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ab316246 Tissue Clearing Kit – CUBIC

This kit enables the easy set-up of rapid tissue clearing for 3D tissue imaging using simple protocols and standard laboratory equipment.

This product is for research use only and is not intended for diagnostic use.

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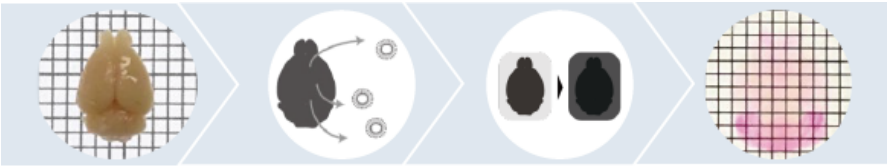
1. Overview

Tissue clearing can be performed with only general lab equipment and does not require special dedicated equipment such as an electrophoresis apparatus.

The process described below uses light-sheet microscopes (LSFM) and confocal laser scanning microscopes to allow imaging of the whole 3D organ with a resolution to the cellular level (please see note below for the 3D tissue staining process).

Workflow



1. 2. 3. 4.

1. PFA fixed sample
2. Remove lipids and pigments with CUBIC-L
3. RI matching with CUBIC-R
4. Transparent sample

Features

- Highly versatile water-soluble CUBIC tissue clearing kit that achieves a high transparency level.
- Compatible with all organs. Fluorescent protein signals can be observed after clearing the sample.
- Our simple and reproducible protocol solves issues pertaining to safety, waste processing, microscope compatibility, fluorescent protein signal retention, etc.

Clearing Reagents

The reagents used allow animal organs to become transparent by immersing them in two types of reagents:

- CUBIC-L for delipidation and decolorization.
- CUBIC-R for clearing (RI matching). High versatility and easy handling.
- These tissue clearing reagents are using CUBIC technology.

Δ Note: *CUBIC-R causes each axis to swell 1.5 times to increase transparency and performance resolution during microscope imaging.*

Δ Note: *3D Tissue Staining Kit – CUBIC (ab316248) will have to be purchased separately to perform 3D tissue staining that allows the observation of all cells in organs (please see protocol outline for further details).*

2. Materials Supplied and Storage

Store kit at ambient temperature on receipt. Kit can be stored for 1 year from receipt.

Item	Quantity 10 tests	Quantity 20 tests
CUBIC-L	250 mL	500 mL
CUBIC-R	250 mL	500 mL

3. Materials Required, Not Supplied

These materials are not included in the kit but will be required to successfully perform this assay.

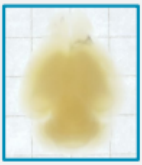
Materials required

Heparin
PBS
Formaldehyde
Sodium azide (NaN_3)

4. Assay Procedure

4.1 Practical Example of Mouse Organ Clearing.

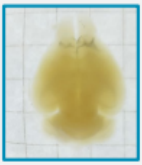
Adult mouse brain after extraction and PFA fixation



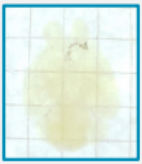
After replacement with 50% CUBIC-L
(Treated overnight at 37°C)

After delipidation and decoloration with 100% CUBIC-L
(Treated for four days at 37°C)

After washing with PBS

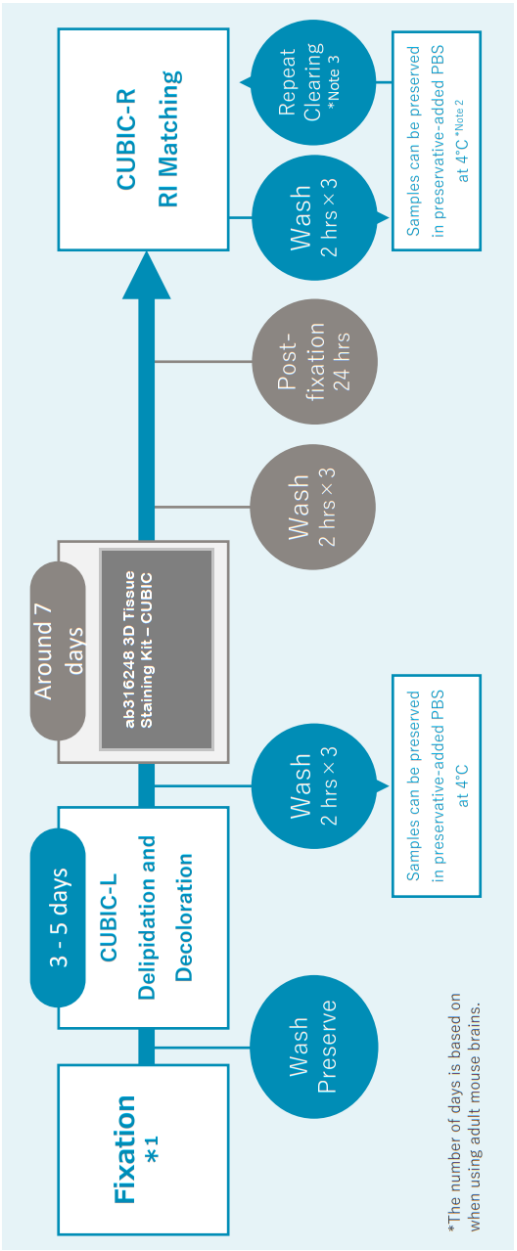


After shaking in 4 mL 50% CUBIC-R
(Treated overnight at room temperature)



After shaking in 4 mL CUBIC-R (overnight at room temperature),
Immersed in mounting agent for observation (RI = 1.522)

4.2 Basic Process of Clearing and Staining Mouse Organs.



*Note 1 We recommend using 4% paraformaldehyde (pH 7~7.5) for the fixative. Transparency is greatly affected by fixation conditions. It is important to minimize the difference between controlled conditions, such as creating large amounts of paraformaldehyde and storing them in the freezer. Overfixation and yellowing may occur if the pH is alkaline.

*Note 2 Transparency samples can be embedded with agarose gel. This allows stable preservation for a long time.

*Note 3 Frequently repeating clearing results in tissue damage.

4.3 Mouse Organ Clearing Procedure (Basic Protocol).

Step	Reagent	Temperature	Time	Notes
Extract organ				After perfusion fixation.
Post-fixation	4% PFA in PBS	4°C	1 day	
Wash x 3	PBS	RT	Up to 2 hrs x 3	Shake gently (the same applies to the steps below).
Before replacement	50% CUBIC-L	RT Or 37°C	6 – 24 hrs	Optional step. Mix an equal amount of CUBIC-L and water.
Delipidation and decoloration	CUBIC-L	37°C	> 2 days	Change CUBIC-L to a new solution every two days.
Wash x 3	PBS	RT	Up to 2 hrs x 3	Exchange or wash the tube every time to prevent carry-over of the solution.
Staining	Staining buffer from ab316248	RT	Up to 7 days	Optional step.
Wash x 3	from ab316248	4°C	Up to 1.5 hrs x 3	Conduct if staining.
Post-fixation	1% formaldehyde	4°C	1 day	Conduct if staining. Use 37% formaldehyde diluted with PBS.
Post-fixation	1% formaldehyde	37°C	1 hr	Conduct if staining. Transfer the solution treated overnight at 4°C to the solution treated at 37°C.
Wash x 3	PBS	RT	Up to 2 hrs x 3	Conduct if staining.
Before replacement	50% CUBIC-R	RT Or 37°C	1 day	Mix an equal amount of CUBIC-R and water.
Clearing	CUBIC-R	RT Or 37°C	> 1 day	

4.4 Tissue clearing protocol.

Collection of mouse organs

1. Anesthetize the mouse.

2. Transcardially perfuse 10 mL (4 mL/min) of ice-cold PBS containing 10 U/mL of Heparin.

3. Transcardially perfuse 20 mL (6 mL/min) of ice-cold 4% (w/v) PFA.

4. Dissect the organs.

5. Post-fix the dissected organs in 4% (w/v) PFA in PBS (~10 mL/whole organ) for overnight (8-24 h) at 4°C with gentle shaking (40-50 rpm/min).

6. Wash the sample in PBS (+0.05% NaN_3) for 2 h × 3 times at room temperature with gentle shaking (40-50 rpm/min).

Delipidation with CUBIC-L

1. Immerse the fixed sample in 10 mL of 0.5× CUBIC-L (1:1 dilution with water) in the 30 mL tube and incubate it for overnight at 37°C with gentle shaking (40-50 rpm/min).

2. Replace with 10-15 mL of 1× CUBIC-L in the 30 mL tube and delipidate for 3-5 days at 37°C with gentle shaking (40-50 rpm/min).
Replace CUBIC-L every 2 to 3 days if the duration of treatment exceeds 3 days.

3. Wash the sample with 20 mL of PBS containing 0.05% NaN_3 for 2 h × 3 times (or 2 h ×1, overnight ×1, 2 h ×1) at 37°C with gentle shaking (40-50 rpm/min).
After each use, the tubes should be washed or replaced in order to remove Triton X-100 thoroughly.
The delipidated sample can be stored in PBS/0.05% NaN3 at 4°C at least for several months.

Optional

Cryosection of CUBIC-L-treated tissues

1. Immerse the CUBIC-L-treated sample in 40% (w/v) sucrose/PBS and maintain it at 4°C until the sample settles at the bottom of the tube (typically it takes over several hours to overnight).

2. Also, for cryopreservation of the fixed sample (without CUBIC-L treatment), immerse the sample in 10% (w/v) sucrose/PBS and keep at 4°C until the sample settles at the bottom of the tube. Subsequently, exchange the reagent to 25% (w/v) and maintain it at 4°C until the sample settles again. Typically, overnight incubation will be needed.

3. Embed the samples in O.C.T. compound.

4. Use a cryostat to prepare sections of these samples, cutting them at a thickness of 50 µm (for CUBIC-L-treated samples) or 30 µm (for fixed samples). Gather and wash the sections in PBS. The prepared sections can be stored in PBS/0.05% NaN_3 at 4°C for at least several months.

Δ Notes:

Amount of reagent and treatment time varies depending on the organ.

Please use a slightly larger tube wider than the organ's diameter. Most of the organ should be immersed in the reagent when the tube is laid on its side.

Shake the organ immersed in the reagent at a speed that shakes the whole organ.

We do not recommend rotation as it will create bubbles.

5. Frequently Asked Questions

Questions on Clearing.

Q: What types of containers should be used during clearing?

A: CUBIC tissue clearing is designed to improve transparency and microscope resolution by slightly expanding the tissue. Thus, we recommend using tubes, containers, etc. with a diameter wider than the tissue. For example, when a tube is filled halfway with the reagent, the sample should not come out of the liquid surface when the tube is laid on its side. CUBIC reagents are aqueous, so polypropylene and polyethylene containers can be used safely.

Q: Will the expansion of tissues affect the experiment?

A: Various cells may expand, but the physical relationship of the cells is maintained. The rigidness of the structure may vary depending on the tissue (nerves, veins, etc.) and may cause artifacts, so confirmation is required according to the situation.

Q: Is fixation still required even when clearing is done right after the organ is extracted from the animal?

A: CUBIC tissue clearing agents are optimized for PFA fixation. Lysis will occur on samples that are not fixed, so please fix the samples.

Q: Can samples be cleared even if some time has passed after dissection and fixation?

A: Samples that have been immersed in fixative for a long time (over several weeks) can still be cleared. However, since the fixation condition is a parameter that greatly affects the transparency condition, we recommend maintaining the preparation condition and fixation period of the fixative as much as possible. In our protocol, we recommend washing with PBS within 24 hours after perfusion fixation of the animal, and promptly starting the CUBIC-L treatment.

Q: Can paraffin-embedded samples become transparent?

A: Paraffin-embedded samples can become transparent after deparaffinization treatment. Please refer to the following treatment method for more details.

Reference: CUBIC pathology: three-dimensional imaging for pathological diagnosis S. Nojima et al., Sci. Rep. 2017, 7, 9269 https://www.nature.com/articles/s41598-017-09117-0

Q: How much reagent is required for clearing?

A: When making the whole mouse body transparent, the amount of reagent should be enough to immerse the whole body. For organs, choose a tube so that when the tube is filled halfway with the reagent, the whole organ is immersed when the tube is laid on its side. For example, for mouse brains, pour 10 to 15 mL of CUBIC-L into a 20 to 30 mL tube. The actual amount will vary according to the sample size and container used. As an example, 20 to 40 mL of CUBIC-L and 10 to 20 mL of CUBICR is required to clear each mouse organ (approx. $1cm^3$).

Q: What is the reason if clearing does not go well?

A: It may be due to the following reasons. Please consider the measures below.

a) **The pH level of the PFA solution used in fixation is high:** When the pH level is over 8, over-fixation occurs, and the organ becomes yellow, causing clearing to slow down. Please set the pH level around 7 to 7.5. In addition, the transparency level differs depending on the PFA pH level and processing time. We recommend maintaining the fixation time and fixative preparation procedure as much as possible.
b) **Delipidation is not complete:** Either extend the delipidation step or consider the exchange frequency of CUBIC-L. We recommend exchanging to a new CUBIC-L solution every two days and shaking at 37ºC.
c) **Clearing is not complete:** When CUBIC-R attracts moisture, the RI drops, and the transparency decreases. Please exchange CUBIC-R.

Q: How long does delipidation take?

A: For adult mouse tissue, delipidation takes three to seven days. The length of delipidation

depends on the organ. Set the delipidation time according to the type and size of the organ, and experiment purpose. For example, complete transparency is required for light-sheet microscope observation, but less time and incomplete transparency are acceptable for partial observation using two-photon microscopes.

Questions on After Clearing.

Q: What should we do with the liquid waste after the experiment?

A: Dispose of the liquid waste according to the specified disposal method after consulting with the person in charge of waste in your institution. Furthermore, in general, samples and reagents used in the experiment should be disposed of as medical waste and infectious waste, and unused CUBIC reagents should be disposed of as organic waste and fire-resistant waste because it has a high water content.

Q: How should samples be stored after clearing?

A: Samples can be stored at room temperature with the used CUBIC-R. Furthermore, the sample may harden if the water in the CUBIC-R solvent evaporates. Seal the container with parafilm, etc. Samples can also be stored at room temperature in agarose gel. This method is more stable.

To archive, samples can be stored at 4℃ inside the preservative-added PBS, such as sodium azide after washing CUBIC-R with PBS.

Agarose Gel Storing Procedure

In an appropriate tube, add 2% agarose gel to the CUBIC-R reagent used in the clearing. Heat and dissolve the solution. Insert the transparent sample into the agarose solution before it hardens. Cool enough to harden.

For more details, refer to the article or website below.

Advanced CUBIC tissue clearing for whole-organ cell profiling K. Matsumoto et al., Nat. Protoc. 2019, 14, 3506. https://www.nature.com/articles/s41596-019-0240-9

Website: http://cubic.riken.jp/

Q: The transparent sample cannot be observed well.

A: We recommend observing with light-sheet microscopes for tissue clearing observation (LSFM), confocal laser scanning microscopes (CLSM) with objective lenses compatible with high RI, and two-photon microscopes. When observing, immerse the sample in the mounting agent for observation, and use objective lenses compatible with the RIs. In recent years, affordable light-sheet microscopy for tissue clearing is developed.

Reference: descSPIM: Affordable and Easy-to-Build Light-Sheet Microscopy for Tissue Clearing Technique Users K. Otomo et al., bioRxiv. 2023

Technical Support

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