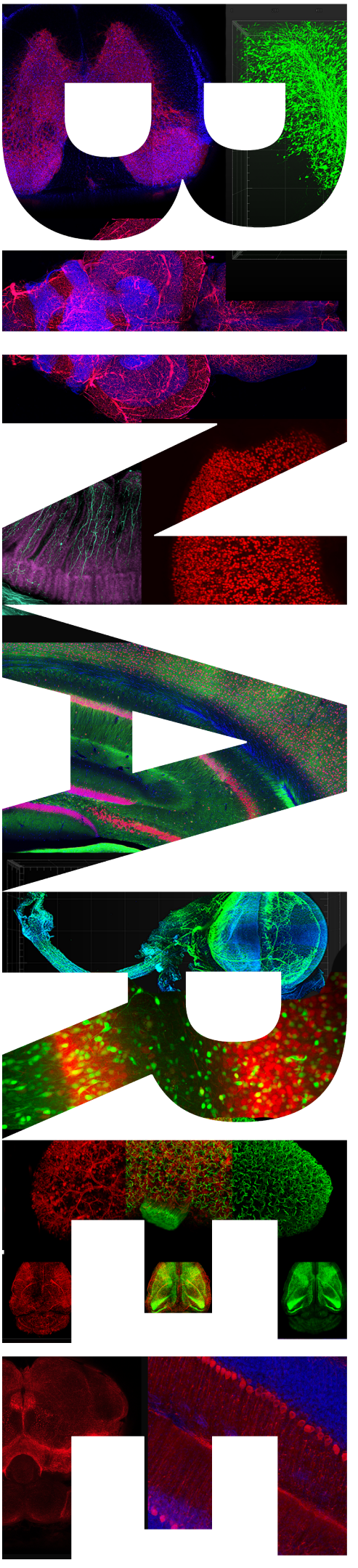
BINAREE

make **visible**

IMMUNOSTAINING

PROTOCOL



NAME OF PROTOCOL **Binaree Tissue Clearing™ Kit for Immunostaining** (Cat.No. HRTI-001)

[A] - Notes Before Starting

A-1. Check Tissue Clearing Solution A & B and Mounting & Storage Solution for crystallization or precipitation before each use. Redissolve any precipitation by warming the solution at 37℃ for 1-2 h and then use.

A-2. 1 mm - thick sections will provide the best results.

A-3. During imaging, the temperature should be above 25℃. At low temperatures, crystallization or precipitation can form.

A-4. After imaging, the samples are stored at room temperature in the Mounting & Storage Solution. Never refrigerate.

[B] - Tissue Preparation

B-1. Perfuse mouse with 4% PFA using transcardial perfusion.

B-2. Post-fix sample with 4% PFA overnight (12-16 h) at 4℃. Do not over-fix the sample.

B-3. Wash the sample with 1 x PBS while shaking at 4℃ for 20 min X 3 times.

B-4. Cut tissues into 1 mm - thick sections using Mouse Brain Slicer.

[C] - Tissue Clearing (1mm-thick tissue)

C-1. Incubate the sample with 6 ㎖ Starting Solution while shaking at 4℃ until the sample sinks (at least 24 h or more).

C-2. Incubate the sample with 3 ㎖ Tissue Clearing Solution A in a shaking incubator at 42℃ for 4 days.

C-3. Wash the sample with distilled water while shaking at 4℃ for 1 h X 4 times.

NOTE: Tissue may become opaque and swell at step 3.

C-4. Incubate the sample with 3 ㎖ Tissue Clearing Solution B in a shaking incubator at 42℃ for 2 days.

[D] - Permeabilization and Blocking

You can perform standard immunostaining procedures.

D-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): 0.1 x PBS or TBS containing 0.2 - 0.5% Triton X-100, 10 - 20% DMSO, Serum.

[E] - Antibody labeling

E-1. Incubate the sample with primary antibody in a shaking incubator at 37℃ for 3-6 days.

E-2. Wash the sample with 0.1 x PBS or TBS while shaking at 4℃ for 20 min X 6 times.

E-3. Incubate the sample with secondary antibody in a shaking incubator at 37℃ for 3-6 days.

E-4. Wash the sample with 0.1 x PBS or TBS while shaking at 4℃ for 20 min X 6 times.

E-5. (optional) Add nuclear stain solution (e.g. DAPI, 20-40 μg/㎖) while shaking at 4℃ for 1 h.

E-6. Wash the sample with 0.1 x PBS or TBS while shaking at 4℃ for 10 min X 3 times.

E-7. Incubate the sample with 5 ㎖ Mounting & Storage Solution in a shaking incubator at 37℃ for at least 12 h or more.

E-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: a 1st antibody dilution of 1 : 50 to 1 : 200 and a 2nd antibody dilution of 1 : 100 to 1 : 500 are required.

b. Usually, a 1 : 100 dilution of the 2nd antibody works well.

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

d. Antibody dilution buffer (recommendation): 0.1 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 2nd 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.

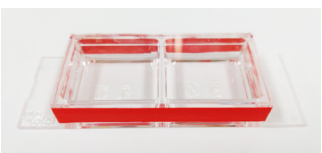


Figure 1. When taking images through confocal microscopy, the image chamber must be seal by label tape.

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

[F] - Preparation | Taking the solutions

F-1. All of the solutions should be stored at 4℃.

F-2. Crystallization or precipitation can occur while solutions are stored at 4℃; This does not affect the clearing process. It will become liquid again after incubating the solutions at 37℃.

F-3. Do not use the individual solutions from the other kit. Even if the names of solutions are the same, the component compositions are not the same. Each solution has a unique component composition depending on the purpose of the kit.

F-4. The Mounting & Storage Solution (Cat.No. SHMS-060) is not included in the kit (Cat.No.HRTI-001).

F-5. The solutions may become crystallized or precipitated. If this occurs, incubate it at 37℃ for 1-2 h before use.

Item	1 mm x 4 pieces (Reagent volumes required for immunostaining)
Starting Solution	6 ㎖
Tissue Clearing Solution A	3 ㎖
Tissue Clearing Solution B	3 ㎖

[G] - Protocol summary

Fix sample with 4% PFA ▶ Starting Solution ▶ Tissue Clearing Solution A ▶ Tissue Clearing Solution B ▶ Permeabilization and Blocking ▶ Stain with 1st antibody ▶ Stain with 2nd antibody ▶ Mounting & Storage Solution

The following protocol describes our protocol for clearing and immunolabeling for specific antibodies.
Refer to the Table for incubation times. Penetration/ Permeabilization and antibody incubation are performed at 37℃.

G-1. GFAP (DAKO, Z0334) - 1st antibody & AffiniPure Fab Fragment Goat Anti-Rabbit IgG (H+L) - 2nd antibody.

	Clearing		Permeabilization	Staining		Mounting
Thickness	Tissue Clearing Solution A	Tissue Clearing Solution B	Penetration/ Permeabilization (0.2% Triton X-100, 20% DMSO in 0.1 x PBS)	1st antibody incubation	2nd antibody incubation	Mounting & Storage Solution
500 ㎛	3 days	1 day	1 day	3 days	3 days	1 day
1 mm	4 days	2 days	4 days	6 days	4 days	1 day
				Antibody diluted buffer		
				0.2% Tween 20, 5% DMSO in 0.1 x PBS		

G-2. Recombinant Anti-MAP2 antibody [EPR19691] (Alexa Fluor®488 or 647) (abcam, ab225316 or ab225315)

	Clearing		Permeabilization	Staining		Mounting
Thickness	Tissue Clearing Solution A	Tissue Clearing Solution B	Penetration/ Permeabilization (0.2% Triton X-100, 20% DMSO in 0.1 x PBS)	1st antibody incubation	2nd antibody incubation	Mounting & Storage Solution
1 mm	4 days	2 days	NONE	6 days	NONE	1 day
				Antibody diluted buffer		
				1% Triton X-100, 5% DMSO in 0.1 x PBS		

[G] - Protocol summary

G-3. Tyrosine Hydroxylase (Abcam, ab112) - 1st antibody & AffiniPure F(ab')₂ Fragment Goat Anti-Rabbit IgG (H+L) - 2nd antibody

	Clearing		Permeabilization	Staining		Mounting
Thickness	Tissue Clearing Solution A	Tissue Clearing Solution B	Penetration/ Permeabilization (0.2% Triton X-100, 20% DMSO in 0.1 x PBS)	1st antibody incubation	2nd antibody incubation	Mounting & Storage Solution
1 mm	4 days	2 days	2 days	5 days	5 days	1 day
				Antibody diluted buffer		
				1% Triton X-100, 5% DMSO in 0.1 x PBS		

G-4. Recombinant Anti-NeuN antibody (Abcam, ab177487) - 1st antibody & AffiniPure F(ab')₂ Fragment Goat Anti-Rabbit IgG (H+L) - 2nd antibody

	Clearing		Permeabilization	Staining		Mounting
Thickness	Tissue Clearing Solution A	Tissue Clearing Solution B	Penetration/ Permeabilization (0.2% Triton X-100, 20% DMSO in 0.1 x PBS)	1st antibody incubation	2nd antibody incubation	Mounting & Storage Solution
500 ㎛	3 days	1 day	1 day	3 days	3 days	3 days
1 mm	4 days	2 days	2 days	5 days	5 days	1 day
				Antibody diluted buffer		
				0.2% Tween 20, 5% DMSO in 0.1 x PBS		

G-5. AQP2 (Santa cruz, sc-515770) - 1st antibody & AffiniPure F(ab')₂ Fragment Goat Anti-mouse IgG (H+L) - 2nd antibody

	Clearing		Permeabilization	Staining		Mounting
Thickness	Tissue Clearing Solution A	Tissue Clearing Solution B	Penetration/ Permeabilization (0.2% Triton X-100, 20% DMSO in 0.1 x PBS)	1st antibody incubation	2nd antibody incubation	Mounting & Storage Solution
1 mm	4 days	2 days	2 days	5 days	5 days	1 day
Whole	4 days	2 days	2-4 days	7 days	5 days	2 days
				Antibody diluted buffer		
				0.2% Tween 20, 5% DMSO in 0.1 x PBS		

[H] - Contact Us | Technical support

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