BINAREE

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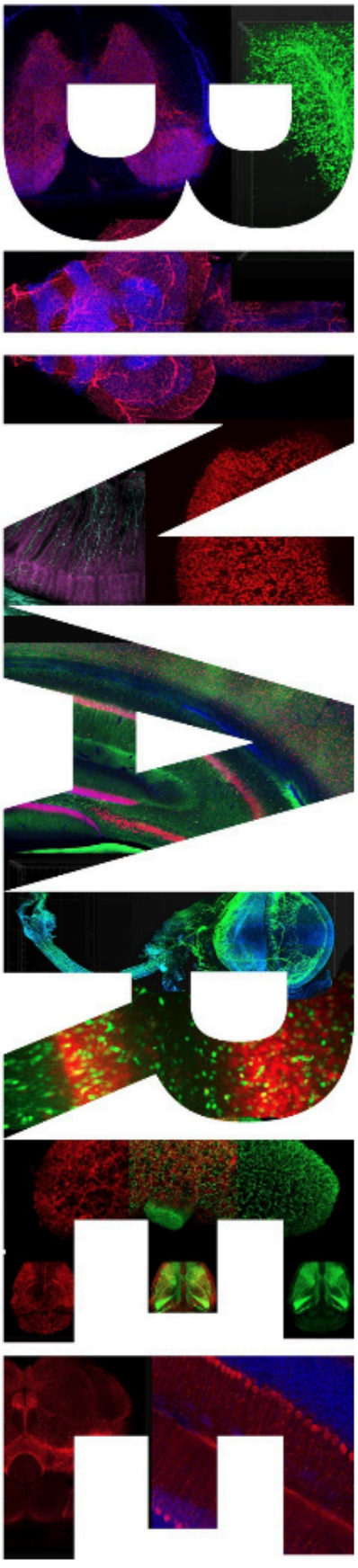
TISSUE CLEARING

PROTOCOL

BRTC-XXX



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Contact us

For technical support with this protocol or other Binaree® products, please contact your distributor. You may also contact Binaree directly:

Email: sales@binaree.com
Tel: +82-(0)53-291-5012
Fax: +82-(0)53-382-5012

Binaree, Inc.
5th floor, 12-2, Gonghang-ro, Buk-gu, Daegu, 41526, Republic of Korea

Solution storage and use

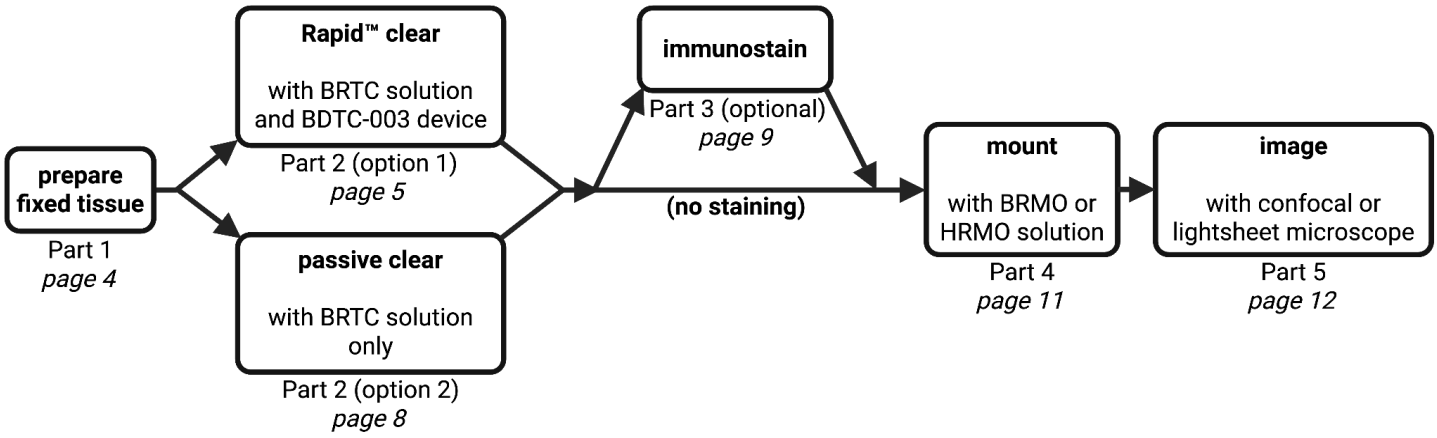
Solution	Shipping	Storage	Preparation
Binaree® Tissue Clearing Rapid™ (BRTC)	RT (24 ºC)	-20 °C	Pre-warm to 37 °C about 1-2 hours prior to use and check for crystallization or precipitation.
Binaree® Rapid™ Mounting (BRMO)	RT (24 ºC)	RT (24 ºC)	No preparation needed before use.
Binaree® Tissue Clearing™ Mounting (HRMO)	RT (24 ºC)	-20 °C	Pre-warm to 37 °C about 1-2 hours prior to use and check for crystallization or precipitation.

Overview

This protocol covers Binaree® Rapid™ and passive clearing of various tissue types and sizes with Binaree® Tissue Clearing Rapid™ (BRTC) solution, and mounting with Binaree® Rapid™ Mounting (BRMO) or Binaree® Tissue Clearing™ Mounting (HRMO) solution. Do not use Binaree® Tissue Clearing™ HRTC solution with this protocol, as it has a unique component composition not intended for Binaree® Rapid™ use.

There are two ways to clear tissue using the BRTC clearing solution: the Rapid™ method is much faster and involves the Binaree® Rapid™ System, but may not be suitable for smaller samples like spheroids or organoids. On the other hand, the passive method takes longer but is simpler and higher throughput, as it involves only incubating samples in the BRTC solution.

This protocol also includes recommendations on fixing, immunostaining, and imaging samples. The general composition of the protocol, from fixing to imaging, is shown here:



Immunostaining is optional but, if included, must be done between clearing and mounting. Mounting can be achieved with either BRMO or HRMO mounting solutions, which can be determined based on the refractive index (RI) needed, either 1.52 or 1.46. Confocal imaging is recommended for smaller or thinner tissue and when high resolution is needed at the cost of imaging depth, while light sheet microscopy is better for larger tissue or when looking deeper into tissue at the expense of image resolution.

Part 1: Tissue fixing and preparation

1.1. For best results during clearing and imaging, use tissue that has been collected recently, and ensure that it is clear of blood by transcardially perfusing with PBS thoroughly before the fixation step. Alternatively, tissue can be washed with heparin after perfusion to ensure removal of blood.

1.2. Following transcardial perfusion with 4% paraformaldehyde (PFA), formalin, or other fixatives, collect the sample and incubate in 4% PFA at 4 °C for 12 to 24 hours. Spheroids or organoids can be incubated for 15 minutes to 1 hour.
Note: for rat or mouse brain, add 10% sucrose in the 4% PFA solution to increase tissue strength.

1.3. Wash the sample 3 times with 1X PBS while agitating at 4 °C for 20 minutes per wash.

1.4. Incubate the sample with 10 mL 30% (w/v) sucrose in 1X PBS at 4 °C until it sinks. See recommended incubation times for different sample types and sizes below.
Note: if the sample does not sink after 2 days, proceed to clearing anyway.

Sample type	**Incubation time**
≥ 7 mm thick tissue: whole brain, lung, kidney, heart, etc.	24 to 48 hours
≤ 7 mm thick tissue: half brain, spleen, spinal cord, etc.	24 hours
3 mm thick mouse brain section or equivalent volume tissue	12 to 24 hours
1 mm thick mouse brain section or equivalent volume tissue	2 to 12 hours
Spheroids or organoids	2 to 12 hours

Pause point

If you need to pause the process here, leave samples at 4 °C in the 30% sucrose solution for no more than 1 month to avoid mold growth.

Part 2 (option 1): Rapid™ clearing

Rapid™ clearing is not recommended for spheroids or organoids. Instead, clear these with the passive clearing method described in Part 2 (option 2) on page 8.

2.1.1. Pre-warm Binaree® Tissue Clearing Rapid™ (BRTC) solution at 37 °C for about 1-2 hours prior to use.

2.1.2. Connect the BDTC-003 Binaree® Rapid™ System's clear-walled Binaree® Tissue Clearing Rapid™ Chamber to the electric terminals at the back of the system's Control Bath.

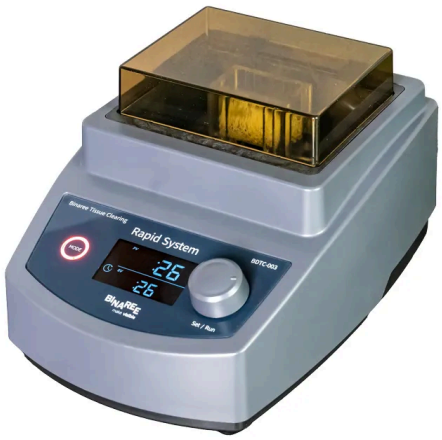


Figure 1. BDTC-003 Binaree® Rapid™ System for Rapid™ clearing using Binaree® Tissue Clearing Rapid™ (BRTC) solution.

2.1.3. Depending on the sample type and size, place the sample into the Binaree® Tissue Clearing Rapid™ Chamber as follows:

Sample type	**Placement in clearing chamber**
Whole rat, mouse, or zebrafish brain	Olfactory bulb oriented toward the red (+) terminal (left side), place sponge on top to hold tissue in place
Half rat, mouse, or zebrafish brain	
Lung, kidney, heart, other larger tissue	Place tissue in any orientation and place sponge on top to hold tissue in place
Spleen, spinal cord, other medium tissue	
Brain sections or smaller tissue	Place tissue in provided cassette in any orientation
Spheroids or organoids	*Rapid™ clearing not recommended*

2.1.4. Fill the BDTC-003 Binaree® Rapid™ System's standard Binaree® Tissue Clearing Rapid™ Chamber with 20 mL (or the larger chamber with 50 mL) BRTC solution. Turn the system ON using the red switch on the back panel. With the top value on the system's display flashing, turn the Jog Dial to select clearing program 001 or 002. Select the clearing solution and program depending on the sample type:

Sample type	**Use clearing solution…**	**Set clearing program…**
Mouse brain (whole, half, or sections)	BRTC-001	Program 002
All other tissue types, including rat brain	BRTC-002	Program 001
Spheroids or organoids	*Rapid™ clearing not recommended*	

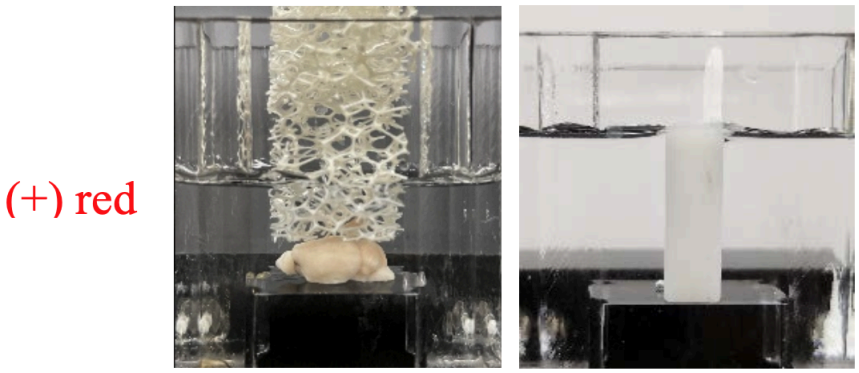


Figure 2. Place larger tissue under the sponge to hold it in place in the Binaree® Tissue Clearing Rapid™ Chamber, or smaller tissue in the provided cassettes. If clearing a brain, orient the olfactory bulb left toward the red (+) terminal.

2.1.5. Close the lid of the system's Control Bath and press the MODE button until the bottom value on the display flashes. Turn the Jog Dial to set the clearing duration in hours. Depending on the sample type and size, set the duration as follows:

Sample type	**Clearing duration**
Whole rat brain	24 to 48 hours
Mouse liver	Up to 48 hours
Whole mouse brain and other whole mouse tissue	Up to 6 hours
Half mouse brain	1 to 6 hours
Whole zebrafish brain, Mouse brain sections, or small tissue	1 hour
Spheroids or organoids	*Rapid™ clearing not recommended*

2.1.6. Press the Jog Dial in to start the clearing process, The Binaree® Rapid™ System can be paused by pressing the Jog Dial in again. Check on the sample periodically, and stop clearing as soon as the sample is fully cleared.
Note: It is normal for the clearing solution to froth and become syrupy during this process. However, excessive clearing may irreversibly caramelize the sample.

2.1.7. For tissue larger or denser than the mouse brain, such as liver or rat brain, clearing solution may have to be replaced every 6 hours or as soon as it turns dark brown and opaque.
Note: denser or bloody tissue will brown the clearing solution faster.

2.1.8. If the sample contains air bubbles after clearing, place the sample in a tube (do not use a chamber slide) at room temperature (24 °C) and either agitate gently or centrifuge at 3,000 rpm for 1 minute.

Pause point

If you need to pause the process here, leave samples at room temperature in fresh Binaree® Tissue Clearing Rapid™ (BRTC) solution for no more than 6 months, or in distilled water for no more than two weeks to avoid mold growth.

Part 2 (option 2): Passive clearing

Passive clearing is not recommended for whole rat brain, since it will take a very long time. Instead, clear whole rat brains with the Rapid™ clearing method described in Part 2 (option 1) starting on page 5.

2.2.1. Incubate the sample in a tube filled with BRTC solution and agitate at 50 rpm at 37 °C. Depending on the sample size and type, incubate as follows:

Sample type	**Clearing solution**	**Incubation time**
Large/dense tissue (lung, liver, kidney, heart, etc.)	20 mL BRTC-002	3 to 6 days
Whole mouse brain	20 mL BRTC-002	3 to 6 days
Half mouse brain	20 mL BRTC-002	48 to 96 hours
Medium tissue such as spleen or spinal cord	20 mL BRTC-002	48 to 96 hours
Small tissue such as whole zebrafish brain	5 mL BRTC-002	48 to 72 hours
3 mm thick mouse brain sections	5 mL BRTC-002	48 to 72 hours
1 mm thick mouse brain sections	5 mL BRTC-002	12 to 24 hours
Spheroids or organoids	1 mL BRTC-002	4 to 24 hours
Whole rat brain	*Passive clearing not recommended*	

2.2.2. If the sample contains air bubbles after clearing, keep the sample in the same tube used for clearing (do not use a chamber slide) at room temperature (24 °C) and either agitate gently or centrifuge at 3,000 rpm for 1 minute.

Pause point

If you need to pause the process here, leave samples at room temperature in fresh Binaree® Tissue Clearing Rapid™ (BRTC) solution for no more than 6 months, or in distilled water for no more than two weeks to avoid mold growth.

Part 3 (optional): Immunostaining

You can perform standard immunostaining procedures after clearing. The specific depth of penetration for each antibody will be dependent upon the sample type, the dilution, as well as the incubation time. Antibodies, especially larger ones, have trouble permeating whole rat and mouse brains and larger tissue, making deep tissue staining and imaging difficult. This is the case regardless of clearing method or protocol. Make sure your desired primary and secondary antibodies have been validated for whole brain staining.

Note: If simply staining nuclei rather than a full immunostaining protocol, wash the sample with distilled water while agitating at 50 rpm at 4 °C for 1 minute, then add nuclear staining solution (e.g., 20 to 40 µg/mL DAPI in distilled water) while agitating at 4 °C overnight, then wash 3 more times with distilled water while agitating at 50 rpm at 4 °C for 10 minutes per wash.

3.1. Incubate the sample with permeabilization solution while agitating in an incubator at 37 °C for 2-3 days. The sample may become opaque during this step, which is normal.

Recommendations for permeabilization

- Optimize the conditions for this permeabilization step depending on the sample type and antibodies used.
- Recommended solution: 1X PBS or TBS containing 0.2-0.5% Triton X-100, 10-20% DMSO, and serum.

3.2. Antigen retrieval can be performed as normal at high temperatures. Although “boiling” of cleared mouse brains in citrate buffer specifically has not been tested, it seems possible to do so to remove antibodies without issues with clearing and tissue integrity.

3.3. Incubate the sample with primary antibody while agitating in an incubator at 37 ºC. This should take 3-6 days for 1 mm thick samples. For best results while staining spinal cord, centrifuge samples for about 3 to 5 hours at ~2000 rpm during antibody incubation.

Recommendations for primary antibody staining

- Before staining thicker samples, optimize the process using a smaller sample.
- Consider using a fluorescence-conjugated primary antibody to save time.
- Optimize primary antibody dilution between 1:50 and 1:200.
- Recommended dilution buffer: 1X PBS or TBS containing 0.2-0.5% Tween 20, 5% DMSO, and 5% serum.

3.3. Wash the sample 6 times with 1X PBS or TBS while agitating at 4 ºC for 20 minutes per wash.

3.4. Incubate the sample with secondary antibody while agitating in an incubator at 37 ºC for 3-6 days.

Recommendations for secondary antibody staining

- Consider using an immunoglobulin fragment (e.g., Fab, $F(ab')_2$) to save time.
- Optimize primary antibody dilution between 1:100 and 1:500, with best performance usually closer to 1:100 dilution.

3.5. Wash the sample 6 times with 1X PBS or TBS while agitating at 4 ºC for 20 minutes per wash.

3.6. (*optional*) Add nuclear staining solution (e.g., 20 to 40 µg/mL DAPI in distilled water) while agitating at 4 ºC. See recommended incubation times below depending on sample type:

Sample type	**Incubation time**
≥ 7 mm thick tissue: whole brain, lung, kidney, heart, etc.	24 to 48 hours
≤ 7 mm thick tissue: half brain, spleen, spinal cord, etc.	12 to 24 hours
3 mm thick mouse brain section or equivalent volume tissue	6 to 12 hours
1 mm thick mouse brain section or equivalent volume tissue	1 hour
Spheroids or organoids	1 hour

DO NOT PAUSE

Do not pause the process here, and instead continue to mounting immediately after immunostaining. This is crucial to preserve fluorescence and sample integrity.

Part 4: Mounting

Mounting is necessary to give the sample a uniform refractive index (RI) that matches that of the lens used for imaging. This is the step at which the sample should become truly clear. The mounting solution BRMO has an RI of 1.52, while HRMO has an RI of 1.46.

4.1. Wash the sample 3 times with 1X PBS or TBS while agitating at 4 ºC for 10 minutes per wash.

4.2. Incubate the sample in BRMO or HRMO solution while agitating at 50 rpm in an incubator at room temperature (24 ºC) using the same volume of mounting solution as used for clearing. Recommended incubation times are shown here:

Sample type	**Incubation time**
≥ 7 mm thick tissue: whole brain, lung, kidney, heart, etc.	at least 48 hours
≤ 7 mm thick tissue: half brain, spleen, spinal cord, etc.	at least 24 hours
3 mm thick mouse brain section or equivalent volume tissue	at least 24 hours
1 mm thick mouse brain section or equivalent volume tissue	up to 24 hours
Spheroids or organoids	at least 6 hours

Pause point

If you need to pause the process here, leave samples at room temperature in the mounting solution you used for no more than 1 month, or in distilled water for no more than two weeks to avoid mold growth. If the sample contains a fluorophore, protect it from light during storage.

Part 5: Imaging

For the best results, image the sample within 7 days of clearing. Use a Light Sheet Fluorescence Microscope (LSFM) for larger tissue where depth of imaging is more important than very high resolution, or a Confocal Laser Scanning Microscope (CLSM) for samples less than 1 mm thick where higher resolution is more important.

5.1. If using LSFM, place the sample into the cuvette. If using CLSM, place the sample into a 2- or 4-well slide chamber, such as the one shown below, and seal the chamber with label tape to prevent drying.

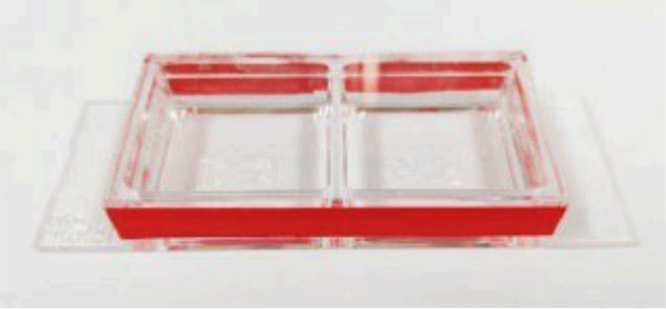


Figure 3. If using a chambered slide for confocal microscopy of smaller tissue, seal the chamber using label tape to prevent drying of the sample.

5.2. Pour the solution used for mounting into the cuvette or slide chamber, ensuring that the refractive index (RI) of the solution matches that of the lens. BRMO has an RI of 1.52, while HRMO has an RI of 1.46.
Note: Avoid creating bubbles in the solution as they can disrupt imaging. To prevent 1 mm thick tissue from floating freely in the slide chamber, use no more than 200 µL mounting solution.

5.3. Load the cuvette or chambered slide with the sample into the microscope and image the sample.

Long-term sample storage

After imaging, do not refrigerate the sample and instead store it at room temperature (24 ºC) in mounting solution indefinitely. If the sample contains a fluorophore, protect it from light during storage.