Zebrafish Module

MBL Embryology Course 2010
June 29th – July 3rd

Manual modified from original version written by Marnie Halpern and Josh Gamse, with many contributions from other instructors and TAs over the years.
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Introduction
In this module, you’ll learn techniques for molecular and embryological manipulation of zebrafish. First you’ll get familiar with staging and dechorionating the embryos, then we’ll show you techniques that you can use to design and execute some experiments of your own!

Throughout this manual, you’ll find EXAMPLES of experiments that can be done with zebrafish embryos, but we encourage you to think of your own experiments to try. When thinking about designing your experiments try to keep in mind the advantages of zebrafish as a model system. Here we provide a list of all the reagents available to you as well as their potential uses. The Instructors and TAs are very happy to discuss ideas for your experiments.

2010 TAs
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2010 Faculty
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Online resources
www.zfin.org  This is the Zebrafish Information Network site, run by the University of Oregon – find information about mutants, transgenics, protocols, genomics, expression patterns, etc.
www.ensembl.org/Danio_rerio/  This is the Zebrafish Genome Project web site. You can look for your favorite gene in zebrafish using the SSAHA search program.

Schedule
Except for Tuesday, lab starts at 9:00 am, when the fish here and at the Marine Resource Center begin to lay. Lectures are after lunch, beginning at 1:00 pm. We will shoot for 60-75 minutes of lecture, followed by questions, so that we can return to the lab by 3:45 pm. Newly fertilized fish embryos for your experiments will be available Wednesday-Saturday mornings and around 3:30 pm on Wednesday-Friday afternoons. *(FYI: embryo production will be more robust in the morning.)* Every day we’ll have different stages of wild-type embryos available, and (hopefully!) an assortment of transgenic embryos on Thursday and Friday mornings. A list of available transgenics will be posted on the chalkboard. If you have special embryo requests, please ask Sharon or one of the other TAs. Embryos will initially be placed in the injection room, then moved to fish incubators. Dishes will be labeled with day and time laid, so that you can find older embryos as you need them for experiments. Reagents such as RNAs, dyes, and morpholinos for injection will be available in aliquots in the injection room (most on ice, MOs at RT).
Tuesday, June 29
On Tuesday evening, embryos at different stages will be available for you to examine. Please familiarize yourselves with developmental staging and how to move embryos around and remove their “eggshell”, the chorion, using forceps. Look over the lists of reagents and suggested experiments in this handbook and dream up experiments you’d like to try. We will give a short presentation to introduce some of the available reagents and zebrafish strains and to provide tips on handling and raising the embryos. Embryos from the NTR lines will be born this morning and available for experiments beginning this evening.

Wednesday, June 30
Lab starts at 9:00 am with a brief lab introduction. We’ll demonstrate how to microinject fish embryos – it’s similar but not exactly the same as in frog. We need to do this in waves, so the first group will start injecting right at 9:30, others will follow as embryos are delivered to the injection room. In the afternoon (3:30-4:30 pm), there will be more embryos for a second, smaller round of injections and experiments. In the afternoon and evening, we’ll demonstrate how to transplant cells from blastula and gastrula stage embryos and how to photoconvert Kaede from green to red for fate mapping experiments.

Thursday, July 1
There will be wildtype embryos and transgenics in the morning and wildtype embryos in the afternoon for injections. However, there is a 4 pm Ethics class after sweatbox that will limit afternoon 1-cell injections. A second round of NTR embryos will be collected this morning – let us know if you want them for injections; otherwise they will be available from blastula stages onward. Catch the Kaede photoconversion and transplantation demos in the afternoon and evening. Continue your ongoing experiments. Start your in situ hybridizations today if you want results by Saturday.

Friday, July 2
There will be embryos for injections in the morning and afternoon – transgenics will be available in the morning only. We will help you with Kaede photoconversion and transplantations in the afternoon and evening.

Saturday, July 3
In the morning, we will demonstrate in vitro fertilization to generate haploid and diploid zebrafish embryos. It’s time to finish up your experiments!

**Lab Schedule Calendar (see board for details about times)**

<table>
<thead>
<tr>
<th></th>
<th>1-cell wild types</th>
<th>Fluorescent Transgenics</th>
<th>NTR transgenics</th>
<th>Transplants</th>
<th>Kaede photoconversion</th>
<th>In vitro fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tues</td>
<td></td>
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</tr>
<tr>
<td>Wed</td>
<td>AM and PM</td>
<td></td>
<td></td>
<td>PM and late PM</td>
<td>PM and late PM</td>
<td></td>
</tr>
<tr>
<td>Thur</td>
<td>AM (and PM?)</td>
<td>Born AM</td>
<td>Born AM</td>
<td>late PM</td>
<td>PM and late PM</td>
<td></td>
</tr>
<tr>
<td>Fri</td>
<td>AM and PM</td>
<td>Born AM</td>
<td></td>
<td>PM and late PM</td>
<td>PM</td>
<td></td>
</tr>
<tr>
<td>Sat</td>
<td>AM only</td>
<td>By request?</td>
<td></td>
<td>By request?</td>
<td>By request?</td>
<td>Late AM</td>
</tr>
</tbody>
</table>

Also, Sarah or Vicky can demonstrate neuronal backfills if you’d like. Just let them know.
Some helpful hints for working with zebrafish embryos!

**General advice**

- Check the board for important information!
- Use the illuminated base when viewing live embryos. Fish embryos are transparent, and you won’t appreciate the details of the embryo if you’re using incident lighting. However, when viewing stained embryos, it’s best to use incident lighting, not transillumination.
- The amount of time for 1-cell injections is somewhat limiting, so please keep to your signed-up injection time. Please leave the area is clean and ready for the next user.
- Please turn off the scopes when you are done.

**Handling embryos**

- Use fish water or embryo medium (provided in the squirt bottles) for embryos and larvae. **IMPORTANT:** for dechorionated embryos < 1 day old, use embryo medium.
- Use the wide-bore glass pipets and rubber bulbs to pipet your embryos. For dechorionated embryos < 1 day old, use fire polished pipets (at front of room, marked with a black ring).
- Use the pokers (provided to you at the start of the module) to push embryos (in chorions) around. Use loops to gently nudge dechorionated embryos (<10 hpf) around.
- Keep your embryos in the 28°C incubators when you’re not working with them. Alternatively, you can keep them at RT or 32°C to slow or speed development, respectively. Zebrafish embryos won’t develop normally if kept at very low temperature for extended times.
- To dechorionate embryos: grab the chorion with one pair of sharp forceps. Grab nearby with a second pair. Carefully tear the chorion open to release the embryo. If you’re squashing the embryos, you’re pulling too hard/fast. You’ll want to dechorionate embryos for manipulations like transplants and after fixation before processing for in situ or antibody staining (if embryos are older than 24 hpf, you need to dechorionate before fixation). Before segmentation stages, be sure to dechorionate embryos on agarose-coated dishes in embryo medium.
- Prior to the end of gastrulation, dechorionated embryos will disintegrate if exposed to air.
- Overcrowding in dishes can cause developmental asynchrony and/or delay. Guidelines: 100 embryos per 100 mm dish, 50 embryos per 60 mm dish, and 25 embryos per 35 mm dish.

**Embryos for experiments**

- Fish lay on a circadian cycle; we have two separate “dawns” for wildtype fish that occur at 9 am and again at 3 pm. Fertilization begins soon after “sunrise”.
- There will be lots of wildtype embryos for you to inject beginning at 9:30 am (until about 11:30 am) and at 3:30 pm (until about 4:30-5 pm). On some days, we will also have transgenic lines, but in the morning only (see later in this manual for a list).
- Embryos will be on trays in the injection room and in the fish incubator. Take only what you need, not what you want – don’t be greedy. Remember, transgenes are dominant and will be expressed in half or all the embryos; they will often segregate independently from parents containing multiple transgenes.
- We cannot guarantee that we will get embryos from all transgenic lines on both days, so please have several equally-exciting possible experiments in mind when you come to the injection room!
Experimental advice

- **Use your staging guide to help you plan your experiments!** For example, most of you will want to do microinjections (MOs, lineage tracers, GFP) to uniformly label the embryo; to do this you must inject embryos before the 4-cell stage (1 hpf). For mosaic labeling, you will inject slightly later. Blastula/gastrula transplantations are performed between 4-6 hpf. Pharmacological inhibitors can be added at various times.

- MOs, RNAs, lineage dyes, inhibitors, etc. will be available in aliquots in the injection room.

- Keep fluorescently labeled embryos in the dark.

- We will provide transplantation pipets. You may learn how to make them as well.

- If you want to image embryos 30 hpf or older, remember to put the embryos into PTU-embryo medium between 18-24 hpf to inhibit pigmentation.

- To fix embryos/larvae, dechorionate them and put them into an eppendorf tube (embryos <24 hpf can be dechorionated after fixation). Prepare 4% paraformaldehyde (PFA) fixative by diluting 32% PFA 8-fold in PBS. WEAR GLOVES! Fix overnight at 4°C or at room temp for 4 hours. Store fixed embryos at 4°C in PBS. Special fixes are required for some staining procedures; please check specific protocols.

- Plan ahead for in situ hybridization – it will take 2-3 days to finish.

- To photograph embryos/larvae:

  You can get good side views of live >1 day old embryos by mounting on 2-, 3-, or 4-high bridged cover slip (at front of room). For top views, you can mount in agar drops or methylcellulose. Mounting media will be at the front of the room.

  For younger than 1 day, you can mount with methylcellulose in a depression slide.

  For fixed embryos, you’ll want to dehydrate them in glycerol and mount either the whole embryo or use forceps to cut off the part you’re interested in, and photograph on a bridged glass slide. Several protocols for mounting can be found later in the manual.
### Zebradish staging table and pictures

Based on Kimmel et al, 1995, Developmental Dynamics.

**Pictures and drawings from Haffter et al, 1996, Development.**

Abbreviations: hpf: hours post fertilization at 28.5°C, EL: embryo length, PF: pectoral fin, HB: approximate stage number in the Hisoaka & Battle (1958) zebradish staging series (reasonably accurate through HB stage 20), HD: head diameter in dorsal view, HTA: heart trunk angle, OVL: otic vesicle length, NO: Nomarski optics, H/W: height/width, Prim: Prim stages refer to the number of the myotome to which the leading end of the posterior lateral line primordium has advanced. YB: yolk ball, YE: yolk extension, YSL: yolk syncytial layer.

<table>
<thead>
<tr>
<th>Stage</th>
<th>hpf</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zygote period</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-cell</td>
<td>0</td>
<td>Cytoplasm streams towards animal pole to form the blastodisk</td>
</tr>
<tr>
<td><strong>Cleavage period</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-cell</td>
<td>3/4</td>
<td>partial cleavage</td>
</tr>
<tr>
<td>4-cell</td>
<td>1</td>
<td>2 x 2 array of blastomeres</td>
</tr>
<tr>
<td>8-cell</td>
<td>1 1/4</td>
<td>2 x 4 array of blastomeres</td>
</tr>
<tr>
<td>16-cell</td>
<td>1 1/2</td>
<td>4 x 4 array of blastomeres</td>
</tr>
<tr>
<td>32-cell</td>
<td>1 3/4</td>
<td>2 regular tiers (horizontal rows) of blastomeres, sometimes in 4 x 8 array</td>
</tr>
<tr>
<td>64-cell</td>
<td>2</td>
<td>3 regular tiers of blastomeres</td>
</tr>
<tr>
<td><strong>Blastula period</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>128-cell</td>
<td>2 1/4</td>
<td>5 blastomere tiers; cleavage planes irregular</td>
</tr>
<tr>
<td>256-cell</td>
<td>2 1/2</td>
<td>7 blastomere tiers</td>
</tr>
<tr>
<td>512-cell</td>
<td>2 3/4</td>
<td>9 tiers of blastomeres; NO: YSL forms</td>
</tr>
<tr>
<td>1k-cell</td>
<td>3</td>
<td>11 tiers of blastomeres; NO: single row of YSL nuclei; slight blastodisk cell cycle asynchrony</td>
</tr>
<tr>
<td>High</td>
<td>3 1/3</td>
<td>&gt;11 tiers of blastomeres; beginning of blastodisk flattening; NO: YSL nuclei in two rows; substantial division asynchrony</td>
</tr>
<tr>
<td>Oblong</td>
<td>3 2/3</td>
<td>Flattening produces an elliptical shape; NO: multiple rows of YSL nuclei</td>
</tr>
<tr>
<td>Sphere</td>
<td>4</td>
<td>Spherical shape; flat border between blastodisk and yolk</td>
</tr>
<tr>
<td>Dome</td>
<td>4 1/3</td>
<td>Shape remains spherical; yolk cell bulging (doming) towards animal pole as epiboly begins</td>
</tr>
<tr>
<td>30%-epiboly</td>
<td>4 2/3</td>
<td>Blastoderm an inverted cup of uniform thickness; margin reaches 30% of distance between the animal and vegetal poles</td>
</tr>
<tr>
<td><strong>Gastrula period</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50%-epiboly</td>
<td>5 1/4</td>
<td>Blastoderm remains uniform in thickness</td>
</tr>
<tr>
<td>Germ-ring</td>
<td>5 2/3</td>
<td>Germ ring visible from animal pole, still 50%-epiboly</td>
</tr>
<tr>
<td>Shield</td>
<td>6</td>
<td>Embryonic shield visible from animal pole, still 50%-epiboly</td>
</tr>
<tr>
<td>75%-epiboly</td>
<td>8</td>
<td>Dorsal side distinctly thicker; epiblast, hypoblast, evacuation zone visible</td>
</tr>
<tr>
<td>90%-epiboly</td>
<td>9</td>
<td>Brain rudiment thickened; notochord rudiment distinct from segmental plate</td>
</tr>
<tr>
<td>Bud</td>
<td>10</td>
<td>Tail bud prominent; notochord rudiment distinct from neural keel; early polster; midsagittal groove in anterior neural keel; 100%-epiboly</td>
</tr>
</tbody>
</table>
### Segmentation period

<table>
<thead>
<tr>
<th>Stage</th>
<th>hpf</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-somite</td>
<td>10 1/3</td>
<td>First somite furrow</td>
</tr>
<tr>
<td>5-somite</td>
<td>11 2/3</td>
<td>Polster prominent; optic vesicle, Kupffer's vesicle</td>
</tr>
<tr>
<td>14-somite</td>
<td>16</td>
<td>EL = 0.9 mm; otic placode; brain neuromeres, v-shaped trunk somites; YE barely forming; NO: pronephric duct</td>
</tr>
<tr>
<td>20-somite</td>
<td>19</td>
<td>EL = 1.4 mm, YE/YB &gt; 0.5 and &lt; 1; muscular twitches; lens, otic vesicle, rhombic flexure; hindbrain neuromeres prominent; tail well extended</td>
</tr>
<tr>
<td>26-somite</td>
<td>22</td>
<td>EL = 1.6 mm; HTA =125 degrees; Side-to-side flexures; otoliths; Prim-3</td>
</tr>
</tbody>
</table>

### Pharyngula period

<table>
<thead>
<tr>
<th>Stage</th>
<th>hpf</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prim-5</td>
<td>24</td>
<td>EL = 1.9 mm; HTA =120 degrees; OVL = 5; YE/YB = 1; early pigment in retina and skin; median fin fold; red blood cells on yolk, heart beat (can often be seen earlier)</td>
</tr>
<tr>
<td>Prim-15</td>
<td>30</td>
<td>EL = 2.5 mm; HTA = 95 degrees; OVL = 3; YE/YB &gt; 1; YB/HD = 2; early touch reflex and reduced spontaneous movements; retina pigmented; dorsal stripe to somite 12; caudal artery halfway to end of tail; caudal vein braided; shallow pectoral fin buds; straight tail; NO: cellular degeneration at end of tail; circulation in aortic arch 1</td>
</tr>
<tr>
<td>Prim-25</td>
<td>36</td>
<td>EL = 2.7 mm; HTA = 75 degrees; OVL = 1; PF(H/W) = 3/4; early motility; tail pigmentation and ventral stripe filling out; strong circulation; single aortic arch pair; caudal artery is 3/4 of the way the to the end of tail; pericardium not swollen; NO: PF apical ectodermal ridge</td>
</tr>
<tr>
<td>High-pec</td>
<td>42</td>
<td>EL = 2.9 mm; HTA = 55 degrees; OVL &lt; 1 and &gt; 1/2; YE/YB = 1.5; YB/HD &lt; 1.3; PF(H/W) = 1; dechorionated embryos rest on side after swimming; YE remaining cylindrical; PF apical ridge prominent; early lateral stripe; complete dorsal stripe; xanthophores in head only; iridophores in retina only; pericardium prominent; HO: heart chambers; segmental blood vessels; mandibular and hyoid arches; foregut developments olfactory cilia; thickened otic vesicle walls</td>
</tr>
</tbody>
</table>

### Hatching period

<table>
<thead>
<tr>
<th>Stage</th>
<th>hpf</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-pec</td>
<td>48</td>
<td>EL = 3.1 mm; HTA = 45 degrees; OVL = 1/2; PF(H/W) = 2; resting dorsal up; YE beginning to taper; PF pointed; dorsal and ventral stripes meet at tail; ca. 6 melanophores in lateral stripe; iridophores plentiful on retina; distinct yellow cast to head; NO: circulation in 2-4 aortic arches and in segmental vessels; olfactory cilia beating; semicircular canals; neuromasts</td>
</tr>
<tr>
<td>Pec-fin</td>
<td>60</td>
<td>EL = 3.3 mm; HTA = 35 degrees; movements too rapid to resolve; YB tapering into YE; up to 10 melanophores in lateral stripe; PF flattened into fin shape with prominent circulation; iridophore retinal ring fills out; iridophores in dorsal stripe; NO: PF cartilage and actinotrichia; gut tract; 2 chambers in otic vesicle; early jaw cartilages; circulation in 5-6 aortic arches; mouth remaining small and open at ventral location midway between eyes</td>
</tr>
<tr>
<td>Protruding mouth</td>
<td>72</td>
<td>EL = 3.5 mm; HTA = 25 degrees; wide open mouth protruding anterior to eye; iridophores in yolk stripe; eye half covered by iridophores; dorsal body as yellow as head; NO: gill slits and filament buds; cartilage in branchial arch 1 and 5; operculum covers the branchial arch 1 or 2; cleithrum</td>
</tr>
</tbody>
</table>
4-cells 1 h  sphere 4 h  shield 6 h
80% epiboly 8 1/3 h 1 somite 10 1/3 h 19 somites 18 1/2 h

pharyngula period 29 h

hatching period 48 h

swimming larva 5 d
Stages of heart development:

16 somite  18s  19s  21s  24s  24hpf

24hpf  36hpf  36hpf  48hpf  day3  day5
Demonstrations
Various techniques will be demonstrated during the zebrafish module. The schedule for demonstrations will be announced each day and may change from the initial schedule depending upon strain availability. Groups of 4-6 people are ideal for the demos, although everyone should watch the microinjection demonstration early in the module. See Protocols section beginning on page 27 for specific protocols.

Microinjection
Zebrafish embryos can be microinjected with RNA, DNA, morpholinos, and/or lineage dyes. There are some important tips for successful microinjection that we’ll demonstrate. All your TAs will help you get started – be sure to watch the demos on Wednesday morning.

Generation of embryos by in vitro fertilization (IVF)
Haploid generation is a useful technique for screening fish for mutations, because all embryos are hemizygous (only one set of chromosomes) and thus exhibit the phenotype of any mutant genes inherited by the parents. We plan to demonstrate IVF to generate diploid embryos and will be happy to demonstrate how to generate haploids if there is interest. Sharon will lead the demonstration.

Cell Transplantation
Cell transplantations can be done at blastula or gastrula stages, to determine what fates mutant/morphant cells can adopt in a wild type host, or vice versa, or to examine cell behaviors of labeled cells among unlabeled neighbors. Label the donor with GFP or fluorescent dextran, then transplant cells at the appropriate stage. Many of your TAs are experts at this procedure and can answer questions; Sharon, Dave, and Vicky will be available for demonstrations.

Kaede Photoconversion
The native Kaede fluoresces green, but when cleaved upon exposure to UV light, the resulting shorter form fluoresces bright red and readily labels cell processes such as axons and dendrites (Ando et al., 2002). Inject anytime between the 1 to 128-cell stage to get varying degrees of mosaicism, or use the huc:Kaede transgenics. Elke will head up these demonstrations.

Neuronal Backfills
A simple technique that allows labeling of neuronal processes and cell bodies. There is no formal demonstration scheduled, but Sarah and Vicky will be happy to help you if you would like to try this technique. This is best done with 5 day-old larvae; we can try it at 3-4 days, but be sure to plan ahead.
Morpholinos (MOs)
Start site MO antisense nucleotides prevent translation of their target RNAs, and can be used to reduce gene function in a reverse genetics approach (Nasevicius and Ekker, 2000). In other words, MOs specifically reduce gene function. Splice-blocking morpholinos block mRNA processing and often can be used to distinguish between maternal and zygotic gene function (Draper et al., 2001). MO-injected embryos with specific phenotypes are often referred to as “morphants” or “deficient” or “depleted” embryos. Try injecting different MOs into wildtype embryos (or transgenic strains) and examine phenotypes in live embryos and by in situ hybridization or immunocytochemistry. Each MO has a short description of the expected phenotype (for published MOs), of gene function in other organisms (for untried MOs), or of the gene expression pattern.

Abelson murine leukemia viral oncogene (Abl2)
Abl tyrosine kinases have been implicated in many developmental processes, including growth cone dynamics and axon guidance. In Drosophila, abl has been shown to regulate actin dynamics; in zebrafish, abl2 is expressed in adaxial cells and somitic cells. Expression pattern: http://zfin.org/cgi-bin/webdriver?MIval=aa-fxallfigures.apg&OID=ZDB-PUB-010810-1&fxallfig_probe_zdb_id=ZDB-EST-020809-12

Cardiac troponin T2a (Tnnt2a, silent heart)
In silent heart mutants, cardiomyocyte sarcomere assembly is defective and the heart is not contractile (Sehnert et al., 2002). Try this MO in the Tg(cmlc2:gfp) line to analyze contractility, or in the Tg(flk1:gfp; gata1:dsred) line to assess whether heart contractility and/or blood flow affects vasculature development.

Casanova (Cas)
Cas is a sox transcription factor required for endoderm formation (Dickmeis et al, 2001; Kukuchi et al., 2001). Intact endoderm is required for both heart formation and for left-right patterning – can you use a low MO dose, or targeted injection, to separate the two phenotypes?

Caudal type homeobox genes (Cdx1a and Cdx4)
Cdx1 and Cdx4 are caudal family transcription factors that have important requirements in axis formation, spinal cord patterning, kidney and pancreatic islet and blood development. Cdx genes are thought to act upstream of Hox genes and also interact with retinoic acid signaling. Cdx genes have some redundant functions, so knocking down cdx1 and cdx4 together causes much more severe phenotypes. See Skromne et al., 2007 and Wingert et al., 2007 for examples. What happens if you knock down cdx genes and alter retinoic acid signaling, either with RA, DEAB, or Cyp26a1 MO? Compare to raldh2 MO injected fish.

Chemokine (C-X-C motif), receptor 4b (Cxcr4b)
Cxcr4b is a G-protein coupled receptor that gets activated by the chemokines Sdf1a and Sdf1b. Cxcr4b is expressed in numerous neural and mesodermal tissues and has been shown to mediate the migration of placodal-derived sensory organs such as the trigeminal ganglion (Knaut et al., 2005), lateral line primordium (Gilmour et al., 2004) as well as germ cells (Doitsidou et al., 2002; Knaut et al. 2003). Compare lateral line primordium migration in wildtype and cxc4b morphants injected with membrane or nuclear labels or in embryos injected with germline-targeted (nanos 3’UTR) GFP. Is neural development changed when cxc4b MO is injected in one of the transgenic lines?
Chordin (Chd)
Chd is a secreted protein expressed in the embryonic organizer (“shield”) that binds to BMPs and prevents induction of ventral fates on the dorsal side of the embryo. The dino mutation disrupts the chordin gene (Schulte-Merker et al., 1997) and dino mutants are ventralized (described in Hammerschmidt et al., 1996). Try different MO doses to see if you can generate different phenotypes consistent with increased BMP signaling on the dorsal side.

Cyclops (Cyc)
Cyc is a secreted Nodal ligand expressed in the organizer (shield) and in mesodermal precursors (Sampath et al., 1988; Rebagliati et al., 1998). cyc mutants have prechordal plate and floor plate deficiencies and secondary neural phenotypes. Inject cyc MOs into huC:Kaede embryos to examine effects on neuronal specification (or inject wildtypes and label motoneurons with islet riboprobe or antibody). Nodal signals act in a dose-dependent way to affect different cell fates; try different doses to determine which tissues are most and least sensitive to Nodal loss.

Cytochrome P450, subfamily XXVIA, polypeptide 1 (Cyp26a1)
Cyp26a1 is an intracellular retinoic acid metabolizing enzyme that is expressed in the gastrula margin and tail bud, neural tube, and many other tissues. Inhibition of cyp26a1 function affects eye, pancreas, fin bud, and somite development and hindbrain and spinal cord patterning (see ZFIN for 30 publications about this gene). Try in combination with the cdx MOs!

Dead end (Dnd)
Dead end is a novel vertebrate-specific RNA-binding protein that is required for germ cell migration and survival and binds and protects some germline mRNAs from miRNA-mediated repression (Weidinger et al., 2003; Kedde et al., 2007). Using this MO, you can ablate the host germ line and completely replace it with cells transplanted from donor embryos, a technique used to generate maternal and maternal-zygotic mutant embryos (Ciruna et al., 2002). Try it!

DeltaC (Dlc, beamter)
DeltaC is a Notch ligand that is strongly expressed in a dynamic oscillating fashion in the presomitic mesoderm (Jiang et al., 2000). DeltaC mutations are responsible for the beamter (bea) mutant phenotype (Julich et al., 2005; Shaw et al., 2005) and a combination of 2 MOs nicely mimics the segmentation and vasculature defects. Unlike the 3 other delta genes, deltaC is not expressed in primary motoneurons, but is expressed in the early retina, cranial ganglia, sensory epithelia, blood vessels and pronephros. Try it in fli:GFP or alpha-actin:GFP embryos.

DeltaD (Dld)
The zebrafish after eight (aei) gene encodes the Notch ligand DeltaD (Holley et al., 2000). deltaD is strongly expressed in the presomitic mesoderm and in developing neurons and is required for both processes. Try analyzing the neurogenic phenotype by islet1 in situ hybridization or by combining with Notch1a MOs and doing neuronal backfills at later times.

Fibroblast growth factor 8 (Fgf8)
Fgf8 is a growth factor expressed in the forming midbrain/hindbrain boundary and in posterior mesoderm. The acerebellar (ace)/fgf8 mutant lacks a cerebellum, consistent with strong fgf8 expression at the midbrain/hindbrain boundary (Reifers et al., 1998). Fgf8 is strongly expressed in posterior mesoderm and functions together with fgf24 to specify posterior mesoderm development (Draper et al., 2003; see below).
Fibroblast growth factor 10a (Fgf10a; daedalus)
Inhibition of fgf10a leads to gut, fin bud, and lateral line primordium defects (e.g. see Norton et al., 2005; Dong et al., 2007; Manfroid et al., 2007; Nechiporuk et al., 2008). Use this MO in the gut:gfp or alpha-actin GFP transgenic backgrounds and/or combine it with other fgf-blocking MOs or lines to enhance the phenotype.

Fibroblast growth factor 24 (Fgf24)
The zebrafish ikarus mutant carries a mutation in the fgf24 gene (Fischer et al., 2003). fgf24 mutants lack pectoral fins, consistent with strong expression in forming fin buds. fgf24 is also expressed in mesodermal precursors in a domain highly overlapping with fgf8, and depletion of both fgf8 and fgf24 leads to severe reduction of posterior mesoderm, indicating that these genes function together during early mesoderm development (Draper et al., 2003). Try the experiment!

Floating head (Flh)
Flh is a homeobox transcription factor required for specification of notochord, floor plate, and pineal cells (Talbot et al., 1995); flh mutants have a reduced floor plate and pineal organ and flh mutant notochord cells transform to a muscle fate (Halpern et al., 1995; Melby et al., 1996). The flh:GFP transgenic line labels pineal and notochord – try the MO in these embryos. Also, notochord loss alters left-right asymmetry of the digestive system – try injecting flh (or ntl) MOs into gut:GFP transgenics.

Fused somites (Fss) / Tbx24
Fss is a T-box transcription factor (Tbx24) expressed in the presomitic mesoderm required for somite segmentation (Nikaido et al. 2002). Notch pathway mutants also have segmentation defects; compare papc expression in fss MO-injected embryos with that of DAPT-treated embryos (deficient in Notch signaling) and investigate the differences.

Heart and neural crest derivatives expressed transcript 2 (Hand2)
Hand2 is a bHLH transcription factor required for the development of the heart and the pectoral fins. For information on the mutant phenotype, see Yelon et al., 2000. Fgf24 and Pbx4 are also required for fin development. Does Hand2 interact with these factors? Try injecting in cmic2:GFP transgenic embryos.

Laminin beta 2 (Lamb2, softy)
Laminin B2 is an extracellular basement membrane protein prominent within skeletal muscle. lamb2 (softy) mutations lead to muscle fiber detachment within the newly formed myotome, and a dystrophic phenotype (Jacoby et al., 2009). Timelapse imaging of morphants using the α-actin GFP transgenic embryos, or immunolabeling with myosin-specific antibodies (F59), will reveal the muscle fiber detachment events between 24-72 hpf (Hall et al., 2007; Jacoby et al., 2009). Transplants of membrane-labeled morphant cells into wild-type hosts might produce isolated muscle fibers for timelapse observation.

Lim homeobox 5 (Lhx5)
Lhx5 is expressed in the rostral neuroectoderm. Lhx5 function promotes forebrain development and inhibits Wnt signaling by promoting the transcription of secreted Wnt antagonists, Sfrp1a and Sfrp5 (Peng and Westerfield, 2006). Do lhx5 morphants display opposite phenotypes to embryos with activated Wnt signaling [by treatment with GSK-3 inhibitor SB216763 or injected with Sfrp1a MO]?
Meis/Pbx genes
Meis/Pbx proteins represent homeodomain-containing transcriptional regulators that act as cofactors together with other proteins controlling embryogenesis and tumorigenesis. They often interact with Hox proteins setting up regional identity in derivatives of the different germ layers.

**meis2.1 (myeloid ecotropic viral integration 2.1)**
meis2.1 shows dynamic expression in the eye, the brain, the somites, and ventral mesoderm.

**meis3 (myeloid ecotropic viral integration3)**
meis3 expression can be detected in forming neural structures, somitic tissue and in a restricted pattern in the endoderm. Loss of Meis3 function leads to defects in the correct positioning of the pancreatic islet (for MO phenotype see diIorio et al., 2007).

**meis4.1a (myeloid ecotropic viral integration4.1a)**
meis4.1a expression can initially be detected in the forming somites and later in various domains of the brain, olfactory placodes, pharyngeal arches, lateral line, pectoral fins and potentially organ-forming endoderm.

**meis1 (myeloid ecotropic viral integration1)**
meis1 is expressed dynamically in specific domains of the brain, spinal cord, otic vesicle, pharyngeal arches, subset of the endoderm and pectoral fins.

**pbx4 (pre-B-cell leukemia transcription factor 4)**
pbx4 expression can be found in the forming central nervous system. lazarus mutants, lacking functional Pbx4, exhibit specific defects in formation of rhombomere 3, a subset of branchial arches, pectoral fins and correct anterior-posterior positioning of the pancreatic endocrine islet (Popperl et al., 2000).

**Mind bomb (Mib)**
Mind bomb is an E3 RING ubiquitin ligase that promotes ubiquitylation and internalization of Delta and is required in the signaling cell for efficient activation of Notch in neighboring cells (Itoh et al., 2003). Loss of mib function leads to a classic neurogenic phenotype. Inject this MO into neural transgenics and watch the madness! Grow them up and do neuronal backfills, or compare mib, dlD or notch1a morphants using neural in situ hybridization and immunostaining.

**Netrin1a (Ntn1a) and Netrin1b (Ntn1b)**
Netrins are diffusible guidance molecules that can either attract or repel growth cones, depending on the constellation of receptors on the target axon. In addition to their roles in the nervous system, Netrins may play roles in the development of other organ systems. Inject Netrin MOs singly or together in different transgenic lines to observed effects on neuron pathfinding and vasculature (Lu et al., 2004; Suli et al., 2006; Wilson et al., 2006; Kastenhuber et al., 2009).

**Neurogenin1 (Ngn1)**
ngn1 is a basic helix-loop-helix (bHLH) gene related to the Drosophila pro-neural gene atonal. Expression initiates in the neural plate at the end of gastrulation and defines broad domains of cells that possess an ability to develop as neurons (Korzh et al., 1998). ngn1 is required for the development of spinal, cranial sensory, and epiphysial neurons, as demonstrated by MO knockdown (Andermann et al., 2002; Cornell and Eisen, 2002; Cau and Wilson, 2003). Overexpression of ngn1 induces ectopic expression of HuC (Kim et al., 1997).
**NK2 transcription factor related 2a (Nkx2.2a)**

*nkx2.2a* encodes a class II homeobox protein that is expressed within the p3 neural progenitor domain (Ericson et al., 1997; Shimamura et al., 1995), which is located dorsolateral to the floor plate and ventral to the motoneuronal precursor domain (pMN; Briscoe et al., 1999). High levels of ectopic Shh dorsally expand *nkx2.2a* expression in early neural plate explants, while elimination of Shh signaling blocks *nkx2.2a* expression (Briscoe et al., 2000; Ericson et al., 1997). Knockdown of zebrafish *nkx2.2a* function affects floor plate development, glial specification, and pancreas development (Pauls et al., 2007; Kucenas et al., 2008).

**No tail (Ntl) (Lissamine-labeled)**

No tail/Brachyury is a T-box transcription factor required for notochord and tail specification; *ntl* mutants lack a notochord and tail, but have expanded floor plate (Halpern et al., 1993; Schulte-Merker et al., 1994). The lissamine (red) tag fluorescently labels MO-injected cells. Try injecting *ntl* MO into embryos and then transplanting *ntl* MO cells into wild type embryos or wildtype cells into *ntl* morphants. Or try it in the *flh:GFP* line and follow “notochord” development over time. Also, notochord loss alters left-right asymmetry of the digestive system – try injecting *ntl* (or *flh*) MOs into *gut:GFP* transgenics.

**No tail b (Ntlb)**

This ortholog of Brachyury is expressed very similarly to *ntla* (above), but has no overt phenotype when function is depleted with MOs. However, when *ntlb* MOs are injected into *ntl* mutants, or when co-injected with *ntla* MOs, the *ntla* mutant phenotype is dramatically enhanced, and embryos lacking both *ntla* and *ntlb* appear virtually identical to mouse Brachyury mutants (Martin and Kimelman, 2008).

**Notch1a**

The zebrafish *deadly seven (des)* mutant carries a mutation in the *notch1a* gene and has somite boundary and neurogenic defects (Gray et al., 2001; Holley et al., 2002). Analyze MO-injected embryos with somitic or neural markers – the expansion of Mauthner neurons in hindbrain rhombomere 4 is particularly dramatic. See the description for Mind bomb above.

**Oligodendrocyte lineage transcription factor 2 (Olig2)**

*olig2* encodes a basic helix-loop-helix (bHLH) gene expressed in the central nervous system. MO mediated-knockdown affects specification of motor neurons and oligodendrocytes (Park et al., 2002; Zannino and Appel, 2009). Overexpression of *olig2* promotes formation of excess primary motor neurons and oligodendrocytes; evidence suggests that Hedgehog signaling is required for *olig2* expression and oligodendrocyte development (Park et al., 2002).

**Pax genes (Pax3b and Pax7b)**

Pax3b and Pax7b are two recently identified homeodomain proteins in zebrafish, and are duplicates of zebrafish Pax3a and Pax7a, respectively. Pax3 and Pax7 genes are expressed in the brain, in neural crest cells, and in the somites, probably in early muscle stem cells. See Minchin and Hughes (2008). Loss of Pax3a or Pax7a can cause defects in neural-crest derived pigment cells. Does loss of Pax3b or Pax7b also cause pigmentation defects? Or muscle or brain defects?

**p53**

Knockdown technologies can have “off-target” effects, and in zebrafish, the effects are often mediated through p53 activation (Robu et al., 2007). If you observe non-specific cell toxicity with MO injections, try co-injecting p53 MO to eliminate cell death cause by off-targeting.
Retinal homeobox gene 3 (rx3/chokh) – Bangla word for ‘eye’
Rx3 is related to the Drosophila paired-like Rx gene. In zebrafish, rx3 is expressed primarily in the ventral forebrain, and injection of rx3 MO leads to ventral forebrain defects and lack of eyes (Tessmar-Raible et al., 2007). Try injecting into wild-type embryos and then immunostaining for acetylated tubulin to see if there are remnants of the eye and ventral forebrain neural circuits. Is it possible to rescue eye formation by transplanting wild-type cells into rx3 morphants?

Retinaldehyde dehydrogenase 2 (Raldh2)
Raldh2 is involved in retinoic acid biosynthesis and is dynamically expressed in a large number of tissues including the paraxial and lateral plate mesoderm and pectoral fin and in neural tissues, such as the hindbrain and the eye. neckless and no fin mutants lacking functional raldh2 exhibit defects in the development of the fins, heart, craniofacial and neural development, as well as endodermal organogenesis (liver and pancreas) (Begemann et al. 2001; Grandel et al. 2002; Stafford and Prince, 2002; Keegan et al. 2005). Inject raldh2 MO into various transgenic lines. How does complete loss-of-function compare to timed treatments with either DEAB or RA?

Secreted frizzled related protein1a (Sfrp1a)
Sfrp proteins are secreted proteins that have the capacity to bind Wnt ligands, to modulate Wnt signaling and to signal directly via the Wnt receptor Frizzled. They are involved in various processes during vertebrate development. sfrp1a is regionally expressed in the brain, eye, posterior endoderm/gut, lateral line primordium and interneuromast cells, forming somites, otic vesicle (Pezeron et al., 2006).

SRY-box containing gene 10 (Sox10)
The Sox gene family encodes a large family of transcription factors, and vertebrates have ≥20 Sox genes each (Wegner, 1999). Sox10 is an important transcriptional regulator in neural crest cell (NCC) development. Zebrafish colourless (cls) mutants harbor a mutation in the sox10 gene. cls mutants have defects in the peripheral and enteric nervous systems, pigmentation, sensory and sympathetic neurons, putative satellite glia and Schwann cells, supporting the proposal that cls functions in specification, proliferation, or survival of a progenitor(s) for all non-ectomesenchymal crest derivatives (Dutton et al., 2001).

T-box gene 2a (Tbx2a) and T-box gene 3b (Tbx3b)
Several T-box (Tbx) transcription factor genes are specifically expressed during embryonic development and have been implicated in development of many organs and tissues. Loss- and gain-of-function analyses show that tbx3b and tbx2a are required to repress the chamber genetic program in the non-chamber myocardium (Ribeiro et al, 2007). Together, they are required to control cell proliferation in the atroventricular canal. Try injecting MO (alone or together) into cm1c2:gfp transgenic embryos for live imaging to assay heart beat and heart looping, or, inject into wild-type embryos and immunostain for MF20 (labels all cardiac muscle) and S46 (labels atrium).

T-box gene 6 (Tbx6)
This T-box gene is expressed similarly to spt, a closely related T-box gene, but comes on slightly later, between shield stage and 60% epiboly (Hug et al., 1997). No tbx6 loss-of-function phenotype has been described. You might try co-injecting tbx6 MOs with spt MOs to examine whether these genes share functional redundancy (as has been shown for two ntl T-box genes; see Martin and Kimelman, 2008).
Vang-like 2 (Vangl2, trilobite, strabismus)
Vangl2 (Strabismus) is a transmembrane component of the Wnt planar cell polarity pathway. vangl2/trilobite mutants disrupted mediolateral cell polarity, leading to impairment of cellular intercalation and directed migration events, and ultimately morphogenetic defects in the convergence and extension tissue movements of gastrulation. In addition, neuronal movements in the hindbrain are affected (Jessen et al., 2002). Transplant experiments can reveal whether the gene acts cell-autonomously, while in situ hybridization and/or timelapse imaging of membrane-labeled embryos demonstrate the dramatic gastrulation defects.

Pharmacological agents

DAPT (see Protocol on page 32)
DAPT is a gamma-secretase inhibitor that blocks Notch signaling in zebrafish (Geling et al., 2002). In the original study, DAPT treatment (100 uM) from cleavage stages to 24 hpf phenocopied the somitic and neurogenic phenotypes of Notch pathway mutants. Think about adding DAPT at different developmental times to test when Notch signaling is important for these processes. Use huC:Kaed or flk1:GFP fish, or fix and stain your embryos for neural markers, presomitic mesodermal markers, vessel markers, etc. In most Notch pathway mutants, the first few 4-8 somites are unaffected – is the same true in DAPT-treated embryos?

DEAB (see Protocol on page 32)
DEAB (4-Diethylaminobenzaldehyde) is a small molecule inhibitor that inhibits Retinaldehyde dehydrogenase (Raldh2), the enzyme that is responsible for retinoic acid synthesis in the embryo. The zebrafish neckless (nls) mutant disrupts raldh2 function (Begemann et al., 2001). Manipulating RA levels with RA inhibitors affects hindbrain patterning, somite size and bilateral symmetry, and cardiac, pectoral fin, and pancreas development. Try treating embryos at different developmental stages to find the critical period for patterning RA-sensitive tissues.

Retinoic Acid (all-trans-Retinoic Acid) (see Protocol on page 32)
As mentioned above, retinoic acid is important for patterning many tissues and organs in the embryo.

GSK3 inhibitor (SB 216763) (see Protocol on page 32)
SB 216763 is a small molecule inhibitor of GSK3 kinase activity that has been used most extensively in cell culture. Addition of this inhibitor enhances Wnt signaling in zebrafish embryos when applied early (during cleavage stages) – it has not been tested extensively at later stages.
Transgenic lines

These transgenic lines can be used to visualize specific tissues in living embryos, using a GFP filter set on a dissecting or compound scope. Available transgenic embryos will be listed on the board. Remember that transgenes are dominant markers, and that all or half the progeny will express the transgene. Experiments to try using these fish are peppered throughout the manual, but dream up your own experiments as well!

\textit{h2A.F/Z:GFP}

GFP is fused to the coding sequence of the histone variant, H2A.F/Z and driven by the H2A.F/Z promoter. Nuclear localization of the fusion protein occurs around MBT (~3 hpf) in all embryonic cells. Transplant these cells into hosts and then follow them by confocal timelapse microscopy (Pauls et al., 2001).

\textit{flh:GFP}

This transgene is expressed in the organizer (shield), then notochord, spinal cord, and pineal. In the c162 (but not the c161) line, GFP is also seen in hindbrain rhombomeres 2 and 3 (perhaps due to enhancer trapping) (Gamse et al., 2003; Pineda et al., 2006; Aquilina-Beck et al., 2007).

\textit{cmlc2:GFP}

GFP is expressed under the control of the \textit{cardiac myosin light chain 2} promoter. The \textit{cmlc2} promoter drives GFP expression in the myocardium beginning around 16 hpf, with robust expression around 24 hpf (Huang et al., 2003). Watch how the heart tube matures or how the rate of contraction changes.

\textit{flk1:GFP} (\textit{kdrl:GFP}) or \textit{fli1:GFP}

The \textit{fli1} promoter drives GFP expression in all blood vessels throughout development, starting around the 12 somite state (15 hpf) (Lawson and Weinstein, 2002). The \textit{flk1} transgene is expressed similarly (Beis et al, 2005). We have a line segregating the \textit{flk1:gfp} and \textit{gata1:dsred} transgenes (Buchner et al., 2007; Fish et al., 2008).

\textit{gata1:dsred}

The \textit{gata1} promoter drives dsRed expression in erythroid cells starting around 24 hpf, and likely earlier (Traver et al., 2003).

\textit{alpha-actin GFP}

GFP is expressed under the control of the skeletal muscle-specific \textit{alpha-actin} promoter (Higashijima et al., 1997).

\textit{gut:GFP}

This line was generated by random integration, and GFP expression is apparently under the control of nearby \textit{foxA3} regulatory elements (Ng et al., 2005). GFP is initially expressed ubiquitously throughout the embryo and becomes restricted to the digestive tract endoderm around 24 hpf (Field et al., 2003). The hatching gland also expresses GFP.

\textit{huC:Kaede}

Kaede is a fluorescent protein with a photoconversion property allowing conversion from green to red fluorescence following irradiation with UV or violet light. \textit{huC} promoter elements of this transgene drive Kaede expression in all post-mitotic neurons in the embryo (Sato et al., 2006).
**gfap:gfp**
GFP is expressed under control of the *gfap* promoter. The transgene is expressed in CNS and PNS glial cells (Bernardos and Raymond, 2006).

**nkx:gfp;olig2:dsred**
In this double transgenic line, *nkx2.2a* regulatory elements drive expression of membrane-tethered EGFP in oligodendrocyte progenitor cells (OPCs), interneurons, floor plate cells and perineurial glia and where *olig2* drives expression of DsRED2 in OPCs and motor neurons (Kucenas et al., 2008). Expression of these transgenes starts early, at ~12 hpf and persists until late larval stages.

**Lines that temporally inhibit or activate FGF signaling**
In zebrafish, manipulation of FGF signaling affects mesoderm formation, lateral line primordium migration, hindbrain and retinal patterning, regeneration, liver specification, and cardiac chamber size and proportionality, to name just a few.

**hs:dnfgfr-egfp** (dominant-negative Fgf receptor)
Use this line for heat-shock inducible, temporally-regulated knockdown of Fgf signaling. Search “pd1” in ZFIN to find references to papers referencing this transgene.

**hs:cafgfr1; cryaa:dsred** (constitutively active Fgf receptor; Dsred expressed in transgenic eyes)
Use this line for heat-shock inducible, temporally-regulated activation of Fgf signaling. Search “pd3” in ZFIN to find references to papers referencing this transgene.

**NTR/Mtz system – a conditional cell ablation system**
The bacterial Nitroreductase (NTR) enzyme, that converts the prodrug Metronidazole (Mtz) into a cytotoxic DNA crosslinking agent, has been adapted for temporally-controlled cell ablation (Curado et al. 2007; Pisharath et al., 2007). Tissue-specific NTR expression restricts the cell damage to a tissue of choice. Fusion of NTR to Cyan Fluorescent Protein (CFP) allows visualization of the tissue (and the extent of tissue ablation) before and after Mtz addition to the embryo medium.

**ins:NTR-CFP; ins:Kaede**
In this line, the insulin promoter drives Kaede and NTR expression in pancreatic β-cells. Mtz treatment ablates β-cells but they can ‘regenerate’ after about 35 hpf (Curado et al. 2007; Pisharath et al., 2007). Does changing RA signaling alter β-cell regeneration? Can β-cells that normally form in ectopic anterior positions after RA treatment regenerate? Photoconvert Kaede prior to ablation to assess ablation efficiency and to distinguish ‘old’ versus ‘new’ β-cells.

**cmlc2:NTR-CFP**
This line drives NTR in cardiomyocytes and Mtz-mediated cell ablation alters the cardiac rhythm. Can RA/DEAB treatment or treatment with Fgf protein alter the recovery process? Does depletion of proapoptotic p53 by MO-p53 injection dampen the effect?
Sense RNAs to inject
RNAs are expressed in all cells inheriting it, making mRNA injection useful for overexpression studies and as lineage tracers. You can inject RNA into the yolk at 1-4 cell stage – it will be transferred to the embryo during cytoplasmic streaming. Or you can inject blastomeres directly. Phenol red is added to the RNA so that you can see that your injection was successful.

Membrane-localized fluorescent protein RNA
These fluorescent proteins label membranes. If injected early, they will label all cells; if injected into only one blastomere at later stages (~16-32 cell stage), they are useful for fate mapping and/or observing cell behavior, i.e. gastrulation movements. Using them in combination with cell transplantation will give you additional information about morphology of transplanted cells.

- **membrane-citrine** – yellow-green fluorescent
- **membrane-tomato** – red fluorescent

Histone 2B-RFP (H2B-RFP) RNA
Can be visualized in living embryos. This RFP localizes to the nucleus and is wonderful in combination with membrane-GFP for imaging.

Kaede FP RNA
The native Kaede fluoresces green, but when cleaved upon exposure to UV light, the resulting shorter form fluoresces bright red and readily labels cell processes such as axons and dendrites. Inject anytime between the 1 to 128-cell stage to get varying degrees of mosaicism. Keep embryos in the dark and photoconvert those that are brightest green (Ando et al., 2002).

Lineage labels
Lineage labels are used for keeping track of particular cells. You can label an entire embryo and use it as a donor for cell transplantation or you can label a subset of cells in the embryo by injecting 1 cell of a 16-cell-stage embryo. As with RNA, inject into the yolk or blastomeres.

- **Rhodamine dextran and Fluorescein dextran**
  Can be visualized in living tissue. Rhodamine is red so it contrasts with GFP and fluorescein.

- **Membrane-localized FP RNA and nuclear-localized RFP (H2B-RFP) RNA**
  See above.

Stains (see Protocols on page 39)

- **Alexa 488 Phalloidin**
  This fluorescent (green) phalloidin binds to F-actin in fixed embryos. Can use it to examine cell morphology in transplants where transplanted cells are labeled with rhodamine-dextran.

- **SYTOX Green nucleic acid stain**
  Can use to label all nuclei of an embryo following antibody staining (make sure the antibody is in red) or possibly an in situ. This dye fluoresces in the green channel. Could possibly be used on live embryos to stain for dead/dying cells?

- **Acridine Orange**
  This stain will label apoptotic cells (Robu et al., 2007).
Antibodies (see Protocols beginning on page 36)
Antibody labeling is relatively fast and simple. See the Protocols section for information on using particular antibodies. You'll have to dechorionate and fix your embryos in paraformaldehyde for antibody staining, except for MF20 and S46 double-label for the heart (see Protocols).

Anti-acetylated tubulin antibody (mouse monoclonal IgG1)
This antibody labels neuronal processes/axons/nerve fascicles through the brain and spinal cord, starting around 24 hpf. Use at 1:500.

zn-12 antibody (mouse IgG1)
This antibody nicely marks commissural, retinal, and abducens neurons. Use at 1:4000.

Engrailed/Invected antibody (4D9) (mouse IgG1)
Nipam has challenged you to figure out what this antibody stains in zebrafish… Hint: the 4D9 antibody recognizes zebrafish eng1, eng2, and eng3, which are expressed in a variety of tissues, including muscle pioneer cells and the midbrain-hindbrain boundary. Use at 1:10.

F310 antibody (mouse IgG1)
This antibody marks fast muscle cells. Use at 1:10.

F59 antibody (mouse IgG1)
This antibody marks early slow muscle cells and can be used to label them during migration. Use at 1:10.

MF20 (mouse IgG2b)
Labels atrium and ventricle of heart. It recognizes a general epitope on sarcomeric myosin heavy chains. This antibody recognizes ventricular myosin heavy chain, atrial myosin heavy chain, and skeletal myosin heavy chain, so will label segmented myotomes as well. Use at 1:10.

Pan-islet antibody (39-4D5) (mouse IgG2b)
Labels Islet-expressing cells (encoded by islet1 and islet2 genes), including motoneurons and pancreatic endocrine cells. Use at 1:20.

Pax7 antibody (mouse IgG1)
This antibody marks specific populations of cells in the neural tube and somites. Use at 1:10.

Phospho-Histone H3 antibody (rabbit IgG)
This antibody labels mitotic cells. Use at 1:1000.

S46 (mouse IgG1)
Labels atrium of heart. Can be used for very nice double labeling (with anti-MF20) to distinguish atrium versus ventricle of the heart. It recognizes an epitope specific for atrial myosin heavy chain. Use at 1:10.
RNA in situ probes (see Protocol on page 34)
In situ hybridization can be used to monitor gene expression. We’ve brought a selection of in situ probes to mark specific embryonic tissues and cells. Dechorionate and fix your embryos in 4% paraformaldehyde (in PBS) before in situ hybridization. The early patterning mix works from the beginning to mid-segmentation stages; the late patterning mix works from mid-segmentation until 24 hpf. Both mixes are listed at the very end of this section.

Neuronal markers

*islet 1*
Expressed in certain differentiating neurons, including motor neurons and photoreceptors from the 5 somite stage on.

*olig2*
Expressed in ventral neural tube and specified motor neurons and oligodendrocytes. Expression begins at 1 somite stage.

*nkx2.2a*
Expressed in head mesoderm, lateral floor plate of ventral spinal cord, oligodendrocytes, spinal cord interneurons and perineurial glia. Expression begins at ~ 80% epiboly.

*sox10*
Expressed in migratory neural crest and some differentiated lineages including Schwann cells and satellite glia. Expression begins at ~1 somite.

*foxa2*
Expressed in ectoderm early and floor plate later. Expression begins at ~ 75% epiboly.

*mbp*
Expressed by myelinating glial cells including oligodendrocytes and Schwann cells. Expression begins at ~ 48 hpf.

*foxd3*
Expressed by neural crest early and glial cells late. Expression begins at 1 somite stage.

*ngn1*
Expressed in proneural cells and primary neurons. Expression begins at 1 somite stage.

*neurod*
Expressed in proneural cells. Expression begins at ~ 1 somite stage.

*gbx2 (gastrulation brain homeo box 2)*
Expressed in the posterior midbrain-hindbrain boundary, as well as eye otic placode, posterior lateral plate mesoderm and fin fold. Expression begins ~ 1 somite stage.

*dlx2*
Dynamically expressed in parts of the diencephalon and telencephalon, branchial arches and pectoral fins. Expression is initiated around 18 hpf.

*pax2*
Dynamically expressed in the midbrain-hindbrain boundary, hindbrain, pharyngeal arches, eye, otic vesicle, pronephric duct, hindbrain. Expression is initiated at late gastrulation stages.
valentino (val), mafba = v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B
Expressed in rhombomeres 5 and 6 during stages of hindbrain patterning and segmentation,
starting at the end of gastrulation (~10 hpf) until 20s (19 hpf). Past 19 hpf expression is detected
in other structures.

krox20
Expressed in rhombomeres 3 and 5 of the hindbrain (between 12-26 hpf) and transiently in the
migrating r5 neural crest around 14 hpf.

Mesodermal markers

no tail (ntl)/Brachyury
Expressed in notochordal precursors and in mesodermal precursors at the blastoderm margin
during gastrulation and in the developing notochord and tailbud during segmentation stages.

paraxial protocadherin (papc)
Expressed in the presomitic mesoderm (PSM), resolving into 1-2 stripes in the anterior PSM and
1-2 stripes marking the anterior compartment of the most recently formed somites. Expressed
transiently in the prechordal plate mesoderm during gastrulation and early segmentation stages.

myoD
Expressed in the paraxial mesoderm during early gastrulation, and by adaxial cells and in
posterior somite compartments from ~11 hpf; and later in newly forming myoblasts throughout
the embryo and larvae.

Endodermal markers

forkhead 7 (fkd7) – pan-endodermal marker beginning early to mid-somitogenesis; also
hypochord and floor plate of spinal cord and ventral brain

ceruloplasmin – liver-specific expression beginning ~36 hpf

trypsin – exocrine-specific pancreas expression beginning ~46 hpf.

insulin – endocrine-specific pancreas expression beginning at 12 somites in bilateral stripe,
merging to form a single pancreatic islet at ~16-18 somites.

Hox genes (expression at 20-somite stage, check ZFIN)

hoxd4a – expressed throughout spinal cord, with anterior border in hindbrain rhombomere 7,
also pectoral fin bud, posterior arches, posterior somites

NOTE:
If you are not sure what phenotypic consequences to expect, you might consider trying one
of the hybridization mixes listed on the next page. These mixes are “tried and true”
combinations that will label several developing tissues.
Early Segmentation Patterning mix
This mix marks a variety of axial positions in the early to mid-segmentation stage embryo. Fix your embryos between 10-16 hpf, with early segmentation stages being optimal.

- hgg1 (prechordal plate, hatching gland cells)
- pax2 (midbrain-hindbrain boundary)
- valentino (rhombomeres 5 and 6 of the hindbrain)
- sonic hedgehog (midline)
- her1 (2-3 stripes in the presomitic mesoderm, tailbud)

Late segmentation patterning mix
This mix marks a variety of axial positions in the mid- to late segmentation stage embryo. Fix your embryos at ~18 somites (18 hpf), with later stages (up to 24 hpf) being less optimal.

- sonic hedgehog (midline)
- gbx2 (midbrain)
- krox20 (rhombomeres 3 and 5 of the hindbrain)
- myoD (somites)
Protocols

Injection of RNA/MOs/lineage tracers into zebrafish embryos
Rule of thumb – the injected droplet (~2 nl) should be about 1/10 the volume of the embryo.

Day before injections:
We’re using program #42 or 43 on the Sutter box-filament needle puller and we’ll pull injection pipets for you (they will be in the injection room). Once pulled, the needles are stored in a petri dish, with one strip of modeling clay to hold the glass.

Day of injections:
1) Cut injection needles so that they have an open tip. For the needles you’ll be using, you’ll just break off the tip of the needle with your forceps under the scope.

2) Get the morpholino, RNA or lineage label from the ice buckets at the front of the room. Phenol red (0.2% final concentration) has been added to the RNA and MO mixes so that progress of injections is easier to follow.

3) Back load needles by pipetting 0.5 ul into the back of each needle, using the special skinny pipet tips. If you load your pipets well ahead of time, make sure that you put a piece of wet kimwipe in the petri dish/needle holder at this point, so your needles won’t dry out.

4) To inject using the micromanipulator, put the embryos into an agarose dish with a molded trough. You’ll find these by the microinjectors. Roll the embryos into the trough with forceps or pokers.

5) Inject the RNA/OLO/lineage label into the region directly under the blastomere. This is where cytoplasmic streaming is occurring - contents of the yolk are being delivered into the blastomeres. The phenol red will stay red if you hit the right place, but will turn yellow elsewhere [Alternatively, you can inject “freehand”, without a micromanipulator in a normal Petri dish. Under the scope, roll the embryos on its side with forceps. The blastomeres should be to your left (for right handed people). Holding the needle holder in your hand, jab the needle through the chorion, into the yolk cell. Your needle has to go through the chorion to get to the embryo – use a smooth rapid motion so that you penetrate the chorion rather than collapse it.]

6) A few hours after injections are finished, take out any infertile eggs, or embryos that were damaged by the injection. If the water in the dish is cloudy, transfer the embryos to a new dish. GFP fluorescence can be detected early, usually by sphere stage (4 hpf).

(For DNA injections (unlike RNA or MOs), it is important to inject the DNA directly into the blastomere. Note that you can use similar techniques to inject one of 16 blastomeres to produce mosaic embryos.)
Cell Transplantation
Protocol adapted from Robert Ho

Some example transplants to try:
Label donor embryos with rhodamine-dextran. Transplant cells at the blastula stage with the transplant rig into a host. What fates can/cannot be adopted by donor cells (use in situ or Ab staining)? Label donor cells with membrane-FP, transplant into hosts, and watch cell interactions during morphogenesis. Transplant to/from MO-injected embryos and assess fates of the transplanted cells.

Mounting donor and host embryos

Label donor embryos at the 1-4 cell stage by injecting 2.5% rhodamine dextran (can also include 2.5% biotin dextran) in 0.2 M KCl into the yolk. Let labeled donor and unlabeled host embryos continue to develop at 28°C in embryo medium and dechorionate them at blastula stages on agar-coated dishes. Embryos can be divided into donor/host pairs of dishes and placed at different temperatures (between RT and 31°C) to slow and/or speed development and allow you to transplant over a longer period of time. You will take cells from dome-50% donors and transplant to blastula-shield stage hosts. Donors and hosts do not have to be heterochronic if you are doing blastula stage transplants.

At the appropriate stage (early blastula to gastrula), mount the embryos in a depression slide: put down a stripe of ice-cold 3% methylcellulose in EM with a wooden stick, then GENTLY add embryo media and embryos to the side. Use a loop to position the embryos.

Mount a labeled donor and an unlabeled host embryo in the stripe of methylcellulose. Place embryos such that the animal pole faces to the top of the stripe. If doing gastrula stage transplants, orient the shield of the donor embryos so that the region of the fate map you are targeting is facing straight up at you. Since many cells can be taken from one labeled embryo, it is often expedient to mount a single donor embryo and 3-4 host embryos in the same chamber.

Transplantation apparatus

The transplantation apparatus is a Hamilton syringe connected to a worm gear drive or a micrometer drive. This provides very fine control of the suction and pressure required to pick up and expel cells. The syringe is filled with light mineral oil with a length of mineral oil-filled polyethylene tubing connected to it. The tubing is attached to a pressure fitting micropipette holder. The holder must be firmly attached to a micromanipulator, so that the micropipette can be moved from embryo to embryo. It is also important to use a holder with a gasket that tightens by screwing the holder tip, so that the micropipette can be mounted firmly.
Fill the micropipette holder with mineral oil. Backfill the transplantation micropipette to within 5-7 mm of its shoulder with mineral oil using a 2.5 inch, 30 gauge needle, inserted into the micropipette holder. Tighten the pipette into the holder. The pressure of tightening the holder will force the mineral oil into the tip. If mineral oil is expelled from the tip at this point, equalize the pressure by creating a small amount of suction with the micrometer or worm drive. **It is crucial that there are NO air bubbles anywhere in the line from the syringes to the tip of your needle. Bubbles will make it impossible to control the suction.**

**Transplantation**

Mount the transplantation pipette onto a micro-manipulator and position the needle tip next to your donor. Using the stage, gently, but forcefully move the needle into the donor embryo. Draw up cells by turning the syringe control knob as you remove the needle (~100 cells). Withdraw the pipette, now containing labeled donor cells, insert it into the unlabeled host embryo, and expel about 1/3 of the donor cells. Carefully withdraw the pipette from the host embryo and continue with the remaining hosts. Place the depression slide in a large Petri dish and gently cover with embryo media. For long-term experiments, it is important to add pen-strep antibiotic to the embryo medium (50 U/ml penicillin, 50 µg/ml streptomycin final, a 1:100 dilution of the stock).

**Transplant needles: (We will provide these for you)**

- Pull needles with the same program we use for injection needles (see injection protocol for details.) Use capillary tubes without filament (TW100-4 WPI). Pull lots of needles. To break, use a razor blade or a pair of #5 forceps to break off needles at ~20-30 microns for gastrula stage and 50-60 microns for blastula stage transplants. You can use a microforge to soften the glass and pull a sharp point if you choose.

**We used lysine-fixable rhodamine-dextran (“fluor-ruby”), so you can observe fluorescence in fixed samples.**

**Staining to detect labeled donor cells (if biotin-dextran included):**

- Fix in 4% para-formaldehyde o/n at 4°C. Fix some donors for a positive control.
- Incubate embryos for 30 minutes in ABC reagent
- Do the HRP stain: Wash embryos 3x in PBT (Tris buffered saline with 0.1 Tween 20%). Incubate in 0.5mg/ml DAB in PBT (1 DAB tablet in 5 ml PBT) for 30 minutes. To develop stain add 1:100 30% H$_2$O$_2$ (0.3% final) and monitor under the scope. Try to keep the light exposure minimal! Too much light can cause the embryos to turn black in storage. Stop reaction with washes in PBT. Re-fix embryos in 4% PFA, then they can be prepared for photography in any way.
- DAB is a carcinogen. Be careful when using and inactivate all equipment with bleach before discarding.
Generation of gynogenetic haploid embryos
(based on a protocol from Charline Walker)
Also see the Zebrafish Book (Westerfield, 1995)

Preparation of male and female fish:
Females will often “squeeze” well if they are pre-mated about 10 days prior to IVF. The night before the experiment, males and females can be “pseudo-mated” by separating them within the same tank so that they are exposed to each other without being allowed to mate.

Sperm Collection:
Sperm is collected from males first. Anesthetize males in Mesab diluted in fish water (see below). When their gill movements slow, use a plastic spoon to lift them and rinse them in fresh fish water. Place the male in a sponge with a slit cut in it, belly up, and blot the genital region with a Kimwipe to avoid exposing sperm to water. Under a microscope and using flat forceps (not pointy or textured), gently extract sperm by touching the forceps to either side of the belly just anterior to the genital region. Push backwards along the flank of the fish towards the genital/anal pore. If sperm does not come out easily, move on to the next male. Sperm is collected using a capillary mounted in an aspirator tube (Drummond micro-caps; Sigma aspirator tube assembly) – place the microcapillary by the genital/anal pore as you squeeze. Sperm is transferred to Hank’s saline (see below). The final volume of Hank’s used varies depending upon the number of clutches one expects to fertilize (estimate 15-20 µL sperm/clutch of eggs). Collect sperm until the Hank’s solution is moderately cloudy.

Sperm Irradiation:
Place a watch glass on ice – make sure the surface of the watch glass stays dry (watch out for condensation). Put 500 µL sperm solution in the watch glass and irradiate in a Stratalinker set on Auto-crosslink (1200x100mjoules). Using a clean pipet tip and new eppendorf tube, transfer the irradiated sperm. Continue with 500 µL aliquots until all the sperm has been treated.

Egg Collection:
Anesthetize females in small batches (2-3) as for the males. When their gill movements slow, use a plastic spoon to lift them and rinse in clean fish water. Lay the female on a pile of absorbent paper towels and flip her over (always use a head-to-tail movement of the spoon), so that both sides of the fish are blotted on the towels. Place the female in a small plastic dish on her side. Lightly dampen your fingers and blot. Supporting the back of the female with one finger placed against her central spine, gently massage her side with another finger in a front to back motion. Your finger should be on her flank but down near the belly. If she doesn’t release eggs easily, move on to the next female – too much pressure can push out internal organs.

Once you have a pile of eggs next to the female, use a small spatula to pull the eggs away from the female, and then use the spatula to lift the female and drop her into fresh recovery water. You may be trying to genotype the female, in which case you want to place each female into individual containers. We start with tanks labeled with two ID stickers, one of which is immediately transferred to the dish with her eggs while the other labels the female in the tank.

The quality of the eggs can be immediately determined: if the eggs make a nice pile and are slightly yellow in color and granular, then they are probably healthy. If they are very white and/or runny and/or there is a lot of liquid extracted, then they are certainly bad eggs and should not be fertilized.
Fertilization:
Using a standard pipetman, add about 20µL of irradiated sperm (or unirradiated sperm for diploids) to the pile of eggs and stir gently. Add approximately 500µL of fish water to the eggs to activate the sperm and allow fertilization. After about a minute, flood the dish with fish water and leave for later sorting.

Sorting:
Once the embryos have begun cell division, embryos that were fertilized can be separated from unfertilized eggs. Transfer fertilized embryos to a 100 mm petri dish of fish water. You should see about 75% fertilization rates or higher. If fertilization rate is very low (more like 25%), then the sperm may be over-irradiated.

Reticulospinal neuronal backfills

Supplies:
5 dpf zebrafish larvae
Microscope slide with nailpolish ring around edge (creates liquid barrier)
1.2% agarose in embryo media (or Ringers)
Mesab
Vannas spring scissors (Fine Science Tools #15003-08)
10K MW lysinated (fixable) rhodamine dextran (Molecular Probes #D-1817) in 0.2M KCl
4% PFA
Glycerol series (25%, 50%, 70% in Tris pH 9.5)

- Melt agarose and place in 46.5°C heat block.
- Anesthetize embryos in small dish of embryo media + 6 drops of mesab
- Cool agarose a bit in your hand (hot agarose can kink the notocord), then add embryo. Discard excess embryo media from pipette and suck up embryo again.
- Place embryo in drop of agar on slide. Mount dorsal up and angled with tail sticking up a bit.
- Dip Vannas scissors into rhodamine. Snip the tail of the embryo with the dye-covered scissors – at the level about halfway down the yolk extension. Once all embryos are snipped, flood slide with a few drops of embryo media + Mesab.
- Leave embryos mounted about 20 min, then de-mount with insect pins and transfer to dish of embryo media. After about an hour (you can check your fills on UV dissecting scope), fix in 4% PFA at room temperature for 4-5 hours.
- Wash 4X5' in PBST.
- Deyolk to flatmount or dissect out brain with #5 forceps
- Clear through graded series of glycerol; Tris pH 9.5
- Mount for confocal: RSNs are ventral, so mount embryos with ventral side up toward coverslip.
DAPT treatment
(adapted from Geling et al., 2002)
(We prepared a 10 mM stock solution of DAPT by diluting in ultrapure DMSO.)
Dechorionate embryos in embryo medium on agar dishes.
Set up the treatment and control solutions in 35 mm agar-coated dishes. Add 10 µl of 10 mM DAPT stock (or 10 µl DMSO for controls) per ml embryo media (100 µm final concentration).
DAPT will precipitate if not warmed to RT. Swirl dishes to thoroughly mix.
Transfer embryos to the treatment dishes, taking great care not to allow embryos to be exposed to air or air bubbles (they will explode). As a general guideline, Geling et al (2002) began DAPT treatment at sphere stage (4 hpf) and incubated until 4-somite, 10-somite, and 24 hpf stages.
DAPT and DMSO are light sensitive so keep dishes in the dark.
Fix embryos at desired stage or examine live if GFP transgenics used.

RA or DEAB treatment
(adapted from Maves and Kimmel, 2005)
We prepared a 100 uM stock solution of all-trans retinoic acid, and 10mM stock solution of DEAB, by diluting in ultrapure DMSO. These are stored at -20C.
It is not necessary to dechorionate embryos for these treatments.
Set up the treatment and control (DMSO) solutions in petri dishes. RA and DEAB are toxic so take precautions. It takes a while for RA and DEAB stock solutions to thaw at room temp.
For strong DEAB treatment (10uM final): add 10 ul of 10mM DEAB (or 10 µl DMSO for controls) to 10 ml embryo media. Swirl dishes to thoroughly mix.
For weaker DEAB treatment (1uM final): add 1 ul of 10mM DEAB to 10 ml embryo media.
For strong RA treatment (100nM final): Dilute 1 ul of 100 uM stock in 10 mls embryo media.
For weaker RA treatment (10nM final): Dilute 1 ul of 100 nM stock in 10 mls embryo media.
Transfer embryos to the treatment dishes in a small volume of embryo media. As a general guideline, you can begin treatments at sphere stage (4 hpf) for DEAB, and later (about 80% epiboly, 8 hpf) for RA, and incubate until 4-somite, 10-somite, and 24 hpf stages. RA, DEAB and DMSO are light sensitive so keep dishes in the dark.
Fix embryos at desired stage or examine live if GFP transgenics used. Discard RA and DEAB in appropriate waste containers (including used stock solutions).

GSK3 inhibitor treatment
We prepared a 50 mM stock solution by diluting in ultrapure DMSO. These are stored at -20C.
It is not necessary to dechorionate embryos for these treatments.
Set up the treatment and control (DMSO) solutions in petri dishes. SB 216763 is toxic so take precautions. It takes a while for SB 216763 stock solutions to thaw at room temp.
For SB 216763 treatments (20uM final): add 4 ul of 20 mM SB 216763 (or 4 µl DMSO for controls) to 10 ml embryo media. Swirl dishes to thoroughly mix.
Transfer embryos to the treatment dishes in a small volume of embryo media. As a general guideline, you can begin treatments between 4-cell and sphere stage (4 hpf), and incubate until for example 10-somite or 24 hpf stages. So far SB 216763 has not been tested extensively at later stages – you could design an interesting experiment and test the functionality of the drug; maybe double the concentration (also adjust DMSO controls accordingly). SB 216763 and DMSO are light sensitive so keep dishes in the dark.
Cell ablation using the NTR-MTZ system

Set up experimental and control solutions in 35 mm dishes at concentrations indicated below for each line. Make sure to include PTU in incubation solutions from 24 hpf onwards to prevent melanin production, which could interfere with later analysis by obscuring the tissue of interest.

Remove Mtz after the desired treatment time by washing embryos 3-5 times with embryo medium, making sure that the final solution contains PTU.

Treatments can be started as early as 24 hpf, with published times being between 48-72 hpf.

Analyze phenotype and/or recovery in live or fixed embryos.

For the Tg (cmcl2: CFP-NTR) line:
For cardiomyocyte ablation, incubate embryos for 12-24 hours in the dark with 10 mM Mtz (dilute Mtz stock 1:100 into PTU-containing embryo medium containing 1% DMSO).

For the Tg (ins: CFP-NTR; ins:Kaede) line:
For β-cell ablation, incubate embryos for 24 hours in the dark with 10 mM Mtz (dilute Mtz stock 1:100 into PTU-containing embryo medium containing 1% DMSO). If you photoconvert Kaede prior to Mtz treatment, any regenerated green β-cells can be unambiguously distinguished from any lingering, unablated red β-cells.
In situ hybridization
Adapted from Christine and Bernard Thisse protocol. KEEP RNAse-FREE until after the hybridization steps.
You will find stocks of ISH reagents at the front of the lab.

Embryo Fixation and Hybridization (Day 1)
1) Fix embryos in fresh 4% PFA for 4 hours at RT or overnight at 4°C. Young embryos can be fixed in their chorions and dechorionated later, but embryos with extended tails should be dechorionated before fixation).
2) Optional: fixed embryos can be stored in MeOH by washing 10 min at RT in MeOH, then adding fresh MeOH and storing at -20°C. If embryos are fixed in their chorions, they should be dechorionated prior to MeOH dehydration. Embryos stored in MeOH must be rehydrated before continuing to step 3: wash 1 x 5 min in 66% MeOH: 33% PBST followed by 1 x 5 min in 33% MeOH: 66% PBST (or 2:1, 1:1, 1:2 for young gastrula stages).
3) Wash embryos in chorions 3-4 times quickly with PBSTw (PBS + 0.1% Tween-20) and transfer to 35mm petri dishes to remove chorions. Transfer embryos back to microfuge tubes. (To avoid variable staining, use <20 embryos per tube.)
4) Wash 5 x 5 min with PBSTw.
5) Optional: Permeabilize embryos with 10 ug/ml Proteinase K in PBST. This step is only necessary for embryos 24 hpf or older. The amount of time to treat is subjective and depends upon enzyme activity, but some guidelines are: 3-5 somites, 1 min; 8-10 somites, 3 min; 16-20 som, 5 min; 24 hpf, 7 min; 30-36 hpf, 15 min. After ProtK digestion, you must refix the embryos for 20 min, and re-wash 5 x 5 min with PBSTw before continuing.
6) Incubate embryos in prehybridization solution for 1-4 hours at 65°C. Use 200-500 ul per tube. WARM the prehyb solution to RT before adding.
7) Incubate embryos in hybridization solution (50 -100 ul per tube is usually plenty) at 65°C overnight. WARM the hybridization mix to 65°C before adding.

Washes (Day 2)
1) 1 x 5 min in 66% prehyb: 33% 2x SSC at 65°C (prewarm solution)
2) 1 x 5 min in 33% prehyb: 66% 2x SSC at 65°C (prewarm solution)
3) 1 x 5 min in 2x SSC (plus 0.1% Tween-20 to prevent sticking) at 65°C (prewarm solution)
4) 2 x 30 min in 0.2x SSC (plus 0.1% Tween-20) at 65°C (prewarm solution)
5) 1 x 5 min in 66% 0.2x SSC: 33% PBSTw at ROOM TEMP (RT)
6) 1 x 5 min in 33% 0.2x SSC: 66% PBSTw at RT
7) 1 x 5 min in PBSTw at RT

Antibody binding and washes (Day 2)
1) Incubate in blocking solution (PBSTw plus 2% sheep serum, 2 mg/ml BSA) for 1 hour at RT.
2) Incubate embryos in antibody solution for 2 h at RT (or 4°C O/N). Use a 1:5000 dilution of anti-digoxigenin-AP Fab fragments, diluted in blocking solution. (Optional: can “preabsorb” the antibody with “generic” embryos if desired.)
3) Wash 2x rapidly with PBST.
4) Wash 5x 15 min with PBST (can stop during one of these washes and store overnight at 4°C)
**Coloration Reaction (Day 2 or 3)**

(1) Wash 3 x 5 min in coloration buffer. Do not leave embryos in coloration buffer for extended periods of time.

(2) To make coloration solution, add 4.5 ul NBT stock per ml of coloration buffer, then add 3.5 ul BCIP stock per ml of coloration buffer. Add 500 ul per tube and transfer embryos to 9-well dishes. Store dishes in the dark and monitor every 15 min or so, depending upon the signal intensity. You are looking for a blue reaction product. *Use gloves when handling NBT and BCIP; they are toxic.*

(3) Stop reactions by washing 2x with sterile water, then store in PBSTw (for long term, store in fix). It is important to view embryos with epiillumination. *NBT and BCIP are toxic; please store the waste in the waste bottle in the hood.*

*You will find stocks of ISH reagents at the front of the lab.*

**PBST (for 400 mls):**
- 40 ml 10x PBS
- 358 ml sterile water
- 2 ml 20% Tween-20

**Prehybridization solution (for 50 ml, can store at -20°C for 1-2 months):**
- 25 ml formamide *
  **PURE GRADE** * (50% final)
- 12.5 ml 20x SSC (5x final)
- 50 ul 50 mg/ml heparin (50 ug/ml final)
- 500 ul 50 mg/mg yeast tRNA (500 ug/ml final)
- 250 ul 20% Tween-20 (0.1% final)
- sterile water to 50 ml
- 460 ul 1M citric acid (to bring final pH to ~6.0)

**Blocking solution (for 50 ml, can store at 4°C for up to 3 weeks)**
- 100 mg BSA
- 1 ml sheep serum
- PBST to 50 ml

Add BSA to 25 ml PBST, rotate conical slowly to dissolve BSA, then add sheep serum and adjust volume to 50 ml with PBST.

**Coloration Buffer (for 200 ml):**
- 20 ml 1M Tris-HCl, pH 9.5 (100 mM final)
- 10 ml 1M MgCl₂ (50 mM final)
- 4 ml 5M NaCl (100 mM final)
- 1 ml 20% Tween-20 (0.1% final)
- 165 ml sterile water

**Coloration Solution (prepare 0.5 ml per tube):**
Add 4.5 ul NBT (nitroblue tetrazolium) stock per ml, then add 3.5 ul BCIP (5-bromo-4-chloro-3-indolyl phosphate) stock per ml coloration buffer.
General wholemount antibody staining protocol
(adapted from Feng et al., 2006)

This protocol is known to work well for F310, F59, MF20, and Pax7 antibodies. It may also work for other course antibodies. All steps are at room temperature (RT) unless noted, in 1.5 ml eppies on rocking platform. The Ab incubation and washing steps can’t be overdone, the longer the better. See Antibody list on page 23 for recommended primary dilutions. Secondary antibodies from Southern Biotech for anti-IgG2b-TRITC and anti-IgG1-FITC should be used at 1:100 dilution. All other secondary antibodies should be used at 1:500.

Fix dechorionated embryos in 4% PFA overnight at 4°C (better) or 2 hours at room temp.
Transfer to 100% MeOH, place at -20°C for 1 hour or more.
Rehydrate with MeOH/PBS+0.1%Tween (PBSTw) series (75%, 50%, 25%)
Rinse with PBSTw
Block in PBSTw plus 5% goat serum for 1 hour room temp.
Incubate overnight at 4°C in PBSTw + 2% goat serum + primaries (can be done in 100ul volumes with tubes kept upright, less than 20 embryos per tube).
Wash 4x15 min with PBSTw
Incubate in PBSTw +2% goat serum + secondaries for 4hr room temp or overnight at 4°C.
Rinse with PBSTw.
Mount in 80% glycerol.

Alternative wholemount antibody labeling protocol
(from Sarah Kucenas, good for neural and glial markers and cells in “deeper” tissues)

Fix embryos/larave in AB fix for 2 hours @ RT
Rinse in PBS-Triton (0.2%) x 2 - 5 minutes
Wash in ddh20 x n - 30 minutes (n=days post fertilization)
Block 1 hr in 10% BSA/SS PBS-Triton
Incubate in primary antibody diluted in 2% BSA/SS PBS-Triton O/N @ 4°C on nutator
Wash in PBS-Tx every 15 minutes for 1.5 hrs
Incubate in secondary antibody diluted in 2% BSA/SS PBS-Tx O/N @ 4°C on nutator
Wash in PBS-Tx every 15 minutes for 1.5 hrs
Store in 1xPBS
Mount in LMP agarose

AB Fix:
8% Paraformaldehyde
2X Fix Buffer

2X Fix Buffer:
8.0 g Sucrose
30 µl 1M CaCl2
10 ml 10X PBS
80 ml ddh20
**Anti-acetylated tubulin antibody staining**
*(adapted from Zebrafish Book by C. Brosamle)*

Fix O/N in 4% PFA in PBS, store in PBS.
Freeze in acetone, -20°C, 30 min (for 2 day embryos, longer for older embryos) to permeabilize tissue. Acetone should be prechilled.
Wash 4 x 5 min with PBSTriton (PBS+Triton 0.5%)
ProtK digest (10 µg/ml in PBS) (5 min for 24hr, 10 min for 2 day, 20-30 minutes for 4 day larvae)
Postfix in 4% PFA 30 min
Wash 3x in PBSTriton, 30 min minimum
Block 1-2 hr in PBSTriton+10% sheep serum
Incubate O/N, 4°C or RT 4 hours in PBSTriton+10% sheep serum + mouse anti-acetylated tubulin AB at 1:500, 4°C (the longer the better)
Wash at least 4 x 1 hr in PBSTriton (again, the longer the better)
Incubate O/N, 4°C or RT 4 hours in PBT+10% sheep serum+Goat-anti-mouse::Alexa 488 secondary diluted 1:200, 4°C
Wash at least 4x 1 hr in PBT
Store in PBT at 4°C
Mount in 80% glycerol.

**zn-12 antibody staining**
*(Metcalfe et al., 1990; Trevarrow et al., 1990)*

Fix 4 h at RT in 4% PFA in PBS
Wash 2x in PBS, 5 min
Wash 1x in distilled water, 5 min
Freeze in acetone, -20°C for 7 min, to permeabilize tissue. Acetone should be prechilled.
Wash 1x in distilled water, 5 min
Wash 1x in PBS, 5 min
Block 30 min in PBS + 0.5% Triton (PBT) plus 10% sheep serum
Incubate O/N in PBT + 10% sheep serum + zn-12 Ab at 1:4000, 4°C or 4 h RT
Wash 4x15 min in PBT
Incubate in PBT + anti-IgG1-FITC secondary diluted 1:100, O/N 4°C or 4 h RT
Wash 4x15 min in PBT (can store in PBS at 4°C)
Mount in 80% glycerol.
**Anti-MF20/S46 staining of the heart**
Debbie Yelon

1) Dechorionate 20-30 embryos (32-72 hpf) manually. Add 3 ml of 1% formaldehyde in PBS for 50 minutes to 1 hour (no longer). The formaldehyde should be EM grade, methanol free. We use Polysciences 04018, which comes as a 10% solution. Make fresh 1X fix solution every time.

2) Remove most of the formaldehyde and flood the dish with 3 ml PBS. Rinse 1x.

3) Remove most of the PBS wash from the dish, and add about 2 ml of block for 1 hour (or longer) at RT. Make fresh block every time.  
Block solution: PBS with 10% (v/v) sheep serum and 2 mg/ml BSA, and 0.2% (w/v) saponin  
Saponin: Make fresh 2% (w/v) solution every time in water (2% solution available at the front of the room). Dilute this 10-fold into the block solution

4) Transfer embryos (using a cut-off yellow tip and P200) to a square of parafilm stuck to the bottom of a new dish or well (push down parafilm with thumb to stick it to dish). You will get a droplet of liquid and embryos on the parafilm; carefully remove excess block from the droplet

5) Add 200 ul of primary antibody mix to the embryo droplet; the primary antibodies should be mixed together in PBS and this mix should also contain 0.2% saponin. After adding antibodies to the embryo droplet, there should be a stable droplet on the parafilm that is not in danger of contacting the plastic of the plate. Incubate in a 'wet box' in the fridge overnight (some sort of sealable Tupperware or Nalgene box with some wet paper towels on the bottom to prevent evaporation). For MF20/S46 staining, the antibody mix should be: 20 ul MF20, 20 ul S46, 20 ul 2% saponin, and 140 ul PBS.

6) After overnight incubation, add 3 ml PBS/0.2% saponin to each well. Remove the parafilm while the wash is on; I usually use a pair of forceps and shake the embryos off of the parafilm so that they remain in the wash. Tilt dish so that embryos congregate near the bottom edge and it is easier to remove the wash. Remove as much of the wash as possible.

7) Incubate in 500 ul of secondary antibody, by resting the dish on its lid so that it tilts and all the embryos can be covered by this volume. Do this in the dark (e.g. a drawer) at RT for 2 hours.

For MF20/S46 staining, use the anti-IgG2b-TRITC (5 ul) and the anti-IgG1-FITC (5 ul) in 490 ul of PBS/saponin.

8) Wash twice with 3 ml PBS/saponin and leave the embryos in the second wash. I store embryos like this in the fridge for weeks. View under fluorescence at your leisure.
Phalloidin (actin) staining of embryos

Fix the embryos overnight in 4% paraformaldehyde in PBS at 4 °C overnight or 2 h at RT.
Wash 2 x 5 min in PBSTw (PBS + 0.1% Tween-20) or PBSTx (PBS + 0.2% Triton X-100)
Incubate 2 h in PBS + 2% Triton X100 (permeabilization!)
Wash 2 x 5 min in PBSTw or PBSTx
Dilute the Alexa 488 phalloidin stock 1:20 in PBSTw and incubate for 1.5 h at RT. Keep sample from protected from light from now on.
Wash 6 x 10 min in PBSTw or PBSTx.
Transfer through a glycerol series (25%, 50%, 80%). Let the 80% incubation sit for 30 min.
Mount in 80% glycerol.

Acridine Orange staining

See Robu et al., 2007. Immerse live embryos in 5ug/ml Acridine Orange for 10 min at RT, then visualize and image for less than 1 min (the signal gets quenched) using a FITC filter.

SYTOX green staining

We will provide 1:100 dilution stocks of SYTOX green, stored at -20C. Process embryos for antibody staining (or whatever) and rinse into PBS. Incubate in a 1:10,000 dilution (i.e. 1:100 of 1:100 stock) of SYTOX Green (Invitrogen) in PBS overnight at 4°C.
Mounting embryos in bridged coverslips for photography
For live embryos, use a fire polished pipet to transfer the embryo plus EM to the middle of a bridged coverslip. Use higher bridges (3 or 4 high) for more delicate stages. Gently put on a top coverslip from left to right. Move the top coverslip to roll and orient the embryo.

For fixed embryos, dehydrate through a glycerol series in a histology dish. Make an “H” shape on the bridged coverslip with glycerol. Move the embryo plus a little glycerol to the crossbar of the “H”. Gently put on a top coverslip from left to right. Move the top coverslip to orient the embryo.

If the embryo doesn’t roll when you move the top coverslip, try a lower bridged coverslip.

Agar mounting 24 hour and older embryos for timelapse or photos
(This takes longer, so let us know ahead of time if you want to do it – we’ll need little time to help you prepare.)

Use the 1.2% agar dissolved in fish water, stored in 70° C bath. Keep a tube of agar in 42° C heat block next to scope. Use microscope slide with PAP pen or vacuum grease circle on it.

Dechorionate embryos. Use 0.4% Mesab solution to anesthetize embryos so that they stop moving. Don’t overdo it - use about 10 drops for a medium dish. Note that you will need to use more if you want to stop the heart beat.

With fire polished pipet, pipet an embryo with a minimum amount of medium into 42°C agar tube, then pipet out agar plus embryo onto slide, inside the PAP pen circle.

Orient embryo with loop, let agar cool. Cover agar-mounted embryo with embryo media + Mesab.

To release embryo: flood coverslip with system water, use loop/forceps to carefully pull apart agar, and pipet embryo to a dish of system water without Mesab to continue development.

Methylcellulose mounting blastula/gastrula embryos
We have 3% methylcellulose made up for you. Keep the methylcellulose “stiff” on ice!

- Use a wooden applicator to put a stripe of ice-cold 3% methylcellulose down the middle of a depression slide. Add embryo medium to the side to which you will add the embryo.
- Carefully pipet a dechorionated embryos next to the methylcellulose stripe.
- Under your dissecting scope, use a loop to carefully position the embryo. Don’t jam it into the methylcellulose – just carefully lean the embryo against it.
- As the methylcellulose warms up, it will get gooey.
- When done, wash the embryo into a dish with EM. Or even better, submerge the slide+embryo in a 100 mm dish of EM and let the embryo float out.

Vacuum Grease/Coverslip Mounting
An alternative quick way to mount embryos is to create a square or circular well on a slide using vacuum grease. Fill the well with Embryo Medium or Methyl Cellulose and place your embryo inside. Cover with a coverglass and gently press down enough to seal but so much that you squish your sample! Carefully moving the coverslip can sometimes adjust the orientation of the sample. Mesab anesthetization can be used to immobilize older embryos for microscopy.
Other Useful Protocols (not covered in the course)

**Alcian Green Staining of Cartilages**
Fix 5 dpf embryos O/N in 5% TCA (trichloroacetic acid)
Rinse 5’ in Acid Alcohol
Stain O/N at RT in Alcian Green Stain
Differentiate with multiple Acid Alcohol washes changing solution about every hour for half a day or so.

Option 1: whole mount
- Rehydrate in Acid Alcohol/water series: 15 min in 75% AA/water, 50%, 25%, water.
- Clear in series of Glycerol; 1%KOH (25%, 50%, 75%)

Option 2: digest away tissues
- Place stained embryos in 35 mm dish with trypsin solution
- Digest 3-8 min (while watching) until eyes fall out.
- Rinse out of trypsin with a few water washes.
- Clear in Glycerol;KOH as above.

Mount and photograph.

**Solutions:**

- **Acid Alcohol:**
  - 70 ml Absolute EtOH
  - 30 ml Glacial Acetic Acid

- **Acid Alcohol/Alcian stain:**
  - 70 ml EtOH
  - 30 ml Glacial Acetic Acid
  - 0.1 g Alcian Green

- **Trypsin solution:**
  - 30 ml saturated NaBorate
  - 30 ml H2O
  - 1 g trypsin

**BODIPY-ceramide staining to visualize membranes**
Clarissa Henry, modified from a protocol by Mark Cooper. *Important: Make the 10 mM BODIPY-ceramide stock (D3521; Molecular probes) in ultra-pure DMSO*

Dilute the 10 mM BODIPY stock 1:50 –1:100 with embryo medium containing 1% DMSO and 10mM HEPES (to buffer the pH). (You can leave out the HEPES, but you must limit the number of embryos [no more then 3 embryos in 200µL of stain solution]).

Start incubating dechorionated embryos at 30% epiboly in an appendoral cap or microfuge tube and let them stain for 3h in the dark. They will be ready for viewing at 60% epiboly. You can stain through the chorion but you have to allow additional incubation time.

If you want to view them at later stages you can start the staining later, but it takes longer for the embryos to be stained (e.g., to view at 24h, start staining at 20h).
Staining embryos for β-galactosidase activity
(Jennifer Liang/Halpern Lab)

Inject embryos with 50-100 pg of β-galactosidase (β-gal) RNA

Fix embryos in 4% paraformaldehyde (PFA) for 50 minutes or in 1-2% PFA overnight

Wash embryos 4X 10 minutes in 1xPBS + 5mM MgCl₂ (50 ml 1X PBS+ 250 ml 1M MgCl₂).

(Don't rock because embryos will be very fragile.)

During last wash, get the β-gal staining solution:

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock</th>
<th>for 10 ml staining solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X PBS</td>
<td>10X PBS</td>
<td>1 ml</td>
</tr>
<tr>
<td>4 mM K₄Fe(CN)₆*</td>
<td>100 mM K₄Fe(CN)₆</td>
<td>400 ml</td>
</tr>
<tr>
<td>4 mM K₃Fe(CN)₆*</td>
<td>100 mM K₃Fe(CN)₆</td>
<td>400 ml</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>1M MgCl₂</td>
<td>50 ml</td>
</tr>
<tr>
<td>0.01% Deoxycholate</td>
<td>0.1% Deoxycholate</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.02% NP40</td>
<td>10% NP40</td>
<td>20 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>6.6 ml</td>
<td></td>
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</tbody>
</table>

Warm solution to 65 °C (takes about 5 minutes). Once warm, add 0.5 ml 20 mg/ml X-gal*,**, Save extra solution at 37 °C in case you need it. *light sensitive, store in dark, **store at -20 °C

Remove most of last wash and add about 1 ml of staining solution/sample. Move embryos to a dish so that you can monitor progress of staining. Place at 37 °C for 1 hour to overnight. Be careful not to leave at room temperature too long (>10 minutes) or X-gal will precipitate.

When desired amount of staining has been achieved, rinse embryos several times with 1X PBS until solution is no longer yellow.

If you want to do RNA in situ on the β-gal stained embryos, post-fix in 4% PFA overnight and then carry on with in situ protocol as usual. If you are planning to do in situ, all solutions in this protocol should be kept RNase free.
References


Reifers F, Bohli H, Walsh EC, Crossley PH, Stainier DY, Brand M (1998) Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development 125, 2381-2395


program by Cdx factors is required for the specification of the vertebrate spinal cord. Development 134, 2147-
2158

Stafford D, Prince VE (2002) Retinoic acid signaling is required for a critical early step in zebrafish pancreatic


Conserved sensory-neurosecretory cell types in annelid and fish forebrain: insights into hypothalamus
evolution. Cell 129(7): 1389-1400

Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI (2003) Transplantation and in vivo imaging of

May;4(5):669-79.


end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and

Wilson, B.D., Li, M., Park, K.W., Suli, A., Sorensen, L.K., Larrieu-Lahargue, F., Urness, L.D., Suh, W., Asai, J.,
Promote Developmental and Therapeutic Angiogenesis. Science. 313(5787): 640-644

PLoS Genet. 3, 1922-1938

Ogon/Secreted Frizzled functions as a negative feedback regulator of Bmp signaling. Development 130, 2705-
16.

Yelon D, Horne SA, Stainier DY (1999) Restricted expression of cardiac myosin genes reveals regulated aspects of

factor Hand2 plays parallel roles in zebrafish heart and pectoral fin development. Development 127, 2573-
2582.

Zannino DA, Appel B (2009) Olig2+ precursors produce abducens motor neurons and oligodendrocytes in the