Pre-Surgery

1. Make glass electrodes for electrophysiological recording. Stretch glass capillary. Tip diameter should be 4-5µm (chip away, use light microscope with scale bar)
2. Electrodes filled with dye solution (2M NaCl in 2% pontamine sky blue or Fast Green) NO BUBBLES
3. Autoclave surgery pack and instruments (check to make sure you have everything needed including instruments, drapes, sutures, ground wire, and cotton swabs)

Surgery Setup

1. Calculate the amount of anaesthetic and analgesic to be administered. Dosage amounts for pigeons are:
    - Ketalean: 65µg/g (0.36 x bird's weight(g) / 550)
    - Xylazine: 9µg/g (0.26 x bird's weight(g) / 550)
2. Using scissors, cut the head feathers in a reverse motion in the area where the incision will be. Cut the feathers coving the ear holes.
3. Check Pedal reflex – must be very dull
4. I.P. sterile saline injection of 50µL for hummingbird (just in at the base of the head)
5. The bird should now be given an injection of Metacam, to be used as an analgesic. Metacam (0.5% for injection) is to be given as a single subcutaneous injection at a dosage of 0.2µg/g bodyweight (which is a volume of 0.04µL/g). In recovery procedures, this dose should be repeated after 24 hours. At this time, also administer an injection of ampicillin (0.08µL/g, i.m.; which is a dose of 20µg/g). (skipped ampicillin)
6. Using Hibitane, swab earbars and head (bird's scalp where feathers have been removed)
7. Place bird in the stereotax using earbars and beakbar – ensure that needle (to find interaural zero) is raised and is not nudged while bird is positioned. Make sure that the earbars are zeroed again; centered and evenly spaced.

Surgery/Exposure

1. Make an incision in the scalp.
2. Apply a small amount of Marcaine (Bupivicaine 5mg/mL concentration - analgesic) to the region of scalp along the edge of the incision with a q-tip
3. Remove a section of bone overlying the desired brain region. Apply sterile saline periodically to keep the surface of the brain and the scalp moist.
4. Gently stroke the border until the dura is framed and slightly broken. Then peel away the dura.

Electrophysiological Recording and Tracer Injections

5. Insert the recording pipette (tip diameter 5µm) into the arm, connect to a a silver wire. ENSURE WIRE IS DRY AND NO BUBBLES IN PIPETTE.
6. Connect alligators, red to silver wire, black/ground to flesh of bird
7. Align electrode to IAZ.
8. Move electrode to the desired injection target using physiological criteria (i.e. responsiveness of neurons to the hand-held visual stimulus consisting of a white background and black lines, dots and squiggles in a random pattern).

9. Eyelids can be retracted with tape and an artificial tear product must be applied to the eyes to prevent the cornea from drying out.
10. Identify target and collect data
11. Replace recording pipette with injection micropipettes containing the neural tracer. ENSURE WIRE IS DRY AND NO BUBBLES IN PIPETTE.
12. Align to IAZ
13. Move electrode to the desired injection target
14. Injection micropipettes (25µm tip diameter) Dry the area of the injection site with little sertile sponges ***
15. Injected neuro tracer iontophoretically (up to 30 min with +3 µamp) or with a picospritzer (i.e. an air puff). These injections should involve only an extremely small amount of tracer (picolitres).The injection itself will not do any damage to the brain.

Tracer injections include either: the anterograde tracer biotinylated dextran amine (BDA), the retrograde tracer LumaFluor (flourescent latex microspheres) or the bidirectional tracer cholera toxin subunit B (CTB).				
Tracer	Duration	Delay	Stiumlus Rate	Volts
CTB	7 x 1000ms	7x1000ms	1.5 x 0.1	4-5µamp
BDA	1x1000ms	1x1000ms	4.5x0.1	4-5µamp
Lesion	Max 1000 (30 sec)	0	10x1000	1.0-1.5 milliamp?
Marking Lesion	30 µamp (+ve current)	10sec	Electrode –ve	

16. When the injections are finished, remove the pipette and cover the exposure with bone wax. Apply a small amount of Marcaine (Bupivicaine 5mg/ml concentration - analgesic) to the region of scalp along the edge of the incision with a sterile q-tip, being very careful not to let any contact the bonewax/exposure region.
17. Suture the incision with vicryl suture (ethicon cat. no. J451H)
18. Remove the bird from the stereotax

Post-surgery

1. Administer an analgesic (buprenorphine, 0.1 mg/kg, i.m.). As the buprenorphine can suppress food and water intake, apply an i.p. injection of sterile saline (20µl/g) post surgery and again during recovery if there are any signs of dehydration. Return the animal to its home cage for recovery (survival of 2-5 days depending on the tracer used and the site of injection)
2. Monitor bird condition (at least once every 4 hours during normal recovery; every half an hour if the animal shows any signs of distress). Note any problems (i.e., posture, aural discharge, eating, etc.).
3. Transcardial perfusion with saline (0.09%), followed by paraformaldehyde (4%). Subsequently, dissect out the brain, section, and process for the neural tracers and constituent neurochemistry.

Bird anesthetized

1. Deliver Isoflurane until lightly anesthetized

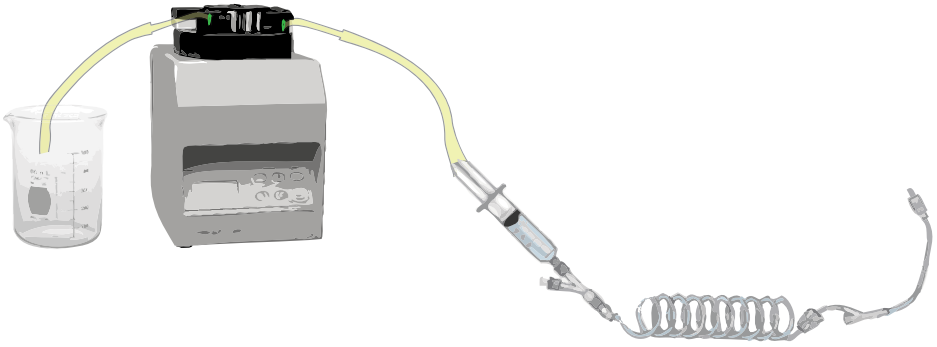
2. Intramuscularly inject Zebra Finches with 0.04ml of Ketamine/Xylazine mixture

NB: Good for 2-3 hours anesthesia

http://www.springerlink.com/content/u63k7t336q760235/
Herrera et al. from Chile recorded electro-retinograms from hummingbirds "Animals were anaesthetized with 75 mg/ml Ketamine and 5.82 mg/ml of Xilazine, 1 microliter per body gram"

Surgery

1. Remove feathers by plucking around the torso and the back of the neck (to expose the jugular)
2. With fine scissors cut along the connective tissue below the pectoralis majors
3. With hard scissors cut medially between the pectoralis major muscles next to the keel
4. Use the retractors to open the chest cavity
5. Flip the bird over and make an incision into the jugular artery on the dorsal side of the next to allow fluid flow or an incision into the fat line on the right side of the heart
6. If using heparin, make an injection into the heart now
7. Insert the pulsing needle (1.25 setting on Minipuls3) with 0.9% saline (if you using heparin, include 2.5mL per 500mL in saline) into the left ventricle.



Transcardial Perfusion (an IV-like system such as above is SO not necessary)

1. First pulse in 0.9 % Saline (~ 3min) at 1.25 setting on Minipuls3 by Gilson (Dave starts with a slow rate, and then after it is in the bird, accelerates the speed)
2. Followed by paraformaldehyde (4% in 0.1M sodium phosphate buffer pH7.4) at 1.25 (don't check the pH)

NB: Perfusing with even low [glutaraldehyde] will generate unacceptably high background.

3. Put skinned head in PFA or skip to step 7

Slice

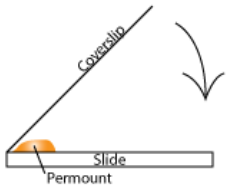
1. Trim the block, ensuring the midline is perpendicular to the plane of slicing (use the area of interest as the primary line to align)
2. Brain stem up and pointing at blade, place on freezing microtome with OCT holding it in place
3. Let block freeze slowly. Fill trays with PBS while you wait.
4. Slice at 40μm
5. Pour small amount of 2-isopropanol into the moat
6. Crush dry ice and fill area (you can use lots of ice after the block is initially fully frozen)
7. Collect sections in PBS

8. Mount on subbed slides
9. Let slides dry

NOTE: for fluorescence, once you get to the tissue of interest, turn the lights off and slice, mount, and store without direct light.

Nissl Staining (Thionin)

1. Place slides in glass racks.
2. Place in chloroform for 45min
3. For 5 seconds each, take through alcohols 100, 100, 95, 95, 70, 50, 30% while gently agitating
4. Leave in buffer solution for 1min
5. Dip in stain for 2.5min for 40µL thick sections
6. Dip in buffer solution for 5sec
7. Bring back through 30 and 50% washes for 15sec/each
8. Place in 70% for 0.5-5 minutes, checking each minute for fading (goal: nearly no pink, slide is clean and stain is only on tissue)
9. Take through alcohols (95, 95, 100, 100%)
   Check colour at each step
10. 2x washes in Hemo-de (Xylene-substitute) for 5min/each – Leave in Hemo-de until coverslipping
11. Coverslip with Permount
    a) Apply a line of permount as thick as the tip of a soft pipette on the bottom edge of the slide
    b) Line up a coverslip along the bottom edge of the slide and slowly allow it to decrease the angle made from the bottom of the slide to the top
    c) Let the slides dry on a smooth surface (not a paper towel)



Notes: Variation in staining times will result from different ages of slides (fresher ones may be darker). The steps you vary are the 70%+glacial acid and the following alcohol rinses. You can work up and down the higher ethanol dilutions, but once in Hemo-de, you're committed. Slides should not dry between steps. The 100% ethanol rinses will dry off fastest, but you do need to stop and look at the darkness of the stains through the 95's and 100's on the way out. These steps will weaken the stain, the glacial acid will weaken stain at a slower rate than background (a good thing). Remember to keep your slides a little dark at the 70%/glacial acid step, as the subsequent 95's and 100's will fade the slide significantly. Hemo-de doesn't.

Waste: chloroform and ethanols into an ethanol waste jar; hemo-de into a separate waste jar

Background: Neurons contain Nissl substance, which is primarily composed of rough endoplasmic reticulum, with the amount, form, and distribution varying in different types of neurons. Because of the RNA content, Nissl substance is very basophilic and will be very sharply stained with basic aniline dyes. By varying the pH and the degree of differentiation, both Nissl substance and nuclei or only Nissl substance may be demonstrated.

RECIPES

Pre-Surgergy:

1. **Dye Solution**

   2M NaCl in 2% Fast Green

   Mix up solution, good for a long time

Bird anesthetized:

2. **Ketamine/Xylazine Mix**

Ketmaine (100mg/mL)	0.1mL
Xylazine (20mg/mL aka 2%)	0.1mL
Deionized H_2O	0.4mL

Use a syringe to take up 0.1ml of 100mg/mL ketamine and release into a 2mL capped-tube
Use a syringe to take up 0.1ml of 20mg/mL xylazine (2% Rompun) add to tube
Use a p1000 pipette and add 200µL dH_2O to tube.
This is your mix solution; total of 0.6mL
Zebra Finches receive 0.04ml of mixture for 2-3 hours anesthesia
Same for hummingbirds, especially small ones

Surgery:

1. **Tracers** *(good for ~ 1 year, according to a comment from Dextran on 10K Red; ships in 2-14 days)*

3000 Red (aka micro-ruby) – bidirectional, stronger anterograde	Dextran	D7162
3000 Green (aka mirco-emerald) – anterograde	Dextran	D7156
10 000 Red (aka micro-ruby) - bidirectional, stronger anterograde	Dextran	D22913
10 000 Green (aka mirco-emerald) – anterograde	Dextran	D22910

* The above for are biotynilated so they can also be sued for light microscopy.

CTB Green (comes in volumes 100-500µg, but 500µg recommended)	C34775
CTB Red (comes in volumes 100-500µg, but 500µg recommended)	C34777

Perfusion:

3. **Normal Saline (0.9%)**

   Filter before use

Sodium chloride	4.5g
Distilled water to make volume to	500mL

Add 4.5g to 250mL ddH_2O
Stir
Then fill to 500mL
Filter to remove all large crystals (filter paper must be folded into ¼ pie, place in funnel)

4. **0.4 M Stock Sodium Phosphate Buffer pH7.4**

   Dilute to 0.1M for use (*ie* 250mL diluted to 1,000, the pH only correct when diluted).

Na_2HPO_4 – Dibasic Anhydrous	9.2	46g	92g
$NaH_2PO4{\cdot}H_2O$ – Monobasic (check formula)	2.10	10.49g	20.98g

Distilled water to make volume to	200mL	1,000mL	2,000mL

Use large Erlenmeyer flask (volumetric flask if it's available)

Add sodium phosphates to 500mL dH_2O

Stir for a very long time

Top up with dH2O to bring to 1,000mL

Fixative & Post-Fix

5. 1.0M sodium hydroxide

NaOH	1.0g	4.0g	10g
Distilled water	25mL	100mL	250mL

CAUTION: This is an exothermic reaction, proceed slowly

Weigh out 1.0g of NaOH and slowly add to 15mL of dH_2O

Wait until solution cools

Top up solution with dH_2O to bring to 25mL

6. Fixative (4% paraformaldehyde in 0.1M sodium phosphate buffer)

Filter before use | Prepare in fume hood

Paraformaldehyde ('high grade')	4g	8g	20g
0.4M stock sodium phosphate buffer	25mL	50mL	125mL
Distilled water to make volume to	100mL	200mL	500mL

Heat dH_2O to 60-70^oC

Suspend the paraformaldehyde in the hot water in the FUME HOOD

Stir for ~ 10min

Add 1.0M NaOH drop-wise until the solution clears/just dissolves paraformaldehyde

Cool below 40^oC on ice slurry

Add 0.4 M phosphate buffer

Bring up to 500mL with dH_20

Filter to remove all large crystals (filter paper must be folded into ¼ pie, place in funnel)

pH to something between 7.4 and 7.5

NB: During the perfusion, the saline is always on the right and the fix is always on the left.

Calibrating the pH Meter

1. Press **MODE** until the display shows measure pH mode.
2. Press the **SETUP** key twice and then the **ENTER** key to clear previous calibration.
3. Immerse the rinsed electrode into the first calibration buffer.
4. Press **STD,** then **STD** again – the meter will automatically recognize the buffer and display it. When the **STABLE** icon appears, the buffer value is entered and meter returns to the **MEASURE** screen.
5. Repeat steps 3 & 4 for the other two buffers.

Turning off the pH Meter just for Now

1. Rinse of the probe with distilled water.
2. Place in pH 7.0 or pH 4.0 buffer solution.
3. Leave the fill hole at the top of the electrode **OPEN.**
4. Press **STBY.**

Turning off the pH Meter for MORE than a day or so

1. Rinse of the probe with distilled water.
2. Press **STBY.**
3. **CLOSE** the fill hole at the top of the electrode.
4. Cover the end of the electrode with the plastic cot. Make sure the cotton in the cot has been wetted with pH4 or pH 7 buffer (**NOT** distilled water).

7. **Phosphate buffered saline pH 7.6, 10X stock solutions (PBS)**

Dilute 1+9 for use

KH_2PO_4	1.0g	2.0g	5.0g	10.0g
Na_2HPO_4 ***	5.75g	11.5g	28.75g	57.5g
NaCl	40.0g	80.0g	200.0g	400.0g
KCl	1.0g	2.0g	5.0g	10.0g
dH_2O to make the volume to	500mL	1,000mL	2,500mL	5,000mL
*** NB: Na2HPO4.2H2O can be used instead			36.05g	72.1g

Directions from Shadwick lab, USE ROB'S QUANTITIES

Dissolve 40g of NaCl, 1.0g KCl, 7.4g of Na_2HPO_4, 1.2g of KH2PO4 in 400mL dH_2O

*Adjust pH to 7.2 to 7.4 with HCl***

Adjust volume to 1L with dH_2O

Sterilize by autoclaving

***NB: July 31, 2012, following Rob's quantities I found the solution before adjusting the volume to be basic instead of acidic, so I added NaOH instead of HCl*

Gelatin Embedding (from Jeremy)

8. **30% Sucrose Solution in 0.1M PBS**

Sucrose	7.5g	15g	150 g
10X PBS	2.5mL	5mL	50mL
dH_2O to make to volume	25mL *(18mL)*	50mL	500mL

Add sucrose and 10X PBS to half of dH_2O volume

Bring volume to dH_2O volume (total should be about 18mL dH_2O for small sample)

Store at 4^oC (refrigerator)

9. **Gelatin solution**

Sucrose	5g	15g
Gelatin	6g	18g
dH_2O to make to volume	50mL	500mL

Dissolve 5g sucrose in 50ml dH_2O

Heat up to 42^OC to dissolve gelatin, but try to keep the temperature as low as possible (the lower the temperature, the harder the block)

While stirring, add 6g gelatin

10. **Post-fix for Gelatin**

Same PFA as before

11. **Sucrose Solution**

Same sucrose as before

Nissl Staining (Thionin) (Large trays are 250mL, small ones are 200mL)

12. 1% Thionin Stock Solution

2g thionin

200mL dH_2O

Bring water to boil and add thionin while stirring

Turn off heat BEFORE ADDING THIONIN (you silly girl!) and allow solution to stir OVERNIGHT

Filter and store in a brown glass bottle

13. 1M Acetic Acid

Acetic acid, Glacial (from Shadwick lab)	28.5mL
dH_2O	471.5mL

Mix, bottle and store

Store in 500mL aliquots at -20^oC

14. 1 M Sodium Acetate

Hydrous sodium acetate (1 mol sodium acetate)	136.08g
dH_2O	1000mL

Add sodium acetate to 800mL dH2O

Stir and bring final volume to 1L

Store in 500mL aliquots at -20^oC

15. Buffer Solution

dH_2O	140mL	280mL	**420mL**
1M Acetic Acid (see above)	24mL	48mL	**72mL**
1M Sodium Acetate (see above)	16mL	32mL	**48mL**

Mix for total volume of 180mL and check pH (near 4.4)

Filter

**Prepare extra for thionin stain.*

16. Thionin Stain

1% Thionin Stock Solution	20mL
Buffer Solution	180mL

17. Washes

Chloroform, 30, 50, 70, 95 (2 trays), and 100% (2 trays) dilutions

Add 5 drops of Acetic Acid, Glacial (from Shadwick Lab) to the 70% tray

18. Substitute for Xylene

Hemo-De

2 trays

1. **0.1% Cresyl Fast Violet (not used in Wylie lab)**

   0.1% Cresyl Fast Violet: Cresyl Echt Violet Acetate disolved in ddH20

   Mix well, filter. For best results allow to "ripen" 48 hours before use. Stable for 1 year.g

   Online directions: Stir on heat (60^{o}C) until majority of crystals are dissolved*. Let the solution cool and store in a dark bottle. Reheat to 60^{o}C and filter before every use.

   From online: 0.1% cresyl violet solution by mixing 0.1 g cresyl fast violet mixed in 100 mL deionized H2O. http://www.scientistsolutions.com/a299-protocol-nissl+staining+for+neurons.aspx

*If possible, stir solution for a couple of days with heat, and then filter again. If you still get specks of solid on sections, use a Bottle Top Filter (rather than a fluted paper) for better filtration

CHEMICAL LIST	**Provider**	**Catalog #-quantity**
Deionized water (Take a flask to Milsom or Jeff Richards lab)		
Sodium chloride NaCl Certified ACS Crystaline	Fisher	S271-3
Sodium Phosphate Dibasic Anhydrous Na_2HPO_4	Fisher	S374-500
Sodium Phosphate Monobasic $NaH_2PO4{\cdot}H_2O$	Fisher	S369-500
Sodium Hydroxide NaOH	Shadwick (under fume hood)	
Hemo-De	Hemo	HD-150G (1L)
Gluteraldehyde (certified 25%)	Fisher	O2957-1
Paraformaldehyde (Formaldehyde, Para | lab grade)	Fisher	T353-500
Sodium Azide NaN_3 (min 99.5%)	Sigma	S-2002 (25g)
Cresyl Fast Violet $C_{18}H_{15}N_3O_3$(certified)	Ted Pella, Inc.	
Sucrose	Sigma	S0389-500G
Ethanol (there's a binder for signing out ethanol)	Stores	outback
Gelatin (Type A laboratory grade powder)	Fisher	G8-500
Potassium Chloride KCl (ACS reagent | 99.0-100.5%)	Sigma	P3911-500G
Fast Green FCF	Fisher	BP-123-10
Tear drops		
Buprenorphine (Opiate, requires special permission)	AVP Orders	
Xylazine (Rompun)	AVP Orders	
Ketamine	AVP Orders	
Saline from IV drip bag for surgery	AVP Orders	
Metacam 0.5% (Injection meioxicam 5mg/mL) Boehringer Ingelheim	DIN 02240463 20mL	
Bone Wax (instead of gel foam)	AVP orders	
(Ethicon W31G, may need an account $46.00 Phone number: 1-905-946-9501)		
Hibiclens 16 oz (1 pt.)		
Marcaine (Bupivicaine 5mg/mL) analgesic 50mL Hospina Cat: 1787	AVP Orders	
3000 Red (aka micro-ruby) – bidirectional, stronger anterograde	Dextran	D7162
3000 Green (aka mirco-emerald) – anterograde	Dextran	D7156
10 000 Red (aka micro-ruby) - bidirectional, stronger anterograde	Dextran	D22913
10 000 Green (aka mirco-emerald) – anterograde	Dextran	D22910
*The above 4 are biotynilated so they can also be sued for light microscopy. Tracers ship between 2 days and 2 weeks.		
CTB Green (volumes of 100-500µg, but 500µg recommended) – retrograde		C34775
CTB Red (volumes of 100-500µg, but 500µg recommended) – retrograde		C34777
Dry Ice (Chem stores, bring a tub, weigh tub, get the key, fill, weigh tub full, record difference n binder)		
Monoclonal Anti-Parvalbumin (Mouse Primary AB)	Sigma	P 3088
Mouse Anti-Zebrin II (from Andy) Primary		
Rabbit anti Calretinin Cedarlane Laboratories Limitied (1-800-268-5058)		7699/3H
Thionin acetate, certified	Sigma	T7025-25G
Normal Donkey Serum (order it from the same place you get the 2^{o}AB)		017-000-121
Permount/toluene solution UN1294	Fisher	SP15-100
Acetone	Sigma	T7025-25G

Chloroform HPLC Grade	Fisher	C606-4
2-Propanol (Isopropanol, into squirt bottle)	Fisher	A416-500
Glycerin	Fisher	G31-500
Sodium Acetate	Fisher	S78229

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TO DO – recipes for 1^{o} and 2^{o} antibody stains + product numbers