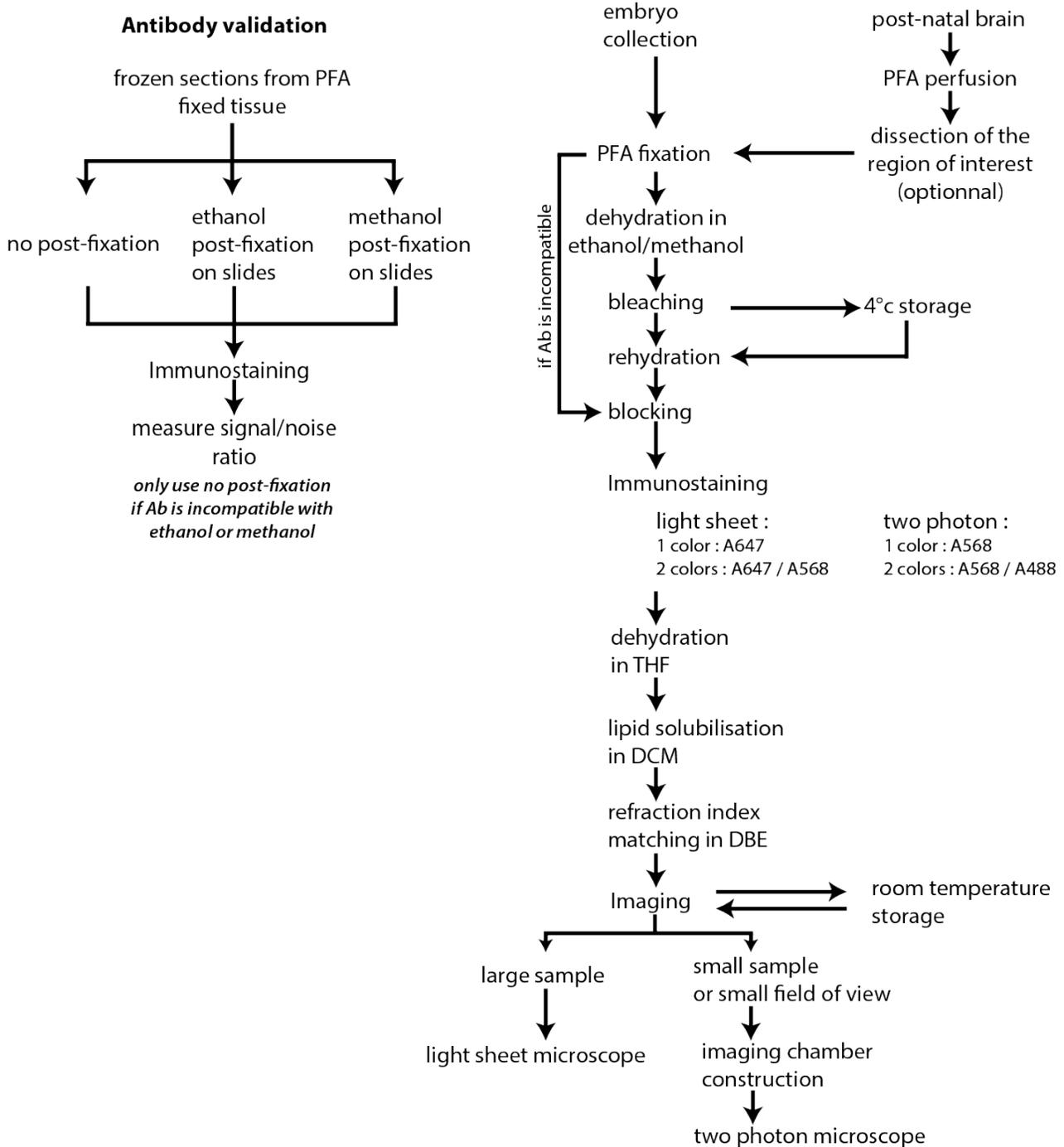


# iDISCO protocol

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## Workflow



## Recommendations for sample handling

Times and volumes are given for as a general guideline and could be slightly shorter or longer for specific applications. We recommend trimming the sample to a size most relevant for the specific biological question to insure the best staining and imaging conditions.

Sample type	Incubation time for Ab (n=)	Antibody solution volume	Wash solution volume
E10 embryo	1d	2ml	2ml
E11 embryo	1.5d	2ml	2ml
E12 embryo	2d	2ml	2ml
E13 embryo	2.5d	2ml	2ml
E14 embryo	3d	2ml	2ml
E15 embryo	3.5d	4ml	4ml
E16 embryo	4d	4ml	4ml
E18 embryo (whole head)	4d	4ml	4ml
Adult kidney	4d	2ml	2ml
<i>Adult brain:</i>			
- hindbrain + cerebellum	3d	2ml	2ml
- Forebrain (without cortex)	3d	2ml	2ml
- Forebrain (half)	4d	2ml	2ml
- Whole brain	4 to 6d	4ml	4ml

## 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.
2. Incubate the slides for 3h in 100% methanol
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 well 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.

## Protocol

### Sample Collection

1. Collect E10.5-E16.5 mouse embryos in ice-cold L15 or PBS.
2. Keep on ice for 5min to drain blood from umbilical cord.  
Or: for adult mouse brains or organs: anesthetize the mouse, and perfuse the animal with 1xPBS/4%PFA. Dissect the brain/organ and trim to the appropriate size
3. Fix in 1xPBS/4%PFA at 4°C, o/n with shaking, then RT 1h.
4. Wash in PBS with shaking: RT 1h x 3times.

### Sample Pretreatment with Methanol

1. Dehydrate in methanol/PBS series (freshly prepared): 50%, 80%, 100%; 1h each at RT.
2. Wash further with 100% methanol for 1h.  
{Samples can be stored in methanol at 4°C.}
3. Chill the sample over ice; bleach in 5%H<sub>2</sub>O<sub>2</sub> in 20%DMSO/methanol o/n at 4°C.

{1 volume 30%H<sub>2</sub>O<sub>2</sub>/1 volume DMSO/4 volume methanol, ice cold}

4. Wash in 100% methanol, RT 1h x3.
5. Incubate in 20%DMSO/methanol, RT 2h.
6. Rehydrate in methanol/PBS series (fresh prepared): 80%, 50%, 0%; 1h each at RT.
7. Wash in 1xPBS/0.2%TritonX-100, RT 1h x2.

### **Sample Pretreatment without Methanol (for antibodies incompatible with MeOH only)**

1. Wash fixed samples in 1xPBS/0.2%TritonX-100, 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 1xPBS/0.2%TritonX-100, RT 1h x 2.

### **Immunolabeling**

1. Incubate pre-treated samples in 1xPBS/0.2%TritonX-100/20%DMSO/0.3Mglycine, 37°C n/2 days.
2. Block in 1xPBS/0.2%TritonX-100/10%DMSO/6% Donkey Serum, 37°, n days. See attached table for recommended times.
3. Wash in 1xPBS/0.2%Tween-20 with 10ug/ml heparin (PTwH), RT 1h x 2.
4. Incubate with primary antibody in PTwH/5%DMSO/3% Donkey Serum, 37°, n days.
5. Wash in PTwH for 10min, 15min, 30min, 1h... then 2h or longer to the next day.
6. Incubate with secondary antibody in PTwH/3% Donkey Serum, 37°, n days. Use a concentration within the same range as the one used for the primary antibody.
7. Wash in PTwH for 10min, 15min, 30min, 1h each; then 2h or longer for 2d.

### **Clearing (adapted from the 3DISCO protocol, Erturk A *et al*, Nat Protoc 2012)**

1. Incubate the sample overnight in 50% Tetrahydrofuran/H<sub>2</sub>O (THF, Sigma **186562-12X100ML**) in a glass vial with a silicon coated cap (Thermo Scientific **C326-0020**). For each step, use between 5 and 10mL per tube.
2. Incubate 1h in 80% THF
3. Incubate 2x1h in 100% THF
4. Incubate in Dichloromethane (DCM, Sigma **270997-12X100ML**) until the sample sinks at the bottom (5min to 1h max). The sample may not sink if air bubbles are trapped inside. DCM improves the clearing in adult tissues, but can increase the shrinkage of soft tissues in embryos (such as the brain), so short incubations for embryos and early post-natal brains are recommended (less than 10min).
5. Incubate in DiBenzyl Ether (Sigma **108014-1KG**) until the sample is clear (20min to 2h). The vial containing DBE should be filled almost completely with DBE to prevent the air inside the vial from oxidizing the sample.

Read attentively the material safety data sheets (MSDS) for the use of THF, DCM and DBE. Work under a hood, collect waste, use glass and not plastics.

## 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 penetration in the sample and very limited light damage.

- 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. A chamber has to be built to confine the DBE and protect the microscope (see attached image). Two methods are suggested to build the chamber:

1) 3D printed imaging chamber, with the provided Script:

- The chamber can be printed with the Visijet M3 Clear resin, which is resistant to DBE.
- Secure the chamber to the microscope slide with Kwik-sil epoxy (VWR). This epoxy is compatible with DBE, cures instantaneously and has no permanent bond, so the sample and spacers can be recovered after imaging. Check the epoxy covers the whole bottom of the chamber with no holes (DBE will leak otherwise).
- Put your sample in the chamber. You can secure the sample with a drop of epoxy.
- Close the chamber by gluing a coverslip to the spacer with the epoxy. Make sure the epoxy is distributed along the whole surface of the spacer.
- Fill the chamber with DBE through the filling inlet with a pipet
- Once filled, plug the hole with the epoxy

2) To build an imaging chamber with dental cement:

- Dental cement is resistant to DBE and can be used to built spacers for the slide and coverslip. Mix the powder and liquid and build a ring spacer of the desired height. Leave a small hole on a side to accommodate a pipet tip (equivalent to the chamber's filling inlet).
- Once the spacer has cured, stick the spacer to a microscope slide with Kwik-sil epoxy (VWR).
- Use the same procedure to assemble the chamber than with the 3D printed chamber (see above).

## **Chamber for two photon or confocal microscope**

