Triton X-100, 10-20% DMSO, Serum.

8-4. Antibody labeling

8-4-1. Incubate the sample with primary antibody in a shaking incubator at 37 ℃ for 3-6 days.
8-4-2. Wash the sample with 1X PBS or TBS while shaking at 4 ℃ for 20 min × 6 times.
8-4-3. Incubate the sample with secondary antibody in a shaking incubator at 37 ℃ for 3-6 days.
8-4-4. Wash the sample with 1X PBS or TBS while shaking at 4 ℃ for 20 min × 6 times.
8-4-5. (optional) Add nuclear stain solution (e.g. DAPI, 20-40 μg/ml in distilled water) while shaking at 4 ℃ for 1 h.
8-4-6. Wash the sample with 1X PBS or TBS while shaking at 4 ℃ for 10 min × 3 times.
8-4-7. Incubate the sample with 5 ml Mounting & Storage Solution in a shaking incubator at 37 ℃ for at least 12 h or more.
8-4-8. Image on a Confocal Laser Scanning Microscope.

Note. The specific depth of penetration for each antibody will be dependent upon the tissue type, the dilution, as well as the incubation time.

Note. Successful immunostaining is strongly dependent on the primary and secondary antibodies. The followings are guidelines for successful immunostaining.

a. Antibody concentration should be optimized: Primary antibody dilution of 1 : 50 to 1 : 200 and secondary antibody dilution of 1 : 100 to 1 : 500 are required.

b. Usually, a 1 : 100 dilution of the secondary antibody works well.

c. Antibody incubation time: for 1 mm-thick tissue, 3-6 days are required.

d. Antibody dilution buffer (recommendation): 1 X PBS or TBS containing 0.2-0.5% Tween 20, 5% DMSO and 5% serum.

e. In order to reduce the total immunostaining time, fluorescence conjugated 1st antibody can be used.

f. In order to reduce the incubation time for the secondary antibody, you can also use an immunoglobulin fragment [e.g. Fab and $F(ab')_2$].

g. Before proceeding to a thicker tissue, immunostaining should be optimized starting with a thinner tissue.

h. To take images of tissue with less than 1 mm thickness via confocal microscope, use a slide chamber (2 wells or 4 wells) like the image below. Sealing the chamber with label tape reduces drying. Too much of the Mounting & Storage Solution can cause the sample in the chamber to shake. The optimal volume for 1 mm-thick tissue is 200 µl.

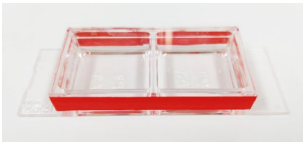

Figure 2. When taking images through confocal microscopy, the image chamber must be seal by label tape.

i. During imaging, the temperature should be above 25 ℃. At low temperatures, crystallization or precipitation can form.

9. Contact Us | Technical support

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