1 Overview

In this project, you will measure the effect of bicoid (bcd) gene dosage on positioning of a morphological feature in the early development of a Drosophila embryo (shown in Fig. 1). In particular, you will measure the position of the cephalic furrow (also known as the head fold, Fig. 1c). You will first mate flies in order to create mutant flies with a single copy of bcd gene. Then, you will measure the cephalic furrow positions in wild type embryos (which contain two copies of bcd) and in the mutant you created. You will use your measurements to assess the French flag model, a prevalent model for morphogenesis, the process by which a developing organism is shaped (see below).

The effect of bcd dosage on the cephalic furrow position was first reported in the following references:


We will not cover these papers in depth, but will discuss some of the important results in class.

2 Background

In this section, we present a brief overview of some background information to help you understand the context and techniques of the experiment.
2.1 Bicoid the French flag model

A **morphogen** is a molecule that determines the fate of cells in a developing organism in a concentration-dependent manner. Through the work in Christiane Nüsslein-Volhard’s lab in the late 1980s, Bicoid was the first morphogen to be identified. Like Nüsslein-Volhard, we will study how Bicoid affects the development of a fruit fly embryo.

How might a morphogen influence cell fate? In 1969, Lewis Wolpert put forward the **French flag model** to describe this phenomenon. The basic idea is that a cell achieves one fate or another if its morphogen concentration is above or below a threshold. The analogy to the French flag is apparent in Fig. 2. It is no coincidence that the image the authors chose in Fig. 2 has the shape of a *Drosophila* embryo, since Bicoid is a classic morphogen.

As shown in the Driever and Nüsslein-Volhard paper, Bicoid protein has a concentration gradient, shown schematically in the top of Fig. 2. The steady state concentration profile is exponential, being described by

\[
[Bcd] = [Bcd]_0 e^{-x/\lambda},
\]

where \(x\) is the position along the anterior-posterior (A-P) axis and \(\lambda\) is the characteristic decay length of the concentration profile. \([Bcd]_0\) is the concentration of bicoid at the anterior.

It is thought that this profile results from the following mechanism. Bicoid protein is produced at the anterior (the end of the embryo with the little “point,” called the **micropyle**) from maternal
mRNA. It then diffuses through the embryo. The protein has a finite lifetime, so it also decays. This combination of localized production at the anterior, diffusion, and decay gives rise to a gradient.

Given this mechanism for gradient formation, the value of \([Bcd]_0\) is set by the amount of maternal mRNA present at the anterior. The amount of mRNA is set by the gene dosage of \(bcd\). So, the relationship between the amount of Bicoid in the eggs of wild type mother and those of the mutants produced from your crosses from a couple weeks ago is \([Bcd]_{0,\text{mut}} = \alpha[Bcd]_{0,\text{wt}}\) with \(\alpha = 1/2\).

### 2.2 Early \textit{Drosophila} embryogenesis

Under the French flag model, when the Bicoid concentration crosses a threshold, we expect to see a morphogenetic feature. The feature we will study is the cephalic furrow. This is one of the first major morphological features to appear in the developing embryo. It is depicted at the lower right of Fig. 3, which shows the very early stages of \textit{Drosophila} embryogenesis, in which the nuclei of the embryo undergo fourteen rounds of divisions. During this time, the nuclei are part of a syncytium, a large, multinucleate cell. After these fourteen divisions, cellularization occurs, in which the nuclei at the periphery of the embryo are surrounded by cell membranes and become part of cells.

The whole process of the 14 nuclear divisions and cellularization takes approximately two hours at room temperature. Immediately after cellularization and cephalic furrow formation, the embryo develops rapidly, and the cephalic furrow is more difficult to see (see Fig. 1). For this reason, we will measure the cephalic furrow when it first forms (lower right of Fig. 3, Fig. 1c) to maintain consistency in the measurements. This stage of development is well-marked because the cellularized blastoderm will look much lighter in color under the microscope immediately before the cephalic furrow forms.

### 2.3 \textit{Drosophila} genetics and creation of a \(bcd\) mutant

In order to produce a flies with a single copy of \(bcd\) (as opposed to two copies in wild type flies), we need to mate, or cross, two flies. In order to understand how this works, we first need to present some background on genetics.

#### 2.3.1 Notation

\textit{Drosophila} have four pairs of homologous chromosomes. The chromosome pairs are written as “fractions,” with the numerator describing what mutations are on one of the homologous chromosomes and the denominator describing the mutations on the other. Wild type (no mutations) is denoted with a “+.” The chromosome “fractions” are separated by semicolons. So, if a fly has mutation \(m\) on one copy of chromosome 3 and the other copy is wild type, the chromosomal composition would be written as \(; ; m/+\). (Note that if both homologous chromosomes are wild type, their “fraction” is typically omitted.) If it had the mutation \(m\) on both chromosomes, it would be written as \(; ; m/m\). A fly with such chromosomal makeup is said to be homozygous. Conversely, a fly with \(; ; m/+\), or \(; ; m_1/m_2\) (where \(m_1\) and \(m_2\) are two different mutations) is heterozygous. Multiple mutations on the same copy of a chromosome are separated by commas, e.g. \(; ; m_1, m_2/+\). Finally, a female fly has two copies of the first chromosome, and a male fly has only one. (The other “first” chromosome for the male is called a Y chromosome and rarely has genes of interest, so it is not written.)

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\footnote{It has only recently been carefully quantified that \(bcd\) gene dosage is in fact directly proportional to the \(Bcd\) concentration. See Liu, Morrison, and Gregor, \textit{PNAS}, 2013.}
2.3.2 Heredity

When two flies are mated, the offspring inherit one copy of each chromosome from each parent. As an example, consider mating a female (♀) fly with genotype ; ; m/b, s with a wild type male (♂) fly. Since chromosomes 1, 2, and 4 are wild type in both parents, all offspring will have wild type chromosomes 1, 2, and 4. For chromosome 3, the mother may donate a chromosome with genotype m or b, s. The male will necessarily donate a chromosome with + genotype.

You can think of the genotype of the offspring as being derived from two draws from urns (Figure 4). The urn corresponding to female chromosome 3 has two types of balls, m and b, s. The male urn has only one type of ball, +. The offspring chromosome pair gets one ball from the female urn and one from the male urn. The offspring have a 50/50 chance of being male or female, so all possible offspring from this example cross are as follows.
2.4 Making a bcd/+ fly

In this experiment, we will cross the following flies.

\[ \text{♂} \ w; \ bcd \times \♀; \ + \]

Here, \( w \) is a mutation that gives white eyes that is unimportant for our cross. All offspring will therefore have a third chromosome with \( bcd/+ \) because the offspring will get one chromosome 3 with \( bcd \) from the father and one wild type from the mother. This is our desired mutant, since the \( bcd \) mutant is not functional, but the wild type (+) is. Note that we cannot see any effects of the \( bcd \) mutation in adult flies. (Next month, we will measure the effects of this mutation on embryos.)

The male flies come from a vial that contains a mixture of \( w; bcd/bcd \) and \( w; bcd/TM3, Sb^1 \). The vial is mixed because a) \( bcd \) is homozygous sterile, meaning that female flies with a \( bcd/bcd \) third chromosome cannot produce offspring, and b) TM3 is homozygous lethal, meaning that we will never see offspring with a \( TM3, Sb^1/TM3, Sb^1 \) third chromosome.\(^2\) The heterozygous flies have the \( Sb^1 \) mutation, which we can see visually in the adult fly (as described below). Therefore, for our cross, we need to find which males are \( w; bcd/bcd \) in our mutant vials, and put them in a vial with virgin wild type flies. The females have to be virgins so we can be sure they did not mate with any other males besides the ones we want them to mate with.

2.5 Mechanics of a cross

In this section, we move from theoretical genetics to the laboratory and describe how to perform a cross in practice.

\(^2\)To check your mastery of the rules of heredity, can you write out all mating pairs that occur in this vial and their offspring to verify that the vial will contain the mixture of genotypes we say it does?
2.5.1 Determining the sex and virginity of a fly

We need to identify male and female flies, as well as female virgins (♀, though the virgin identification will be done by your TAs). Figures 5 and 6 show visual cues. Your TAs will have virgin wild type flies ready for you. You need to pick out the males from the mutant stock.

![FlyMove](image)

Figure 5: Male and female flies, from FlyMove. Males are typically smaller than females. The rear abdomen of males is also darker. The best way to distinguish males from females is to look at their genitalia; see Figure 6.

2.5.2 Identifying phenotypes

To pick out which flies are homozygous in the mutant stock, we need to determine which flies have the $Sb^1$ mutation and which do not. We want to pick out the ones that do not. Figure 7 describes how to identify this phenotype.

2.5.3 Handling flies

Flies are kept in plastic vials with porous cotton plugs on them. The bottoms of the vials contain food. If the plug is removed, the flies will fly away. Therefore, they must be anesthetized. We use carbon dioxide for this purpose. To put the flies to sleep, insert a needle connected to a CO$_2$ source into the vial through the cotton plug. The vial should be inverted (cotton plug down) while you do this to ensure that the flies do not get stuck in the food. When the flies stop moving in the vial, remove the plug and let the flies fall onto a flypad. The flypad is connected to a CO$_2$ source, so the flies continue to sleep on the pad. Push the flies around with a paint brush while looking at them using a dissection microscope. You can brush the flies into fresh vials as needed for crosses. Flies you wish to discard go to the “morgue.” For this purpose, we use a beaker of soapy water in an ice bucket.
Figure 6: The underside of male, female, and virgin flies, from FlyMove. The genitalia of males and females is clearly different. Virgin females are often slightly larger and lighter in color. Importantly, the meconium, a dark spot remaining from the fly’s last meal as a larva, is visible.

Figure 7: Images of the thorax of adult fruit flies from FlyBase. Oregon R is wild type. (We will use Canton S as our wild type strain, but it looks exactly like Oregon R.) The $Sb^1$ phenotype features shorter bristles that lack the taper and point of wild type. The easiest way to identify whether a fly is $Sb^1$ or wild type is to look at the back two bristles, which cross each other in wild type.

3 Protocol

The protocol consists of two parts. First, we must perform the cross to get the flies with the genotype we want. We have to wait three weeks and work with the offspring of the cross. Then, we prepare the embryos for imaging. We finally acquire microscope images to locate the cephalic furrow.
3.1 Crosses: Protocol for making the \textit{bcd/+} fly, week 4

Following the fly handling guidelines of section 2.5, do the following:

1. Your TAs will provide you with vials of wild type virgins.
2. Collect males that have wild type bristles from the vial containing the mutant flies.
3. Place two or three of these males in each vial of wild type virgins.
4. After about 8 or 9 days, adult flies from the cross will begin to eclose (emerge after completion of metamorphosis). Collect the flies.\footnote{You can keep the males, but it is the females that are important, since we are studying the effect of \textit{bcd}. \textit{Bcd} is a \textbf{maternal effect gene}, which means that the genotype of the mother alone determines what happens in the development of the offspring. I.e., the genotype of the offspring is unimportant, and therefore so is the genotype of the father. We will cover this in more depth when we look at the effects of \textit{bcd} on development next month.}

3.2 Imaging: Protocol for locating the cephalic furrow in live embryos, week 7

To measure the cephalic furrow, we need to isolate live embryos at the right stage of development, mount, and image them. There are two crucial points here. First, we need to make sure the embryos are at the correct stage of development for formation of the cephalic furrow (at cellularization). Second, we need to remove the \textbf{chorion}, the protective shell of the embryo, before imaging. The chorion is reflective, and will greatly compromise image quality if not removed. Below, we describe the protocol we will use in lab.

While it differs in some ways from our protocol, you may find it useful to watch the video description of acquisition and imaging of eggs found in this JoVE article: Figard, L., Sokac, A. M., \textit{J. Vis. Exp.} \textbf{49}, e2503, doi:10.3791/2503 (2011).

3.2.1 Protocol for embryo collection and dechorionation

1. Two days before lab, combine females of interest (either wild type for \(2 \times \text{bcd}\) dosage or \(\text{bcd}^{-}\); \(\text{bcd}^{+}\) for \(1 \times \text{bcd}\) dosage) with wild type males. The flies should be about two days old. The cross should be prepared in a collection cup with an apple juice plate. (Done by TAs)
2. On the day of lab, replace the collection plate with a temporary collection plate with stripes of yeast paste. (Done by TAs)
3. Let the collection cup/plate sit for 90 minutes. This allows the flies to flush retained eggs. (Done by TAs)
4. Replace the temporary collection plates (which can be disposed of) with the yeasted apple juice plates. Allow the flies to lay eggs in quiet with light for 1.5 to 2 hours. (Done by TAs)
5. Before doing this step, perform steps 1 through 4 in section 3.2.2 to prepare for embryo mounting. Put the collection cup mesh side-down on a fly pad with CO\(_2\) flowing through it. Tap gently until all the flies are asleep.
6. Remove the collection plate from the collection cup and discard the sleeping flies into a morgue.
7. Using a wet paintbrush, transfer the eggs from the collection plate into a cell strainer. The white eggs, depicted in Fig. 8, are about a half millimeter long and have two arms sticking out of them called \textbf{dorsal appendages}. 

\footnote{You can keep the males, but it is the females that are important, since we are studying the effect of \textit{bcd}. \textit{Bcd} is a \textbf{maternal effect gene}, which means that the genotype of the mother alone determines what happens in the development of the offspring. I.e., the genotype of the offspring is unimportant, and therefore so is the genotype of the father. We will cover this in more depth when we look at the effects of \textit{bcd} on development next month.}
8. To dechorionate the eggs, place the cell strainer into the lid of a petri dish partially filled with a 50% bleach solution. Gently swish the cell strainer through the bleach solution. Do this for 30 to 45 seconds (if you wait much longer than 45 seconds, the embryos will die!).

9. Immediately remove the cell strainer and wash the eggs with deionized water for approximately one minute.

Figure 8: A female fruit fly laying eggs. The eggs are approximately half a millimeter in length. Note the two dorsal appendages on each egg. Image credit: E. Fast and H. Frydman.

### 3.2.2 Protocol for embryo mounting and imaging

Perform steps 1 through 4 **before** dechorionation of the embryos.

1. Put a strip of double sided tape on a glass slide, as shown in Fig. 9a.
2. Trim the overhanging tape with a razor blade, as shown in Fig. 9b. **Safety warning:** be very careful with the razor blades!
3. Place strips of double sided tape immediately to the right and left of the center strip. Trim the excess off so that your slide looks like Fig. 9c.
4. Cut a strip of agarose from an apple juice plate and place it on the lid of a petri dish as in Fig. 9d.
5. Use a wet paintbrush to transfer the dechorionated embryos from the cell strainer to the agarose strip.
6. Use forceps (or a paintbrush, if this is easier for you) to line up the embryos along the agarose strip. For consistency, the embryos should be lying on their lateral side. You can tell they are lying on their side if their shape is roughly as depicted in Fig. 2, with one side (dorsal, top) having less curvature than the other (ventral, bottom). The embryos tend to naturally lay this way.
7. Gently press the slide onto the agarose strip. The line of embryos should be in the middle of the center slip of tape. The embryos will come right off the agarose and onto the tape.
8. Place double sided tape on either side of the embryos. This should make a channel about 3 to 5 mm across.
9. Trim the overhanging tape with a razor blade.
10. Add another strip or tape to either side, making three layers of tape total such that the channel is two layers deep.
11. Place one or two drops of halocarbon oil 27 over the embryos. This is optically clear and oxygen permeable, and will keep the embryos from drying out. You do not need much oil (see Fig. 9e), just enough to cover the embryos.
12. Place a cover slip over the embryos. Gently press the sides of the cover slip using the back of the forceps.

13. Mount the embryos on the microscope and image them. Capture images every 30 seconds. If you have an automated stage, you should program it to capture images of all properly-staged viable embryos on your slide. You may want to bin the images so that the saved images are lower resolution to save on storage.

14. From the time lapse movies, you can find the frame where the cephalic furrow first forms and can determine its position. You can use Fig. 1 as a guide.

![Images of mounting preparation techniques. a) Place a strip of double sided tape in the center of the slide. b) Every time you put on a new strip of tape, you should trim it with a razor blade. It is much easier to trim a single layer of tape than multiple layers. Be very careful with the razor blade so you do not cut yourself. c) Before adding embryos to the slide, you should have three strips of double sided tape adjoining each other. d) Use a razor blade to cut a strip of agar out of a plate and place it on the top of a lid. You will line the embryos up on this strip of agar and then put the slide over them to stick them to the tape. e) After sticking the embryos to the tape, cover them with one or two drops of halocarbon oil. In this image, the overhanging tape should have already be cut off, but is left on here to illustrate approximate channel width.](image)

4 Assignment

Problem 0 (Summary).
Write a summary of this experiment, its objectives, and conclusions between one paragraph and one page in length.

Problem 1 (Predictions based on the French flag model).
In this problem and the next, we will generate predictions for the cephalic furrow position based on the French flag model. This is the power of theory and experiment together. A theory can generate a testable prediction. You will do the theory now, and test its prediction in the lab.

Under the French flag model, we assume that the position of the cephalic furrow is the point where the Bcd protein concentration passes a threshold. We define this threshold concentration to be \([Bcd]_\ast\).

Let \([Bcd]_{0,\text{wt}}\) be the anterior Bcd concentration for 2× \text{bed} dosage (wild type). Let \(x_{\text{wt}}\) be the position
of the cephalic furrow when \( [Bcd]_0 = [Bcd]_{0, \text{wt}} \). Finally, let \( x_\alpha \) be the position of the cephalic furrow when \( [Bcd]_0 = \alpha [Bcd]_{0, \text{wt}} \). Here, \( \alpha \) is the gene dosage factor. E.g., if we have 1× dosage, this is half of wild type, so \( \alpha = 1/2 \).

Derive an expression for \( x_\alpha \) as a function of \( \lambda \), \( \alpha \), \( [Bcd]_{0, \text{wt}} \), and \( [Bcd]_* \). Write down an expression for \( x_\alpha - x_{\text{wt}} \), the shift in furrow positioning due to dosage. This should be a function only of \( \lambda \) and \( \alpha \).

**Problem 2** (Measuring the Bcd gradient).
To check the validity of the French flag model, we need to know the value of the Bcd concentration profile decay length \( \lambda \). Driever and Nüsslein-Volhard measured the concentration profile of Bcd and fit an exponential curve, but did not report the value of \( \lambda \). We will calculate \( \lambda \) from their data so we have it in hand going into our furrow measurements.

Use the digitized wild type Bcd immunostain data from Figure 3A of the Driever and Nüsslein-Volhard paper (in the file `wt_bcd_driever_fig3A.csv`) and perform a curve fit to determine \( \lambda \). The value of \( \lambda \) should be in units of fraction of the total embryo length. *Hint:* The Driever and Nüsslein-Volhard data have a background signal. Therefore, the function you should use to fit the data is

\[
[Bcd] = b + [Bcd]_0 e^{-x/\lambda},
\]

where \( b \) is the background signal. Your three fit parameters are then \( b \), \( [Bcd]_0 \), and \( \lambda \). You can modify the tutorial on nonlinear regression to fit this function.

This this parameter \( \lambda \) in hand, and your result from problem 1, you can now predict how the furrow position will shift in your mutant based on the French flag model. We will check this prediction against your measurements.

**Problem 3** (Alternate crossing scheme).
We produced flies with the genotype \( bcd/+ \) on the third chromosome. We did this by mating

\[
\begin{array}{c}
\text{♀} \\
+ \\
\times \\
\text{♂} \\
\text{bcd/bcd}
\end{array}
\]

so all offspring are \( bcd/+ \). Say instead we crossed

\[
\begin{array}{c}
\text{♀} \\
+ \\
\times \\
\text{♂} \\
\text{bcd/TM3, Sb1}
\end{array}
\]

What would the genotypes of the female offspring be? (Think about the contents of the “urns” from the handout we gave you about fly crosses.) How could we pick the ones that are \( bcd/+ \)?

**Problem 4** (Image processing).
Discuss how you process the images to determine the cephalic furrow position. Justify your choice on how the furrow position is defined.

**Problem 5** (Experiment vs. French flag model).
In our experiment, \( \alpha = 1/2 \) for the mutant flies. In problems 1 and 2, you generated a prediction on how the position of the cephalic furrow should change. Do your experimental results agree with this prediction?
Problem 6 (Checking more dosage levels).
In lab, we only looked at two dosage levels of \textit{bcd}. Driever and Nüsslein-Volhard looked at four dosage levels. Plot your predictive expression from problems 1 and 2 for $x_\alpha - x_{wt}$ versus $\alpha$ on the same plot as Driever and Nüsslein-Volhard’s measurements of the furrow position. Spectulate on the causes of any differences between the predicted and measured furrow positions. For convenience, here are the values for the position of the furrow (in units of fractional embryo length) measured in their paper.

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1× dosage</td>
<td>0.27</td>
</tr>
<tr>
<td>2× dosage</td>
<td>0.35</td>
</tr>
<tr>
<td>3× dosage</td>
<td>0.41</td>
</tr>
<tr>
<td>4× dosage</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Problem 7 (Data and code).
Attach all code, data, and sample images not specifically asked for in the other problems.