F3 Forward Genetic Screen in Zebrafish

by Jennifer Liang

Introduction and History

The goal of this laboratory is to gain hands on experience with what it is like to carry out a forward genetic screen. Genetic screens are one of the most powerful techniques in biology, and they are aimed at identifying genes that are important for the process you are studying. For the purposes of this introduction and this laboratory, let's pretend we are interested in eye development, and that we have chosen zebrafish as our model organism to use in this research (probably a good choice, as zebrafish have eyes).

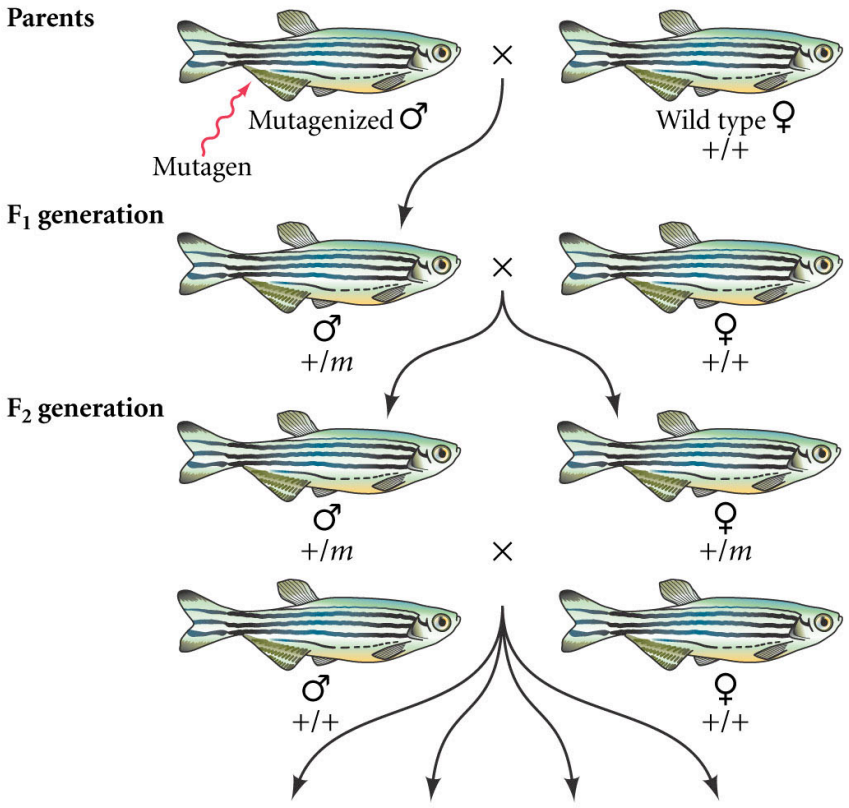
There are two main categories of genetic screens, forward genetic screens and reverse genetic screens. In a forward genetic screen, you would mutagenize zebrafish and then screen fish carrying the mutations for defects in eye development. In a reverse genetic screen, you might start with a gene that you know is expressed in the eye, cause a reduction or absence in the expression of this gene, and then see if the resulting fish have anything wrong with their eyes. We will do a reverse genetic approach in zebrafish later in the semester (in zebrafish, reverse genetic screens are more technically demanding than forward genetic screens).

Zebrafish is a relatively new model system. I am in only the third generation of zebrafish researchers (my post-doctoral advisor (Dr. Marnie Halpern) did her post-doctoral research with one of the founders of the field (Dr. Chuck Kimmel)). When the field started, the first mutations were induced with gamma-irradiation. Gamma irradiation causes large deletions or translocations in the genome (for instance, see Hatta and Kimmel, 1991, Nature v. 350, pp. 339-341). Keeping some of these mutant lines going was really an art form. From your knowledge of meiosis, think of why it might be very difficult to maintain a strain of fish that has a large translocation.

In the mid-1990s (therefore, in your lifetime), zebrafish research began its exponential growth with the completion of two very large forward genetic screens, one in Tubingen Germany and the other in Boston, MA (reviewed in Haffter and Nusslein-Volhard, 1996, Int. J. Dev. Biol. v. 40, pp. 221-227 and Stemple and Driever, 1996, Curr. Opin. Cell Biol. v. 8, pp. 858-864). These screens used a chemical that is abbreviated ENU to induce point mutations in the genome, and then kept every mutant line that had a developmental defect. It is the methods that were developed in these large screens that we are going to follow in this laboratory. If you are interested, there was a whole issue of the journal Development that was completely devoted to the results of these screens (Development v. 123, published in December 1996).

This kind of screen is called an F3 screen because it requires three generations of offspring (F is for filial) from the parental (P) generation. This scheme is represented on the next page. Briefly, the premeiotic sperm of adult males are mutagenized with ENU (very nasty, the fish have to swim around in the mutagen, and the humans have to wear many layers of protective clothing, and its hot because they are tropical fish). The mutagenized fish are crossed to WT females to create the F1 generation. The F1 fish are crossed individually to WT fish to create F2 families.

This is where you all come in. What we have are essentially tanks of F2 families that are a mixture of fish carrying a particular mutation ("m" in the figure on p. 3) and fish not carrying a particular mutation. Each family has a different mutation, but the fish within a family should all have the same one. Make sure you understand why this is so. You are going to carry out blind intercrosses-that means you do not know the genotype of the mother or the father, between the fish in your family. You will then screen the resulting F3 embryos for eye and other phenotypes, count the ratio of normal to abnormal embryos, and then make hypotheses about what kind of mutation it is (recessive, dominant, etc.) and the genotype of the parents. The only difference between this screen and a real F3 screen is that we know our families are carrying mutations that affect development.



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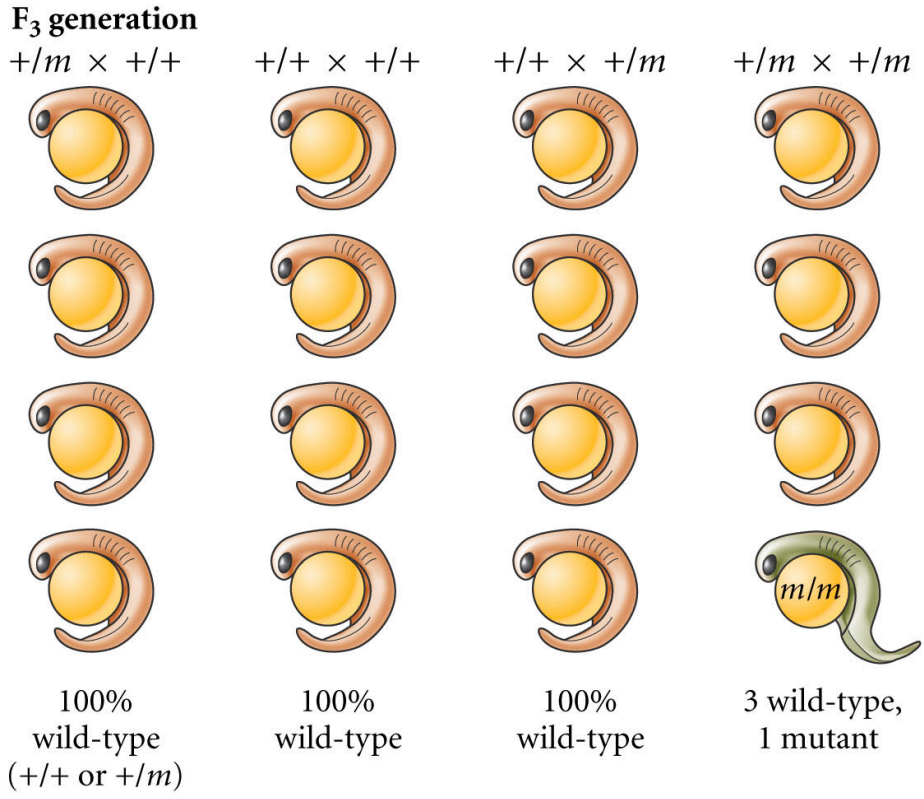

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Summary of Protocol

This is a three part/ three day laboratory.

Part 1: The first step is to set up the adult fish in your F2 family in single pair matings (one female and one male fish). The fish stay in their special mating tanks overnight, and then typically lay their eggs within the first two hours after the lights come on the following morning.

Part 2: The second step is to collect the eggs from each mating and put the adults in holding tanks. You will then sort the good eggs away from debris and bad/infertile eggs, and count the good eggs.

Part 3: You will then screen the resulting clutches (groups of sibling embryos) for eye and other phenotypes. This will be done either with live or fixed embryos, depending on the timing of the spawning and the phenotype of the mutants.

Because we only have labs two days a week, you will not necessarily be doing the parts in order.

Step by step protocol

Part1

1. Take two fish nets, one big, one small, out of the net dip and rinse in tap water for a few seconds. The big net will be for scooping the fish out of their home tank, and the smaller net will be for sorting males from females.

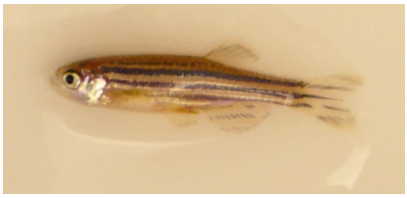
2. Net fish from your assigned tank into a smaller tank containing fish water (to more easily identify males and females). Zebrafish are jumpy, so it is good idea to have the smaller tank close by and to cover the net with your hand while you are moving the fish.

IMPORTANT-ALL OF OUR ADULT ZEBRAFISH LOOK PRETTY MUCH THE SAME. Therefore, it is VERY important to properly label every tank and dish before you move the fish. Please look at the last page of this handout now for detailed instructions on how to label tanks and dishes of embryos.

If you want to show that you are a TRUE zebrafish researcher, you will fold over one edge of the tape to make it easier to get off later. We use a lot of tape.

3. Prepare one additional small tank with fish water (make sure to label it). You will then use your net to sort the males and females into separate tanks. To identify the sex of the zebrafish, keep in mind that females tend to be larger and rounder with a silver-blue streak whereas males are somewhat yellowish and tend to be more active.

Female



Male

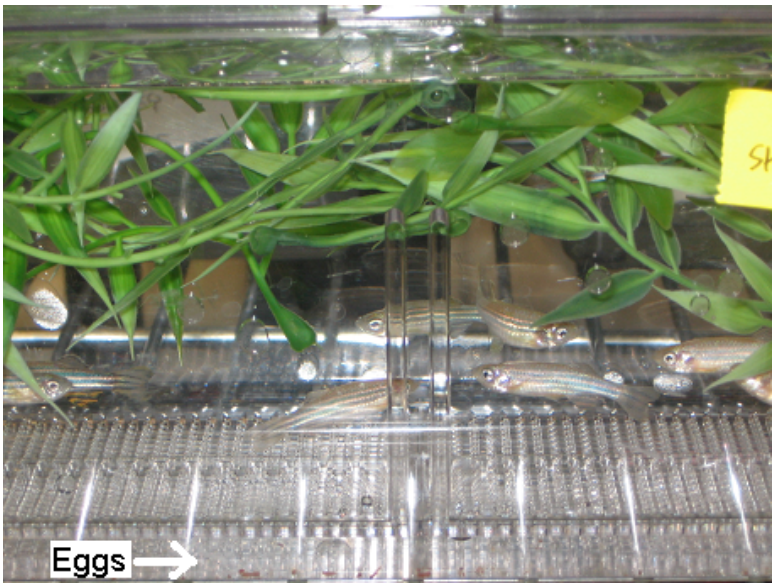
4. Determine how many single pair matings (one male, one female) you will be able to set up from your tank. Set up the appropriate number of crossing tanks. The crossing tanks consist of a top compartment with holes in the bottom for the eggs to fall through, a bottom reservoir, and a cover. Fill these with fish water, some plastic greenery, and label. Each crossing tank should also get a unique number (ex: intercross 1 of 10 (ix1 of 10), ix2 of 10, …ix 10 of 10).

5. Net one female and one male into the top compartment of the mating tank. Put the cover on, and place the mating tank in a place that it can remain undisturbed. The tank will remain in place until the next morning, when it will be checked for eggs.

6. Net any unused fish back into the home tank. Return the net to the net dip.

Part 2

1. Look for eggs in the lower compartment, lift the tank and look from underneath if necessary. If there are eggs, turn the label vertically to indicate the presence of eggs.



2. Fish that have laid eggs will be moved into holding tanks in the fish rack while we finish all of the parts of this experiment. Fish that have not produced any embryos will be left for several hours in the mating cages-sometimes they mate several hours after the lights come on-and then moved back into their home tanks.

3. For each clutch of eggs, label a Petri dish as directed on the last page of this handout and fill it half full with fish water.

4. Let the fertilized eggs settle to the bottom of the mating tank. Drain most of the water by carefully pouring it out of the corner, making sure not to lose any eggs. Water can go down the sink. Pour until the eggs and water cover one corner. Make sure to have the labeled Petri dish nearby.

5. Quickly pour eggs with the remaining fish water into the labeled Petri dish.

6. Sort the fertile eggs into a new Petri dish labeled appropriately. Leave infertile/bad eggs (brown and dead or misshapen) and debris (fish waste, scales, etc.) behind in the original dish. Have the instructor check your sorting to make sure it is correct.

7. Use an embryo loop to count how many fertile eggs are in the dish. A good way to do this is to swirl all of the eggs to the middle, and then use an embryo loop to push eggs to the edge as you count them.

8. Write the number of good eggs in your dish on the top with the other labels, and put the dish into the 28.5°C incubator.

Part 3

Purpose: To learn about Mendelian Genetics by observing phenotypes of real crosses between zebrafish.

In this lab, you will be completing the last step of the F3 screen. You will be looking at various groups ("clutches") of zebrafish embryos containing wildtype and mutant fish, making a hypothesis as to the type of mutations that are present, and testing your hypothesis using a chi-square test.

Each Petri dish you examine contains one clutch, or all of the embryos produced from one single pair mating between adult zebrafish in the F2 generation. Most of these embryos were obtained from the crosses you set up as part 1 of this laboratory. Others were obtained by similar crosses set up by your instructors.

Types of mutations that could be present in your F3 embryos:

1. Dominant – allele or phenotype that is expressed in either the homozygous or the heterozygous state. (AA or Aa)
2. Recessive – allele or phenotype that is expressed in only the homozygous state. (aa)
3. Codominant- when alleles show equal expression of phenotype.
4. Incomplete (partial) dominance- condition that results when the phenotype of one allele in not completely dominant over another allele and the heterozygous form exhibits a phenotype that show traits of both homozygous forms of the alleles.
5. Incompletely penetrant- when some of the embryos having a particular genotype have the trait and others do not.
6. Some clutches may have more than one mutation and therefore more than two phenotypes.

Procedures:

STEP 1: Observations

1) Start with one Petri dish containing a clutch of fixed or live embryos.

2) Observe all the embryos in this clutch for any mutant phenotypes.

3) Separate the embryos based on whether they have a wildtype or mutant phenotype. This is called "scoring" the embryos. It is important to choose a consistent characteristic to use in your scoring. For example: straight tail (WT) vs. curly tail (mutant).

4) Count the number of embryos that have wildtype phenotypes and the mutant phenotypes and record.

5) Compare the numbers of wildtype versus mutant embryos for that clutch.

6) Repeat steps 1-5 for one dish (i.e. clutch) per category. There are 4 different categories of embryos to observe.

STEP 2: Analysis

7) Make your hypotheses – 3 parts for each clutch
   a. Hypothesis about the parental (F2) genotypes
   b. Hypothesis about the offspring (F3) genotypes
   c. Hypothesis about the relationship of genotype to phenotype (+/+ = WT phenotype, etc.)

8) Do the chi-square analysis on your data from all 4 of the Petri dishes (i.e. clutches) that you observed. Use the chi-square worksheets provided.

9) Make sure to record your results in your laboratory notebook. Some things that should be included:
   a. Were there mutants in the dish? If so, what kind of mutants, and how many? How many wild-type embryos were in the dish (if any)?
   b. Describe the mutant phenotypes. What physical features did you use to sort the embryos?
   c. Record detailed methods –Keep good records of what type of microscope was used, what tools you used, how you sorted the embryos, etc. The methods section should include both how you set up the experiment (mating fish, sorting embryos- Days 1 and 2) and how you did the analysis (all methods from Day 3).