Modified iDISCO+ protocol

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Overview

The iDISCO+ protocol (Renier et al., 2016) can be used to clear whole mouse brains with isotropic shrinkage and is compatible with antibody labeling. However, immunolabeling can rapidly decrease in deep brain structures if brains are not halved prior to starting the protocol. Here, we incorporated additional steps to enhance antibody penetration and improve decolorization based on the SHANEL protocol (Zhao et al., 2020). Changes to the iDISCO+ protocol include:

1. Decolorization and permeabilization with CHAPS to remove residual blood, which scatters light.
2. Loosening extracellular matrix with guanidine hydrochloride.
3. Permeabilization with CHAPS before antibody incubation.
4. Adding CHAPS to primary and secondary diluent for greater antibody penetration.
5. Multiple DBE incubations for improved refractive index (RI) matching.

Reagents

Abbreviation	Name	Supplier, cat. no.
10X PBS	Phosphate-Buffered Saline	Gibco, 70011044
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate	Anatrace, C316
DBE	Dibenzyl ether	Sigma, 108014 Alfa Aesar, A18447
DCM	Dichloromethane	Sigma, 270997-12X100mL
DMSO	Dimethyl sulfoxide	Sigma, M81802
Guanidine HCL	Carbamimidoylazanium chloride	Alfa Aesar, A13543
H_2O_2	Hydrogen Peroxide	Sigma, 216763
Heparin	Sodium heparin	Serva, 24590.01
MeOH	Methanol	Fisher, AC268280010
Na_3N	Sodium Azide	Sigma, S2002
NDS	Normal Donkey Serum	Fisher, 50413115
MDEA	N-Methyldiethanolamine	Sigma, 471828
PFA	Paraformaldehyde	Fisher, 50-980-487
Triton X-100	2-[4-(2,4,4-trimethylpentan-2-yl) phenoxy]ethanol	Sigma, T9284
Tween-20	Polyoxyethylene (20) sorbitan monolaurate	Fisher, BP337

Solutions to prepare

10% CHAPS/ 25% MDEA permeabilization / decolorization solution (10 mL)

CHAPS	1 g
MDEA	2.5 mL
10% Na_3N	20 µL

Fill to 10mL with 0.1M PBS (1X)

PTx.2 (1 L)

10X PBS	100 mL
Triton X-100	2 mL
10% Na_3N	2 mL

Fill to 1 L with dH_20

PBS w/ Heparin (1 L)

10X PBS	100 mL
10 mg/mL Heparin (in PBS)	1 mL

Fill to 1 L with dH_20

Blocking solution (50 mL)

NDS	3 mL
DMSO	5 mL
Fill to 50 mL with PTx.2	

4M Guanidine Solution (10 mL)

Guanidine HCL	3.82 g
CHAPS	0.1 g
Fill to 10 mL with 0.1M PBS	

PTwH (1L)

10X PBS	100 mL
Tween-20	2 mL
10 mg/mL Heparin in PBS	1 mL
10% Na_3N	2 mL
Fill to 1 L with dH_20	

5% H_2O_2 in 80% MeOH (30 mL)

30% H_2O_2	5 mL
100% MeOH	25 mL

Antibody Diluent (50 mL)

NDS	1.5 mL
CHAPS	1.25 g
Fill to 50 mL with PTwH	

Notes:

- All steps are performed in 5 mL Eppendorf centrifuge tubes (Eppendorf cat. no. 0030119401). The same tube can be used until RI matching in DBE, where a fresh tube should be used after the first DBE change.
- Solutions containing DCM will cause brains to float to the top of the tube. Since the tubes cannot be filled completely, if brains are shaken with the tubes horizontally, they will be repeatedly exposed to air. This can be prevented by lifting the tapered end of the tube slightly so that the air bubble is trapped in the narrow part of the tube.
- Wherever nutation is specified, do not shake instead. Nutation involves inversion of the tube.
- Solutions containing DCM should be handled in a fume hood with proper PPE.
- DBE is not volatile and can be used on a bench, but it is very difficult to clean up if spilled. 100% ethanol spray can be used to clean spills.
- Peroxide in methanol will bleach skin.
- CHAPS was found to be compatible with anti-DsRed Polyclonal Antibody (Takara cat. no 632496) and anti-GFP (Aves cat. no. GFP-1020). However, we have not tested it with other antibodies. Test your antibodies in tissue sections with the antibody diluent containing CHAPS before starting the protocol. CHAPS concentration may need to be changed on a case-by-case basis.

Antibodies

Primary antibody		Secondary antibody	
Antibody	**Cat no.**	**Antibody**	**Cat no.**
GFP	Aves, GFP1020	Alexa 647	Life Technologies, A32933
DsRed	Takara, 632496	Alexa 546	Life Technologies, A11035

Whole-brain clearing procedures

Animal perfusion (0d)

1. Prepare 4% PFA in 0.1M PBS and 0.1M PBS containing 1uL/mL of 10mg/mL heparin sodium.
2. Perfuse animals transcardially with freshly made PBS w/ heparin, followed by 4% PFA solution
   Note: Including heparin improves perfusion by reducing clotting.
3. Postfix overnight at 4^{o}C in 4% PFA in 0.1M PBS.

Permeabilization and decolorization (+1d)

4. Wash samples in 0.1M PBS for 10 min and transfer to 5 mL centrifuge tubes (Eppendorf, cat. No. 0030119401).
   Note: Samples can be stored at 4^{o}C in 0.1M PBS containing 0.01% sodium azide.
5. Incubate brains in 10% CHAPS/25% MDEA solution w/ nutation at 37^{o}C for 48h.
   Note 1: We use a Bellco Autoblot micro hybridization oven w/ rotisserie for this step.
   Note 2: CHAPS is light-sensitive; wrap samples in foil to protect from light.
   Note 3: Solution should turn green. This is the reaction of MDEA with heme.

Wash (+3d)

6. Wash with 0.1M PBS. Perform three to five solution changes and leave overnight to 24h on an orbital shaker (115 RPM) at room temperature.

Dehydration and delipidation (+4d)

All steps are performed on an orbital shaker (115 RPM) at room temperature.

7. 20% MeOH in dH_20; 1h
8. 40% MeOH in dH_20; 1h
9. 60% MeOH in dH_20; 1h
10. 80% MeOH in dH_20; 1h
11. 100% MeOH; 1h
12. 100% MeOH; 1h
13. 2:1 DCM:MeOH; overnight

Peroxide Bleaching (+5d)

Steps 14 & 15 are performed on an orbital shaker (115 RPM) at room temperature.

14. 100% MeOH; 4h
15. 100% MeOH; 4h
16. 5% H_2O_2 in 80% MeOH; overnight at 4^{o}C, no shaking

Rehydration and extracellular matrix treatment with guanidine (+6d)

Steps 17 to 22 are performed on an orbital shaker (115 RPM) at room temperature.

17. 60% MeOH in dH_20; 1h
18. 40% MeOH in dH_20; 1h
19. 20% MeOH in dH_20; 1h
20. 0.1M PBS; 1h

21. PTx.2; 1h
22. 4M guanidine hydrochloride and 1% CHAPS in 0.1M PBS for 24 hrs

    Note: After guanidine wash, brains will appear swollen and almost clear. They will shrink and turn opaque after PBS washing.

Wash (+7d)

23. Wash 3 x 5 min in 0.1M PBS; leave overnight at room temperature

Permeabilization (+8d)

24. Incubate samples in 10% CHAPS/25% MDEA solution w/ nutation at 37^{o}C overnight

Blocking (+9d)

25. Incubate samples in blocking solution w/ nutation at 37^{o}C overnight

Primary antibody staining (+10d)

26. Incubate samples in antibody diluent containing primary antibody w/ nutation for 7d at 37^{o}C

    Note: CHAPS is light-sensitive; wrap samples in foil to protect from light.

Wash (+17d)

27. Wash 5x in PTwH over 24 h on an orbital shaker (115 RPM) at room temperature.

Secondary antibody staining (+18d)

28. Incubate samples in antibody diluent containing secondary antibody w/ nutation for 7d at 37^{o}C

    Note 1: To reduce secondary antibody aggregates, syringe filter (0.2 μm) the diluent after adding secondary antibody and dispense into a clean centrifuge tube.

    Note 2: CHAPS is light-sensitive; wrap samples in foil to protect from light.

Wash (+25d)

29. Wash 5x in PTwH over 24h an orbital shaker (115 RPM) at room temperature.

Dehydration (+26d)

Steps 30-34 are performed on an orbital shaker (115 RPM) at room temperature.

30. 20% MeOH in dH_20; 1h
31. 40% MeOH in dH_20; 1h
32. 60% MeOH in dH_20; 1h
33. 80% MeOH in dH_20; 1h
34. 100% MeOH; 1h
35. 100% MeOH; overnight at 4^{o}C, no shaking

Clearing (+27d)

36. Wash in 2:1 DCM:MeOH for 3h, followed by two 100% DCM washes for 15 min each.
37. DBE overnight at 4^{o}C. Change 4x DBE, store at 4^{o}C. Samples are ready for imaging after the final DBE change.

    Note 1: Multiple DBE changes are required for proper RI matching. The first DBE wash can be done in the same tube used for the protocol, but use a fresh tube after that. Residual DCM will affect RI matching.

Note 2: Samples are RI matched once the edges of the sample are not visible. However, multiple DBE washes are required for complete RI matching. When tissue is placed into a fresh DBE solution, there should be no fluid turbulence visible and the edges of the tissue should not be visible. This may take two overnight incubations to complete.

Note 3: Use the same DBE used for RI matching to fill the imaging chamber on the microscope.

Note 4: In wash solutions containing DCM, brains will float to the top of the tube. This can result in air bubbles being caught in the ventricles (e.g., if brains are exposed to air). If air bubbles are present after DBE incubation, they can be removed by centrifugation at 500g for 10 min. This can be repeated until bubbles are gone.

Reverse clearing procedures

Cleared samples can be rehydrated and sectioned to perform traditional immunohistochemistry. We have found rehydrated tissue to be compatible with the following antibodies:

Primary antibody		Secondary antibody	
Antibody	**Cat no.**	**Antibody**	**Cat no.**
CHAT	Millipore, AB144P	Alexa 488	Life Technologies, A11039
CTIP2	Abcam, ab18465	Alexa 546	Life Technologies, A11035
DsRed	Takara, 632496	Alexa 546	Life Technologies, A10040
GAD67	Millipore, MAB5406	Alexa 594	Life Technologies, A11058
GFP	Aves, GFP1020	Alexa 647	Life Technologies, A32933
mCherry	Abcam, ab205402		
NEUN	Millipore, MAB5364		
Reelin	Millipore, MAB5364		
WFS1	Protein Tech, 11558-1-AP		

Protocol

1. Remove cleared sample from DBE and place into a clean tube containing 100% DCM.
2. Wash with fresh DCM for 2x 15 min on an orbital shaker (115 RPM) at room temperature.
3. Incubate with 2:1 DCM:MeOH; overnight on an orbital shaker (115 RPM) at room temperature.

Steps 4 to 10 are performed on an orbital shaker (115 RPM) at room temperature.

4. 100% MeOH; 1h
5. 100% MeOH; 1h
6. 80% MeOH in dH_20; 1h
7. 60% MeOH in dH_20; 1h
8. 40% MeOH in dH_20; 1h
9. 20% MeOH in dH_20; 1h
10. 0.1M PBS; 1h
11. Samples can be sectioned on a vibratome in 0.1M PBS containing 0.05% Triton X-100 or stored at 4^{o}C in 0.1M PBS containing preservative (e.g., sodium azide).

Note 1: Rehydrated tissue sections are more hydrophobic than typical PFA-fixed tissue. If rehydrated tissue is placed into PBS without detergent, it will float to the top of the solution.

Note 2: Vibratome sectioning can be performed using PBS containing 0.05% Triton X-100 and cutting speed should be reduced by ~50% of typical speed used for conventional PFA-fixed tissue.

Note 3: We have not tried cryostat sectioning of rehydrated tissue.

Note 4: When mounting tissue onto charged slides (e.g., Fisher Superfrost Plus, cat. No. 12-550-15), it may be helpful to use PBS containing 0.05% Triton X-100 to prevent the tissue from adhering to the glass and/or tearing.

References

1. Renier N, et al. (2016). 165, 1789-1802. PMID: 27238021.
2. Zhou S, etc al. (2020). Cell. 180, 796-812e19. PMID: 32059778.