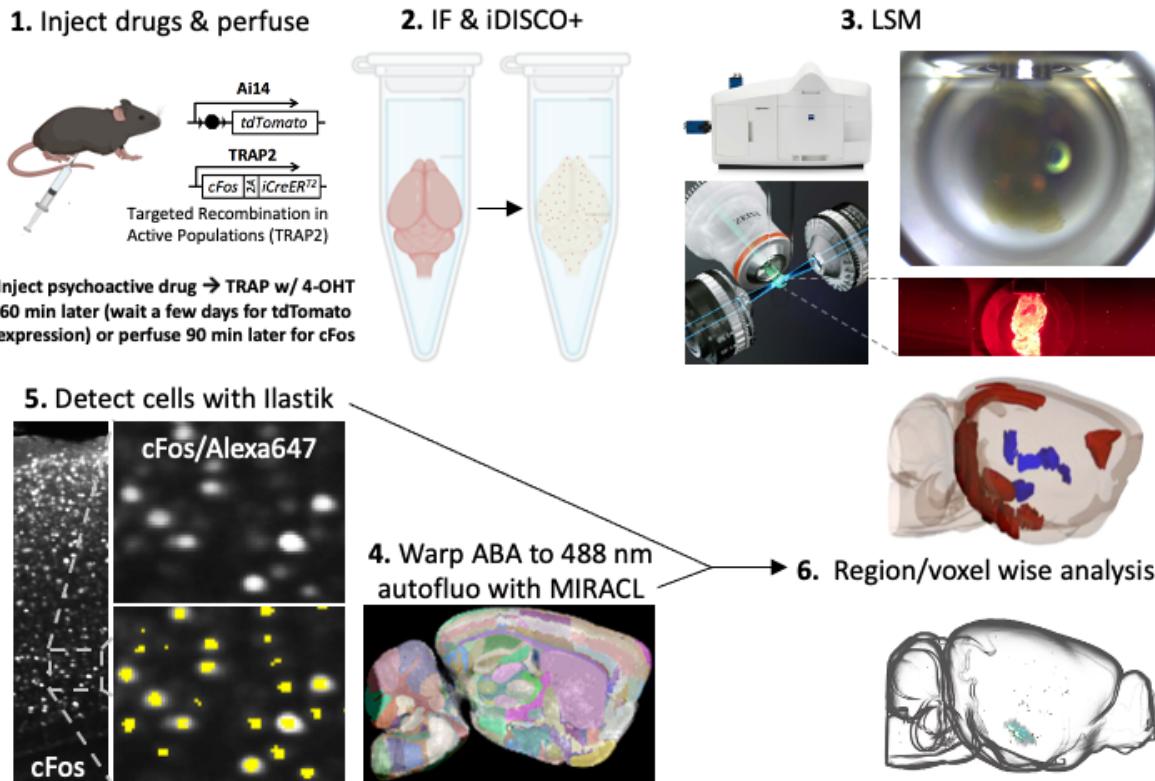


Brain activity mapping in mice

(TRAP2/IF/iDISCO/Lightsheet/Analysis)

Experiment overview



Neurons that are active during an experience are later either immunolabeled for [cFos](#) or [tdTomato](#) if [TRAPing](#) (Targeted Recombination in Active Populations) neurons in [TRAP2:Ai14 mice](#) (we use TRAP+-Ai14+ mice). Brains are cleared with iDISCO+ and imaged with a Zeiss Lightsheet 7 microscope. Autofluorescence (activity-independent background glow with stable anatomical info) is excited with a 488 nm laser line and is used for warping Allen Brain Atlas (ABA) to the tissue (by registration of the 3D autofluorescence image of the brain to an averaged template brain that is aligned with the ABA). This warping transformation can be used to warp to and from native space and Allen space. Fluorescence from previously active cells is excited by a 638 nm laser line. Ilastik is independently trained by 5 raters (1 Ilastik project per rater) to detect active cells, generating a binary segmentation of active cells. If % raters detect a cell, it is preserved in the consensus activity map, which is used for region-based stats (comparing cell densities in each brain region), voxel-wise stats (finding focal changes in activity using the [GLM-based randomise tool](#) in [FSL](#)), and cluster-based stats (measuring cell densities in focal areas with differences in activity [i.e., top hot/cold spots] and validating results by overlaying clusters with raw 638 nm data). Results are used for targeting viral injections with the goal of manipulating activity of the TRAPed ensemble within a specific region during behavioral experiments to gain mechanistic insight into the neural circuits mediating the effects of psychoactive drugs.

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TRAPing active neurons

To image brain-wide activity, TRAP2 mice, in which the neuron firing-dependent Fos promoter drives CreER expression, were crossed to a reporter line (Ai14). In effect, 4-hydroxytamoxifen (4-OHT) transiently enables CreER to translocate to the nucleus where it can trigger permanent tdTomato expression by excising a stop codon that interrupts Rosa26::tdTomato in Ai14+ mice.

4-Hydroxytamoxifen (4-OHT)

Dose: 50 mg/kg injected as 10 mg/ml (e.g., for a 20 g mouse, inject 0.1 ml [1 mg])

*Faster kinetics than tamoxifen, so better temporal precision with TRAPing (e.g., [see](#))

*Make extra due to loss of oil (so if injecting 4 mice, make enough for ~5-6 mice)

*A lot of 4-OHT is lost if you weigh it out. If possible, plan N's for experiments such that you use a full bottle ([SigmaAldrich: h6278](#)). 10 mg or 50 mg are options, but 50 mg is cheaper.

*This involves a vacuum centrifuge, so prep this at Lokey on the same day as your experiment

- 1) **Add 50 µl of EthOH per mg 4-OHT (up to 300 µl per 1.5 ml Eppendorf tube).** If you order 10 mg or 50 mg, add 500 µl or 2500 µl, respectively, to the bottle and aliquot 300 µl/tube. Otherwise, weigh out 6 mg into a tube and add 300 µl of ethanol
- 2) **Put bottle or tubes into 50 ml Falcon tube** and stretch a piece of tape over top to keep them from falling out (don't cap so warm air can get in).
- 3) **Place in 37°C incubator shaker in for at least 30 min** (inverting every few min to dissolve faster). Solution clear before proceeding. Prep oil/tubes during this step.
 - a) The shaker is in the equipment corridor in Lokey (in hall running East/West, North side of hall, near the hallway intersection) and looks ~ like [this](#) and has a tilted tube rack ~ like [this](#).
- 4) **Mix 80 µl sunflower oil and 20 µl castor oil per mg 4-OHT and add to tube(s)**
 - a) Either add oil mix to empty tubes and add the ethanol/4-OHT to this or add oil mixture to tube(s) with ethanol/4-OHT
 - i) 600 µl oil + 300 µl ethanol per tube
 - ii) Oils are at Nick Wall's bench.
 - iii) Cut back pipette tips to draw up oils
- 5) **Evaporate ethanol by putting tubes in vacuum centrifuge and spinning under negative pressure for 20-30 min**
 - a) Balance it w/ tubes on opposite sides, ~match weights, and close lid.
 - b) Close all valves above vacuum manifold.
 - c) Turn on (back left) vacuum manifold and hit vacuum button.
 - d) Also turn on vacuum pump.
 - e) Slowly turn valve to direct negative pressure to centrifuge.
 - f) Turn on centrifuge for 20-30 min
 - g) Stop centrifuge
 - h) Turn off vacuum pump
 - i) Open valve to vent negative pressure to room
 - j) Open centrifuge lid and turn off vacuum manifold
 - k) Log vacuum centrifuge use in notebook
 - i) If vacuum pump smokes, oil may need to be added (not common)

Perfusing mice for brain extraction

e.g., see this [video](#) as an example of the surgery, review these [diagrams](#) and watch others in lab

- 1) In fume hood, deeply anesthetize mice with isoflurane in small chamber**
 - a) w/ paper towel below mouse on pipette tip holder to separate mouse from direct liquid
 - b) Or use 100 mg/kg Sodium Pentobarbital via intraperitoneal injection (inject more if >10 min and not deep enough)
- 2) Verify anesthesia with foot pinch**
- 3) With mouse in supine position, pin limbs down to styrofoam**
 - a) In collection pan that will collect the perfusate
- 4) Spray fur with 70% ethanol to limit fluff during dissection**
- 5) Use medium scissors to cut along/through bottom of rib cage to open chest flap**
 - a) For this use forceps to pinch and raise skin just below sternum and avoid cutting organs and major vessels
- 6) Lift chest flap back and pin**
- 7) Cut diaphragm along inner ribcage**
- 8) Turn on PBS (see recipe) flow with peristaltic pump (or use 60 ml syringe)**
 - a) Some use ice cold solutions for perfusions (chill at 4°C and keep on ice)
- 9) Insert butterfly needle into left ventricle (BEC 367285)**
- 10) Use small/medium scissors to cut right artium (dark red)**
- 11) Perfuse ~10 ml PBS over ~3 min**
 - a) Too much pressure may blow out capillaries, contributing to unwanted autofluorescence in the 638 nm channel, and may enlarge the ventricles
 - b) Ideally, the liver will turn from a dark red to a pale-ish brown as blood is washed out and the perfusate will turn from blood red to clear before the next step
- 12) Switch to 4% PFA (ideally fresh) and perfuse ~10 ml over ~3 min**
 - a) 4% PFA prepared from 8% PFA stock diluted with 1x PBS. The stock should last for 2 months at 4°C. See recipe below. Plan on at least 20 ml of 4%/mouse (make some extra just in case)
 - b) 10% formalin may also work, pilot this first to compare quality
 - c) With a good perfusion the mouse will move around and the tail will curl up and become more stiff than before (This is from the effects of PFA on muscles, not the mouse waking up)
 - d) Discard PFA waste into labeled waste container
- 13) Spray head with 70% ethanol to limit fluff**
- 14) Cut off head with larger scissors**
 - a) Pinch the ears together with one hand (mouse facing palm in prone position) while cutting neck near ears
 - b) Place body in red or black bag and store in -20C in bag. Occasionally bring up to carcus fridge.
- 15) Use medium scissors to cut skin along dorsal midline from neck to nose**
- 16) Use smaller scissors to cut the skull**
 - a) Put one scissor tip into brain stem hole and cut along midline dorsally to top of the cerebellum (where skull flattens out)
 - i) With scissor tip inside skull, always keep tip near skull to minimize damage to the brain
 - b) Put one scissor tip into brain stem hole and make a horizontal cut in both directions
 - c) Insert tips of scissors into the center of the [frontonasal suture](#) with scissors oriented to open laterally
 - d) Open scissors quickly to split skull along midline (if needed cut along the midline of the dorsal skull, being careful not to damage the brain)
 - e) Cut the skull between the center of the [frontonasal suture](#) and the eyes
 - f) Use a spatula or forceps to pry open the left and right flaps of the skull
 - g) Cut strands of pia matter if they are digging into the brain or preventing it from being removed
 - h) Gently scoop brain out of skull ventrally with [spatula](#)
 - i) You may need to cut the optic nerve
- 17) Remove meninges, especially near the olfactory bulbs (otherwise there will be a dark film that does not clear)**
- 18) Place brain in a labeled 20 ml scintillation vial in about 10 ml of 4% PFA**
 - a) Use tape to label so vial is easier to reuse.

19) Store at 4°C overnight

- a) If there are genetically encoded fluorophores that you plan to image without staining, it is important to keep vials in a box to prevent light from bleaching signal

20) Remove sample from 4°C and let warm to room temperature (RT)

21) Wash brains 3x 30 min with 1x PBS with 0.02% Na Azide (NaN_3)

- a) This can occur in the iDISCO tubes for easier mixing on a rotator/nutator (see start of Sample Pretreatment in iDISCO section for more details)
- b) Add 2ml 10% w/v Na Azide to 1 L of 1xPBS
- c) Dump the waste into a beaker while keeping the brain in the vial or tube with a [spatula](#) or [spoon](#). Discard PFA waste into labeled waste container

22) Store at 4°C in 1x PBS containing 0.02% Na Azide until starting iDISCO+

1x PBS for perfusions (1 L)

1) 1 L of MilliQ water

2) 5 tablets of P4417-100TAB from Sigma-Aldrich

3) Heparin (1,000 U/L; Since 1U = ~0.002 mg, 20 mg is needed for 1L)

- a) Heparin comes in 25KU bottles, so they likely contain ~50 mg
- b) Consider also adding sodium nitrite (10 mM) in PBS as suggested [here](#) to more fully wash out blood and reduce autofluorescence

8% PFA stock (1 L)

1) In a fume hood, put 2L beaker on stir plate with 600 ml 1x PBS and mix w/ stir bar

- a) Use clamp to hold thermometer in the solution, but don't let stir bar hit glass

2) Heat at max temp

3) At 40°C add 80 g of PFA powder

- a) Push in powder as needed

4) Wait till temp increases to ~56.5°C and then turn off heat (the temp will continue to rise. PFA dissolves well around 60°C in a basic pH, but do not let temp exceed 65°C as FA will degrade)

- a) If needed, move to RT stir plate or wait for hot plate to cool before using it to stir

5) At ~58°C add 10N NaOH dropwise and stir until solution becomes clear

- a) Monitor pH, which should not exceed pH 10, as this will degrade FA

- i) To use pH meter, remove bottle with high KCl solution (3 or 4 M KCl, sometimes with pH buffer) from the electrode and rinse with MilliQ water. Dab delicate glass bulb dry with KimWipe (do not rub as this will create static and may damage the electrode or make it inaccurate). Talk to Austen about calibrating the pH meter. Suspend electrode in solution and press button to measure pH. Wait till it stabilizes for an accurate reading. When finished, rinse bulb with water and dab dry, then put back into bottle with KCl solution. Refill KCl solution if there is not enough to adequately submerge the glass bulb.

- b) In a pinch, slowly add NaOH pellets

6) QS (*quantum satis*) to 1L with 1x PBS (stop stirring to see meniscus)

7) Stir and let cool to 20-30°C

8) Adjust pH to 7.2-7.4 with drops of 10 N HCl

- a) Use transfer pipette

- b) Slow down when getting close to desired pH as it will start to change faster

9) Vacuum-filter solution

10) Store at 4°C for up to 2 months, but fresh may be better

Immunolabeling & iDISCO+

We follow the [original iDISCO+ protocol](#), with some extra details from [Eliza Adams' protocol](#) (e.g., use 1xPBS instead of water for dehydration/rehydration). [FAQs and troubleshooting](#).



Prep

- 1) Order supplies if needed ([cost/supply calculator](#))
- 2) For whole brains use 4.5 ml of solutions with 5 ml Eppendorf tubes. For hemispheres use 1.6 ml with 2 ml Eppendorf tubes.
 - a) For hemispheres, I prefer to use a razor blade and a 100 mm dish and eyeball the midline cut. Take your time and be as precise as possible. Oblique cuts result in missing tissue for one hemisphere and extra tissue for the other. This can be corrected to some extent later with 3D Slicer, but this is time consuming and you lose data in the missing regions. Another option is to use a [matrix](#) to guide the cut, but the brain is not always perfectly positioned and can shift during cutting. Hence, I think it is more accurate by eye (you can see the ventral side better as you cut). Also cut brain stem just posterior to cerebellum.
- 3) Make a Google Sheet w/ experiment info [in this folder](#), which is in Heifets Lab shared drive 1/Brain activity mapping/ and list sample ID #s or short IDs
 - a) Let us know if you need access
 - b) Ideally, include the experiment start date in the Sheet name and also note this date on the tape or notecard that will be stored with your samples so we can look up the experimental info in the future
- 4) Use a soldering iron to melt the IDs into the tubes in the fume hood and use a solvent resistant marker to make IDs easier to read
 - a) Don't press too hard or you will melt a hole in the tubes (check tubes for leaking during incubations). Some tubes have thin parts, so pay attention to this when deciding what tubes to use for future experiments.

Sample pretreatment

- 1) Warm samples to RT
- 2) Transfer samples to labeled tubes containing **1x PBS w/ 0.02% Na Azide (NaN₃)**
 - a) ALL SOLUTIONS AT RISK OF BACTERIA GROWTH NEED 0.02% NaN₃
 - b) This may be done if you already washed 4% PFA out with PBS
 - c) You can dump the brain in the a petri dish and gently scope it up with a [spatula](#)
- 3) Write down your name, experiment start date, and the experiment on tape or notecard to keep with your samples during processing and storage
- 4) Wash brains **3x 30 min (RT)** with **1x PBS with 0.02% Na Azide** with a nutator/rotator
 - a) This may be done if you already washed 4% PFA out with PBS multiple times
 - b) Samples can be stored at 4°C in 1xPBS with 0.02% Na Azide
 - c) To switch solutions, use a beaker to collect waste (rinsing out between washes). Quickly invert the tube while using a [spatula/spoon](#) to prevent the sample from falling into the waste beaker. If you invert too slowly, the brain may stay at the bottom of the tube and trap some of the solution.
 - d) It is fastest to dump the solution from a few samples and then use a transfer pipette to fill the tubes with the indicated solution. Volumes do not need to be super precise for washes, blocking etc., but volumes need to be precise for antibody incubations. Be more careful with fully changing media when adding antibodies, fully dehydrating or other key steps. Precision is less important for washes and the most of the de/rehydration series
 - e) If dumping solution from multiple samples and then filling all at once, I usually limit this to one tube paddle at a time (up to 7 samples for whole brains or 15 for hemispheres). DON'T LET THE SAMPLES DRY OUT BY SITTING WITHOUT LIQUID FOR TOO LONG.
 - f) I think you need to order the [10mL/15mL paddles](#) for 5 ml tubes. Order [1.5mL/2mL paddles](#) for 2 ml tubes.
 - i) Paddles for 5 ml tubes are more fragile and will break if you push the tube in too far. Just push it in enough to firmly hold the tube. More paddles are in the drawer at the iDISCO+ bench.
 - g) Be careful to recap tubes before rotating. There are a lot of steps so it is easy to eventually make a mistake
 - h) Consider grouping samples of the same condition on paddles (not mixing conditions), because if you accidentally spill the brains, you could still use the samples if they are all from the same condition. But if processing like this, be very careful to identically process samples for all paddles. The other approach is to mix

the tubes, so you have some from each condition on each paddle to limit risk of paddle to paddle variability contributing to differences later.

5) Dehydrate with methanol/1xPBS series: 20%, 40%, 60%, 80%, 100% methanol at RT with constant rotation

- a) ~45-60 min each for hemispheres, 1 hour each for whole brain
- b) Prep the solutions fresh before each step
- c) Each step should be at least 30 min (max 3 hrs)

6) Repeat 100% methanol incubation for 1 hour at RT

- a) Be careful to dump as much solution as possible before adding this
- b) Don't let samples dry out

7) Chill samples at 4°C

8) Incubate with fresh 66%DCM/33%Methanol at RT rotating overnight

- a) Work with DCM in the fume hood
- b) If DCM has syringe cap, the easiest way to remove DCM from the bottle is to insert ~4 large gauge needle tips (e.g., pink ones) and invert above a 50 ml Falcon tube. Remove needles afterwards to prevent evaporation.
- c) Consider wearing an extra pair of gloves.
- d) DCM will dissolve some plastics, so do not use a serological pipette/stripette tip. Transfer pipettes are fine.

9) Wash 2x ~60 min with 100% Methanol at RT

10) Chill samples at 4°C

11) Prepare fresh chilled 5% H₂O₂ bleach (1 volume 30% H₂O₂ to 5 volumes methanol) and incubate the samples overnight at 4°C with cap-side down

- a) Flip all tubes in rack easily by holding piece of cardboard (or another tube rack on tops of tubes while inverting)

12) Warm samples to RT

13) Rehydrate with methanol/1xPBS series: 80%, 60%, 40%, 20%, 1xPBS at RT with constant rotation

- a) ~45-60 min each for hemispheres, 1 hour each for whole brain

14) Incubate in 1xPBS 0.2% Triton (PTx.2), rotating for 1 hr at RT

Immunolabeling

1) Incubate in Permeabilization Solution at 37°C, rotating for 2 days

- a) 400ml 1xPBS 0.2% Triton, 11.5g Glycine, 1ml 10% w/v Na Azide, 100ml DMSO (add DMSO last)
- b) Incubate in cell culture incubators. ~34-36°C is ok. The temp is set a little low because of a tendency to overheat at 37°C with the added heat from the rotators. Adjust temp parameters as needed following manual. Water in tray not needed, but the walls of the incubators have been filled with DI water.

2) Wash sample in PTwH 2x 5 min, rotating at RT

- a) 995 ml 1xPBS, 2ml Tween-20, 1ml of 10mg/ml Heparin, 2 ml 10% w/v Na Azide

3) Block (1xPBS/0.2%Triton/6%Donkey Serum/10%DMSO) at 37°C, rotating for 2 days

- a) Add DMSO last and make sure block solution contains Na Azide

4) Incubate with primary antibody in PTwH/3%Donkey Serum/5%DMSO, at 37°C, rotating for 10 days

- a) Add DMSO last, just before adding primary antibody and make sure solution contains Na Azide
- b) Prepare a fresh master antibody mix for use with all samples
- c) Rb anti-RFP (Rockland; 1:250) or Rb anti-cFos (SynapticSystems; 1:500). Test dilution series if new antibody
- d) Remove as much blocking solution as possible when inverting to avoid diluting 1° antibody variable amounts
- e) 5 days may be sufficient, but we have erred on the side of caution to insure good antibody penetration

5) Wash sample in PTwH 2-3x for 5 min rotating at RT, then every few hours until end of day (4-5 washes total), leave rotating overnight

- a) Make sure block solution contains Na Azide
- b) Samples can be left up to 7 days

6) Incubate with secondary antibody (Donkey anti-Rabbit Alexa647 (1:250)) in PTwH/3%Donkey Serum (syringe-filter) the solution at with 0.2 µm pore size) at 37°C, rotating for 5 days

- a) Prepare a fresh mastermix antibody dilution for all samples and make sure PTwH contains Na Azide
- b) Remove secondary antibody precipitates with syringe filter (0.2 µm pore size)
- c) Remove as much PTwH solution as possible when inverting to avoid diluting primary antibody variable amounts
- d) IMPORTANT: Keep covered from light from here on out

7) Wash sample in PTwH 2-3x for 5 min rotating at RT, then every few hours until end of day (4-5 washes total), leave rotating overnight

- a) Sample can stay in PTwH up to three days, with or without additional wash changes; stainings with high background can benefit from additional washing (Two overnight washes is recommended)
- b) Cover from light (invert a cardboard box over the nutator/rotator)

Embedding in low melting point agarose

1) Line the inside of each well (1 per sample) in 12 well plates with a small piece of aluminum foil

- a) The foil will act like a muffin cup and make it much easier to remove the brain/agarose from the well later.
- b) Use top of a 15 ml Falcon tube to press the foil into the well and make space for the brain to lay flat in the middle of the well. It's ok if the foil rips a little.

2) Label wells to keep track of the samples

3) For each sample add 5 ml of 1xPBS, add 5 µl of 10mg/ml Heparin, 10 µl of 10% w/v Na Azide, and 0.05 g of low melting point agarose (1% w/v) to a beaker

- a) Although PTwH has Triton-X 100, adding this detergent will introduce lots of bubbles, so leave it out.
- b) Make some extra in case of loss. Note how much was used and update here if needed.

4) Mix with stir bar on stir plate

5) Monitor temp with thermometer as you increase the temp to dissolve the agarose

- a) Thermometer held by stand behind hot plate
- b) The melting point is < 65.5°C. If you go above this, it will become cloudy, but it will clear again as it cools

6) Once the solution is 37°C, proceed with remaining steps, reheating as needed

- a) Gel temp is 26±2°C, so do not let cool to this temperature until after embedding the brains

7) Dump PTwH from tube into waster beaker and dump brain onto petri dish

- a) Protect brains from light when not actively working with them.

8) Carefully dry brain with Kimwipe

- a) Residual solution could cause the agarose to not stick well to the brain

9) Use spatula to transfer brain to well in 12 well plate

10) Pour in a warm agarose

11) Stir brain around with paint brush

- a) Stirring helps agarose get into crevices of the brain

12) Center brain in the well (ventral side down)

- a) Accelerate cooling of full plate with 4°C fridge.

13) Using foil cup, pull sample out of well

14) Carefully remove foil

- a) If agarose rips or separates from the sample, it can trap bubbles, which occlude light (detection objective cannot see the far side of a bubble with a lightsheet microscope), so remove agarose and embed again.

15) Place agarose/brain on plastic petri dish and cut excess agarose with razor blade

- a) Cut it into a rectangular cube such that most of the agarose is removed from the left, right, and posterior side, leaving ~1-2 mm of agarose on these sides. These vertical cuts may be angled in toward the olfactory bulbs
- b) Also cut excess from the dorsal side, leaving ~2-3 mm of agarose. This is an oblique cut, since the front of the brain is thinner than the back.
- c) Leave a few mm of agarose on the anterior side to leave room for the spike on the C-clamp, so the spike can be positioned in front of or in between the olfactory bulbs. View the c-clamp tissue mounts to note max length.
- d) Too much agarose may slow down subsequent washes. Too little risks the agarose falling off the brain.

16) Return the sample to the tube and fill with PTwH

Clearing



17) Dehydrate with a methanol/1xPBS series: 20%, 40%, 60%, 80%, 100% methanol @ RT with constant rotation (covered from light)

- a) ~45-60 min each for hemispheres, 1 hour each for whole brain
- b) Cover from light

18) Repeat 100% methanol incubation for 1 hour at RT

- a) Be careful to dump as much solution as possible before adding this. If sample is not fully dehydrated, it may become cloudy instead of clear later
- b) Don't let samples dry out
- c) Cover from light
- d) Samples can be left overnight if convenient

19) Incubate overnight in fresh 66%DCM/33%methanol at RT with rotation

- a) Cover from light
- b) 3-4 hours could be ok

20) Incubate in 100% DCM for 2x 15 min at RT with rotation to remove methanol

- a) Fully change solutions each time. DCM is volatile, so minimize how long samples are not in solution.
- b) Cover from light

21) Remove all DCM and fill tube with DiBenzyl Ether (DBE)

- a) Make sure tube is almost completely filled to prevent air from oxidizing the sample
- b) Sample will clear in ~20min, no rotating; place cap-side down, keep covered from light
- c) Wait at least half a day before imaging the sample, as full clearing may take several hours; invert several times just before imaging to mix fully
- d) Write down your name, experiment start date, and the experiment on tape or notecard to keep with your samples during processing and storage. Ideally, include the experiment start date in the Sheet name and also note this date on the tape or notecard that will be stored with your samples so we can look up the experimental info in the future. After imaging, put a Ziploc bag around tubes/rack to limit DBE fumes and store below hood. Samples can be stored in dark with minimal fading for months. Samples more than 1 yr old can retain decent staining, but image sooner rather than later just in case.

Notes

- Always fill tubes to the top. Too much air in the tube may cause the sample to dry out and lead to tissue damage and incomplete immunolabeling.
- Check samples periodically to ensure they aren't stuck in the cap or conical bottom of the tubes, they should be freely moving with rotation.
- Add sodium azide to all solutions (0.02% NaN₃) to prevent bacterial growth during long incubations; another 0.5 µl of NaN₃ can be added directly to block/primary/secondary solutions if bacterial growth is of particular concern
- Keep all steps after secondary antibody application covered from light exposure
- See end of original iDISCO+ protocol for suggestions on [antibody choice, concentration, and validation](#)

Solutions

*All solutions should have 0.02% sodium azide final concentration

1xPBS 0.2% Triton (PTx.2)

5 PBS tabs
2ml Triton-X100
2ml 10% w/v NaN₃
996 ml ddH₂O

PTwH (1 L)

5 PBS tabs
2ml Tween-20
1ml of 10mg/ml Heparin
2ml 10% w/v NaN₃
995 ml ddH₂O

Permeabilization Solution (500 mL)

400ml PTx.2
11.5g Glycine
100 ml DMSO
1ml 10% w/v NaN₃

Block (50ml)

42ml PTx.2
3ml Donkey Serum
5ml DMSO (add last)

Primary (50ml)

46ml PTwH
1.5ml Donkey Serum
2.5ml DMSO (add last)

Secondary (50ml)

48.5ml PTwH
1.5ml Donkey Serum