Allen Institute Array Tomography Cryoprotection -

Prep:

* Order perfusion

Day 1:

Perfusion

* Keep in post-fix overnight at 4 degrees C

Solution Prep:

* Make up stock solution of 0.1M NaA - 4.1g/500mL H20

* Make up stock solution of 0.1% CaCl2 in 0.1M NaA - 0.1g CaCl2 into 100 ml NaA

* Make up glycerol dilutions (10%, 20%, 30%) in 0.1M NaA

Day 2:

Vibratome

1. Block brain to include V1

2. Mount on vibratome

3. Cut 200um thick slices into cold 0.1M PB

4. Collect sections back into chilled 0.1M PB

Pre-Treatment – All done in chilled Styrofoam box on shaker

1. 3 x 10 minutes in 0.1M NaA

2. 1 hour in 0.1% CaCl2/0.1M NaA

3. 3 x 5 minutes in 0.1M NaA

Cryoprotection – All done in chilled Styrofoam box on shaker; add new ice packs before overnight step

1. 30 min in 10% glycerol/0.1M NaA

2. 30 min in 20% glycerol/0.1M NaA

3. 30 min 30% glycerol/0.1M NaA

4. Overnight in 30% glycerol/0.1M NaA

Prep

* Clean metal dishes and bake overnight

* Cut, clean, and bake Aclar strips overnight

Day 3:

Prep

1. Fill and chill AFS to -90 deg C

2. Chill down metal dishes and mesh-bottomed capsules in acetone

Dissection

1. In 30% glycerol/0.1M NaA, dissect out desired number of V1sections
2. Place back in 30% glycerol/0.1M NaA after dissection

Allen Institute Array Tomography Plunge Freezing Protocols

Forrest Plunge Freezing Method

- ACLAR spears
- cryotubes
- 2%UA in acetone
- filter paper for wicking
- Liquid nitrogen (LN2) in styrafoam container
- transfer pipette

Procedure

1. pipette 1.5mL UA/Acetone into each cryotube, seal
2. freeze cryotube in LN2 or in AFS Chamber (Can fill chamber with LN2 if desired)
3. place tissue on ACLAR spear
4. wick off glyercol
5. insert spear into cryotube and place tissue on top
6. Store in LN2 until placing into AFS Chamber

UNC Plunge Freezing

- Container liner with foil
- Pure methanol
- pure ethanol
- CO2 Chunks (aka dry ice)
- AFS Containers
- Baskets for AFS
- ACLAR Spears

Procedure:

1. Fill contain with EtOH + CO2 chunks
2. Place container in basket in the slurry but do not submerge
3. Fill container with MeOH
4. Place tissue on ACLAR spear and wick off excess glycerol
5. Inset tissue/spear into MeOH basket
6. Once all tissue is frozen, transfer to AFS chamber
7. Switch out MeOH with UA/Acetone solution

Allen Institute Array Tomography AFS Lowicryl HM20 Protocol

Ideally the AFS schedule starts on a Friday

	Mon	Tues	Wed	Thur	Fri
Week 1	**Freeze tissue sometime this week or before**				**<u>Day 1:</u> UA infiltration**
Week 2	**<u>Day 2:</u> Lowicryl infilitration**	**<u>Day 3:</u> UV polymerization**			**<u>Day 4:</u> Clean up and store tissue**
Week 3	**<u>Day 5:</u> Turn off AFS/ finish clean up**				

Friday, Day 1: UA infiltration over the weekend

Preparation:

- 1.5% uranyl acetate (plenty for four chambers): UA dissolve very slowly, so you may want to prepare it few hours in advance or the day before and store in 4C with light protection. Depending on how many chambers you need, you can prepare less UA to reduce the waste.
    - 0.75g in 50 ml anhydrous acetone (*weigh in hood*)
    - make in 50 mL conical, wrap in Al foil
- Filter 1.5% uranyl acetate using 0.2 µm 25 mm syringe filter and 60mL syringe
- If necessary, clean all the metal containers and AFS accessories with acetone in sonicator ~1 min.

PROCEEDURE

1. Fill AFS machine with liquid nitrogen.

1. Draw diagram in notebook detailing tissue organization within metal dishes. Include extra dish for hat removal and tissue sorting.
2. Insert beem capsule holder at bottom of chamber.
3. Assemble metal cans and flow-through capsules in fume hood. Be sure to notch one basket in each group in order to distinguish orientation of tissue map/locations. Fill dishes with 1.5% UA in acetone.

4. Place metal dishes containing flow-through baskets and UA/acetone into AFS chamber before cool-down.
5. Set the AFS program to cool down to -90°C. Let cool for about 2 hours.
6. Once cooled, transfer tissue from cryotubes into designated baskets in AFS.
    a. Transfer cryotubes into Styrofoam box of liquid nitrogen
    b. With cryo-gloved hands, quickly grab, uncap, and dump the tissue into 'sorting dish' within AFS chamber.
    c. Use forceps or paintbrush to separate tissue from caps if possible, and transfer tissue into appropriate dish/basket according to your map.

1. You must wait 2-3 days before going on to Day 2 of AFS.
2. Refill AFS with LN2 over the weekend.

Monday, Day 2: Lowicryl infiltration

1. First thing on Monday, pre-cool anhydrous acetone for at least 20 mins in metal dish in AFS chamber.
2. Wash samples with anhydrous acetone, 3 times, 15 minutes per wash. Use glass pipettes to transfer solutions. Note: if you reuse the pipettes, leave them in the AFS chamber so you do not introduce condensation.
3. Prepare resin as follows during the 1st 15 min wash or during initial cooling. Wear goggles and gloves.

Use the **Lowicry HM20 Embedding Kit**

(Electron Microscopy Sciences- Cat#14340)

# of Cylinders:	1	2	3	4
Crosslinker D	4.47 g	7.45 g	10.43 g	13.41 g
Monomer E	25.53 g	42.55 g	59.57 g	76.59 g
Initiator C	0.15 g	0.25 g	0.35 g	0.45 g

Be extremely careful! Initiator C is a neurotoxin!

-Add each chemical or solution to a plastic beaker on the scale- tare between each reading. Use a plastic pipette cut at an angle as a spoon.

-After adding Monomer E, use a transfer pipette with bulb cut off to ***bubble nitrogen gas*** into the beaker for two minutes to remove oxygen and mix the liquid.

-Then add Initiator C. Again bubble nitrogen gas for two minutes.

-Leave covered solution in hood. Clean the scale well with methanol or acetone after use!

4. To do infiltration with Lowicryl HM20 resin at -45°C, change buffers every two hours as follows. Be sure to pre-cool the buffers for at least twenty minutes each time before changing (You can stack them all in at the same time if space allows).

a.	*Lowicryl/acetone 1:2*	2 hours
b.	*Lowicryl/acetone 1:1*	2 hours
c.	*Lowicryl/acetone 2:1*	2 hours
d.	*Pure Lowicryl*	2 hours
e.	*Pure Lowicryl*	Over Night

Tuesday, Day 3: UV-Polymerization

1. Prepare labels for each block using font size 6. Insert the rolled label for each block into beem capsules
2. Add more LN_2 to the AFS machine.
3. Bubble lowicryl HM20 w/ N2 gas to get rid of O2 in hood. Pre-cool for 20 mins in metal dish in AFS.
4. Change solution to the pre-cooled Lowicryl, and leave for 1 hour.
5. Place beem capsules in available stable location within AFS chamber. Let cool for at least 20 minutes.
6. Once cooled, add a small amount of chilled Lowicryl to each capsule.
7. Using paintbrush or forceps, transfer tissue into beem capsules, trying to ensure that it is centrally located and resting flat against the bottom of the capsule. Add appropriate label to each beem campsule as you go.
    a. To instert, roll up label so text is facing the outside and diameter is smaller than that of beem capsule.
    b. Insert into top of beem capsule using sharp forceps.
8. Put UV light in place, plug in
9. Select program, 24hr at -45°C. Increase temp to 0°C (increase 4°C /hr), 35hr at 0°C.

Friday, Day 4: Clean Up

Take out the specimen and check if the tissue is there. Clean everything with acetone.

1. Set AFS Machine to burn out program (60C) for 3 days to get rid of excess liquid nitrogen.

Monday, Day 5: Clean Up, Cont.

Turn off machine and ensure everything is clean, put back in its place and that there is enough of each reagent for next run.

Agitator used during AFS:

https://www.amazon.com/Cleansing-Waterproof-Rechargeable-Vibrating-Exfoliating/dp/B0B9XTXCWH/ref=sr_1_17?crid=2PLGN9V651BBD&keywords=vibrating%2Bface%2Bscrubber&qid=1684421590&sprefix=vibrating%2Bface%2Caps%2C146&sr=8-17&th=1

Allen Institute Array Tomography Elution Protocol

Supplies:

NaOH

SDS (20% w/v; Roche 1666924)

Elution solution:

0.2 M NaOH

0.1% SDS (0.1% of the 20% SDS, so it's actually 0.02%)

In diH20

1. Flush mounting media (20% DAPI solution) from flow cell using 3 x 1x Tris rinses.
2. Flush with 1 mL diH20
3. Apply elution solution through flow cell for 15-20 minutes at room temperature.
4. Wash well (3x flow cell volume) with 1x Tris
5. Rinse with 10 mL diH20
6. Aspirate remaining liquid off of slide and place on 45 degree C warmer to dry for ~30 min.