SUNJin Lab
Optical Clearing Innovation

Mouse Brain

Which RapiClear reagent is right for your mouse brain samples?

Sample		RapiClear (RC)	Clearing Time	FPs[1] preservation	Immuno -staining	Lipid dye	Nucleic acid dye	Method
Slice (thickness)	<500㎛	RC 1.47	hours	★★★★	✓	✓	✓	Page 1~2
	500~1000㎛	RC 1.49	hours	★★★★	✓	✓	✓	Page 1~2
	>1000㎛	RC CS[2]	weeks	★★★	✓	✗	✓	Page 3~4
Whole brain		RC 1.55[3]	week	★★★	✓[4]	N.D.	N.D.	Page 5~6
		RC CS[2]	weeks	★★★	✓[4]	✗	✓	Page 7~8

[1]FPs, Fluorescent Proteins

[2]RapiClear CS should work with *CLARITY*™ technology

[3]RapiClear 1.55 should work with Sca*l*eB4 solution

[4]Immunostaining for whole mouse brain may be limited by antibody penetration depth

Quick Guide: Slice **RapiClear 1.47/1.49**

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1  room temp. 

Perfusion:
Animal fixed with 4% PFA via cardiac perfusion

4  room temp.

Section:
Brain can be sectioned manually (e.g., 500µm or 1000µm)

7  room temp. 

Clear:
Slices were incubated in 1ml of RapiClear1.47 or 1.49 solution

(on an orbital shaker)

2  4℃ 

Fixation:
Brain subjected to 20ml PFA solution in a 50ml tube

(on an orbital shaker)

5  room temp. 

Fixation :
Slices fixed with 4% PFA again

(on an orbital shaker)

8  room temp.

Mount:
Slices placed in fresh RapiClear between two coverslips separated with iSpacer

3  room temp. 

Wash:
Wash with PBS 3 times, 20min/time

(on an orbital shaker)

6  room temp. 

Permeabilization:
Wash with PBS 3 times and then transfer samples into 2% PBST solution

(on an orbital shaker)

9  room temp.

Image:
Confocal or 2-photon

Option:
Staining of antibody or nucleic acid dyes should follow the protocol in next page

Protocol: Slices **RapiClear 1.47/1.49**

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******To prepare any type of chemical, please read the MSDS book to determine what types of safety clothing and equipment you should wear******

1. Anesthetize and perfuse the mice with ice-cold 1× PBS at ~10 ml/min. Make sure that the PBS becomes colorless by the end of perfusion.
2. Perfuse the mice with ice-cold 4% PFA at ~10 ml/min. Recover the tissues from the mice and place it in the 20 ml PFA solution. (Note: PFA is toxic. Perform all procedures in a fume hood.)
3. Incubate the brains in the PFA solution overnight at 4˚C with gentle shaking.
4. Wash the samples three times with PBS (20min/time) at RT with gentle shaking.
5. Brains can be sectioned manually (e.g., 500μm for RC147 clearing or 1000μm for RC149 clearing) using either vibratome, cryo-section, or a tissue matrix with razor blades.
6. Fix the sample slices in a 12-well plate with 4% PFA solution on an orbital shaker or rocker for 2h at RT.
7. Wash the samples three times with PBS (10min/time) at RT with gentle shaking.
8. Transfer samples into 2% PBST (2% Triton-X100) solution overnight at RT for permeabilization.
9. Option: Immunostaining

   9-1. Keep the specimen in blocking buffer on an orbital shaker or rocker at 4˚C overnight. Blocking buffer (10% normal goat serum, 2% Triton-X 100, and 0.2% sodium azide in PBS): Store this solution for only a short period of time (overnight at most) at 4˚C.

   9-2. Incubate the specimen with primary antibody in a 24-well plate (500 μl/well) on an orbital shaker or rocker at 4˚C for 3~5 days. Ab dilution buffer (1% normal goat serum, 0.2% Triton-X 100, and 0.2% sodium azide in PBS) : Store this solution for only a short period of time (overnight at most) at 4˚C.

   9-3. Wash the specimen with washing buffer for >1hr at room temperature for two times. Then, keep the specimen in washing buffer on an orbital shaker or rocker at 4˚C for overnight. (Note: washing step is quite important for immunostaining!) Washing buffer (3% NaCl and 0.2% Triton-X 100 in PBS): Store this nonhazardous buffer at 4˚C.

   9-4. Incubate the specimen with secondary antibody on an orbital shaker or rocker at 4˚C for 1~2 days.

   9-5. Wash the specimen with washing buffer for >1hr at room temperature for two times. Then, keep the specimen in washing buffer on an orbital shaker or rocker at 4˚C for overnight. (Note: washing step is quite important for immunostaining!)
10. Option: DAPI staining

    11-1. Incubate the sample with DAPI on an orbital shaker or rocker at 4˚C, overnight. DAPI (Invitrogen D3571, preparing 1mg/ml for stocks) working solution, 1:2000 of stocks.

    11-2. Wash the sample three times with PBS (20min/time) at RT. Then keep the sample in fresh PBS on an orbital shaker or rocker at 4˚C, overnight.
11. Kimwipes can be used to remove the residual PBS from the samples surface.
12. Incubate the samples in 1ml of RapiClear1.47 or 1.49 solution (it is reusable, but no more than three times) with gentle shaking and periodically check the clarity of the sample over the next few hours. Pre-warm the RC reagent to 37℃ before mounting can facilitate solution penetration. Once the sample is fully clarified, proceed with imaging preparations. Sample can be stored in RapiClear for several weeks at room temperature without affecting imaging quality.
13. Mount the sample in fresh RapiClear reagent between two coverslips separated by iSpacer. Press gently around the sticker to seal the coverslips.
14. Remove the extra solution at the edges with Kimwipes.
15. Fill the space outside the iSpacer with clear nail polish to seal the edges between the two coverslips.
16. Before RapiClear (or after), the sample may be stored indefinitely in PBS [with 0.1% (wt/vol) sodium azide] at 4˚C.
17. Confocal or 2-Photon microscopy imaging.

Quick Guide: Slices **RapiClear CS**

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1 room temp. · 1hr

Perfusion:
Animal fixed with HM solution via cardiac perfusion

2 4℃ · O/N

Fixation:
Brain subjected to 20ml HM solution in a 50ml tube

(on an orbital shaker)

3 room temp. · 4hr

Degassing & Polymerization:
Oxygen must be removed from the tissue sample before polymerization

4 room temp.

Section:
1~3mm of brain slices can be sectioned manually

5 39℃ · 7~10 days

Passive clarification:
8% SDS is used in clarification

6 37℃ · 2 days

Wash:
Wash with 0.2% PBST and 1X PBS

(on an orbital shaker)

7 room temp. · 1 day

Clear:
Add 2~3ml RapiClear CS (RCCS) solution

(on an orbital shaker)

8 room temp.

Mount:
Mount the sample in fresh RCCS solution between two coverslips separated with iSpacer

9 room temp.

Image:
Confocal or 2-photon

Option:
Staining of antibody or nucleic acid dyes should follow the protocol in next page

Protocol: Slices **RapiClear CS**

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1. Anesthetize and perfuse the mice with ice-cold 1× PBS at ~10 ml/min. Make sure that the PBS becomes colorless by the end of perfusion.
2. Perfuse the mice with hydrogel solution (HM solution) [mixture of ice-cold 4% PFA, 2% acrylamide, 0.125% Bis, 0.025% VA-044 initiator, in 1X PBS)] at ~10 ml/min. Recover the tissues from the mice and place it in the 20 ml of HM solution. (Note: PFA and acrylamide monomer are toxic. Perform all procedures in a fume hood.)
3. Incubate the brains in the HM solution at 4˚C, overnight with gentle shaking.
4. Place sample in the 50-ml conical tube filled with 10ml fresh HM solution.
5. Open the tube cap and place a plastic tube connecting with nitrogen tank main valve into the tube above HM solution (do not touch with HM solution).
6. Open the nitrogen tank main valve and adjust the control valve to fill the tube with nitrogen gas for >2min. Make sure to remove as much residual oxygen as possible.
7. Remove the nitrogen plastic tube and close the tube cap as soon as possible.
8. Transfer the closed conical tube to a 37˚C water bath for 3~4hr. Make sure that the hydrogel has solidified before proceeding to the next step. If not, repeat the degassing step and transfer the tube to 37˚C again. Gentle shaking helps in heat transfer for achieving robust and uniform polymerization.
9. Do not leave the sample in the HM (at 37˚C) for more than 5hr. Over-polymerization of the hydrogel will make tissue clearing and staining very difficult.
10. Carefully extract tissue samples from solidified hydrogel. Use Kimwipes to remove the residual gel from the tissue surface. (Note: residual gel is toxic. Perform all procedures in a fume hood.)
11. Wash the sample twice with 50 ml 200mM Tris-Boric Buffer (pH=8.5) containing 8% SDS for 1 day at RT to dialyze the remaining PFA, initiator and monomer.
12. 1~3mm of brain slices can be sectioned manually using a tissue matrix with razor blades.
13. Wash the slices twice with 50 ml 200mM Tris-Boric Buffer (pH=8.5) containing 8% SDS for 12~24hr at RT.
14. Place samples in a simple combination of 50ml tube and heated stirring plate. 100mM Tris-Boric Buffer (pH=8.5) containing 8% SDS is used to accelerate the clarification (at 39˚C). Detailed setup is available online at http://capture-clarity.org/optimized-clarity/
15. With this setup, 2mm of brain slices can be cleared in 7~10 days. The 8% SDS/100mM Tris-Boric Buffer (pH=8.5) needs to be refreshed once the pH goes below 7.5 or clearing efficiency will drop. Therefore, the buffer may need to be changed every 3~4 days.
16. Check for completion of sample clearing by assessing transparency (easy visualization of high-contrast signals such as a black-and-white grid or printed text through the tissue) and homogeneity (even distribution of transparency across the tissue). Once the tissue is cleared proceed to the next step.
17. Wash the samples twice with 50ml PBS (containing 0.1% sodium azide, NaN3) for 12hr at 37 ˚C.
18. Wash the samples twice with 50ml 0.2% PBST (0.2% Triton-X100 and 0.1% sodium azide, NaN3) for 12hr at 37˚C.
19. Wash the samples twice with 50ml PBS (containing 0.1% sodium azide, NaN3) for 1 day at 37 ˚C.
20. Option: Molecular labeling [following the descriptions in the Nature Protocols 9, 1682–1697 (2014): Advanced CLARITY for rapid and high-resolution imaging of intact tissues]
21. Incubate the samples in RapiClear CS mounting solution and periodically check the visual clarity of the sample over the next few hours. 2mm of brain slices will typically complete the refractive index (RI) homogenization process in 1~2 days. Once the tissue has been fully clarified, proceed with imaging preparations. Sample can be stored in RapiClear CS for several weeks at room temperature without affecting imaging quality.
22. Before RI homogenization (or after), the samples may be stored indefinitely in 0.2% PBST [with 0.1% (wt/vol) sodium azide] at room temperature.
23. Confocal, 2-Photon or Light-sheet microscopy imaging (for Ultramicroscope imaging setup is available online at http://capture-clarity.org/imaging/).

Quick Guide: Whole brain **RapiClear 1.55**

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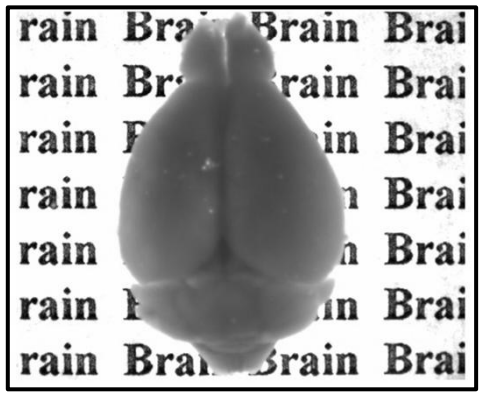
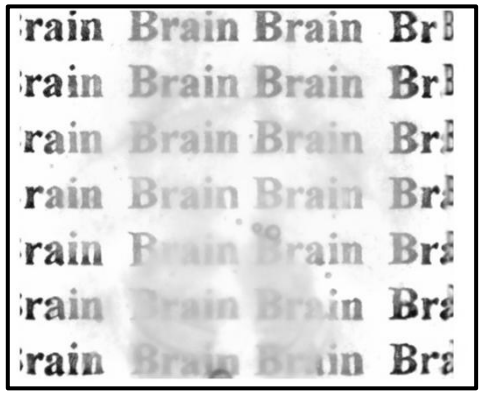
Pre-clearing



Post-clearing



1 room temp. · 1hr

Perfusion:
Mouse fixed with 4% PFA via cardiac perfusion

2 4℃ · O/N

Fixation:
Brain subjected to 20ml 4% PFA solution in a 50ml tube

(on an orbital shaker)

3 4℃ · 24hr

Cryoprotection:
Brain subjected to 50ml 20% sucrose/ PBS solution

4 -20℃ · O/N

OCT embedding:
Brain embedded in OCT and freeze

5 room temp. · 2hr

Fixation:
Brain subjected to 20ml 4% PFA again in a 50ml tube

6 room temp. · 7~12 days

Sca*l*eB4:
Brain subjected to 50ml SCALEVIEW-A2 and Sca*l*eB4 solution

(on an orbital shaker)

7 room temp. · 2~4 days

Clear:
Brain subjected to 10ml working solution prepared by diluting RapiClear 1.55 with Sca*l*eB4

8 room temp.

Mount:
Mount the brain in fresh working solution between two coverslips separated with iSpacer

9 room temp.

Image:
Confocal, 2-photon, or light-sheet microscopy

Protocol: Whole brain **RapiClear 1.55**

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1. Anesthetize and perfuse the mice with ice-cold 1× PBS at ~10 ml/min. Make sure that the PBS becomes colorless by the end of perfusion.
2. Perfuse the mice with ice-cold 4% PFA at ~10 ml/min. Recover the tissues from the mice and place it in the 20 ml of PFA solution. (Note: PFA is toxic. Perform all procedures in a fume hood.)
3. Incubate the brains in the PFA solution overnight at 4˚C with gentle shaking.
4. Wash the samples three times with PBS (20min/time) at RT with gentle shaking.
5. Transfer the samples to 20% sucrose/PBS (wt/vol) at 4˚C for 1 day.
6. Embed the samples in OCT compound and freeze overnight.
7. Wash the samples three times with PBS (20min/time) at RT with gentle shaking.
8. Fix the samples again with 4% PFA solution for 2hr at RT with gentle shaking.
9. Wash the samples three times with PBS (20min/time) at RT with gentle shaking.
10. Treat the samples first with 50ml of SCALEVIEW-A2 solution (Olympus Co., LTD.) for 2 days, then with 50ml of ScaleB4 solution (8M Urea with 0.1% Triton X-100) for the next 5~10 days at RT with gentle shaking. Refresh Sca*l*eB4 solution every 2~3 days. (Note: During the preparation of the Sca*l*eB4 solution, the solution will initially become cold to the touch. A 25~28 ˚C water bath will aid in the dissolution of the powdered urea. Solution can be stirred with a heavy magnetic bar and the temperature should never exceed 30˚C. Do not allow the temperature of the solution to rise over 30 ˚C since urea may begin to breakdown into isocyanate, which is detrimental to the proteins.)
11. Use Kimwipes to remove the residual solution from the tissue surface.
12. Transfer the samples to 5~7ml working solution prepared by 3:2 diluting RapiClear® 1.55 with Sca*l*eB4 in a 50ml tube on to a rotator (Elmi Intelli-Mixer, Rose Scientific Ltd) for 2~4 days at RT. (Note: Pre-warm the RapiClear® to 40℃ before mounting can facilitate solution penetration.)
14. Once the sample has been fully clarified, proceed with imaging preparations. Sample can be stored in working solution for several weeks at room temperature without affecting imaging quality.
15. Mount the sample in fresh working solution between two coverslips separated by iSpacer. Press gently around the sticker to seal the coverslips.
16. Remove the extra solution at the edges with Kimwipes.
17. Confocal, 2-Photon or Light-sheet microscopy imaging.

Quick Guide: Whole brain **RapiClear CS**

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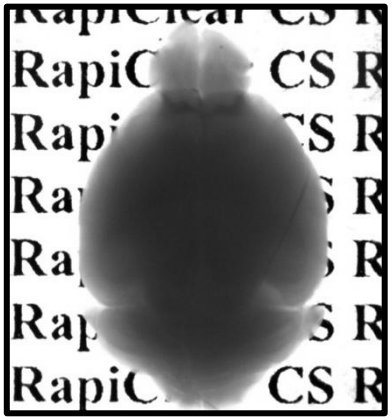
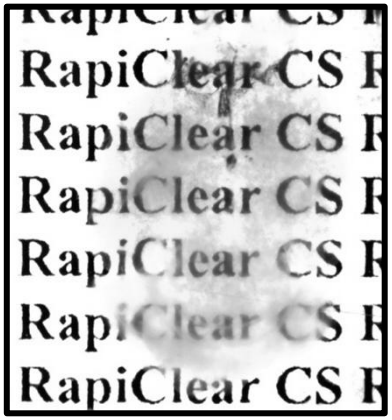
Pre-clearing



Post-clearing



1 room temp. · 1hr

Perfusion:
Animal fixed with HM solution via cardiac perfusion

2 4℃ · O/N

Fixation:
Brain subjected to 20ml HM solution in a 50ml tube

(on an orbital shaker)

3 37℃ · 4hr

Degassing & Polymerization:
Oxygen must be removed from the tissue sample before polymerization

4 39℃ · 12~21 days

Passive clarification:
8% SDS (with conical tube/stir bar) is used in clarification

5 37℃ · 2 days

Wash:
Wash with 0.2% PBST and 1X PBS

(on an orbital shaker)

6 37℃ · 1~3 days

Clear:
Add 5~10ml RapiClear CS (RCCS) solution

(on an orbital shaker)

7 room temp.

Mount:
Mount the sample in fresh RCCS solution between two coverslips separated with iSpacer

8 room temp.

Image:
Confocal, 2-photon, or light-sheet microscopy

Option:
Staining of antibody or nucleic acid dyes should follow the protocol in next page

RapiClear® CS

Protocol: Whole brain **RapiClear CS**

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1. Anesthetize and perfuse the mice with ice-cold 1× PBS at ~10 ml/min. Make sure that the PBS becomes colorless by the end of perfusion.
2. Perfuse the mice with hydrogel solution (HM solution) [mixture of ice-cold 4% PFA, 2% acrylamide, 0.125% Bis, 0.025% VA-044 initiator, in 1X PBS)] at ~10 ml/min. Recover the tissues from the mice and place it in the 20 ml of HM solution. (Note: PFA and acrylamide monomer are toxic. Perform all procedures in a fume hood.)
3. Incubate the brains in the HM solution at 4˚C, overnight with gentle shaking.
4. Place sample in the 50-ml conical tube filled with 10ml fresh HM solution
5. Open the tube cap and place a plastic tube connecting with nitrogen tank main valve into the tube above HM solution (do not touch with HM solution).
6. Open the nitrogen tank main valve and adjust the control valve to fill the tube with nitrogen gas for >2min. Make sure to remove as much residual oxygen as possible.
7. Remove the nitrogen plastic tube and close the tube cap as soon as possible.
8. Transfer the closed conical tube to a 37˚C water bath for 3~4hr. Make sure that the hydrogel has solidified before proceeding to the next step. If not, repeat the degassing step and transfer the tube to 37˚C again. Gentle shaking helps in heat transfer for achieving robust and uniform polymerization.
9. Do not leave the sample in the HM (at 37˚C) for more than 5hr. Over-polymerization of the hydrogel will make tissue clearing and staining very difficult.
10. Carefully extract tissue samples from solidified hydrogel. Use Kimwipes to remove the residual gel from the tissue surface. (Note: residual gel is toxic. Perform all procedures in a fume hood.)
11. Wash the sample twice with 50 ml 200mM Tris-Boric Buffer (pH=8.5) containing 8% SDS for 12~24hr at RT to dialyze the remaining PFA, initiator and monomer.
12. Passive clearing of hydrogel-embedded tissue .
13. Place samples in a simple combination of 50ml tube and heated stirring plate. 100mM Tris-Boric Buffer (pH=8.5) containing 8% SDS is used to accelerate the clarification (at 39˚C). Detailed setup is available online at http://capture-clarity.org/optimized-clarity/
14. With this setup, Brains can be cleared in 12~21 days. The 8% SDS/100mM Tris-Boric Buffer (pH=8.5) needs to be refreshed once the pH goes below 7.5 or clearing efficiency will drop. Therefore, the buffer may need to be changed every 3~4 days.
15. Check for completion of sample clearing by assessing transparency (easy visualization of high-contrast signals such as a black-and-white grid or printed text through the tissue) and homogeneity (even distribution of transparency across the tissue). Once the tissue is cleared proceed to the next step.
16. Wash the samples twice with 50ml PBS (containing 0.1% sodium azide, NaN3) for 12hr at 37 ˚C.
17. Wash the samples twice with 50ml 0.2% PBST (0.2% Triton-X100 and 0.1% sodium azide, NaN3) for 12hr at 37˚C.
18. Wash the samples twice with 50ml PBS (containing 0.1% sodium azide, NaN3) for 1 day at 37 ˚C.
19. Option: Molecular labeling [following the descriptions in the Nature Protocols 9, 1682–1697 (2014): Advanced CLARITY for rapid and high-resolution imaging of intact tissues]
20. Incubate the samples in RapiClear CS mounting solution and periodically check the visual clarity of the sample over the next few hours. An intact mouse brain will typically complete the refractive index (RI) homogenization process in 1~3 days. Once the tissue has been fully clarified, proceed with imaging preparations. Sample can be stored in RapiClear CS for several weeks at room temperature without affecting imaging quality.
21. Before RI homogenization (or after), the tissue may be stored indefinitely in 0.2% PBST [with 0.1% (wt/vol) sodium azide] at room temperature.
22. Confocal, 2-Photon or Light-sheet microscopy imaging (for Ultramicroscope imaging setup is available online at http://capture-clarity.org/imaging/).