

**Data S1. Supplementary clearing worksheet**, related to Before You Begin [Step 6](#) and Step-by-Step Methods [Step 12](#).

**iDISCO+ PROTOCOL**

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<b>SAMPLE NAMES/#:</b> E.g.: 20160624_tp_bl6_lob6a+500R_01	<b>Primary antibody + concentration:</b>	<b>Secondary antibody + concentration:</b>

Day 1: Perfuse with 4% PFA/1x PBS. Fix in 4% PFA/1x PBS at RT for >4 hrs (or o/n with shaking).

Day 2: Dehydration

In 5 ml eppendorf tubes: 30 minutes each R@RT:

1x PBS \_\_\_\_\_, 1x PBS \_\_\_\_\_, 1x PBS \_\_\_\_\_,

Dehydrate in methanol/**H2O series**\*, R@RTx1hr:

20% \_\_\_\_\_, 40% \_\_\_\_\_, 60% \_\_\_\_\_, 80% \_\_\_\_\_, 100% \_\_\_\_\_ . 100% \_\_\_\_\_ .\*\*

5% H2O2 / Methanol (1 part 30% H2O2: 5 parts 100% methanol) O/N @ RT on Rocker \_\_\_\_\_,

\* freshly prepared with ~1.5 hrs for solutions to cool, or mix them the night before

\*\*Samples can be stored long term in 100% methanol at 4°C (no rocking). Timing works the best to start bleaching on Tuesday evening.

Day 3: Rehydration

Rehydrate in methanol/**H2O series**\*, R@RTx1hr:

100% \_\_\_\_\_, 80% \_\_\_\_\_, 60% \_\_\_\_\_, 40% \_\_\_\_\_, 20% \_\_\_\_\_ . 1xPBS \_\_\_\_\_.

0.2% Triton X-100/1x PBS \_\_\_\_\_, 0.2%Triton X-100/1x PBS \_\_\_\_\_,

20%DMSO/0.3M glycine/0.2%Triton X-100/1x PBS, 37°C 2 days. (22.5 mg/ml of glycine). R@37°C \_\_\_\_\_,

\* freshly prepared with ~1.5 hrs for solutions to cool, or mix them the night before

Day 4 Blocking:

Allow sample to reach RT (~1.5 hrs) \_\_\_\_\_,\*

10% DMSO/6% Donkey Serum/0.2% Triton X-100/1x PBS, 2 or 3 days ; R@37°C. \_\_\_\_\_,

Day 5 Primary Antibody:

Sample R@RT to allow sample to reach RT (~1.5 hrs) \_\_\_\_\_,\*

PTwH R@RT 1 hr \_\_\_\_\_, PTwH R@RT 1 hr \_\_\_\_\_, 1° Antibody Solution\*\* \_\_\_\_\_,

PTwH: PBS + 0.2% Tween-20 + 10 µg/ml heparin

\*\*Primary Antibody Solution: 5% DMSO/3% Donkey Serum/PTwH, R@37°C for 7 days. **Spin down antibodies at 4°C for 10 minutes at 17-18g (~14K RPM) before using.**

R@RT=Rocking at Room Temp | R@37°C =Rocking at 37° C

PTwH: 1x PBS/0.2%Tween-20 with 10 µg/ml heparin

**\*IMPORTANT:** allow brains to get to room temperature, this prevents holes from forming.

Day 6 Wash:

Sample R@RT to allow sample to reach RT (~1.5 hrs)\_\_\_\_\_\*

**R@RT for the following\*\*:**

PTwH 10 min\_\_\_\_\_, PTwH 15 min\_\_\_\_\_, PTwH 30 min\_\_\_\_\_, PTwH 1 hr\_\_\_\_\_, PTwH 2 hr\_\_\_\_\_.

\*\*minimum amount of washing listed, adding several extra washes can help.

Day 7: Secondary Antibodies:

2° Antibody Solution\*\* R@37°C for 7 days\_\_\_\_\_

\*\*Secondary Antibody Solution: 3% Donkey Serum/PTwH, R@37°C for 7 days. **Spin down antibodies at 4C for 10 minutes at 17-18g (~14K RPM) before using.**

\*\*\*Brains are now like vampires: sensitive to the light!

Day 8 Wash:

Sample R@RT to allow sample to reach RT (~1.5 hrs)\_\_\_\_\_\*

**R@RT for the following\*:**

PTwH 10 min\_\_\_\_\_, PTwH 15 min\_\_\_\_\_, PTwH 30 min\_\_\_\_\_, PTwH 1 hr\_\_\_\_\_, PTwH 2 hr\_\_\_\_\_.

Then leave R@RT until next day.

\*minimum amount of washing listed, adding several extra washes can help.

Day 9 Clearing:

Dehydrate in methanol/**H2O series** (freshly prepared, make early to allow solutions to reach RT), R@RTx1hr:

20%\_\_\_\_\_, 40%\_\_\_\_\_, 60%\_\_\_\_\_, 80%\_\_\_\_\_, 100%\_\_\_\_\_. 100%\_\_\_\_\_\*\*

66% DCM / 33% Methanol R@RT 3hrs\_\_\_\_\_\*\*\*

100% DCM R@RT 15min\_\_\_\_\_\*\*\* 100% DCM R@RT 15min\_\_\_\_\_\*\*\*

100% DBE **NO ROCKING** (but do invert several times) >1 hr\_\_\_\_\_.

Final Solution 100% DBE **NO ROCKING**\_\_\_\_\_\*\*\*\*

\*\*Can be left overnight R@RT.

\*\*\* **DCM: WEAR DOUBLE NITRILE GLOVES; WORK UNDER THE HOOD/SNORKEL.** Avoid "blasting" DCM out of syringe.

\*\*\*\*Wipe outside of 5ml tubes to remove DBE w/ 70-100% ETOH.

R@RT=Rocking at Room Temp | R@37°C =Rocking at 37° C

PTwH: 1x PBS/0.2%Tween-20 with 10 µg/ml heparin

\***IMPORTANT:** allow brains to get to room temperature, this prevents holes from forming.

## REAGENTS

Name	Provider	SKU/Order NO	Link
DBE (benzyl ether)	Sigma	108014-1KG	<a href="http://www.sigmaaldrich.com/catalog/product/aldrich/108014?lang=en&amp;region=US">http://www.sigmaaldrich.com/catalog/product/aldrich/108014?lang=en&amp;region=US</a>
PBS pH 7.4, 6 x 1000 ml	ThermoFisher	14190136	<a href="https://www.thermofisher.com/order/catalog/product/14190144">https://www.thermofisher.com/order/catalog/product/14190144</a>
PROTOCOL™ 10% Buffered Formalin, Fisher Diagnostics	Fisher Scientific	245-685	<a href="https://www.fishersci.com/shop/products/protocol-10-buffered-formalin-fisher-diagnostics-5/p-21703">https://www.fishersci.com/shop/products/protocol-10-buffered-formalin-fisher-diagnostics-5/p-21703</a>
Methanol, Laboratory Grade, 20L	Carolina	874195	<a href="http://www.carolina.com/specialty-chemicals-m-o/methanol-laboratory-grade-20-l/874915.pr?question=">http://www.carolina.com/specialty-chemicals-m-o/methanol-laboratory-grade-20-l/874915.pr?question=</a>
Dichloromethane - 2 Liter	Sigma	270997-2L	<a href="http://www.sigmaaldrich.com/catalog/product/sial/270997?lang=en&amp;region=US">http://www.sigmaaldrich.com/catalog/product/sial/270997?lang=en&amp;region=US</a>
Hydrogen peroxide solution	Sigma	H1009-100ML	<a href="https://www.sigmaaldrich.com/catalog/product/sigma/h1009?lang=en&amp;region=US">https://www.sigmaaldrich.com/catalog/product/sigma/h1009?lang=en&amp;region=US</a>
Glycine	Sigma	410225-50G	<a href="https://www.sigmaaldrich.com/catalog/product/sial/410225?lang=en&amp;region=US">https://www.sigmaaldrich.com/catalog/product/sial/410225?lang=en&amp;region=US</a>
Triton X-100, 50 mL	Sigma	T8787-50ML	<a href="http://www.sigmaaldrich.com/catalog/product/sigma/t8787?lang=en&amp;region=US">http://www.sigmaaldrich.com/catalog/product/sigma/t8787?lang=en&amp;region=US</a>
Tween 20	Sigma	P9416-50ML	<a href="https://www.sigmaaldrich.com/catalog/product/sigma/p9416?lang=en&amp;region=US">https://www.sigmaaldrich.com/catalog/product/sigma/p9416?lang=en&amp;region=US</a>
Dimethyl sulfoxide (DMSO), 1L	Fisher Scientific	D128-1	<a href="https://www.fishersci.com/shop/products/dimethyl-sulfoxide-certified-acs-fisher-chemical-4/p-215904">https://www.fishersci.com/shop/products/dimethyl-sulfoxide-certified-acs-fisher-chemical-4/p-215904</a>
S30-100ml Donkey Serum	EMD Millipore	S30-100ml	<a href="http://www.emdmillipore.com/US/en/product/Donkey-Serum%2C-100-ml,MM_NF-S30-100ML?ReferrerURL=https%3A%2F%2Fwww.google.com%2F">http://www.emdmillipore.com/US/en/product/Donkey-Serum%2C-100-ml,MM_NF-S30-100ML?ReferrerURL=https%3A%2F%2Fwww.google.com%2F</a>
Heparin sodium salt from porcine intestinal mucosa	Sigma	H3149-100KU	<a href="https://www.sigmaaldrich.com/catalog/product/sial/h3149?lang=en&amp;region=US">https://www.sigmaaldrich.com/catalog/product/sial/h3149?lang=en&amp;region=US</a>

## ABBREVIATIONS

1° - primary

2° - secondary

DBE - dibenzyl ether (also known as benzyl ether)

DCM - dichloromethane

DMSO - dimethyl sulfoxide

H<sub>2</sub>O - water

H<sub>2</sub>O<sub>2</sub> - hydrogen peroxide

PFA - paraformaldehyde

PTwH - PBS + 0.2% Tween-20 + 10 µg/ml heparin

PBS - phosphate-buffered saline

RT - room temperature

R@RT - rocking at room temperature

R@RT=Rocking at Room Temp | R@37°C =Rocking at 37° C

PTwH: 1x PBS/0.2%Tween-20 with 10 µg/ml heparin

**\*IMPORTANT:** allow brains to get to room temperature, this prevents holes from forming.