Basic Stereotaxic Surgery & Histological Processing Protocol

Anaesthesia for recovery experiments (zebra finch):

- Take 0.1 ml of 100mg/ml ketamine and add 0.2 ml dH2O.
- Then do the same for 2% xylazine.
- Mix A + B. This is now the K/X stock solution.

Give ZFs 0.04 ml i.m. (40 uL) of that mixture for 2-3 hours of stable anesthesia.

Anaesthesia delivery protocol:

- Fill insulin syringe with 40 uL (0.04 mL) K/X stock solution.
- Feel for breast bone/sternum; inject to left of bone. Can use alcohol to wet feathers so that injection site can be better visualized. Go in at a 45-degree angle. Can push the entire needle in – it will hit bone before entering something important.
- Pull back on syringe to make sure no blood enters syringe (seeing blood could indicate that you are in a blood vessel). If no blood, inject the K/X.
- Bird should be ready for surgery in 10 min.

Pain management:

- Marcaine (local anesthetic)
- Metacam (0.5%) injection (0.2 ug/g s.c.) – repeat this dose 24 h after surgery (I made up a 5% working solution, so give 10 uL of this s.c.).

Making glass electrode:

- Pull pipette – want a long shank
- Clip the tip with angled corneal scissors
- Measure tip under the microscope – for localizing nuclei with ephys and making tracer injections, we want an ID of ~10-20 um.
- Load the electrode onto the stereotax

Other surgical prep notes:

- Use a thin strip of masking tape to tape down the wings just below the shoulder joint.
- Clip feathers from around ears and scalp
- Line up the electrode with the center of the far (secured) ear bar. This is interaural zero (IAZ). Note these coordinates and then move electrode caudally 0.3 mm (this should line up with the Y commissure bifurcation)
- When placing the head in the frame: want ear bars in contact with bony part of ear on the anterior side. Secure beak.
- Swab area with iodine
- Make incision in scalp with #22 scalpel blade, making sure to pull skin tight.

- Apply Marcaine.
- Chip away at the skull above the bifurcation – identify this point using the tungsten or glass microelectrode. If the electrode does not lower to the correct rostro-caudal location, change the angle of the head until it lines up with the Y (should be close to 45°). IAZ is the zero point for coordinates, but the bifurcation is used to set the head angle.

Filling electrode

- Fill by suction using large (60 mL) syringe attached to PE tubing and silastic (silastic gets attached to electrode)
- Make a small well by cutting the tip off of a 1.5 mL eppendorf tube (using a scalpel). Place the well in some blu-tak. Put 3 uL of desired tracer (Martin uses 10% BDA 3K in PBS or CTB) in well. Use forceps to place well on bird's head, lower electrode into well and then use the syringe to draw up some tracer into electrode (need very small amount). Remove well.
- Use twisted Kim-wipe dipped in saline to clean off tip of electrode.

Preparing for recording/injection:

- Remove bone in area of injection using #4 or #5 forceps.
- Clean of tip of microelectrode.
- Make slit in dura using insulin syringe needle.
- Attach reference/ground electrode (from the headstage) to bird's skin. Place thick platinum or silver wire in electrode, trying to submerge as deeply as possible in tracer. Bend the wire over the edge at the top of the electrode. Clip the positive lead from the headstage to the wire.
- Use aluminum foil for shielding around the bird. Start lowering electrode.
- Turn on the amplifier and try to record cells (Martin uses following parameters: Filter 300-5K Hz; Gain 10,000x; doesn't chloridate the wire)
- Iontophoresis parameters; Apply current for 15-30 min:

Tracer	Duration (ON)	Delay (OFF)	Current
BDA	1 s	1 s	4-5 µAmp
CTB	7 s	7 s	4-5 µAmp

- Leave micropipette undisturbed for 5 min post-injection
- Glue edges of incision together (super glue or tissue glue)

Recovery:

- Administer buprenorphine (0.1 mg/kg, i.m.) – opiates suppress food and water intake – so may want to give saline injection and keep an eye on the bird's intake during recovery. Martin doesn't give opiates.
- Leave animal on heat as it wakes up (could get a small separate cage for recovery). Place food and water dishes on floor of cage. Keep separated for first night, then reintroduce to home cage with other animals.
- Recover animals for 3-5 days to allow for adequate transport.

Perfusion:

- Perfusing with cold saline and 4%PFA in 0.1 M phosphate buffer pH 7.4 (see perfusion protocol).
  - Not necessary to make fresh 4% PFA (should be kept cold)
  - 0.9% saline (ice cold for perfusion)
  - Animals perfused with even low concentrations of glutaraldehyde will generate results with unacceptably high background staining.
- Set up perfusion equipment, run 0.9% saline through tubes (no air bubbles), and draw up heparin in insulin syringe (0.1-0.2 mL) – I think it was 35,000 units/mL – large vial anyway
- Administer overdose of K/X (~0.1-0.15 mL) i.m.
- Squirt EtOH on bird's chest
- Use scalpel (#22) to scrape away feathers.
- Cut down midline on either side of sternum with the scalpel, then across the abdomen under the ribcage.
- Use scissors to cut through any remaining muscle/connective tissue in the abdominal incision, then cut along midline through the rib cage on either side of the sternum, being careful not to open the scissors to widely and nicking the heart. Cut the sternum at the top so that it can be completely removed.
- Insert spreaders as close to the head as possible and open wide.
- Cut the pericardium.
- Inject the heparin into the heart.
- Cut the right atrium (Martin may have cut the left – so maybe side doesn't matter) using the #10 scalpel blade.
- Insert the perfusion needle into the left ventricle and turn the pump on.
- Remove the spreaders, and put a pair of forceps in the bird's beak to prop open.
- Saline should pump through bird until the outflow runs clear. Look for feet to clear and inside of beak to run clear.
- Turn off the pump and switch over to PFA. Turn pump back on and pump about 30 mL of PFA through bird, or until legs and neck are stiff. Remove tube from PFA bottle and let rest of tube contents run through bird, switching pump off just before the air reaches the heart.
- Cut the head off and cut open the scalp. Use rongeurs to remove the dorsal part of the skull – if the dorsal surface of the brain is important for the experiment, then do this under the microscope so as to not nick the surface of the brain.
- Post-fix brain for 6-8 h (or o/n if preferred) in 4% PFA in 0.1M sodium phosphate buffer pH7.4.

Block brain in stereotax:

- Put large scalpel (#22) in the holder. Make sure that it lines up straight with the ear bar.
- Place bird in the stereotax, beak in beak bar.

- Lower the blade to cut off part of the forebrain – want to make the cut anterior to the optic chiasm – maybe the first quarter of the telencephalon? How the brain is blocked depends on the tracing study.
- Raise and move blade laterally, then lower again until brain is completely cut in coronal plane.

Cryoprotect:
Place tissue in 30% sucrose (in PBS) to cryoprotect. Leave until brain sinks to bottom of tube (usually o/n) at room temperature, or at 4°C.

Section:
Section brain on a freezing microtome at 30 to 50µm and collect sections in PBS or PBS-azide (0.1%) for long-term storage. Make two 24-well trays (one in PBS, the other in PBS azide) and serial section alternating between tray 1 and tray 2.
Instructions for using the microtome:

- Squirt some 95% EtOH in the well surrounding the stage, then add a small amount of dry ice.
- Put OCT on the stage so it gets into the cracks before freezing.
- Add some more ice to the well.
- Leave until OCT is almost hard.
- In the meantime, put the brain on a paper towel to dry off.
- Put OCT on a scalpel blade (#22)
- Put brain on the blade – flat surface down. Slide the brain off the blade and onto the OCT platform.
- Crush some dry ice and cover the brain with the crushed ice so that the brain freezes – this only takes a few minutes (2-4 min).
- Clear off some of the ice, and make a few slices until you reach the area where you want to start collecting slices. Use an insulin syringe to poke a pinhole to mark the right side of the brain (or front if doing sagittal sections).
- If the brain is too cold, then you'll get high frequency noise and chatter in your sections.
- If the brain is too warm, you'll get curved rather the flat sections.
- Use finger to rub the brain surface briefly, warming up the cutting surface, so that the section will come off nicely. As the brain gets too cold it will be hard to cut through.
- No need to put OCT over top of brain.

Processing: **BIOTINYLATED DEXTRAN AMINE (BDA) CYTOCHEMISTRY**

Wash: sections in PBS for 3x10 minutes.

Bleach: sections (in 50% methanol with 1% hydrogen peroxide) for 20 minutes.

Bleach recipe (20 mL)

- *Methanol* *9 mL*
- *PBS* *9 mL*

- *30% H_2O_2* *700 µL*

Wash: sections in PBS for 3x10 minutes.

Incubate: in streptavidin complex:

1. Streptavidin-peroxidase (diluted typically 1:1,000) in PBS-TX (PBS-TritonX-100 [0.4%]) to produce an opaque reaction product (black or brown).
2. Streptavidin-fluorescein (diluted 1:250), rhodamine (diluted 1:250), Cy2 (diluted 1:250-1:500) or Cy3 (diluted 1:500) for a fluorescent signal in PBS-TX.

Wash: sections in PBS for 3x10 minutes.

Incubate: sections incubated with streptavidin-peroxidase with DAB/H202 medium to produce a brown reaction product, or with cobalt chloride added for a black reaction product.

Wash: sections in PBS for 3x10 minutes.

**After processing BDA, if there is also a CTB injection, continue on to Ab incubations to detect CTB

Processing: **CHOLERAGENOID (CTB) IMMUNOREACTIVITY**

Wash: sections in PBS for 3x10 minutes.

Bleach: sections (in 50% methanol with 1% hydrogen peroxide) for 20 minutes.

Bleach recipe (20 mL)

- *Methanol* *9 mL*
- *PBS* *9 mL*
- *30% H_2O_2* *700 µL*

Wash: sections in PBS for 3x10 minutes.

Incubate: in anti choleragenoid medium (diluted 1:30,000) with 2% normal serum (and sodium azide). Incubated typically overnight, either at room temperature, or at 4°C.

Wash: sections in PBS for 3x10 minutes.

Incubate:

1. In biotinylated anti-goat medium (1:200) IN PBS-TX for 1 to 2 hours at room temperature.

2. In anti-goat Texas red (typically diluted 1:200-1:500 in PBS-TX with 2% normal horse serum) for red fluorescent visualisation of the choleragenoid.

**With Martin, we detected BDA with black DAB rxn, and CTB with brown DAB rxn.

Wash: sections in PBS for 3x10 minutes.

Incubate: in streptavidin complex in PBS-TX for sections previously incubated in biotinylated anti-goat medium. Incubate for 1 to 4 hours (usually 1 hour).

1. Streptavidin-peroxidase (typically diluted 1:1,000) is used to produce an opaque reaction product (black or brown).
2. Streptavidin-fluorescein (diluted 1:250), rhodamine (diluted 1:250), Cy2 (diluted 1:250-1:500) or Cy3 (diluted 1:500) for fluorescent signals.

Wash: sections in PBS for 3x10 minutes.

Incubate: sections reacted with streptavidin peroxidase with DAB/H202 medium to produce a brown reaction product, or with cobalt chloride added for a black reaction product.

Wash: sections in PBS for 3x10 minutes.

DAB STAINING PROTOCOL (Work in fume hood)

- Prepare waterbath containing bleach to neutralize spills and to de-react DAB solution after experiment is finished
- Measure 50 mL 1xPBS into Erlenmeyer flask (see reagents list below for exact recipe)
    + If black reaction product is desired, add 1.5 mL Cobalt Chloride (if the product is wanted brown, leave it out)
    + Add 12.5 mg of DAB and mix
    + Add 7.5 ul of hydrogen peroxide, mix and pour into appropriate reaction container (lid of 24 well plate)
- Incubate sections until reaction is considered complete (approx. 10 seconds to 2 minutes). Place section tray into PBS (use tweezers to move tray – wear gloves).
- Wash sections 3x10 minutes in 1xPBS.
- Mount on gelatin-coated slides (use PBS-TX in petri dish to help with mounting) and leave to dry o/n.
- Dehydrate through alcohol series (70, 90 and 100% EtOH, 3 minutes each).
- Dewax in Xylene for 20 minutes.
- Coverslip with permount.

Reagents

Phosphate buffered saline pH 7.4, 10X stock solutions (PBS)

Dilute 1+9 for use

KH_2PO_4	5.0 g	10.0 g
Na_2HPO_4 ***	28.75 g	57.5 g
NaCl	200.0 g	400.0 g
KCl	5.0 g	10.0 g
Distilled water to make the volume to	2,500 mL	5,000 mL

*** <NB> Na2HPO4.2H2O can be used instead	36.05 g	72.1 g

Composition of 10X PBS
1370 mM Sodium chloride
27 mM Potassium chloride
81 mM Disodium hydrogen phosphate
14.7 mM Potassium dihydrogen phosphate

25-30% Sucrose in PBS pH 7.4

PBS with 0.4% Triton X-100 (PBS-TX)
Phosphate buffered saline
Triton X-100 (Sigma)

Bleach (50% methanol in distilled water with 1% hydrogen peroxide)

Methanol	45 mL
Distilled water (we used PBS)	45 mL
30% H_2O_2	3 mL

With Martin I made this according to following recipe:

Methanol	9 mL
PBS	9 mL
30% H_2O_2	700 uL

10% sodium azide

Anti-choleragenoid stock solution
Goat anti-choleragenoid (List Labs) diluted 1:1,000
Can make a 1:10 stock with PBS that you then dilute 100x for this incubation

Anti- choleragenoid incubating medium *WITHOUT* sodium azide for incubations overnight at 4°C

Anti-choleragenoid stock solution 1:1,000	33 µl
Normal rabbit serum (Sigma)	25 µl
10% sodium azide	10 µl
PBS-TX	942 µl

Anti-choleragenoid incubating medium *WITH* 0.1% sodium azide for incubations at room temperature or at 4°C

Anti-choleragenoid stock solution 1:1,000	33 µl
Normal rabbit serum (Sigma)	25 µl
10% sodium azide	10 µl
PBS-TX	932 µl

Biotinylated anti-goat medium (1:200)

Biotinylated rabbit anti-goat (Sigma)	5 µl
PBS-TX	1.0 mL

Streptavidin peroxidase medium (1:1,000)

Streptavidin peroxidase *Molecular Probes (1mg/ml)*	2 µl
PBS-TX	2 mL

Diaminobenzidine peroxidase reaction medium for <u>BROWN</u> reaction product

Diaminobenzidine (Sigma)	12.5 mg	25 mg
PBS	50 mL	100 mL
30% H_2O_2	7.5 µl	15 µl

Diaminobenzidine peroxidase reaction medium for <u>BLACK</u> reaction product

Diaminobenzidine (Sigma)	12.5 mg	25 mg
PBS	48.5 mL	97 mL
0.5% Cobalt chloride	1.5 mL	3 mL
30% H_2O_2	7.5 µl	15 µl