Adapted from Eljo van Battum
Modification by Youri Adolfs
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Modification by
Lieke van de Haar
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Youri adolfs
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Youri adolfs
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3DISCO Immuno and Clearing protocol
(According to Alain Chedotal & Morgane Belle)

Note: If there are any question about this protocol or if you see that some improvement can be made, do not hesitate to contact me. (y.adols@umcutrecht.nl)

Immuno protocol

Dissection / Fixation

Before E15, preferably use entire embryo and include bleaching protocol.
From E15 onwards, dissect the brain carefully. Bleaching protocol is not needed.

Optional: From E11.5 on the skin of the skull can be removed to improve the permeabilization.(This is depending on your antibody)

Adult brain should be dissected in target areas and bleaching protocol should be used.

Fix tissue by immersion in 4% PFA in PBS (pH 7.4) or transcardial perfusion (in case of adult mice).
Note1: don't fix tissue too long (no longer than 4^{o}C ON or 3hrs at RT for embryo's; for postnatal brains don't do post-fixation after perfusion).
Note2: keep samples in fridge in 1xPBS (for longer storage add 0,01 thimerosal or NaAzide) after fixation.

Dehydration-Bleaching-Rehydration

This is used for adult brains or whole embryo.
(brain-only samples from E16, E18, P0 don't need this steps)

Solutions:

- Methanol (Merck Millipore: 1060092500)
- H_2O_2 (Merck Millipore: 1072091000)

Method

Dehydration	*Bleaching*	*Rehydration*
1xPBS ≥1.5h RT on shaker	90% methanol (100%vol) + 10% H_2O_2 (30%vol) (so add 1ml 30% H_2O_2 to 9ml 100% methanol) **<u>4°C O/N!!!</u>** **<u>not on shaker!!!</u>**	100% methanol ≥1h RT shaker
50% methanol ≥1.5h RT shaker		100% methanol ≥1h RT shaker
80% methanol ≥1.5h RT shaker		80% methanol ≥1h RT shaker
100% methanol ≥1.5h RT shaker		50% methanol ≥1h RT shaker
		1x PBS ≥1h RT on shaker

Keep samples in the fridge (4°C) after the Dehydration-Bleaching-Rehydration.

Blocking and antibody incubations

Solutions:

PBSGT

- 0.2% gelatin (VWR-2460.233)
- 0.5% triton-X-100 (SIGMA: x100-500ml)
- 0.01% thimerosal** (GERBU #1031/ USP35) or 0.01% Natrium azide.
- 1x PBS

**thimerosal contains mercury, beware to get rid of the waste in the right manner.

PBSGT + 0,1% saponin

- 0.1 % saponin (SIGMA: S-7900)

Method

1. Samples are incubated in blocking solution at RT on a horizontal shaker (70rpm) in PBSGT.

2. Primary antibodies are diluted in PBSGT + 0,1% saponin and incubated on horizontal shaker (70rpm) at 37°C according to the table, or at RT when small pieces of tissue are used or when the antibody is not compatible with prolonged 37°C incubation. (Fill a complete Eppendorf 2ml tube.)

3. Samples are washed 6x **1hr** in 15ml PBSGT at RT, while rotating. (In a 15ml tube)
4. Secondary antibodies are diluted in PBSGT + 0,1% saponin and incubated on horizontal shaker (70rpm) at 37°C or at RT according to the table. (Filtrate the antibody / PBSGT with a 0.20 uM filter. To avoid having secondary antibody precipitates)
5. Samples are washed 6x **1hr** in 15ml PBSGT at RT, while rotating. (In a 15ml tube)
6. The samples can be stored in PBS at 4°C until clearing.

Age	*blocking*	*primary*	*secondary*
E12*	3h	3 days	2days
E14-E18*	24h	7 days	2days
P0	36h	10 days	4 days
P5, P8	48h	14 days	4 days
Adult	48h	14 days	8 days

*Entire embryo's take equally long incubation times as brain-only samples

Notes:
1: Our lightsheet has the following lasers and Combinations.

	Laser	Emission Filters	Optimal to use with the following secondary Alexa's
1	488nm	525/50	Alexa 488
2	561nm	615/40	Alexa 568
3	647nm	676/29	Alexa 647
4	647nm	716/43	Alexa 680 (but preferably use Alexa 647 with filter combination 676/29)
5	730 nm	775/50	Alexa 750

2: Use far red dyes (like Alexa 647) for the most important staining. In the far red channel (647nm) you will have the strongest fluorescence and less background.

3: The 488 channel is the weakest and in this channel you will have most autofluorescent background! So use this one for the least important staining. You could also use this channel to make an image without any staining, so you have an image of the structure of your tissue.

4: The 750nm is weaker than the 647nm laser it has a good signal to noise ratio! (Low autofluorescent background)

5: TO-PRO®-3 (T3605) can be used as a nuclear counterstaining. Use for this dye laser 647 nm with filter set 676/29nm.

6: Anti-GFP anti-bodies seems to give more issues that the TD tomato antibodies.

Clearing protocol (3Disco)

Agarose embedding

If you use tissue that might twist during clearing process (like spinal cord), or samples smaller than E11, agarose is used before entering clearing steps.
(Do not use it on bigger tissues, since the agarose shrinks less than the tissue during the dehydration, it will damage your tissue!!!)

Cook 1% general purpose agarose in 1xTAE and let it cool down. Embed sample in agarose and let it set completely. Cut nice block of the agarose surrounding your tissue in proper orientation.
(The cutting should be always performed before the clearing!!!)

Solutions

THF: tetrahydrofurane (sigma 186562-1L)

- make 50%, (*optional 70%)* and 80% vol/vol dilutions in MQ.
  Store brown glass bottles in dark, RT, max 2 weeks.

  (Always use THF in which BHT is included as inhibitor. This is an antioxidant)
  (Use a needle and syringe to get the THF out of the bottle to prevent the THF from getting into contact with air.
  Only if you use all THF within 2 weeks that you could open the lid.)

DCM: dichloromethane (sigma 270997-1L)

- Use at 100%
  Store in brown glass bottles in dark, RT

DBE: dibenzyl ether (sigma 108014-1kg)

- use at 100%, store in brown glass bottles in dark, RT.

Note:
Before every step, blot the tissue sample dry on paper to get rid of excess liquid.
All incubations are performed at RT in falcon tubes wrapped with tin foil to protect against light, on a rotator (14rpm) in the fume hood.

Be carefully with DCM !!!!!!!!!!!
It can dissolve you cloves!!!!!!
All clearing steps should be performed in the fume hood and with protection.

Perform the clearing in a glass file or in TPP falcon, as this is DBE resistant.

Method

1. Tissue samples are incubated sequentially in 50%, 80%, 100% and 100% solutions of THF for dehydration. Time of incubation varies across sample-sizes (table), longer incubations do not harm clearing process.

	Adult mouse brain	*E11 mouse embryo*	*E12-P0*	*P5, P8*
THF 50%	≥1 night	1 night	1 night	1 night
*Optional: THF 70%**				
THF 80%	≥2h	≥30min	≥1h	≥90min
THF 100%	≥1h	≥30min	≥1h	≥90min
THF 100%**	≥1h	≥30min	≥1h	≥90min

**For a slower dehydration.*
*** Because THF is re-used, use one bottle always for the first step of the clearing and the other one for the last. Then the last flask stays "pure" 100% THF.*

2. Dissolve tissue lipids by incubating samples in 100% DCM

	Adult mouse brain	*E11 mouse embryo*	*E12-P0*	*P5, P8*
DCM 100%	45min (or until they sink)	10min (or until they sink)	20min (or until they sink)	40min (or until they sink)

Note: THF-dehydrated tissue floats when it is placed in DCM, at the end of the DCM step the samples must have sunk. If samples have not sunk, lengthen DCM step (but never more than 4h. Then you probably have an air bubble in your sample).

3. The final clearing step is done by incubating in 100% DBE.

	Adult mouse brain	*E11 mouse embryo*	*E12-P0*	*P5, P8*
DBE 100%	> 3h (or ON)	20min	ON	ON

After DBE incubation the samples should be transparent, having the same refraction index as DBE (1.56)

4. Tissue samples must be conserved at RT in Falcon tubes wrapped with tin foil, or brown glass vials with DBE. (Never store samples in the fridge!!!! Otherwise the sample will loose its clarity)