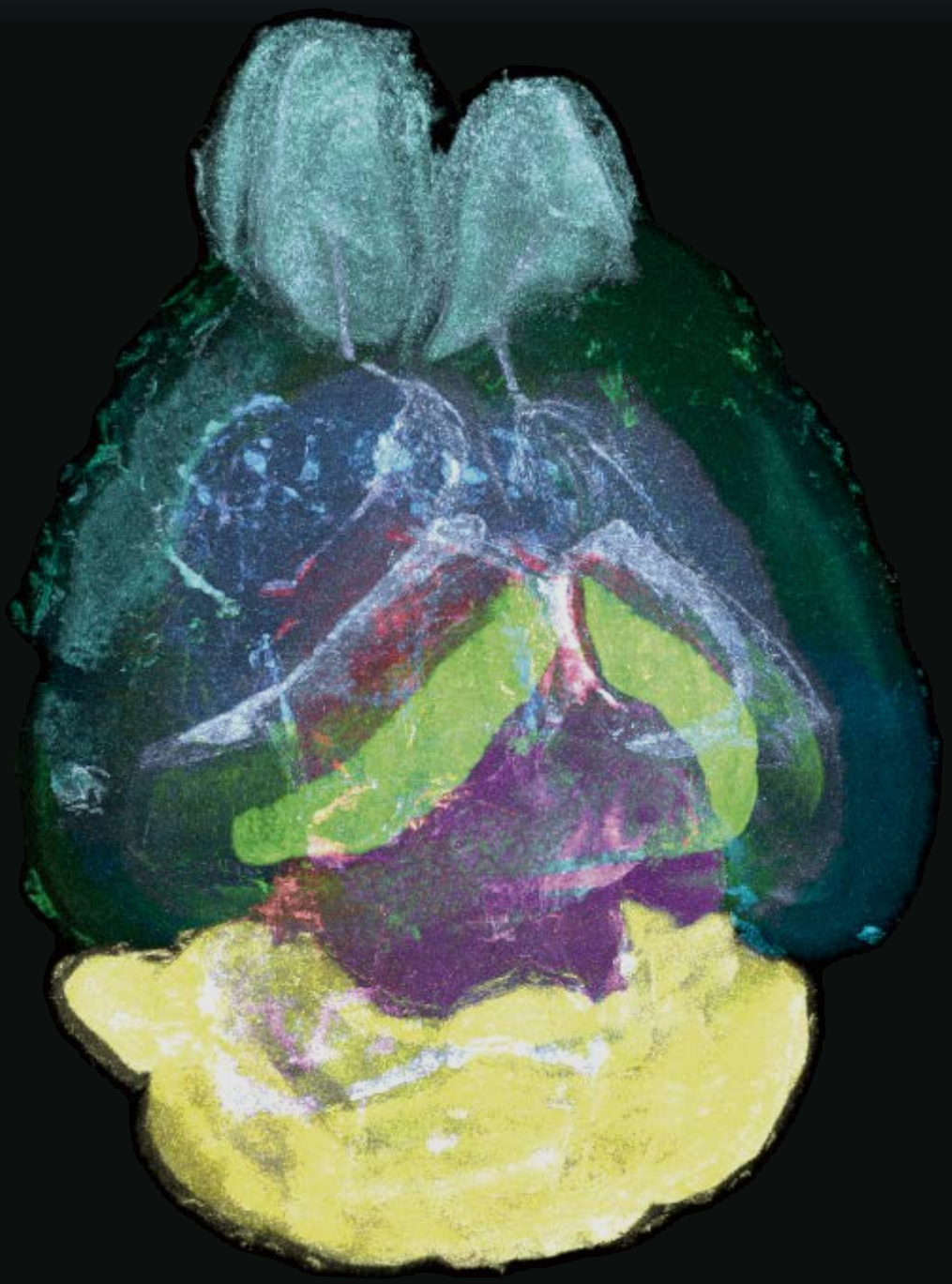
LIFE SCIENCE

TCI

CUBIC

– Animal Tissue-Clearing Reagents –
Technical Guidebook



Provided by
The University of Tokyo
Graduate School of Medicine
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TOKYO CHEMICAL INDUSTRY CO., LTD.

What to Clear ?

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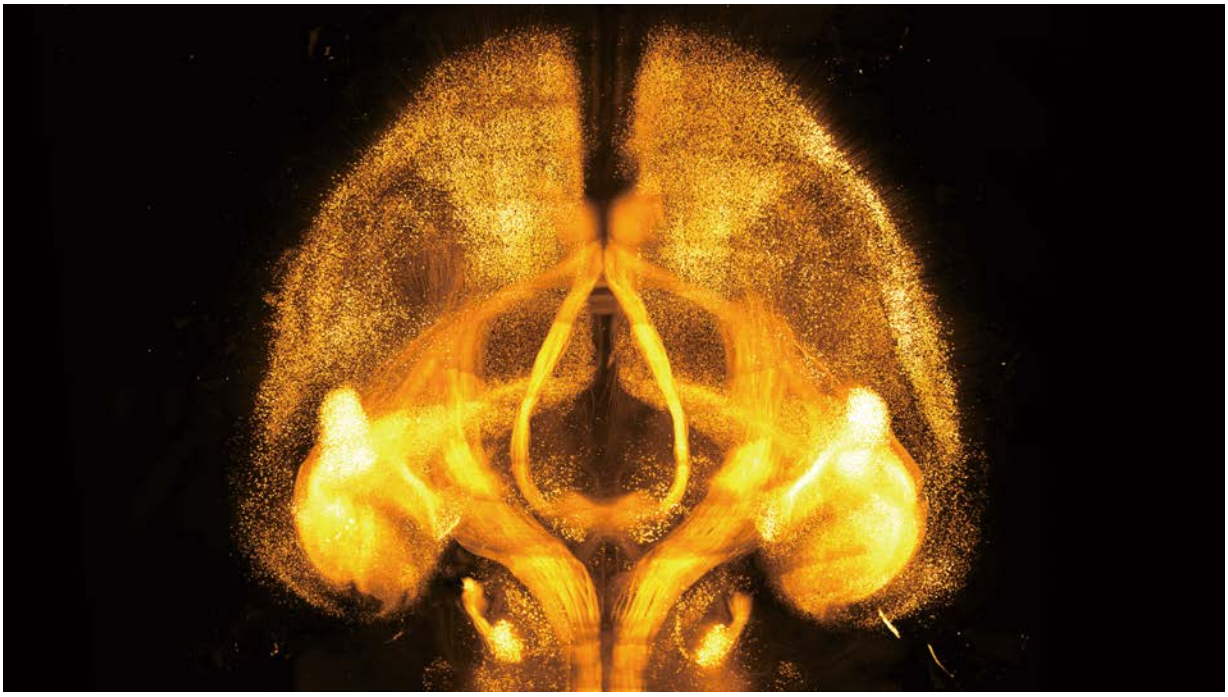
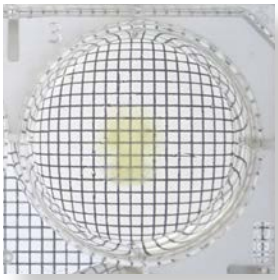


Product Introduction

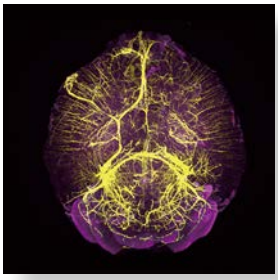
CUBIC-L	**(for delipidation and decoloring)**	25mL / 100mL [T3740]
CUBIC-R+	**(for RI matching)**	25mL / 100mL [T3741]
CUBIC-B	**(for decalcification)**	25mL / 100mL [T3780]
CUBIC-HL	**(for delipidation strongly and quenching autofluorescence)**	25mL / 100mL [T3781]
CUBIC-P	**(with perfusion before tissue excision)**	25mL / 100mL [T3782]
CUBIC-X1	**(for expansion)**	25mL / 100mL [T3866]
CUBIC-X2	**(for RI matching with expansion)**	25mL / 100mL [T3867]

- **Basic protocol ;**
  Mouse whole-body or animal organ clearing is achieved by using two reagents, CUBIC-L [T3740] for delipidation and CUBIC-R+ [T3741] for RI matching.
- **Optional protocol ;**
  The following products can easily clear tissues, such as bones or highly fatty tissues which were previously difficult to clear.
  CUBIC-B [T3780] for bone, CUBIC-HL [T3781] for highly fatty tissues
- **CUBIC-P [T3782] for mouse perfusion efficiently aids with perfusion fixation.**
- **Expansion protocol;**
  The following products can clear tissues with expansion.
  CUBIC-X1 [T3866] for expansion tissues
  CUBIC-X2 [T3867] for RI matching with keeping the expanded size of tissues
- **Tissue expansion enables acquisition of images easy.**
- **Preserve the fluorescent protein signals except CUBIC-HL [T3781].**
- **Using light-sheet fluorescent microscopy (LSFM) or confocal laser-scanning microscopy (CLSM) enables the whole-organ / body imaging at a cellular resolution.**

These products were developed by Prof. Hiroki R. Ueda (The University of Tokyo / RIKEN) and are under invention licenses by RIKEN, Japan.



Whole-brain clearing



Whole-body clearing with nuclei staining and immunostaining

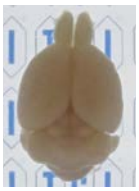
Direction for Use : Mouse whole-organ clearing protocol

Fix	Wash x 3	Pre-treatment	Delipidation	Wash x 3	(Staining)	(Wash x 3)	Pre-treatment	RI match
4% PFA	PBS	50% CUBIC-L	CUBIC-L	PBS	Stains*	PBS	50% CUBIC-R+	CUBIC-R+
1 day	> 2 hr x 3	6 - 24 hr	> 2 days	> 2 hr x 3	> 3 days	> 2 hr x 3	1 day	> 1 day

Process	Reagent	Temp.	Time	Notes
Tissue excision				After perfusion fixation
Tissue Fix	4% PFA in PBS	4℃	1 day	
Wash x 3	PBS	RT	> 2 hr x 3	Shake gently (Same in following steps). Total 1 day
(Pre-treatment)	50% CUBIC-L	37℃ or RT	6 - 24 hr	1:1 mixture of water and CUBIC-L. Optional
Delipidation	CUBIC-L	37℃	> 2 days	Refresh CUBIC-L on day 1, day 2 and every 2 days after day 4.
Wash x 3	PBS	RT	> 2 hr x 3	Total 1 day
(Staining)	Staining reagents*	RT	> 3 days	Optional
(Wash x 3)	PBS	RT	> 2 hr x 3	Total 1 day, optional
Pre-treatment	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
RI matching	CUBIC-R+	RT	> 1 day	

Application

- **An adult mouse brain after excision**



- **After pre-treatment step of 4 mL 50% CUBIC-L at room temperature overnight**

- **After delipidation step of 4 mL CUBIC-L at 37°C for 4 days**
  (Refresh CUBIC-L on day 1, day 2)

- **After pre-treatment step of 4 mL CUBIC-R+ at room temperature overnight**

- **After RI matching of 4 mL CUBIC-R+ at room temperature overnight**

Total
- CUBIC-L : 14 mL
- CUBIC-R+ : 6 mL

Each sample of these images is immersed in each reagent.

- Light penetrates the organ.
- CUBIC-L does not get colored after treatment.
  Above points are the signs of end of delipidation.

The reagent volumes of the left example is in the case of usage in a 5 mL-tube.
Work in a tube whose diameter is a little larger than that of organs and the volume of reagents is half of that of tubes.
* For nuclear staining, use 30 μg/mL Propidium iodide (PI) and 1.5 M NaCl in PBS.
Since the expanded brains are fragile, careful handling is required after the swelling step.

PFA : paraformaldehyde, RT : room temperature

Direction for Use : Mouse whole-body clearing protocol

Pre-treatment	Delipidation	Wash x 3	(Staining)	(Wash x 3)	Pre-treatment	RI match
50% CUBIC-L > 6 hr	CUBIC-L > 5 days	PBS > 2 hr x 3	Stains* > 3 days	PBS > 2 hr x 3	50% CUBIC-R+ 1 day	CUBIC-R+ > 1 day

Process	Reagent	Temp.	Time	Notes
Perfusion fixation	PBS 4% PFA in PBS			After perfusion, the mouse needs to be perfused with 50% CUBIC-L (1:1 mixture of water and CUBIC-L : water).
Perfusion	PBS 50% CUBIC-L			
(Pre-treatment)	50% CUBIC-L	37℃	> 6 hr	Completely immerse the whole body of the mouse with gentle shaking (same in following steps). Optional
Delipidation	CUBIC-L	37℃	> 5 days	Refresh CUBIC-L on day 1, day 2 and every 2 days after day 4.
Wash x 3	PBS	RT	> 2 hr x 3	Total 1 day
(Staining)	Staining reagents*	RT	> 3 days	Optional
(Wash x 3)	PBS	RT	> 2 hr x 3	Total 1 day, optional
Pre-treatment	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
RI matching	CUBIC-R+	RT	> 1 day	

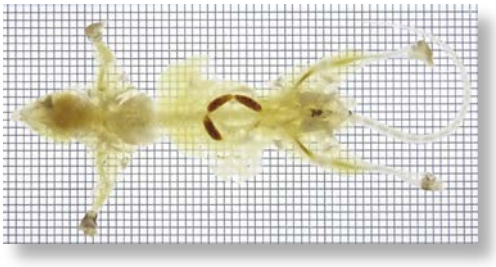
Application

- **Example of usage of adult mouse whole-body clearing**
- **Pre-treatment step of 200 mL 50% CUBIC-L at 37°C overnight**
- **Delipidation step of 200 mL CUBIC-L at 37°C for 5 days**
  (Refresh CUBIC-L on day 1, day 2 and day 4)
  - Light penetrates the organ.
  - CUBIC-L does not get colored after treatment.
    Above points are the signs of end of delipidation.
- **Pre-treatment step of 200 mL CUBIC-R+ at room temperature overnight**
- **RI matching of 200 mL CUBIC-R+ at room temperature overnight**

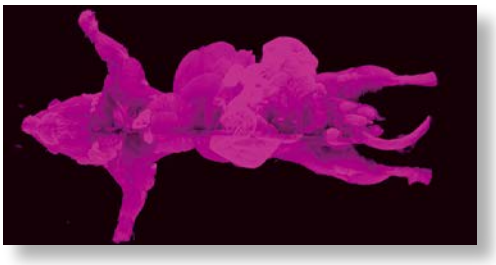
Total
- CUBIC-L : 700 mL
- CUBIC-R+ : 300 mL

The reagent volumes of the above example is in the case of usage in a 12 cm x 8 cm x 6 cm container.
Use a tube wherein the whole body can be submerged.
*For nuclear staining, use 30 μg/mL Propidium iodide (PI) and 1.5 M NaCl in PBS.

PFA : paraformaldehyde, RT : room temperature



Whole-body clearing



Whole-body clearing with propidium iodide staining

Direction for Use : Mouse whole-brain clearing with expansion protocol

Fix	Wash x 3	Pre-treatment	Delipidation	Wash	Staining	Wash	Swelling	RI match
4% PFA 1 day	PBS > 2 hr x 3	50% CUBIC-L 3 hr	CUBIC-L 5 – 14 days	PBS 1 day	Stains* 3 days	PBS 1 day	CUBIC-X1 2.5 day	CUBIC-X2 1.5 day

Process	Reagent	Temp.	Time	Notes
Tissue excision				After perfusion fixation
Tissue Fix	4% PFA in PBS	4℃	1 day	
Wash x 3	PBS	RT	> 2 hr x 3	Shake gently (Same in following steps). Total 1 day
Pre-treatment	50% CUBIC-L	37℃	3 hr	1:1 mixture of water and CUBIC-L.
Delipidation	CUBIC-L	37℃	5 – 14 days	Refresh CUBIC-L every 4 days. 5 days for 1-week-old mice 7 days for 3-week-old mice 14 days for 8-week-old and 6-month-old mice
Wash	PBS	RT	1 day	
Staining	Staining reagents*	RT	3 days	
Wash	PBS	RT	1 day	
Swelling	CUBIC-X1	4℃	2.5 days	
RI matching	CUBIC-X2	RT	1.5 days	Refresh CUBIC-X2 every 12 hours.

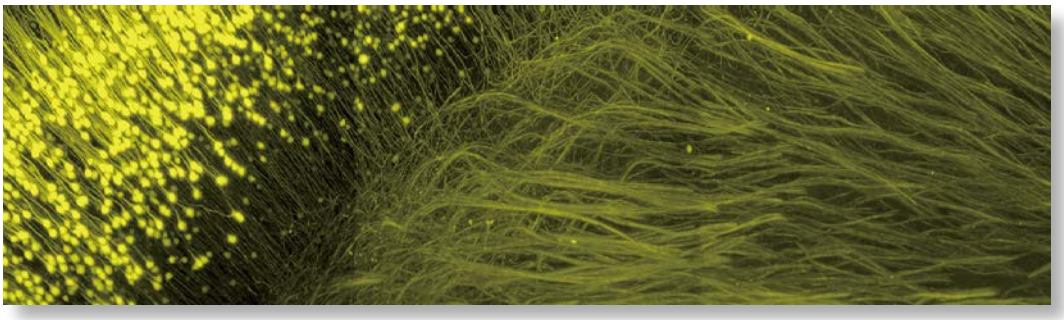
Application

- **Example of usage of mouse brain clearing and expansion**
- **Pre-treatment step of 3 mL 50% CUBIC-L at 37°C for 3 hours after PBS wash**
- **Delipidation step of 3 mL CUBIC-L at 37°C for 14 days**
  (Refresh CUBIC-L on day 4, day 8 and day 12)
- **Wash by PBS, staining by staining reagents and wash by PBS**
- **Expansion step of 30 mL CUBIC-X1 at 4°C for 2.5 days**
- **RI matching of 40 mL CUBIC-X2 at room temperature for 1.5 days**
  (Refresh CUBIC-X2 every 12 hours)

Total
- CUBIC-L : 10.5 mL
- CUBIC-X1 : 30 mL
- CUBIC-X2 : 120 mL

*For nuclear staining, use 30 μg/mL Propidium iodide (PI) and 1.5 M NaCl in PBS.
Since the expanded brains are fragile, careful handling is required after the swelling step.

PFA : paraformaldehyde, RT : room temperature



Magnified view of a transgenic mouse brain after clearing-expansion protocol

Direction for Use : For the efficient clearing of adult mouse (more than 6-week-old) whole-body or organ samples

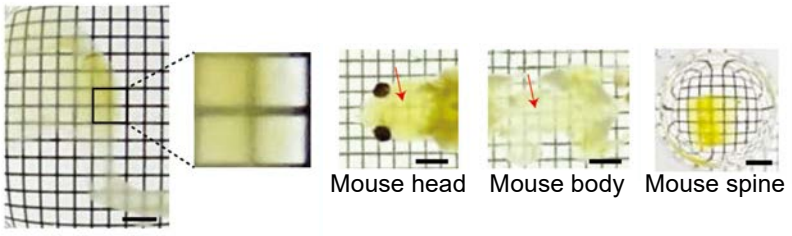
Perfusion	Delipidation	Wash	(Staining)	(Wash)	Pre-treatment	RI matching
CUBIC-P	CUBIC-L 3 – 7 days*	PBS 1 day	Stains 5 – 7 days	PBS 1 day	50% CUBIC-R+ 1 day	CUBIC-R+ 1 – 2 days

Process	Reagent	Temp.	Time	Notes
Sacrifice	Pentobarbital			Overdose of pentobarbital
Perfusion fixation	15mL PBS 20mL 4% PFA in PBS 15mL PBS 100mL CUBIC-P	4℃		After perfusion of CUBIC-P, the organs are dissected.
Delipidation	CUBIC-L	37℃	3 – 7 days*	Shake gently (same in following steps).
Wash	PBS	RT	1 day	
(Staining)	Staining reagents	RT	5 – 7 days	Optional
(Wash)	PBS	RT	1 day	Optional
Pre-treatment	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
RI matching	CUBIC-R+	RT	1 – 2 days	

*If the immersion period is longer than 4 days, the CUBIC-L should be replaced at least once.

Direction for Use : For mouse body or tissues including bone

Clearing examples

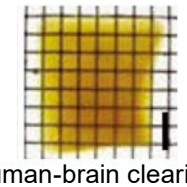


Delipidation	Wash	Decalcification	Wash	Delipidation	Wash	(Staining)	(Wash)	Pre-treatment	RI matching
CUBIC-L 3 – 7 days*	PBS 1 day	CUBIC-B 5 – 7 days	PBS 1 day	CUBIC-L 2 – 4 days	PBS 1 day	Stains 5 – 7 days	PBS 1 day	50% CUBIC-R+ 1 day	CUBIC-R+ 1 – 2 days

Process	Reagent	Temp.	Time	Notes
Tissue fixation	4% PFA in PBS	4℃	1 day	
Delipidation	CUBIC-L	37℃	3 – 7 days*	Shake gently (same in following steps).
Wash	PBS	RT	1 day	
Decalcification	CUBIC-B	37℃	5 – 7 days	The CUBIC-B should be refreshed at least once.
Wash	PBS	RT	1 day	
Delipidation	CUBIC-L	37℃	2 – 4 days	
Wash	PBS	RT	1 day	
(Staining)	Staining reagents	RT	5 – 7 days	Optional
(Wash)	PBS	RT	1 day	Optional
Pre-treatment	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
RI matching	CUBIC-R+	RT	1 – 2 days	

*If the immersion period is longer than 4 days, the CUBIC-L should be replaced at least once.

Direction for Use : For large blocks of human brain tissue



Human-brain clearing

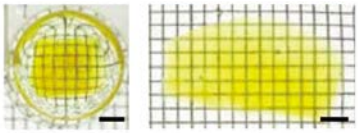
Wash PBS 1 day	Delipidation CUBIC-L 1 – 2 weeks	Wash PBS 1 day	Pre-treatment 50% CUBIC-R+ 1 day	RI matching CUBIC-R+ 1 – 2 days

Process	Reagent	Temp.	Time	Notes
Fixation	Formalin	4℃		Stored until use.
Wash	PBS	RT	1 day	Shake gently (same in following steps).
Delipidation	CUBIC-L	45℃	1 – 2 weeks	The CUBIC-L should be refreshed at least once.
Wash	PBS	RT	1 day	
Pre-treatment	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
RI matching	CUBIC-R+	RT	1 – 2 days	

The autofluorescence of cerebral cells decreased as the delipidation period increased. In order to preserve sufficient autofluorescent signals, delipidation should be finished within approximately one week.

Direction for Use : For aggressive human tissue clearing

Clearing examples



Human heart Human kidney

Wash PBS 1 day	Delipidation CUBIC-HL 1 – 2 weeks**	Wash PBS 1 day	(Staining) Stains 5 – 7 days	(Wash) PBS 1 day	Pre-treatment 50% CUBIC-R+ 1 day	RI matching CUBIC-R+ 1 – 2 days

Process	Reagent	Temp.	Time	Notes
Fixation	Formalin	4℃		Stored until use.
Wash	PBS	RT	1 day	Shake gently (same in following steps).
Delipidation	CUBIC-HL	37℃ or 45℃	1 – 2 weeks**	37 ℃ for human brain or kidney, 45 ℃ for human heart, liver, lung or spleen
Wash	PBS	RT	1 day	
(Staining)	Staining reagents	RT	5 – 7 days	Optional
(Wash)	PBS	RT	1 day	Optional
Pre-treatment	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
RI matching	CUBIC-R+	RT	1 – 2 days	

The immersion period is based on the sample size. As delipidation progresses, the apparent opacity inside the sample disappears. During delipidation, the CUBIC-HL should be replaced at least once. If delipidation needs to be prolonged beyond 2 weeks, the subsequent delipidation is recommended to be done at a lower temperature or in CUBIC-L **[T3740].

Q&A : About Staining Reagents

Q: What kind of staining reagents can be used?

A: a) Antibodies: direct fluorescent-labeled antibodies are preferred. For example, the antibodies are diluted adequately in PBS containing both 0.5% Triton X-100 and 0.01% NaN_3.
b) Nuclei staining reagents: propidium iodide can be used. Propidium iodide is diluted to 10 μg/mL with 0.1 M phosphate buffer (pH 7.4), containing 0.5 M NaCl.

Q: Can antibodies from my laboratory be used?

A: Some proteins do not change their antigenicity during fixation or tissue-clearing procedures. However, this is not conformed for all the proteins. It is recommended to initially test the antibodies you are already using.

Q: Can fluorescent-labeled secondary antibodies be used?

A: We do not have information about protocols using secondary antibodies. It takes significant amounts of time for the two-step treatment with both primary and secondary antibodies. Therefore, we recommend directly labeling your primary antibody with fluorescent reagents.

Q: What kind of fluorescent proteins can be used?

A: We assessed the retention of fluorescence intensity in GFP. Some fluorescent proteins, such as, EGFP, EYFP, mCherry, and mKate2, have been confirmed to retain their fluorescence signals (*Cell* **2014**, *157*, 726-739.)

Q&A : During Clearing Steps

Q: What kind of container is suitable?

A: Any container that is slightly larger than the organism specimen being cleared is suitable. For mouse organ clearing, a container that is slightly bigger than the organ itself is recommended because the organ may expand during the clearing procedure. CUBIC products are aqueous-based reagents, thus they can be used safely with any laboratory plasticware such as polypropylene or polyethylene.

Q: Does tissue swell? If yes, will this influence the experiment in any way?

A: The tissue or organs may expand during the clearing; However, it has been reported that relative cell position remains the same. Thus, expansion is linear and uniform.

Q: Could the sample fixation step be omitted because the organs undergo the clearing steps as soon as they are excised?

A: In the samples without fixation, the structure of cells may be destroyed, thus, they should be fixed before clearing steps.

Q: Is it possible to clear the samples after they were excised and fixed?

A: The samples which were soaked in fixing solution for several weeks or which are stored at -80°C for several months after fixation can still get cleared.

Q: What quantity of these reagents are required for tissue clearing?

A: For mouse whole-body clearing, the volume of reagents used must be sufficient to submerge the entire specimen. For example, for the whole-body clearing of a mouse, 200 to 400 mL of CUBIC-L and 100 to 200 mL of CUBIC-R+ are needed. For mouse tissue clearing, the volume of reagents needed is half the volume of the organ being cleared. For example, 20 to 40 mL of CUBIC-L and 10 to 20 mL CUBIC-R+ are needed.

Q:The clearing of organs or bodies was interrupted and did not occur successfully.

A: There are a few possible reasons. Consider the following troubleshooting options.

a) The pH of PFA solution for fixing organs or bodies is too high.
When the pH is more than 8, organs and bodies become over-fixed and are less cleared, thus, the pH should be adjusted between 7 – 7.5.

b) Delipidation is incomplete.
Samples are immersed in CUBIC-L with gentle shaking at 37 °C for 2 – 5 days or more, and fresh CUBIC-L should be used daily.

c) Clearing is incomplete.
The clearing time period can be extended. Additionally, consider replacing and using a fresh CUBIC-R+ solution.

Q: How long does it take to delipidate samples?

A: Approximately 3 days are required to delipidate the lung, intestine, pancreas and spleen of an adult mouse, and approximately 5 days to delipidate the heart, brain, liver and kidney.

Q&A : After Clearing Samples

Q: How should the reagents be disposed of following use?

A: Please dispose of the reagents according to the regulations of your institution. Reagents used to soak animal or organ samples are typically treated as medical waste. The unused CUBIC-L, and CUBIC-R+ reagents are non-flammable waste liquids. Please refer to the included package insert for reagent descriptions and constituencies.

Q: How should clearing samples be stored?

A: Clearing samples can be stored at room temperature in CUBIC-R+ or CUBIC-X2. CUBIC-R+ and CUBIC-X2 contain many solutes and a little water as a solvent. Thus, the samples should be stored sealed by parafilm or other means to prevent reagents from evaporation. The agarose gel embedding sample can also be stored at room temperature.

[How to embed in agarose gel]
Add agarose powder to the used CUBIC-R+ to a final concentration of 2%(w/v) in a tube, and dissolve it by heat. Embed samples into the mixture before gelation, and prepare the gel by cooling it. This agarose gel can be stored at room temperature, and if required, the head of the tube can be cut and the gel pushed out. The surface of the gel becomes dry and white when it is pushed out; therefore, it should be used immediately after pushing.

Q: Clearing samples cannot be observed well.

A: Light-sheet fluorescent microscopy (LSFM) or confocal laser-scanning microscopy (CLSM) is recommended for the observation of the samples. Clearing samples become gel-like and it may be difficult to cut them into thin slices. For microscopic observation, embed samples in agarose gel, cut them out into an appropriate shape, and then observe them use.

Q: What is the refractive index (RI) of CUBIC reagents?

A: The RI of CUBIC-R+ is 1.520 and that of CUBIC-X2 is 1.467. The objective lens or the immersion oils which are suitable for these RIs should be used. They should not be mixed with other solvents such as water in order to change their RIs.

Q: CUBIC-1, CUBIC-2 have been described in some papers, are they the same as CUBIC-L, CUBIC-R+?

A: CUBIC-1, CUBIC-2 differ from CUBIC-L, CUBIC-R+ in terms of their clearing ability, as CUBIC-L, CUBIC-R+ is superior. CUBIC-1 and CUBIC-L play the same role in delipidation and decoloring, and CUBIC-2 and CUBIC-R+ play the same role in RI matching. CUBIC-R also differs from CUBIC-R+. CUBIC-R is composed of nicotinamide and CUBIC-R+ is composed of *N*-methylnicotinamide. CUBIC-R+ is superior to CUBIC-R in terms of maintaining fluorescent signals.

*The clearing or staining result differ according to the samples or staining reagents. Please examine the treatment time or the concentration of staining reagents.

References

Using CUBIC-X1 and CUBIC-X2, Mouse Brain Expansion

A three-dimensional single-cell-resolution whole-brain atlas using CUBIC-X expansion microscopy and tissue clearing
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Using CUBIC-L, CUBIC-R+, CUBIC-B, CUBIC-HL, CUBIC-P, Mouse Whole Body, Brain, Lung, Liver, Leg, Kidney, Marmoset Brain, Human Brain, Kidney, Liver, Lung Clearing [Immunohistochemistry after CUBIC protocol]

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Mouse Whole Body, Brain, Lung Clearing

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With CUBIC Perfusion, Mouse Whole Body, Heart, Lung, Kidney, Liver Clearing

Whole-Body Imaging with Single-Cell Resolution by Tissue Decolorization
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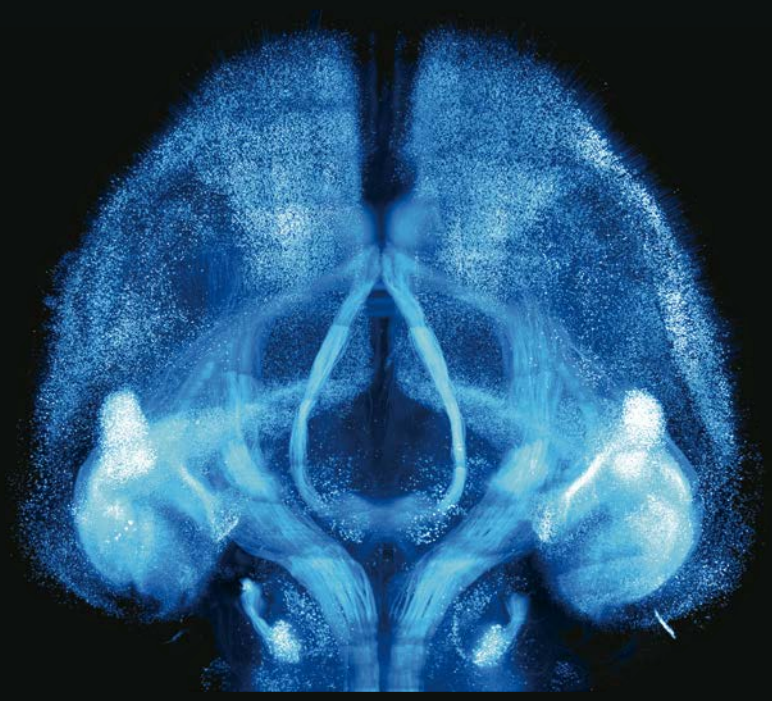
Application to Pathological Tissue Diagnosis

CUBIC pathology: three-dimensional imaging for pathological diagnosis
S. Nojima, E. A. Susaki, K. Yoshida, H. Takemoto, N. Tsujimura, S. Iijima, K. Takachi, Y. Nakahara, S. Tahara, K. Ohshima, M. Kurashige, Y. Hori, N. Wada, J. Ikeda, A. Kumanogoh, E. Morii, H. R. Ueda, *Scientific Reports* **2017**, *7*, 9269.



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