iDISCO+ protocol

Recommendations for sample handling

The following are given as a general guideline and may vary for specific applications.

Sample type	Incubation time (n)	Solution volume
Embryonic:		
E10-E11 embryo	1d	1.6mL
E12 embryo	2d	1.6mL
E13-E14 embryo	3d	1.6mL
E15-16 embryo	4d	4mL
E18 head	4d	4mL
Adult organ	4d	1.6mL
Adult brain:		
hindbrain + cerebellum	3d	1.6mL
cut hemisphere	5d	1.6mL
Whole brain	7d	4.5mL

To insure the best staining and Imaging conditions we recommend trimming the sample to a size most relevant for the specific biological question.

Buffers

PTx.2 (1L)

- 100mL PBS 10X
- 2mL TritonX-100

PTwH (1L)

- 100mL PBS 10X
- 2mL Tween-20
- 1mL of 10mg/mL Heparin stock solution

Permeabilization Solution (500mL)

- 400mL PTx.2
- 11.5g of Glycine
- 100mL of DMSO

Blocking Solution (50mL)

- 42mL PTx.2
- 3mL of Donkey Serum
- 5mL of DMSO

Add .02% NaN_3 to all stock solutions to prevent microbial growth.

Bench Protocol

Sample Collection

Embryo:

1. Collect E10.5-E16.5 mouse embryos in ice-cold Leibovitz L-15 or PBS.
2. Keep on ice for 5min to drain blood from umbilical cord.
3. Fix in 1xPBS/4%PFA at 4°c, o/n with shaking.
4. Fix at room temperature (RT) 1h (optional).
5. Wash in PBS with shaking: RT 30min x 3times.

Can store in PBS with .02% NaN_3 at 4°.

Adult:

1. Anesthetize the mouse.
2. Perfuse with 20mL PBS.
3. Perfuse with 20mL 4%PFA/PBS.
4. Dissect the brain/organ and trim to the appropriate size.
5. Fix in 1xPBS/4%PFA at 4°c, o/n with shaking, then RT 1h.
6. Wash in PBS with shaking: RT 30min x 3times.

Can store in PBS with .02% NaN_3 at 4°.

Sample Pretreatment with Methanol

Before staining make sure antibodies are compatible with methanol pretreatment. Instruction on how to do so are listed on the last page.

1. Dehydrate with methanol/H_2O series: 20%, 40%, 60%, 80%, 100%; 1h each.
2. Wash further with 100% methanol for 1h and then chill the sample at 4°c.
3. Bleach in chilled fresh 5%H_2O_2 in methanol (1 volume 30% H2O2 to 5 volumes MeOH), overnight at 4°c.
4. Rehydrate with methanol/H2O series: 80%, 60%, 40%, 20%, PBS; 1h each at RT.
5. Wash in **PTx.2** RT 1h x2 at RT.

Centrifuging secondary antibody solution at 20000g for 10 minutes can prevent formation of precipitates in the sample. Alternatively, you can syringe-filter the solution at 0.2µm.

Alternative Pretreatment (<1mm (adult) or embryos only)

1. Wash fixed samples in **PTx.2**, RT 1h x2.
2. Incubate in 1xPBS/0.2%TritonX-100/20%DMSO, 37°C, o/n.
3. Incubate in 1xPBS/0.1%Tween-20/0.1%TritonX-100/0.1%Deoxycholate/0.1%NP40/20%DMSO, 37°C o/n.
4. Wash in **PTx.2**, RT 1h x 2.

Immunolabeling

Perform all steps in closed tubes. Fully fill tubes to prevent oxidation

1. Incubate samples in **Permeabilization Solution**, 37°C $^{n}/_{2}$ days (max. 2 days)
2. Block in **Blocking Solution**, 37 °, $^{n}/_{2}$ days (max. 2 days).
3. Incubate with primary antibody in **PTwH**/5%DMSO/3% Donkey Serum, 37°, **n** days.
4. Wash in **PTwH** for 4-5 times until the next day.
5. Incubate with secondary antibody in **PTwH**/3% Donkey Serum, 37°, **n** days.
6. Wash in **PTwH** for 4-5 times until the next day.

Clearing

1. Dehydrate in methanol/H_2O series: 20%, 40%, 60%, 80%, 100%, 100%; 1hr each at RT. Can be left optionally overnight at RT at this point.
2. 3H incubation, with shaking, in 66% DCM / 33% Methanol at RT
3. Incubate in 100% DCM (Sigma **270997-12X100ML**) 15 minutes twice (with shaking) to wash the MeOH.
4. Incubate in DiBenzyl Ether (DBE, Sigma **108014-1KG**) (no shaking). The tube should be filled almost completely with DBE to prevent the air from oxidizing the sample. Before imaging, invert the tube a couple of time to finish mixing the solution.

Read the safety data sheet (MSDS) for the use of DCM and DBE. Incubations and storage can be done in plastic Eppendorf tubes.

Imaging

Light sheet microscope

The use of a light sheet microscope (e.g. LaVision Biotec.) is recommended. The sample is ready to be directly imaged in the microscope chamber filled with DBE. Light sheet allows fast imaging of a large field of view, with very deep optical penetration and limited photobleaching.

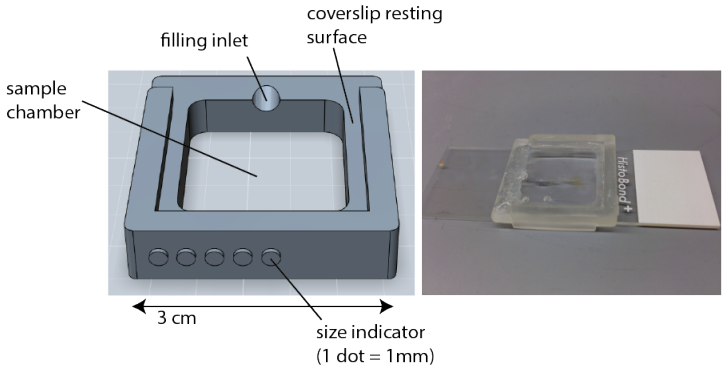
Scanning microscope (confocal or 2-photon)

Upright confocal and 2-photon microscopes can image the sample with a depth up to the working distance of the objective at high resolution. A chamber has to be built to confine the DBE and protect the microscope. To build an imaging chamber:

1. The chamber (Script provided at iDISCO.info) can be 3D printed with Visijet M3 Crystal resin, which is resistant to DBE.
2. Secure the chamber to the microscope slide with Kwik-sil epoxy (VWR). This epoxy does not permanently bond so that sample and spacers can be recovered after imaging. Make sure there are no gaps that the DBE can leak through.
3. Place a drop of epoxy in the center of the chamber and place sample on drop.
4. Close the chamber by gluing a coverslip to the spacer with the epoxy. Do not glue over filling inlet.
5. Fill the chamber with DBE with a pipet through the filling inlet.
6. Plug the inlet with epoxy.

A chamber can also be built up using dental epoxy if access to a 3D printer in not available. Also, online 3D printing services can provide a good alternative. http://www.3dhubs.com is a good way to start.

Chamber for two photon or confocal microscope



Antibody validation

To assess the methanol compatibility of untested antibodies, we recommend doing the following:

1. Collect 20µm frozen sections of the PFA fixed tissue of interest on superfrost slides. Floating vibratome sections also work.
2. Incubate the sections for 3h at least in 100% methanol at room temperature
3. Rehydrate in PBS directly and proceed with the immunostaining normally. Use non methanol treated slides as a positive control.

If the antibody yields a good signal to noise ratio, the antibody is then compatible with the methanol treatment and should work in whole-mount. If the signal is strongly diminished after the methanol treatment, one can use the non-methanol protocol, or test alternative antibodies against the target protein.

Antibody Concentration and Choice

Antibody concentration is the most important parameter to optimize for a successful staining.

- If you notice a "ring" background around the edges of the sample, the antibody is causing a non-specific staining, indicating that the concentration is too high. This will also cause poor diffusion. Reduce the concentration.
- If you don't have any staining deep in the sample AND don't have a ring background, the antibody is depleted by the antigens and the concentration should be increased. If increasing the concentration leads to the formation of a ring background, or is not economically viable, another reference should be used, or the antigen is too concentrated for whole-mount labeling.
- If you have a weak staining at the center, but still visible, the incubation time should be increased.
- Never use antibodies raised in mouse on mouse tissue, as the secondary IgGs will bind to the endogenous mouse IgG in the vasculature.
- It is very often a good investment to spend time screening antibodies from different vendors for a given antigen. Not all antibodies are made equal!
- Secondary antibody concentrations are less critical, but concentrations should be within the same range as for the primary IgG concentrations.

Questions?

You can visit http://www.idisco.info to get the latest public updates on the protocol and validated antibodies.

Reagents list

We use the following reagents for iDISCO+. Most reagents may be replaced by similar products from other vendors, but those replacement should be carefully considered. It is critical to use very high quality organic solvents to prevent oxidation of the sample during clearing.

We use double distilled water (MilliQ system).

Reagent	Reference
PBS 10X	Ambion AM9624
Triton-X100	Sigma X100-500ML
Tween-20	Sigma P9416-100ML
DMSO	Fisher D128-4
Sodium Azide	58032-100G
Donkey Serum	Jackson Immunoresearch 017-000-121
Glycine	Sigma G7126-500G
Heparin	Sigma H3393-50KU
Methanol	Fisher A412SK-4
Hydrogen Peroxide 30%	Sigma 216763-100ML
DiChloroMethane	Sigma 270997-12X100ML
DiBenzylEther	Sigma 108014-1KG or 3KG
ParaFormAldehyde 16%	EMS 15710-S

Consumables and hardware

Reagent	Reference
Tubes (small samples)	Eppendorf 2mL
Tubes (large samples)	Eppendorf 5mL
Orbital shaker	VWR nutating mixer
Hybridization oven	VWR 5420
	With carousel 47746-112