

Animal Tissue Optical Clearing Kit

Introduction

Animal Tissue Optical Clearing Kit is a highly effective kit that enables simple, convenient, and rapid high-quality and high-transparency clearing of whole animal bodies, whole organs, tissue blocks, or organoids. Animal samples cleared with this kit can be combined with subsequent immunofluorescence, immunohistochemistry, routine or fluorescent staining, bioluminescence, and other techniques to achieve three-dimensional imaging of tissue blocks, organoids, organs, or even entire animal bodies, making it widely applicable in biomedical research.

CUBIC (Clear, unobstructed brain/body imaging cocktails and computational analysis) is a simple and effective method for animal tissue clearing. The Animal Tissue Optical Clearing Kit achieves clearing effects essentially comparable to conventional CUBIC methods.

The Solution I provided in this kit not only promotes delipidation of tissues and organs but also decolorizes heme in tissues and organs. Solution II adjusts the refractive index of tissues to match that of the solvent, minimizing light scattering within the tissue and facilitating optical imaging.

Components and Storage

Components	25 mL	100 mL	500 mL	Storage
Solution I	25 mL	100 mL	500 mL	-20°C away from light
Solution II	25 mL	100 mL	500 mL	-20°C away from light
Wash Buffer	1 Package	1 Package	3 Packages	Room temperature
Mounting Solution	10 mL	40 mL	200 mL	-20°C away from light
Shipping: Blue ice		Shelf life: 12 months		

Protocol

1. Preparation of tissue samples

a. Preparation of reagents and equipment.

a) Fixative: 4% paraformaldehyde fixative or immunostaining fixative.

b) Anesthetic: Choose an appropriate anesthetic based on experimental requirements, such as sodium pentobarbital or ketamine.

- c) Perfusion solutions: 1X PBS/heparin sodium (10 U/mL) solution or 1X PBS, 4% paraformaldehyde fixative.
 - d) Equipment: Surgical scissors, ophthalmic forceps, hemostatic forceps, infusion set needles (6 or 7 gauge recommended for mice, 10–12 gauge for rats).
- b. Animal anesthesia: For example, anesthetize mice by intraperitoneal injection of 1% sodium pentobarbital (recommended dosage: 80 mL/kg body weight).
- c. Make a transverse incision along the body surface and abdominal wall below the ribs, quickly cut the diaphragm, lift the sternum, and expose the heart.
- d. Insert the needle starting from the apex of the heart, angling from the lower left obliquely toward the upper right until reaching the ascending aorta. If resistance is encountered, try changing direction and reinsert; do not force the needle in.
- e. Use hemostatic forceps to clamp the ventricle (or hold by hand) to secure the needle and prevent leakage. Make a small incision in the right atrium.
- f. Perfuse with 1X PBS/heparin sodium (10 U/mL), initially at a slow flow rate followed by a faster flow rate, to flush blood vessels and remove blood. The standard for adequacy is clear fluid flowing from the right atrium and a grayish-white liver.
- g. Perfuse with 4% paraformaldehyde fixative for fixation, initially at a fast flow rate followed by a slower flow rate. The endpoint is stiffening of the muscles; typically, the volume used is approximately equivalent to the body weight of the experimental animal. Alternatively, dissect the required tissues/organs and then fix them.
- h. Dissect the required tissues/organs and immerse them in 4% paraformaldehyde fixative. Fix overnight on an orbital shaker.
- 2. Preparation of the clearing reagent**
- a. Preparation of 50% Solution I: Mix Solution I and distilled water in a 1:1 ratio. The mixture can be stored at room temperature for up to 1 month.
 - b. Preparation of 50% Solution II: Mix Solution II and 1X PBS in a 1:1 ratio. The mixture can be stored at room temperature for up to 2 weeks.
 - c. Preparation of 1X Wash Buffer: Dissolve 1 package of Wash Buffer in 2 L of water, then filter. The buffer can be stored at room temperature for several months.
- 3. Delipidation and decolorization of tissues/organs**
- a. Discard the fixative, add 1X Wash Buffer, and wash 3 times for 2 hours each time on a room temperature shaker (60 rpm).

- b. Add an appropriate amount of 50% Solution I to immerse the sample. Incubate at 37°C on a shaker (60 rpm) for 4–24 hours. Gradual clearing at the edges of the tissue can be observed.

***Note:** The volume of 50% Solution I can be adjusted according to sample size, ensuring the sample remains fully immersed during shaking.

- c. Add an appropriate amount of Solution I to immerse the sample. Incubate at 37°C on a shaker (60 rpm), replacing with fresh solution every 4 days, until the tissue/organ sample becomes completely clear.

***Note:** The volume of Solution I can be adjusted according to sample size, ensuring the sample remains fully immersed during shaking.

- d. Add an appropriate amount of 1X Wash Buffer to immerse the sample. Wash at room temperature on a shaker (60 rpm) at least 3 times for 2 hours each time.

4. Immunofluorescence staining (optional)

- a. Primary antibody incubation: Dilute with immunostained primary antibody dilution, immunostained primary antibody dilution, or other appropriate primary antibody dilution according to the recommended dilution ratio of the primary antibody. Incubate in a 37 °C shaker (60 rpm) for 3 days in appropriate shade.

***Note:** The dilution ratio of the primary antibody can be adjusted appropriately according to the actual dyeing effect.

- b. Recover the primary antibody and wash it 6 times at room temperature for 2 hours with 1X Wash Buffer.
- c. Secondary antibody incubation: Dilute with immunofluorescence staining secondary antibody dilution, immunofluorescence staining secondary antibody dilution, or other appropriate secondary antibody dilution according to the recommended dilution ratio of the secondary antibody. Incubate in a 37 °C shaker (60 rpm) protected from light for 3 days.

***Note:** The dilution ratio of the secondary antibody can be adjusted appropriately according to the actual staining effect.

- d. Recover the secondary antibody and wash it at least 3 times at room temperature for 2 hours with 1X Wash Buffer.

5. Nuclear staining (optional)

- a. DAPI incubation: 1X Wash Buffer dilute DAPI to 0.5-10 µg/mg working solution and incubate in a 37 °C shaker (60 rpm) protected from light for 2-3 days.

***Note:** The dilution ratio can be adjusted appropriately according to the actual dyeing effect.

- b. With 1X Wash Buffer, wash at least 3 times at room temperature for 2 hours each.

6. Adjust the refractive index of the sample

- a. Add an appropriate amount of 50% Solution II immersion sample, place it upright (bubbles are likely to form when placed horizontally), and incubate in a horizontal shaker at 37°C (60 rpm) for 6-24 hours until the sample sinks to the bottom.

***Note:** The dosage of 50% Solution II can be adjusted appropriately according to the sample volume; It is recommended to use at least 2-3 times the sample volume each time, ensuring that the sample is completely submerged during shaking.

- b. Add an appropriate amount of Solution II immersion sample, place it upright (bubbles are easy to produce when placed horizontally), and incubate for 24 hours on a horizontal shaker at 37 °C (60 rpm).

***Note:** The dosage of Solution II can be adjusted appropriately according to the sample volume; It is recommended to use at least 2-3 times the sample volume each time, ensuring that the sample is completely submerged during shaking.

- c. Repeat step 6b once.
- d. Remove Solution II, add Mounting Solution to submerge the sample for 10 minutes, carefully remove bubbles from the surface of the sample, and use it for photography.

FAQs

1. Is it necessary to use a special type of container for animal tissue clearing?

A: No special type of container is required. Polypropylene or polyethylene plasticware or glassware commonly used in laboratories can be used. Since animal tissues and organs can swell during the clearing process, it is recommended to use a slightly larger container.

2. Does the sample expand during clearing? Does expansion have any negative effects on experiments?

Answer: Samples such as tissues and organs are likely to expand during the clearing process. However, this expansion is nearly linear and relatively uniform, and the relative position of cells remains constant. Except for the expansion of the overall shape, there will be no obvious negative effects.

3. Can fresh tissue be cleared directly without the fixation step?

A: No. Tissue clearing without fixation may cause disturbances in the relative position of cells and may affect the effect of subsequent immunostaining. Therefore, the sample needs to be fixed before clearing.

4. Can it be cleared using a fixed sample that has been stored for a long time?

A: Yes. This product can be used for samples that have been soaked in a fixation solution for several weeks, as well as those that have been fixed and stored at -80°C for several months. However, if samples are planned to be immunostained, long-term storage of fixed samples may experience a decrease in the immunostaining effect of the protein of interest.

5. How do I get a rough estimate of how much each reagent I need for my sample?

A: For mouse systemic clearing, the volume of reagents used must be sufficient to submerge the entire specimen (200-400 mL of Solution I and 100-200 mL of Solution II are typically required). For organ clearing, the sample should be soaked in Solution I at least 5 times the sample volume. For example, for a 1 cm³ specimen, a total of 20-50 mL of Solution I and 10-30 mL of Solution II are required. When tissue blood residues are high, lipids are high, or tissues are thick, the total amount of reagents required for clearing may be appropriately increased.

6. Can different tissues and organs or several identical tissues and organs be placed in a tube for transparency?

A: Several tissues and organs can be processed in one tube. For example, the heart, lungs, kidneys, spleen, pancreas and a piece of liver can be placed in the same tube, and several appropriate small organs such as the heart, lungs, kidneys, spleen, and pancreas can also be placed in the same tube. But the stomach and intestines should be separated into separate tubes, and the contents of the gastrointestinal tract should be removed as much as possible.

7. Does it necessarily need to be soaked in the Mounting Solution for specimen observation after clearing?

A: Since Solution II is an aqueous solution, it may evaporate over a longer period of time, leading to changes in refractive index and solute deposition, making image acquisition difficult. For short observations and photographs (<1 hour), samples can be immersed in Solution II. For longer observation times (>1 h), it is recommended to soak the sample with the Mounting Solution.

8. It is difficult to observe the sample after transparency.

A: It is recommended to use light sheet fluorescence microscopy (LSFM) or laser confocal scanning microscopy (CLSM) to observe the cleared sample. In addition, it is recommended not to cut the sample to be cleared too thin to prevent the gel properties from distorting the tissue structure. Transparent samples are recommended to be photographed in the Mounting Solution (Refractive Index/RI = 1.467) and required to be viewed with objectives suitable for these RIs.

9. What is the refractive index (RI) of Solution II?

A: The RI of Solution II is 1.48–1.49. It is recommended to use objectives and immersion oils compatible with these refractive indices. Do not mix the Optical Clearing Reagents in this kit with other solvents (e.g., water) to avoid altering their refractive index and affecting the observation of cleared samples.

10. Why is the sample not sufficiently transparent?

A: The possible causes and suggestions are as follows:

- a) PFA fixative pH is too high: pH above 8 can lead to overfixation, making it more difficult to transparent the sample. It is recommended to try adjusting the pH level to between 7-7.5.
- b) Incomplete degrease: Try to extend the degreasing time or replace fresh Solution I more often. It is recommended to soak the sample in Solution I at 37 °C for at least 2-5 days, changing the fresh reagent daily.
- c) Refractive index mismatch: When using, ensure that Solution II is completely dissolved and free of precipitation. Try extending the time and/or replacing it with a fresh Solution II.
- d) Use of older animals: Clearing in younger animals is generally better than in older animals.

11. How long does a sample need to be degreased?

A: For adult mice, it takes about 2-3 days for the intestines, pancreas, and spleen, and about 1-2 weeks for the lungs, heart, brain, stomach, muscles, liver, and kidneys.

12. What types of tissue staining reagents can be used with clearing kits?

A: For example, large molecule staining such as immunofluorescence staining and small molecule staining such as nuclear fluorescence staining (e.g., DAPI, Hoechst, propidium iodide) are compatible with transparent samples.

13. What kind of nuclear staining reagents can be used for clearing samples?

A: DAPI, Hoechst 33342 Staining Solution, Hoechst 33258 Staining Solution, Propidium Iodide, etc., are recommended.

14. Do I need to purchase special antibodies for cleared samples?

A: While many proteins do not lose their antigenicity during fixation, fat removal, and decolorization, there are exceptions. It is recommended that the antibodies used be properly validated.

15. Can I use fluorescently labeled secondary antibodies?

A: Yes. However, considering the time required for each antibody in the sample to be incubated, it is recommended to use fluorescently labeled primary antibodies to shorten the experimental cycle.

16. What fluorescent proteins are compatible with?

A: This product has good fluorescent protein compatibility and can be compatible with common fluorescent proteins such as GFP, EGFP, EYFP, mCherry, and mKate2.

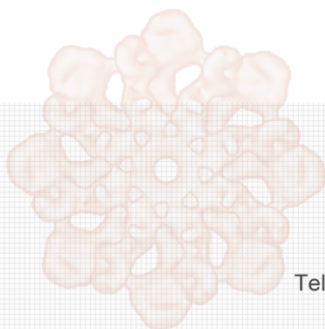
17. What kind of fluorescent dyes can be used?

A: Common fluorescent probes such as FITC, Rhodamine, Cy3, Alexa Fluor 488, Alexa Fluor 555, Alexa Fluor 594, and Alexa Fluor 647 are all compatible.

Note

1. Before using Solution I and Solution II, let it sit at room temperature until completely melted, ensuring that the liquid is transparent and free of sedimentation. If there is crystal precipitation or the solution is opaque, it can be placed in a water bath at 55°C for a short time to dissolve until completely transparent and free of precipitation.
2. Solution I and Solution II fail at high temperatures (>75°C), so be careful not to treat them at high temperatures.
3. Solution I The tissue will become soft after treatment, please use a spoon to sample carefully and do not use forceps to avoid damage to the tissue.

4. Solution I easily removes oily ink marks. When using, please close the lid as tightly as possible and seal the lid with Parafilm to prevent leakage. It is recommended to label the sample on both the cap and the tube body to avoid loss of sample information.
5. If there is a strong ammonia odor during Solution I treatment, it indicates that the solution may have failed, please replace it with fresh Solution I in time to avoid affecting the transparency effect.
6. Do not shake Solution II vigorously to avoid bubbles. If a large number of bubbles are inadvertently generated in Solution II, ultrasonic cleaner or vacuum (~0.1 MPa, ~30 minutes) to remove bubbles, or let them sit at room temperature (at least 7-8 hours) to eliminate bubbles on their own to avoid bubbles around or inside tissues and organs.
7. Solution II is more viscous, please use a large-mouth tip (you can cut off the tip of the gun tip with scissors) to suck slowly, and slowly add it to the sample, do not blow vigorously to avoid a large number of bubbles.
8. The tissue and organ samples after photo and video acquisition can be removed from the Mounting Solution and then soaked in Solution II and stored at room temperature for 1 week. The longer the time, the more transparent the sample becomes, but it can also cause tissue swelling. Samples after photo and video capture can be washed with a 1X Wash Buffer, dehydrated with 30% (w/v) sucrose solution, then embedded with O.C.T compounds, and stored at -80°C for a long time.
9. For thicker sections (>2 mm) or whole organs, the incubation time for each step can be appropriately extended according to the actual clearing effect.
10. To ensure long-term effectiveness of Solution I and Solution II, it needs to be stored at -20°C in a dark place. However, in order to facilitate the observation of the tissue clearing effect, the sample can be cleared under non-strict light protection conditions.
11. This product is for scientific use only.



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