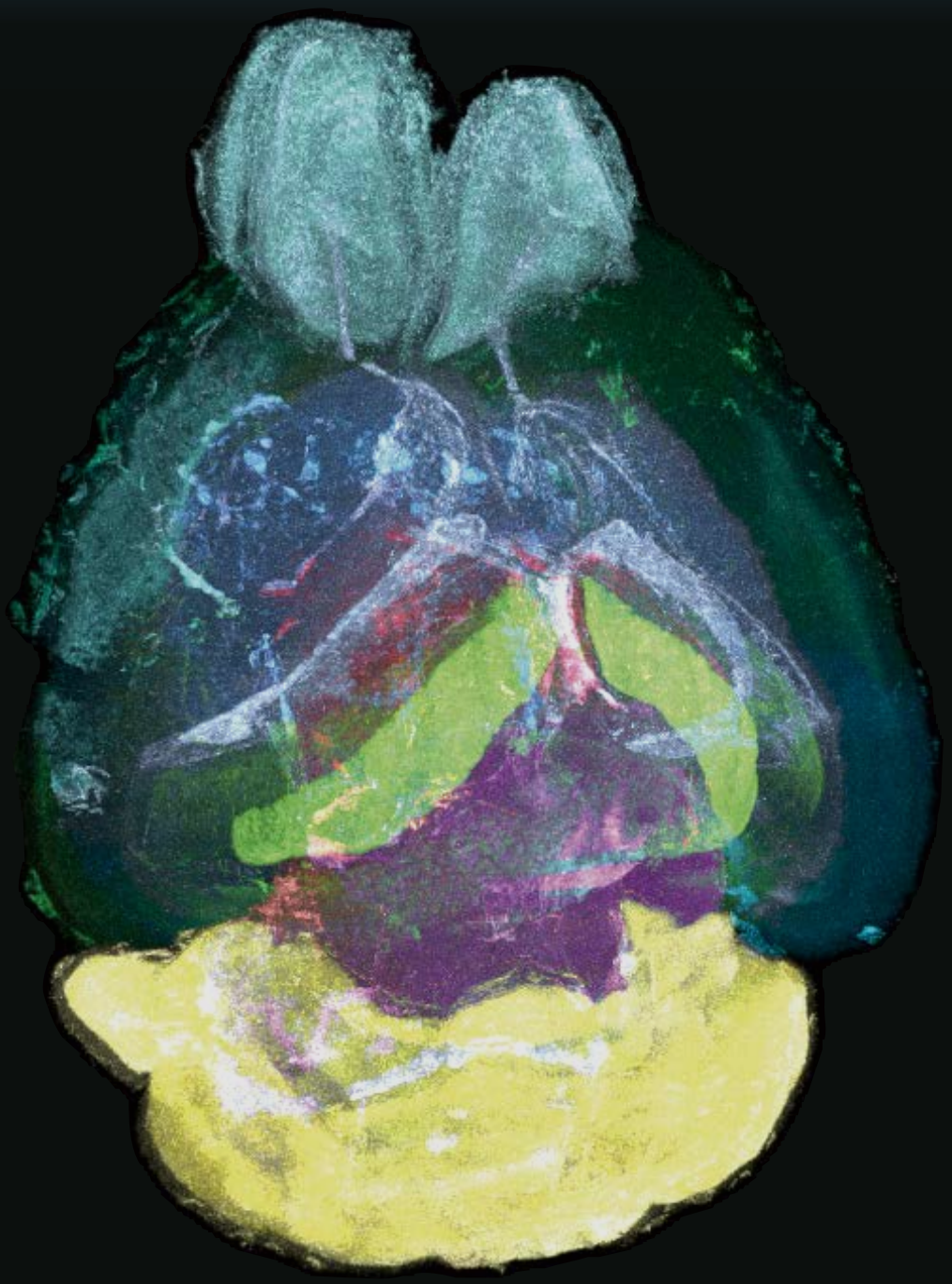
LIFE SCIENCE

TCI

CUBIC

– Animal Tissue-Clearing Reagents –
Technical Guidebook



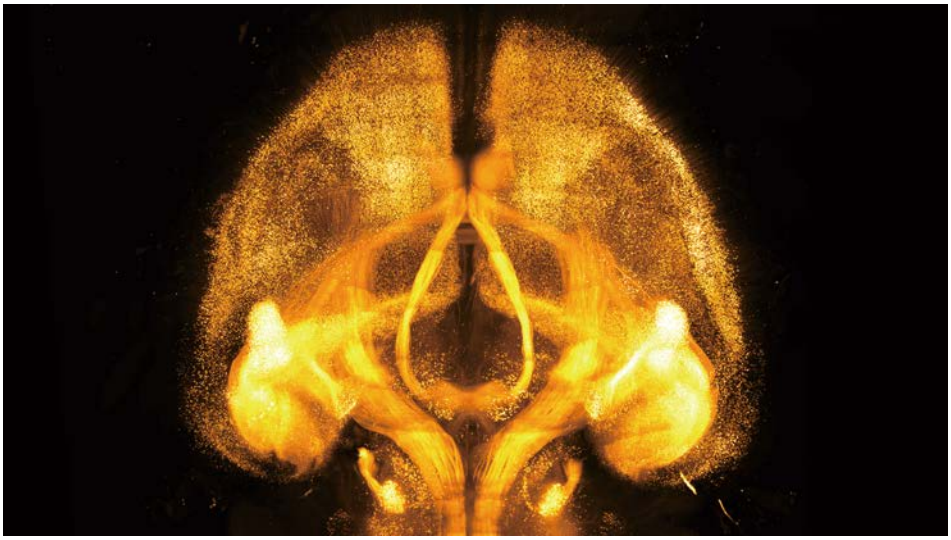
Provided by
The University of Tokyo
Graduate School of Medicine
Prof. Hiroki R. Ueda and Tomoyuki Mano

TOKYO CHEMICAL INDUSTRY CO., LTD.

What to Clear ?

For more information including CUBIC reagent tutorial videos, visit the following:

Scan or Access the URL **http://bit.ly/37lO9TD**



Please refer to your local sales representative for pricing and availability.

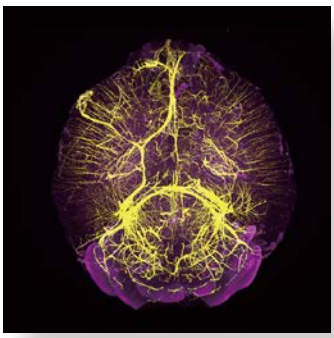
The CUBIC Lineup

CUBIC trial kit (including mounting solution) ;All-in-One basic tissue clearing kit containing CUBIC-L (50mL), CUBIC-R+(M) (50mL), and RI-Matched Mounting Solution (RI = 1.520, 50mL)	1kit	[C3942]
CUBIC-L (Delipidation and Decoloring)	25mL / 100mL / 500mL	[T3740]
CUBIC-R+(N) (RI matching)	25mL / 100mL / 500mL	[T3983]
CUBIC-R+(M) (RI Matching)	25mL / 100mL	[T3741]
CUBIC-B (Decalcification)	25mL / 100mL	[T3780]
CUBIC-HL (For Hard-to-Clear / Autofluorescent Tissues)	25mL / 100mL	[T3781]
CUBIC-P (Pre-Excision Perfusion)	25mL / 100mL	[T3782]
CUBIC-X1 (Expansion)	25mL / 100mL	[T3866]
CUBIC-X2 (Post-Expansion RI Matching)	25mL / 100mL	[T3867]
CUBIC-HV™1 3D immunostaining kit (Casein separately)	1kit	[C3717]
CUBIC-HV™1 3D nuclear staining kit	1kit	[C3709]

Related Products

Mounting Solution [RI 1.520] (for use with CUBIC-R+)	50mL	[M3294]
Mounting Solution [RI 1.467] (for use with CUBIC-X2)	50mL	[M3292]
Mouse Anti-NeuN Monoclonal Antibody	0.1mg/vial	[M3586]
Goat Anti-Mouse IgG_1 Fab Fragment Cyanine 3 Conjugate	0.05mg/vial	[G0598]

Whole-brain clearing



Whole-body clearing
with nuclear staining and immunostaining

These products were developed by
Prof. Hiroki R. Ueda (The University of Tokyo / RIKEN)
and are under invention licenses by RIKEN, Japan.
*CUBIC-HV™ is a registered trademark of
CUBICStars Co.

Advantages

- **Get started with tissue clearing with this All-in-One CUBIC trial kit [C3942].**
- **Basic protocol ;**
  Clearing of whole mouse bodies as well as animal organs can be achieved by using two reagents in sequence: CUBIC-L **[T3740]** for delipidation and either CUBIC-R+(N) **[T3983]** or CUBIC-R+(M) **[T3741]** for RI matching.

  The difference between CUBIC-R+(N) **[T3983]** and CUBIC-R+(M) **[T3741]**:
  CUBIC-R+(N) is inexpensive and easier to handle because it raises less precipitation.
  The fluorescence signal may decay, but the fluorescence signals of samples in CUBIC-R+(N) can be observed for several days after immersion. CUBIC-R+(M) is superior in retaining the fluorescence signal. However, at low temperatures such as in winter, it may precipitate. In that case, it can be resolved by placing the sample at 37°C for a few days.
  For these reasons, it is recommended to try CUBIC-R+(N) first and then use CUBIC-R+(M) if fluorescence signal cannot be found.
- **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
- **For efficiently aiding with perfusion fixation for mouse perfusion ;**
  CUBIC-P **[T3782]**
- **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
- **For staining thick and large specimens uniformly ;**
  CUBIC-HV™1 3D immunostaining kit **[C3717]** for 3D immunostaining
  CUBIC-HV™1 3D nuclear staining kit **[C3709]** for 3D nuclear staining
- **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.**

Directions for Use: Mouse Whole-Organ Clearing (The Basic Protocol)

Fixation	Wash x 3	Pre-Delipidation	Delipidation	Wash x 3	(Staining)	(Wash x 3)	(1st post-stain fixation)	(2nd post-stain fixation)	(Wash x 3)	RI match	RI match
4% PFA	PBS	50% CUBIC-L	CUBIC-L	PBS	Stains	PBS	1% FA	1% FA	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 hr	> 2 hr x 3	1 day	> 1 day

	Process	Reagent	Temp.	Time	Notes
	Tissue excision				After perfusion fixation
	Tissue Fixation	4% PFA in PBS	4°C	1 day	
	Wash x 3	PBS	RT	> 2 hr x 3	With gentle shaking (applies to all subsequent steps) Aim to perform all wash steps for a total of 24 hours (e.g. 2hr x 2 followed by once O/N)
	Pre-Delipidation	50% CUBIC-L	37°C or RT	6 – 24 hr	1:1 mixture of water and CUBIC-L (Optional)
	Delipidation	CUBIC-L	37°C	> 2 days	Refresh CUBIC-L on days 1, 2, and every other subsequent day
	Wash x 3	PBS	RT	> 2 hr x 3	
‡	(Staining)	Stains	RT	> 3 days	
‡	(Wash x 3)	PBS	RT	> 2 hr x 3	
‡	(1st post-stain fixation)	1% FA	4°C	1 day	Diluted down from 37% FA with PBS
‡	(2nd post-stain fixation)	1% FA	37°C	1 hr	
‡	(Wash x 3)	PBS	RT	> 2 hr x 3	
	RI match	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
	RI match	CUBIC-R+	RT	> 1 day	

‡Optional. Only required when performing immuno / nuclear staining.

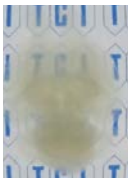
Application: Whole Adult Mouse Brain

- **Post-Excision**

- **Post- RT O/N CUBIC-L Pre-Treatment**

***All images show samples immersed in the reagent for that step.**

- **Post- 37°C 5-day CUBIC-L Delipidation**
  (CUBIC-L refreshed on days 1, 2, and 4)



Delipidation has reached its endpoint when:

- Partial transparency is acheived
- CUBIC-L remains colorless after incubation with sample

- **Post- RT O/N CUBIC-R+(M) Pre-Treatment**

- **Post- RT O/N CUBIC-R+(M) RI Matching and Immersion in Mounting Solution (RI = 1.520)**

Reagent Totals (for a 5 mL tube):

- CUBIC-L : 14 mL
- CUBIC-R+(M) : 6 mL

*Use a tube whose diameter is slightly larger than that of your sample. Reagent volumes used at each step should be half the volume of this tube.

FA: formaldehyde, O/N: overnight,
PFA: paraformaldehyde, RT: room temperature

Directions for Use: Large Samples (e.g. Mouse Whole-Body Clearing)

Pre-Delipidation	Delipidation	Wash x 3	(Staining)	(Wash x 3)	(1st post-stain fixation)	(2nd post-stain fixation)	(Wash x 3)	RI match	RI match
50% CUBIC-L	CUBIC-L	PBS	Stains	PBS	1% FA	1% FA	PBS	50% CUBIC-R+	CUBIC-R+
6 hr	> 5 days	> 2 hr x 3	> 3 days	> 2 hr x 3	1 day	1 hr	> 2 hr x 3	1 day	> 1 day

	Process	Reagent	Temp.	Time	Notes
	Perfusion fixation	PBS			After perfusion fixation, specimens need to be perfused with a 1:1 mixture of CUBIC-L and water
		4% PFA in PBS			
	Perfusion	PBS			
		50% CUBIC-L			
	Pre-Delipidation	50% CUBIC-L	37°C	> 6 hr	(Optional) Completely immersed with gentle shaking (applies to all subsequent steps)
	Delipidation	CUBIC-L	37°C	> 5 days	Refresh CUBIC-L on days 1, 2, and every other subsequent day
	Wash x 3	PBS	RT	> 2 hr x 3	Aim to perform all wash steps for a total of 24 hours (e.g. 2hr x 2 followed by once O/N)
‡	(Staining)	Stains	RT	> 3 days	
‡	(Wash x 3)	PBS	RT	> 2 hr x 3	
‡	(1st post-stain fixation)	1% FA	4°C	1 day	Diluted down from 37% FA with PBS
‡	(2nd post-stain fixation)	1% FA	37°C	1 hr	
‡	(Wash x 3)	PBS	RT	> 2 hr x 3	
	RI match	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
	RI match	CUBIC-R+	RT	> 1 day	

‡Optional. Only required when performing immuno / nuclear staining.

Application: Whole Adult Mouse

- **Pre-Treatment: 200 mL 50% CUBIC-L at 37°C O/N**
- **Delipidation: 200 mL CUBIC-L at 37°C for 5 days**

(CUBIC-L refreshed on days 1, 2, and 4)

Delipidation has reached its endpoint when:
- Partial transparency is acheived
- CUBIC-L remains colorless after incubation with sample

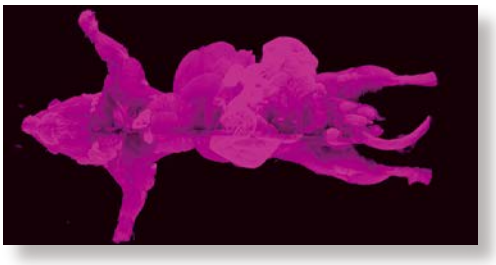
- **Pre-Treatment: 200 mL 50% CUBIC-R+(M) at RT O/N**
- **RI Matching: 200 mL CUBIC-R+(M) at RT O/N**

Reagent Totals (for a 12 cm x 8 cm x 6 cm container):
- CUBIC-L : 700 mL
- CUBIC-R+(M) : 300 mL

†Use a container that allows you to submerge your whole specimen.
†For nuclear staining, we recommend using a solution of 30 µg/mL propidium iodide (PI) and 1.5M NaCl in PBS.

FA: formaldehyde, O/N: overnight, PFA: paraformaldehyde, RT: room temperature

Whole-body clearing



Whole-body clearing with propidium iodide staining

Directions for Use: Clearing Mouse Tissue with Expansion

Fixation	Wash x 3	Pre-Delipidation	Delipidation	Wash	Staining	Wash	Fixation	Fixation	Wash x 3	Expansion	RI match
4% PFA	PBS	50% CUBIC-L	CUBIC-L	PBS	Stains	PBS	1% FA	1% FA	PBS	CUBIC-X1	CUBIC-X2
1 day	> 2 hr x 3	3 hr	5 - 14 days	1 day	3 days	1 day	1 day	1 hr	> 2 hr x 3	2.5 days	1.5 days

Process	Reagent	Temp.	Time	Notes
Tissue excision				After perfusion fixation
Tissue Fixation	4% PFA in PBS	4°C	1 day	
Wash x 3	PBS	RT	> 2 hr x 3	With gentle shaking (applies to all subsequent steps) Aim to perform all wash steps for a total of 24 hours (e.g. 2hr x 2 followed by once O/N)
Pre-Delipidation	50% CUBIC-L	37°C	3 hr	1:1 mixture of water and CUBIC-L
Delipidation	CUBIC-L	37°C	5 - 14 days	Refresh CUBIC-L every 4 days for a total of: • 5 days for 1 week-old mice • 7 days for 3 week-old mice • 14 days for greater than 8 week-old mice
Wash	PBS	RT	1 day	
Staining	Stains	RT	3 days	
Wash	PBS	RT	1 day	
Fixation	1% FA	4°C	1 day	Diluted down from 37% FA with PBS
Fixation	1% FA	37°C	1 hr	
Wash x 3	PBS	RT	> 2 hr x 3	
Expansion	CUBIC-X1	4°C	2.5 days	
RI match	CUBIC-X2	RT	1.5 days	CUBIC-X2 refreshed every 12 hours

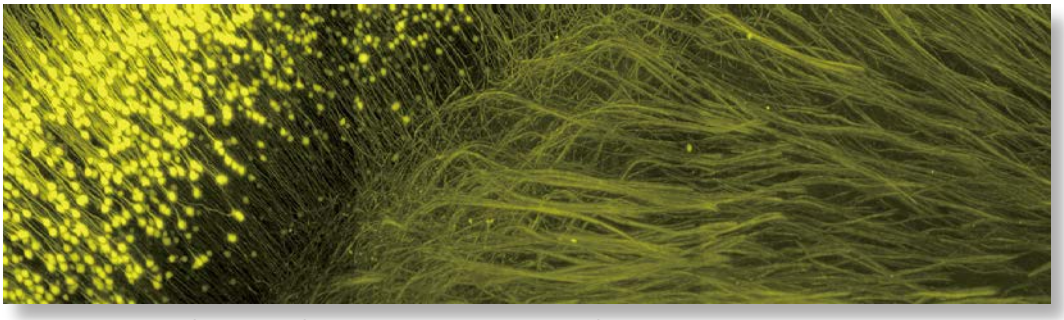
Application: Adult Mouse Brain

- **Pre-Treatment: 3 mL 50% CUBIC-L at 37°C for 3 hours (post-wash)**
- **Delipidation: 3 mL CUBIC-L at 37°C for 14 days**
  (CUBIC-L refreshed on days 4, 8, and 12)
- **Wash (PBS) → Staining → Wash (PBS)**
- **Expansion: 30 mL CUBIC-X1 at 4°C for 2.5 days**
- **RI Matching: 40 mL CUBIC-X2 at RT for 1.5 days**
  (CUBIC-X1 refreshed every 12 hours)
  Mounting: Mounting Solution (RI = 1.467)

Reagent Totals (for a 50 mL tube):
- CUBIC-L : 10.5 mL
- CUBIC-X1 : 30 mL
- CUBIC-X2 : 120 mL

†For nuclear staining, we recommend using a solution of 30 µg/mL propidium iodide (PI) and 1.5M NaCl in PBS.
†Expanded brains are fragile; careful handling is required after the expansion step.

FA: formaldehyde, O/N: overnight, PFA: paraformaldehyde, RT: room temperature



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

Directions for Use: When You Need to Make Things Even Clearer (Perfusion)

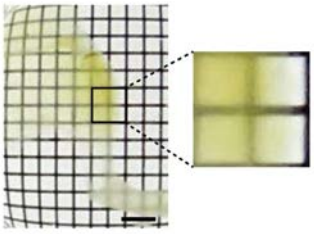
Perfusion	Delipidation	Wash	(Staining)	(Wash)	(1st post-stain fixation)	(2nd post-stain fixation)	(Wash x 3)	RI match	RI match
CUBIC-P	CUBIC-L 3 - 7 days*	PBS 1 day	Stains 5 - 7 days	PBS 1 day	1% FA 1 day	1% FA 1 hr	PBS > 2 hr x 3	50% CUBIC-R+ 1 day	CUBIC-R+ 1 - 2 days

	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°C		Wait to dissect until after perfusion fixation
	Delipidation	CUBIC-L	37°C	3 - 7 days*	With gentle shaking (applies to all subsequent steps)
	Wash	PBS	RT	1 day	
‡	(Staining)	Staining reagents	RT	5 - 7 days	
‡	(Wash)	PBS	RT	1 day	
‡	(1st post-stain fixation)	1% FA	4°C	1 day	Diluted down from 37% FA with PBS
‡	(2nd post-stain fixation)	1% FA	37°C	1 hr	
‡	(Wash x 3)	PBS	RT	> 2 hr x 3	
	RI match	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
	RI match	CUBIC-R+	RT	1 - 2 days	

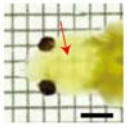
*If the immersion period is longer than 4 days, CUBIC-L should be replaced at least once.
‡Optional. Only required when performing immuno / nuclear staining.

Directions for Use: Decalcification (Whole Mouse)

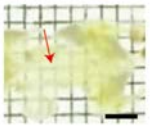
Clearing examples



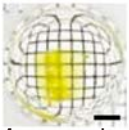
Mouse leg



Mouse head



Mouse body



Mouse spine

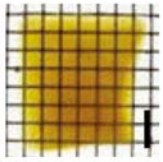
Delipidation	Wash	Decalcification	Wash	Delipidation	Wash	(Staining)	(Wash)	(1st post-stain fixation)	(2nd post-stain fixation)	(Wash x 3)	RI match	RI match
CUBIC-L	PBS	CUBIC-B	PBS	CUBIC-L	PBS	Stains	PBS	1% FA	1% FA	PBS	50% CUBIC-R+	CUBIC-R+
3 - 7 days*	1 day	5 - 7 days	1 day	2 - 4 days	1 day	5 - 7 days	1 day	1 day	1 hr	> 2 hr x 3	1 day	1 - 2 days

	Process	Reagent	Temp.	Time	Notes
	Tissue fixation	4% PFA in PBS	4°C	1 day	
	Delipidation	CUBIC-L	37°C	3 - 7 days*	With gentle shaking (applies to all subsequent steps)
	Wash	PBS	RT	1 day	
	Decalcification	CUBIC-B	37°C	5 - 7 days	CUBIC-B should be refreshed at least once
	Wash	PBS	RT	1 day	
	Delipidation	CUBIC-L	37°C	2 - 4 days	
	Wash	PBS	RT	1 day	
‡	(Staining)	Stains	RT	5 - 7 days	
‡	(Wash)	PBS	RT	1 day	
‡	(1st post-stain fixation)	1% FA	4°C	1 day	Diluted down from 37% FA with PBS
‡	(2nd post-stain fixation)	1% FA	37°C	1 hr	
‡	(Wash x 3)	PBS	RT	> 2 hr x 3	
	RI match	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
	RI match	CUBIC-R+	RT	1 - 2 days	

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

‡Optional. Only required when performing immuno / nuclear staining.

Directions for Use: Human Tissue (the Brain)



Human-brain clearing

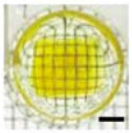
Wash	Delipidation	Wash	RI match	RI match
PBS	CUBIC-L	PBS	50% CUBIC-R+	CUBIC-R+
1 day	1 - 2 weeks	1 day	1 day	1 - 2 days

Process	Reagent	Temp.	Time	Notes
Fixation	Formalin	4°C		Store until ready for processing
Wash	PBS	RT	1 day	With gentle shaking (applies to all subsequent steps)
Delipidation	CUBIC-L	45°C	1 - 2 weeks	CUBIC-L should be refreshed at least once
Wash	PBS	RT	1 day	
RI match	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
RI match	CUBIC-R+	RT	1 - 2 days	

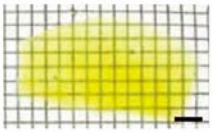
Cerebral cell autofluorescence irreversibly decreases over time in CUBIC-L due to leeching out of lipids. To preserve a level of autofluorescence sufficient for observation, delipidation should not be allowed to proceed for longer than approximately one week.

Directions for Use: Human Tissue (Hard-to-Clear / Autofluorescent Tissues)

Clearing examples



Human heart



Human kidney

Wash	Delipidation	Wash	(Staining)	(Wash)	(1st post-stain fixation)	(2nd post-stain fixation)	(Wash x 3)	RI match	RI match
PBS	CUBIC-HL	PBS	Stains	PBS	1% FA	1% FA	PBS	50% CUBIC-R+	CUBIC-R+
1 day	1 - 2 weeks**	1 day	5 - 7 days	1 day	1 day	1 hr	> 2 hr x 3	1 day	1 - 2 days

	Process	Reagent	Temp.	Time	Notes
	Fixation	Formalin	4°C		Store until ready for processing
	Wash	PBS	RT	1 day	With gentle shaking (applies to all subsequent steps)
	Delipidation	CUBIC-HL	37°C or 45°C	1 - 2 weeks**	37 °C for human brain or kidney, 45 °C for human heart, liver, lung or spleen
	Wash	PBS	RT	1 day	
‡	(Staining)	Stains	RT	5 - 7 days	
‡	(Wash)	PBS	RT	1 day	
‡	(1st post-stain fixation)	1% FA	4°C	1 day	Diluted down from 37% FA with PBS
‡	(2nd post-stain fixation)	1% FA	37°C	1 hr	
‡	(Wash x 3)	PBS	RT	> 2 hr x 3	
	RI match	50% CUBIC-R+	RT	1 day	1:1 mixture of water and CUBIC-R+
	RI match	CUBIC-R+	RT	1 - 2 days	

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

‡Optional. Only required when performing immuno / nuclear staining.

3D Tissue Staining

3D Tissue Staining Kits CUBIC-HV™

Introduction

- **Stain bulky specimens uniformly.**
  (Includes two nuclear stains and an antibody control)
- **CUBIC-L [T3740] and CUBIC-R+(M) [T3741] (sold separately) required for upstream / downstream sample processing.**

Components

CUBIC-HV™1 3D immunostaining kit (Casein separately) 1kit [C3717]

- 2 x Immunostaining Buffer (without Casein) (for 10 tests)
- Casein Sodium from Milk (Subdivided)
- 1 x Immunostaining Washing Buffer (for 10 tests)
- 10 x Immunostaining Additive (for 10 tests)
- Anti NeuN Mouse IgG1 Antibody (1mg/mL) (for 2 tests)
- 10 packs of 15mL tube

(The casein should be mixed to the buffer just before use and after mixing, the buffer should be used as soon as possible. It takes about 2 hours to dissolve the casein.)

CUBIC-HV™1 3D nuclear staining kit 1kit [C3709]

- 1 x 3D Nuclear Staining Buffer (for 10 tests)
- 100 x 3D Nuclear Staining Washing Buffer (for 10 tests)
- 200 x DAPI 2HCl (1mg/mL in Water) [for Cell Staining] (for 10 tests)
- 100 x Propidium Iodide (1mg/mL in Water) [for Cell Staining] (for 10 tests)
- 10 packs of 5mL tube

Individual component volumes optimized for use with adult mouse brains.

Contents subject to change without notice.

Reference E. A. Susaki, H. R. Ueda, *et al.*, *Nat. Commun.* **2020**, *11*, 1982. https://doi.org/10.1038/s41467-020-15906-5

※CUBIC-HV™ is manufactured by CUBICStars Co.

3D Staining Protocol (Mouse Brain)

Note: Adjustment of experimental conditions may be required to better suit your specific samples and experimental goals.

Deplipidation

Tissue dissection		
Post-fixation	: Gently shake in 4% PFA in PBS (included).	4°C, 1 day
Wash	: Gently shake in PBS +0.05% NaN_3.	RT, 3 hrs x 3
Pre-treatment	: Gently shake in 50% CUBIC-L [T3740] (v/v) in distilled water.	RT, 1 day
Delipidation	: Gently shake in CUBIC-L [T3740].	37°C, 3-5 days
Wash	: Gently shake in PBS +0.05% NaN_3.	37°C, 2 hrs x 3

Nuclear staining by 3D nuclear staining kit [C3709]

Nuclear staining	: Incubate with rotation in 1x CUBIC-HV™1 3D nuclear staining buffer (included), containing either DAPI (included) at 37°C for 5 days or Propidium Iodide (included) at 37°C for 3 days.	37°C, few days
Wash	: Gently shake in 3D nuclear staining wash buffer (included).	25°C, 2 hrs x 3

Enzyme reaction (faster and more cleanly, optional process)

Pre-treatment	: Gently shake in hyaluronidase reaction buffer (included).	4°C, 1 day
Enzyme reaction	: Gently shake in enzyme solution (included).	37°C, 1 day
Wash	: Gently shake in hyaluronidase wash buffer (included).	37°C, 2 hrs x 3

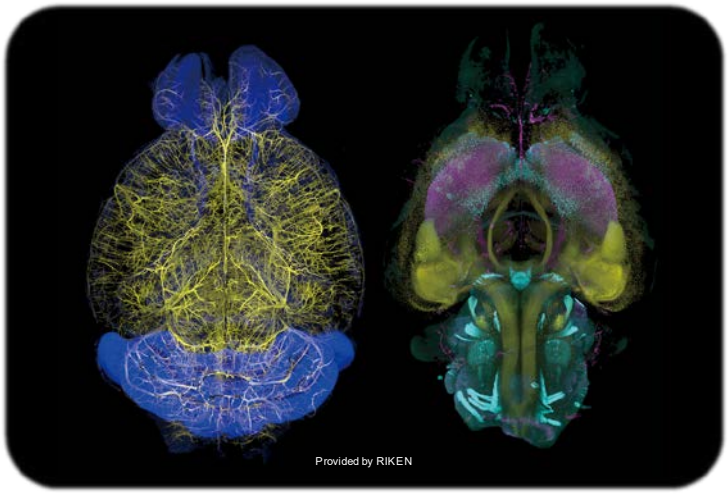
Immunostaining by 3D immunostaining kit [C3717] (Times given assume prior treatment with enzyme reaction)

Antibody Preparation	: Prior reaction with primary antibody required if using secondary antibody. (refer to included instructions for help determining reaction conditions and antibody dilutions): Mouse IgG1 Anti-NeuN primary antibody (positive control) is Included. Other primary antibodies or secondary antibodies arenot included.	37°C, 1.5 hrs
Pre-treatment	: Gently shake in CUBIC-HV™1 3D immunostaining buffer (included).	32°C, 1.5 hrs
Immunostaining	: Gently shake in premixed antibody staining solution (see below). Premixed antibody staining solution is composed of; 1x CUBIC-HV™1 3D immunostaining buffer (included), 1x CUBIC-HV™1 additive (included), and antibody mixture (prepared above).	32°C, 1 week And then 4°C, 1 day
Wash	: Gently shake in pre-chilled 1x CUBIC-HV™1 3D immunostaining wash buffer (included).	4°C, 30min x 2
Fixation	: Gently shake in 1% formaldehyde. (37% formalin solution diluted with distilled water)	4°C, 1 day And then 37°C, 1 hr
Wash	: Gently shake in PBS.	25°C, 2 hrs

RI matching

Pre-treatment	: Gently shake in 50% CUBIC-R+(M) [T3741] (v/v) in distilled water.	25°C, 1 day
RI matching	: Gently shake in CUBIC-R+(M) [T3741].	25°C, 2 days
Observation	: Samples ready for observation.	

Examples



Technical information is also available on the CUBICStars website.
https://www.cubicstars.com/cubic-hv/index.html

CUBIC-HV

Q&A : Staining Reagents

Q: What kind of tissue staining reagents can be used with CUBIC?

A: When immunostaining cleared samples, it is possible to use only fluorescent-labeled primary antibodies in the vast majority of cases, i.e. secondary antibodies are usually not required. Exact dilutions must be determined on a case-by-case basis. Dilute antibodies in PBS containing 0.5% Triton™ X-100 and 0.01% NaN_3. For nuclear staining, use propidium iodide diluted to 10 µg/mL in 0.1 M phosphate buffer (pH 7.4) with 0.5 M NaCl.

Q: What kind of nuclear staining reagents can be used in conjunction with CUBIC-HV™ kits?

A: We recommend using DAPI, SYTOX®-G, Propidium Iodide (PI), or RedDot™2. The CUBIC-HV™1 3D nuclear staining kit comes with DAPI and PI included.

Q: Do I need to purchase special antibodies?

A: Current research suggests that while a good number of proteins do not lose their antigenicity during fixation or tissue clearing, this has not been confirmed true for all proteins. Initial tests of all antibodies under consideration should be carried out.

Q: Can I use fluorescent-labeled secondary antibodies?

A: As primary antibodies are generally sufficient, we do not have information / procedures regarding the use of secondary antibodies. However, considering the time required for equilibration of each antibody within samples, we highly recommend labeling your primary antibody with fluorescent reagents in lieu of using secondary antibodies.

Q: What kinds of fluorescent tag proteins can be used for labeling?

A: Here at TCI, we test to ensure minimal loss of fluorescent signal upon tissue clearing using GFP. Minimal loss of fluorescent signal upon tissue clearing has also been demonstrated in the literature for EGFP, EYFP, mCherry, and mKate2 (*Cell* **2014**, *157*, 726.)

Q: What kinds of fluorescent dyes can be used for labeling?

A: The available literature (*Cell* **2014**, *159*, 911. and *Cell Reports* **2018**, *24*, 2196.) reports success using dyes such as FITC, Rhodamine, Alexa Fluor® 594, and Alexa Fluor® 647.

Q&A : During Clearing

Q: Do the clearing steps require use of any special kind of container?

A: As specimens – especially organs – may expand during clearing, we recommend using a container slightly larger than the specimen being cleared. Additionally, because CUBIC reagents are aqueous, they can be used safely with any laboratory plasticware, such as those made from polypropylene or polyethylene.

Q: Will my specimen swell? If so, will this have any impact on my experiment?

A: Yes, tissues / organs may expand during clearing. This expansion however is linear and uniform, meaning relative cell positions remain the same.

Q: I plan on clearing my samples as soon as they have been excised. Will this allow me to skip the fixation step?

A: Performing tissue clearing without prior fixation may result in substantial perturbation of relative cell position. As such, we highly recommend fixing any and all specimens prior to clearing.

Q: Is it possible to clear already fixed samples that have been stored for extended periods of time?

A: Yes, it is possible to use samples that have been soaked in fixing solution for several weeks, as well those that have been fixed and stored at -80°C for up to several months. However, if you are planning on immunostaining samples, be aware that storage at -80°C may cause a reduction in the antigenicity of your target protein.

Q: Is it possible to clear paraffin embedded and sectioned samples?

A: Yes, it is possible to clear paraffin-embedded samples, however they must be thermally deparaffinized prior to clearing. Samples prepared this way should be sectioned to at least 1 mm in thickness; clearing causes samples to become fragile and thicknesses of only a few µm are prohibitively difficult to work with. For more details on how to clear paraffin-embedded samples, refer to the following report: *Sci. Rep.* **2017**, *7*, 9269.

Q: What is a rough estimate of how much of each reagent I'll need?

A: For mouse whole-body clearing, the volume of reagents used must be sufficient to submerge the entire specimen (in general 200 to 400 mL of CUBIC-L and 100 to 200 mL of CUBIC-R+ are required). For organ clearing, the necessary volume of reagents works out to roughly half the volume of the organ being cleared. For example, for a 1 cm^3 specimen, 20 to 40 mL of CUBIC-L and 10 to 20 mL CUBIC-R+ are needed.

Q: Why won't my specimen clear?

A: Find common troubleshooting methods below.

a) PFA fixation solution pH too high:
A pH of greater than 8 may result in over-fixation, making it harder for samples to be cleared; try adjusting the pH to between 7 – 7.5.

b) Incomplete delipidation:
Try extending delipidation time or refreshing CUBIC-L more frequently. We recommend shaking samples immersed in CUBIC-L at 37 °C for at least 2 - 5 days, and replacing CUBIC-L with fresh reagent daily.

c) Incomplete clearing:
Try extending clearing time and/or consider changing out CUBIC-R+ reagent for fresh reagent partway through RI matching.

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

Q: Is Mounting Solution [M3294] or [M3292] necessary for the observation of cleared samples?

A: For short observation periods (< 1hr), soak lenses and samples in CUBIC-R+ or CUBIC-X2. For longer observation periods (> 1hr), we highly recommend the use of Mounting Solution. As CUBIC reagents are water-based, they tend to evaporate over the course of longer periods of observation, resulting in changing RIs and solute deposition, which in turn lead to difficulties in image acquisition.

Q: How should I dispose of CUBIC reagents?

A: Please dispose of CUBIC reagents according to the institutional regulations. Reagents used to soak animals / organ samples are typically treated as medical waste. Treat unused CUBIC-L and CUBIC-R+ reagents as non-flammable, water-containing organic waste. Please refer to the included package insert for reagent descriptions and components.

[How to embed in agarose gel]

Dissolve agarose powder into used CUBIC-R+ to a final concentration of 2% (w/v) and heat to dissolve. Immerse samples into this mixture as it cools to embed them inside. Samples prepared this way can be stored at room temperature. Cutting off of the tip of the tube (see insert) allows for the quick and easy extraction of samples, though difficult samples may require gentle heating. Following removal from the tube, the surface of the gel has a tendency to dry out and become white, hampering observation; gels should be observed immediately after removal.

Q: I'm having trouble observing my cleared samples.

A: We recommend using light-sheet fluorescent microscopy (LSFM) or confocal laser-scanning microscopy (CLSM) to observe cleared samples. Additionally, we recommend against cutting cleared samples too thinly as their gel-like nature makes them prone to distortion. Cleared samples should be observed in Mounting Solution (RI = 1.520) **[M3294]** or Mounting Solution (RI = 1.467) **[M3292]** and observed through objective lenses suited to these RIs.

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

A: The RI of CUBIC-R+ is 1.52 and that of CUBIC-X2 is 1.467. Objective lenses and immersion oils suitable for these RIs should be used. We highly discourage mixing CUBIC reagents with other solvents such as water in an attempt to change their RIs.

Q: Are CUBIC-1 and CUBIC-2 the same as CUBIC-L and CUBIC-R+?

A: CUBIC-1 and CUBIC-2 differ from CUBIC-L and CUBIC-R in terms of their clearing ability, with CUBIC-L and CUBIC-R+ being more effective. CUBIC-1 and CUBIC-L play the same role - delipidation and decoloring, and CUBIC-2 and CUBIC-R+ play the same role - RI matching. Please be aware that CUBIC-R is not the same as CUBIC-R+. While CUBIC-R contains nicotinamide, CUBIC-R+ contains *N*-methylnicotinamide and is superior to CUBIC-R in terms of its ability to maintain fluorophore fluorescence over long periods of time.

**Results may vary by sample or staining reagent. Be sure to carefully consider appropriate treatment times and staining reagent concentrations.

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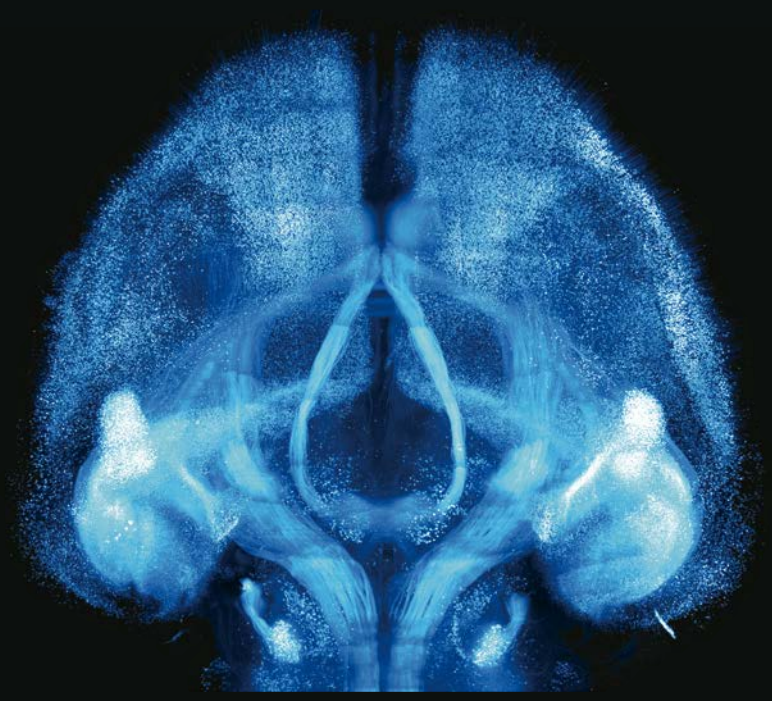

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