Instructor's Key for the F_3 Screen Protocol

Summary of Supplemental Materials

Supplemental Material 1	Animal training homework
Supplemental Material 7	Rubric for grading worksheet/laboratory notebook
Supplemental Material 8	F_3 Screen Instructor's Key
Supplemental Material 9	Powerpoint introduction to F_3 Screen
Supplemental Material 10	Student Guide to F_3 Screen
Supplemental Material 11	Worksheet for chi-square analysis
Supplemental Material 12	Easier homework for F_3 Screen
Supplemental Material 13	More difficult homework for F_3 Screen

Time line

Fish needed for laboratory

Most research laboratories using zebrafish maintain several lines of fish that are carrying recessive lethal mutations (m). The next generation of fish is generated by crossing adult fish heterozygous for the mutation, and then raising the progeny with a WT phenotype to adulthood. These WT progeny will be a mixture of fish heterozygous for the mutation (2/3rds) and fish that are homozygous WT (1/3rd) (Figure 1 below). In this protocol, these sibling groups are called "mock F_2 families" as they are similar to the F_2 families in an F_3 genetic screen (see introduction power point for this laboratory, Supplemental Material 9). Protocols for raising fish from embryos to adulthood can be found at:
http://www.zfic.org/common%20techniques/Raising%20baby%20fish.html and
http://www.zfic.org/common%20techniques/SOP.html.

Week before the laboratory

Because this laboratory uses vertebrate animals, students need to complete any training required by the institution's Institutional Animal Care and Use Committee before starting the laboratory. These requirements will vary from institution to institution. In our case, students complete three online training modules as a homework assignment, and then turn the signed homework to document completion of this training (Supplemental Material 1).

First day of laboratory

Move materials into the teaching laboratory:
1. Tanks containing the mock F_2 families
2. Mating tanks
3. Many liters of fish water for filling mating tanks (each mating tank takes ~1L)
4. 4-5 nets for each mock F_2 family
5. In class copies of the guide to this laboratory "F_3 Forward Genetic Screen in Zebrafish" (Supplemental Material 10)
6. In class copies of the protocol for setting up single pair matings:
http://www.zfic.org/common%20techniques/mating.html
7. In class copies of the protocol for labeling tanks and Petri dishes:
http://www.zfic.org/common%20techniques/tank%20labels.pdf
8. In class copies of the guide for determining gender of adult fish:
http://www.zfic.org/common%20techniques/Gender%20identification%20guide.pdf

Students will set up several blind intercrosses from each mock F_2 family following the steps in the handout "F_3 Forward Genetic Screen in Zebrafish" and the protocols listed above. We try to have several families available, each carrying a different mutation that affects early development. These crosses are called "blind" because they contain one female and one male, and the genotype of both of these fish is unknown. As you can see from Figure 1, a little bit less than ½ of the crosses will yield embryos that are homozygous for the mutation and thus expressing a mutant phenotype. Therefore, it is best to set up several blind intercrosses from each family to ensure that there are mutants to examine. See "Common Problems" on page 3 for ways to simplify this step.

Figure 1: Outline of fish and crosses

Parents: m/+ X m/+

Mock F_2 family: $1/4^{th}$ +/+ (WT phenotype)
1/2 m/+ (WT phenotype)
$1/4^{th}$ m/m (embryonic lethal)

Mock F_2 family as adults: $1/3^{rd}$ +/+ & $2/3^{rd}$ m/+

Possible combinations of blind intercrosses between adults from the F_2 family:
A) +/+ X +/+ ($1/9^{th}$ of crosses)
B) m/+ X +/+ ($4/9^{th}$ of crosses)
C) m/+ X m/+ ($4/9^{th}$ of crosses)

Progeny from blind intercrosses (F_3 generation):
A) 100% embryos with a WT phenotype
B) 100% embryos with a WT phenotype
C) 75% embryos with a WT phenotype, 25% embryos with a mutant phenotype

Second day of laboratory

Move materials into the teaching laboratory:
1. Tanks containing the mock F_2 families
2. Mating tanks containing the single pair matings set up the day before
3. Dissecting microscopes with illuminated bases
4. Fish water
5. Fish nets
6. Transfer pipets
See http://www.zfic.org/common%20techniques/embryo%20swirl.html for instructions for the "embryo swirl" to move zebrafish to the center of a Petri dish for observation and sorting.
7. Embryos loops
http://www.zfic.org/common%20techniques/Embryoloops.html
8. In class copies of "F_3 Forward Genetic Screen in Zebrafish" (Supplemental Material 10)
9. In class copies of the protocol for setting up single pair matings:
http://www.zfic.org/common%20techniques/mating.html
10. In class copies of the protocol for labeling tanks and Petri dishes:
http://www.zfic.org/common%20techniques/tank%20labels.pdf

Students harvest embryos and place adults back in their home tanks according to the handout "F_3 Forward Genetic Screen in Zebrafish" and the protocol for setting up single pair matings. After they are finished sorting, the students should place the Petri dishes containing their embryos into secondary containment, and then into an incubator or fish room with a temperature at approximately 28.5 ºC until the next meeting of the class.

Note that the embryos must be collected and the adult fish put back in their home tank the day after the fish are set up in the single pair matings, as the adult fish can only remain in the mating tanks for one day. Thus, it may be difficult for the students to do this step if the class does not meet every day. In our case, each section of the laboratory only meets once per week, and so the instructors of the course usually put away the adult fish and collect and sort the embryos. We then set up different crosses the day before the following week's laboratory so that the students can gain experience in sorting embryos.

Third day of the laboratory

Move materials into teaching laboratory:

1. Petri dishes containing F_3 progeny. Petri dishes are coded with colored tape to mark the family of the embryos parents (ex; red tape for mock F_2 family containing *cyclops*, green tape for each mock F_2 family containing *squint*). Depending upon the time available, we sometimes bias these towards Petri dishes that contain mutants.
2. Empty Petri dishes for counting embryos and sorting them into groups with different phenotypes
5. In class copies of "F_3 Forward Genetic Screen in Zebrafish" (Supplemental Material 10)
3. Copies of the chi-square worksheet for students to use in their laboratory notebooks (Supplemental Material 11)
4. A few liters of fish water for filling Petri dishes
5. Embryo loops
6. Transfer pipets

The third day of the laboratory can be held as soon as the F_3 embryos have a morphological phenotype. Students are required to gather data from at least one Petri dish containing mutants in it for each mock F_2 family. Since not all Petri dishes will have mutants, they will often have to look through several dishes before they find one to score. Typically, we will have 5 or 6 different mutants for them to examine in one four hour laboratory period. Students follow the directions in the handout "F_3 Forward Genetic Screen in Zebrafish" and use the chi-square worksheets as part of their laboratory notebook entry.

Because our class meets only once a week, for some mutants we slow down development by keeping the F_3 fish at room temperature. If we do this, they are in early larval stage (the equivalent of ~4 dpf) by the third day of the laboratory. The phenotypes of most of our mutants are very severe by this stage, making it quite easy for students to distinguish between WT and mutant fish. In addition, using early larval stage fish avoids complications from having fish at different embryonic stages, which can cause confusion about what constitutes a WT phenotype.

Pedagogy

Learning Objectives

1. Students begin to learn the rules for doing research with vertebrate animals.
2. Students begin to develop skills on how to efficiently and accurately gather data
3. Students gain experience with light microscopy
4. Students begin to develop the fine motor skills needed to manipulate samples under a microscope

5. Students gain hands on experience with the relationship between genotype and phenotype
6. Students learn how to form a hypothesis based on phenotypes present in the F_3 progeny
7. Students learn how to test their hypothesis using chi-square analysis
8. Students begin learning how to interpret a P-value

Summary of Assessments
1. Animal training homework (Supplemental Material 1)
2. Laboratory notebook entry for each day of the laboratory (Supplemental Material 7)
3. One or both F_3 Genetic Screen homeworks (Supplemental Materials 12 and 13)

Common Problems

1. Using a microscope

For many students, this will be the first time that they have used a microscope, and they will not be familiar with how to set it up properly. If this is the case, there is a useful guide at http://www.zfic.org/common%20techniques/steromicroscope.html. We also typically walk around the room and look in student's eye pieces to make sure their microscope is focused and they are getting even illumination across the field of view.

2. Difficulties scoring phenotypes and carrying out chi-square analysis

There are many questions that emerge as students score the Petri dishes of embryos. A mechanism to hook one of the microscopes up to a projector can be very useful for enabling everyone to see the embryos while answering these questions, and thus for facilitating group discussions. Students may need help generating a plan to gather their data. Instructors can help lead them to the idea of working as a group to score and count dishes with large numbers of progeny, moving the embryos with different phenotypes to separate dishes to make it easier to confirm phenotypes and count, and using embryo loops to move embryos to different orientations to make the phenotypes easier to observe.

Very often there will be embryos that are not normal but also do not have the phenotype typical of the mutants in the clutch. Students will often need guidance in deciding how to deal with these embryos in their chi-square analysis. Instructors can help lead them to the idea that (1) these embryos should be included in their total number of embryos for their calculation of the "expected" numbers for each phenotype, as there is no basis for discarding these embryos from the analysis, and (2) the phenotype of these atypical embryos should not be included in the "phenotype" column of their chi-square table unless they are predicted by the hypothesis. The later idea will also come out as the chi-square analysis progresses, as any phenotype listed that has an expected number of zero will not be analyzable as there will be a zero in the denominator of column 6 of the chi-square table (Supplemental Material 11).

3. Difficulty getting enough clutches of embryos for the laboratory.

Laboratories that require large numbers of live zebrafish embryos can be stressful for the instructor, as it is difficult to know ahead of time if you are going to get enough material for the class. One solution is to set up some group matings of fish already known to be heterozygous for the mutation. Group matings are more likely to spawn than single pair matings, and crosses of known heterozygotes will always produce clutches with mutant embryos. Another solution is to build up a bank of clutches that have been fixed in paraformaldehyde and stored in methanol using standard procedures

(http://www.zfic.org/common%20techniques/fixing.html). Clutches of fixed embryos can be stored for years at -20 ºC and used as backups if live embryos are not produced. The disadvantage of this approach is that the developing fish become opaque after fixation. This can make it more difficult to indentify mutant phenotypes. In addition, the embryos and larva are less beautiful and the laboratory is less engaging for the students.