

Lab 4A. Preparation of *Drosophila* Polytene Chromosome Squashes

A response is required for each item marked: (#___). Your grade for the lab 4 report (lab 4A and 4B combined) will be the fraction of correct responses on a 50 point scale[(# correct/# total) x 50].

In this week's lab, you will prepare squashes of polytene chromosomes from *Drosophila* salivary glands, similar to those used by Thomas Hunt Morgan and students in demonstrating the relationship between chromosomes and the hereditary information. Morgan chose chromosomes from the *Drosophila* larval salivary gland for his studies, because of their unusually large size and clearly visible banding (Compare Figures 1 and 2). The large size of these chromosomes results from an atypical cell cycle in which they undergo ten rounds of DNA replication (S phase) without intervening mitoses. As you recall from Chapter 14 of the text, the typical cell division cycle consists of a single DNA replication cycle (S phase) to generate a pair of sister chromatids, which are then segregated into individual daughter nuclei during mitosis (M phase). The polytene cell cycle of the salivary gland produces chromosomes consisting of 1000 (2^{10}) sister chromatids each. This amplification of the genetic material allows the larvae to produce the large quantities of gene products needed for it to undergo a rapid growth in size as it progresses from the first to third instar stages of development. The genetic experiments of Morgan showed that the *Drosophila* genome is subdivided into four linkage groups, which correspond to the four pairs of chromosome homologues observed in squashes of chromosomes from a tissue that undergoes a typical cell cycle of a single S phase and M phase (Figure 2).

As shown in Figure 1, the four chromosomes of the *Drosophila* genome are held together at their centromeres that do not undergo polytenization

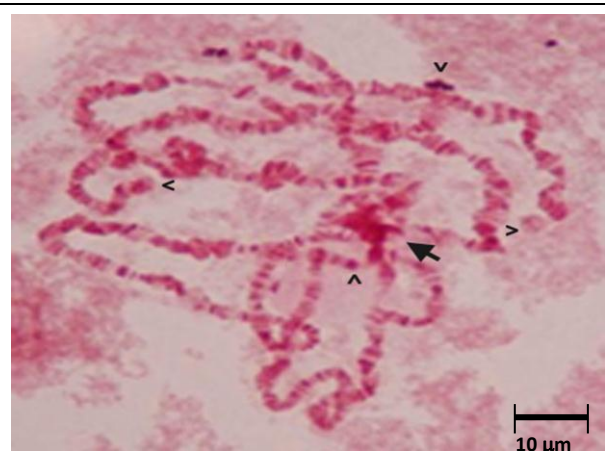


Figure 1. Squash of Polytene Chromosomes from *Drosophila* larval salivary gland. Note the light and dark banding pattern along each chromosome arm, the darkly staining chromocenter containing centromeric heterochromatin in the center of the squash (large arrow), and the telomeres (small arrowheads).

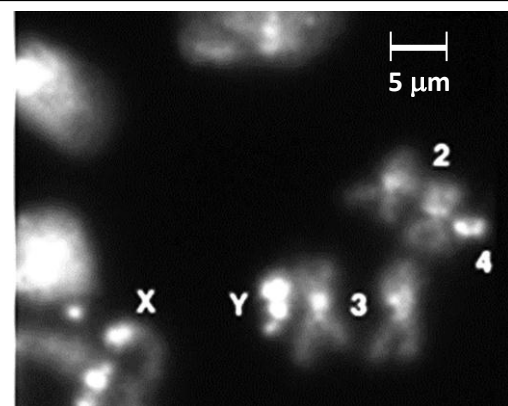


Figure 2. Example of a High Quality Squash of Chromosomes from the Mitotically Active *Drosophila* Larval Brain. The individual chromosomes were identified on the basis of their characteristic shapes and sizes.

and, therefore remain tightly adhered to one another to form what is known as the chromocenter. In a well spread polytene chromosome squash such as the one in Figure 1, it is possible to identify five chromosome arms (the left and right arms of chromosomes 2 and 3 and the single long arm of the X chromosome). The very small 4th chromosome is also sometimes visible. The genes along the sister chromatids of each visible arm are held in register along the arms to form a characteristic and reproducible banding pattern on each arm. Also note that the polytene nucleus of the salivary gland is diploid, and each arm contains a pair of homologous chromosomes aligned in register along their lengths. The precise and reproducible banding pattern for each arm provided a mechanism for Morgan and his students to correlate changes in the physical properties of the chromosomes to changes in a heritable trait. A comparison of the banding pattern of one homologue to that of its partner allowed these changes to be recognized. Morgan and students treated animals to high energy radiation (X-rays and γ -rays) and other mutagenic agents to generate alterations in the characteristic banding patterns. Cytological evidence of these chromosome rearrangements provided evidence that chromosomes are the physical entities responsible for the laws of Mendelian genetics. In this week's lab, we will prepare polytene chromosome squashes, similar to those of Morgan and students.

Lab Exercise

The following reagents and supplies will be sitting on your bench:

- 1) Dissecting scope (one per Lab Group)
- 2) Bottle of wild type *Drosophila* stock
- 3) 10 ml Saline (0.7% NaCl)
- 4) 1 ml 45% Acetic Acid
- 5) 0.5 ml Orcein stain
- 6) Kimwipes
- 7) H₂O wash bottle
- 8) In Lab Bench Box:
 - Microscope slides
 - Coverslips
 - 2 pairs of Forceps

Before you begin your squash preparation, watch the video demonstration and/or live demonstration of *Drosophila* larval dissection and squash preparation by your instructor. Then use the following procedure to prepare your own squashes. The dissections and manual manipulations of salivary glands require some technical proficiency that is possible to acquire with some practice. Be patient and just do your best.

Salivary Gland Dissections

1. Use your forceps to remove 8-10 actively crawling larvae from the sides of the cultivation bottle. Place them on a dry microscope slide (slightly to one side).
2. Use a Pasteur pipette to place a drop of saline solution in the middle of the same microscope slide. Use your forceps to transfer one of the larvae to the saline drop, with its tracheal tubes facing up (See Figure 3).
3. Use one pair of forceps to hold the anterior end of the larva in place at the spot just above the neural ganglion in Figures 3 and 4, while using a second pair of forceps to grab the top layer of cuticle from a position on the larva at about one third body length from the anterior end (Figure 4).
4. The salivary glands and ventral ganglion (brain) will usually remain attached to the head region, separate from the rest of the body, after a clean dissection. A diagram of this is shown in Figure 5, and a photograph is shown in Figure 6. Figure 7 shows a photograph of salivary glands after clean dissection from the larval body and head regions. You may have to use your forceps to search for the salivary glands among the dissected material. If this fails, simply start over with a new larva. These dissections are difficult to master, but reasonable results can be obtained with a little practice.
5. As you dissect the salivary glands, you can either keep them set aside in a separate saline drop on the slide used for your dissections or transfer them to a separate saline drop on a different slide. In handling the salivary glands, either grab them from their less fragile smallest diameter base (Figure 7, arrow) or use your forceps to scoop

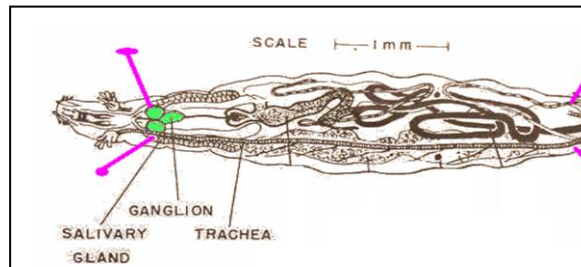


Figure 3. Diagram of *Drosophila* larva, showing the positions of the tracheal tubes, salivary glands ventral ganglion (brain).

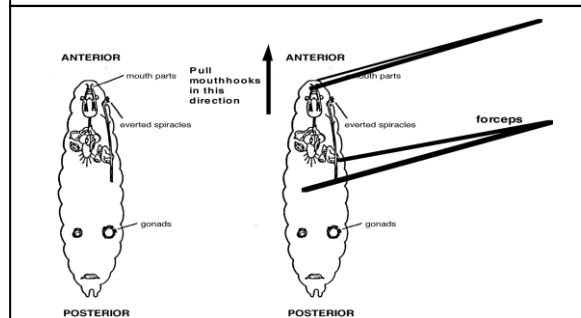


Figure 4. *Drosophila* larval dissections

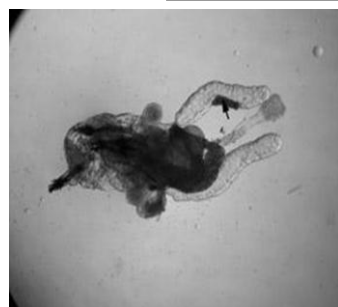
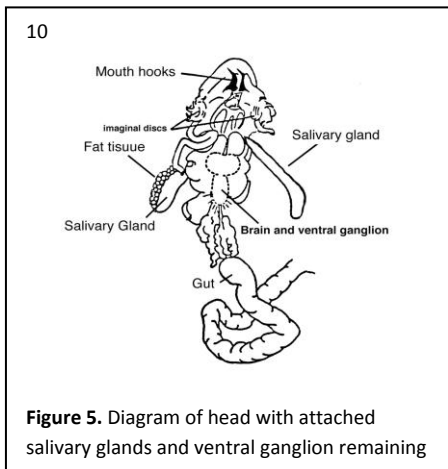


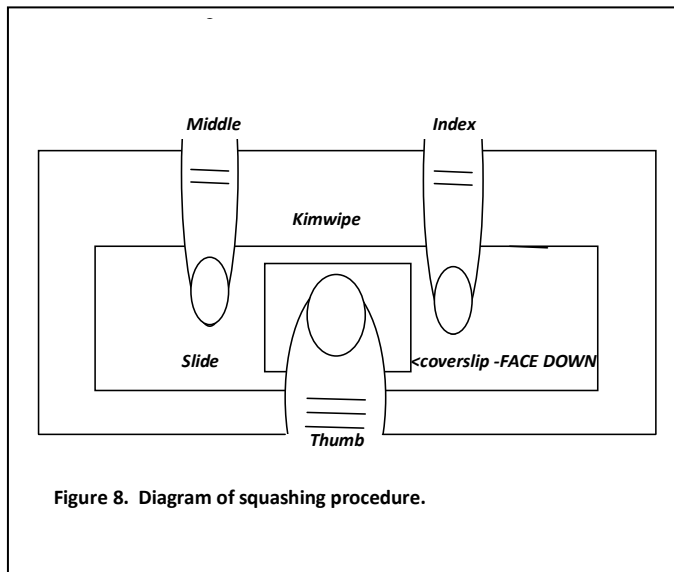
Figure 6. Photograph of head with attached salivary glands and ventral ganglion remaining after clean dissection.



Figure 7. Photograph of salivary glands after clean dissection from the larval body and head regions.

them from underneath.

6. After you have accumulated 5-6 salivary glands, prepare a slide for a squash of 1-2 glands. Use a Kimwipe to wipe its surface clean, then blow any dust from its surface before placing a 10 μ l drop of **Lacto/Aceto Orcein stain** on the slide. One partner of the lab group can handle this task while the other (probably the partner who did the dissection) handles step # 7 below.
7. Then place a drop of **45% Acetic Acid (HOAc)** fixative on the slide next to the drop of saline holding your salivary glands and use your forceps to transfer the salivary glands from the saline to it and **hold in place for ~30 seconds**. Then immediately transfer the glands to the drop of **Lacto/Aceto Orcein stain** on the slide from step # 6.
8. Place a clean coverslip (dust blown from its surface) onto the surface of the glands and use your forceps to gently tap the surface of the coverslip in a circular pattern. **The coverslip should rotate somewhat freely on the surface of the microscope slide; this is needed to burst the membranes of the polytene nuclei and allow the chromosome arms to spread.**
9. Place the slide on your microscope. Using the lowest power objective and standard light source, scan around the slide until you find the squashed material. It is often useful to first locate the squashed material on the slide by eye. Then place the slide on the microscope stage with this material centered in the light beam. This will make it easier to find your chromosomes under the microscope. First locate the focal plane of your chromosomes by adjusting the focus knob until the fluid background material becomes visible. You can then begin to scan around the slide to find the chromosomes.
11. If the chromosomes look sufficiently well spread (See Example in Figure 1), you can now invert the slide and coverslip onto a sheet of Kimwipe. Then use your thumb to apply pressure to the coverslip while using the middle and index fingers of your other hand to secure the position of the slide and coverslip (Diagram in Figure 8). This squashing action will bring about further spreading of the chromosomes and remove excess liquid between the slide and coverslip to allow maximal attachment of the chromosomes to the slide.



12. If the nuclei were found to be still intact, you can attempt to rupture them if there is evidence of liquid between the slide and coverslip as evidenced by movement of background material. To rupture the nuclei, use your forceps to tap the surface of the coverslip as described in Steps 8-10. It may be necessary to start over with some newly dissected salivary glands. Preparing polytene chromosome squashes is part science/part art. It takes a lot of practice and a little luck to get the quality of squash shown in Figure 1.

Questions

- 1. Draw a sketch of your best polytene chromosome spread. Are you able to see all five major chromosome arms (X, 2L, 2R, 3L, 3R)? Are you able to see the small 4th chromosome? Can you locate the chromocenter in your spread? Are you able track along a single chromosome arm from its centromere to telomere? (#1)*
- 2. Compare the scale bars for the photomicrographs of the polytene chromosomes in Figure 1 and the metaphase chromosomes from a mitotically active cell in Figure 2. What can you conclude about their relative sizes from this? (#2)*
- 3. How would you describe the differences in banding patterns for the two types of chromosomes? Is it easier to see the bands in one type than in the other? (#3)*
- 4. The polytene chromosomes are both longer and wider than the chromosomes in the larval brain squash. The atypical cell cycle of the salivary gland cell can account for them being wider than a metaphase chromosome? What might account for them also being longer? [Consider the effect of cell cycle on chromosome organization. (Figure 12.12)] (#4)*

5. Figure 8 shows an example of how polytene chromosomes were used to provide cytological evidence of chromosome rearrangements. Observation of such physical defects in chromosomes from mutant animals provided solid evidence for chromosomes as the carriers of the hereditary information to Morgan and his students. The photomicrograph shows a section of an aligned pair of chromosome homologues in an individual that was heterozygous for a chromosomal aberration. Compare the banding patterns of the paired chromosome homologues in the region indicated by the arrows.

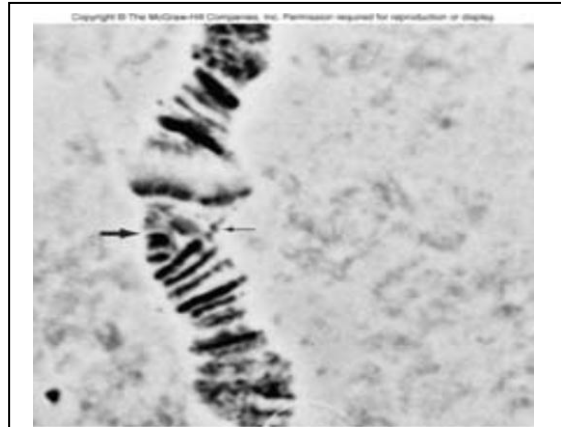


Figure 8. Photomicrograph of a chromosomal aberration in an individual that is heterozygous for a mutant chromosome. Large and small arrows indicate the wild type and mutant chromosome homologues, respectively.

What type of chromosomal aberration seems to have occurred (deletion, insertion, inversion)? (#5)

6. Figure 9 shows polytene chromosomes from larvae that have been subjected to high temperature to induce transcription of heat shock genes located at bands designated as 87A, 87C, and 93D. *How would you compare the banding patterns of the chromosomes from larvae that were not subjected to heat shock (panel A) to those from larvae that were subjected to heat shock (panel B)? (#6)*

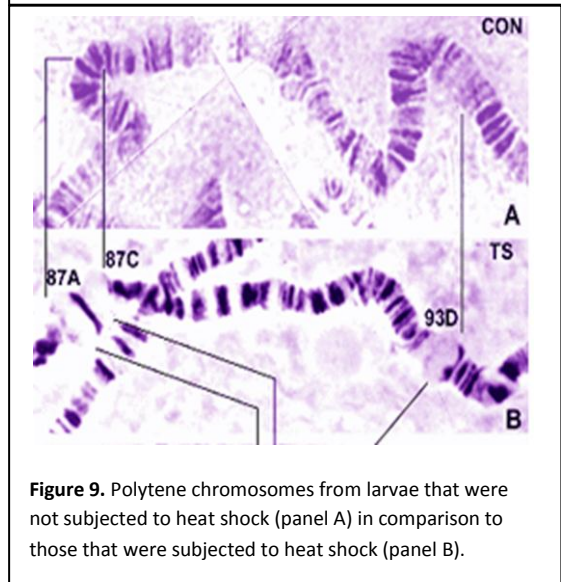


Figure 9. Polytene chromosomes from larvae that were not subjected to heat shock (panel A) in comparison to those that were subjected to heat shock (panel B).

7. *How do you think the chromatin structure at these bands differs in to the samples? Give your answer in terms of the levels of chromosome organization depicted in Figure 12.12 of your text. (#7)*

8. Figure 10 shows immunostaining of a polytene chromosome spread with an antibody against a chromosomal protein. A color version of this photomicrograph will be projected on the screen during class. *How would you describe the immunolocalization pattern of this protein? Which region of the chromosome is most highly stained with the Texas Red-labeled antibody? Hint: See the figure legend for Figure 1 above. (#8)*



Figure 10. Immunostaining on a Polytene Chromosome Spread

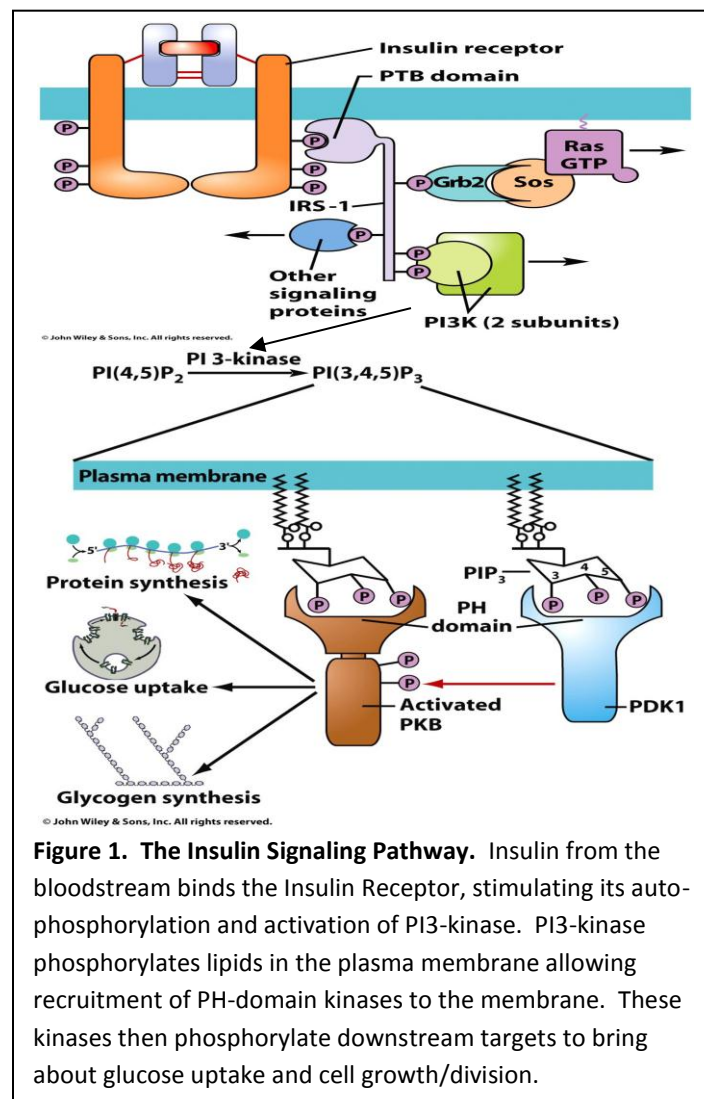
9. *Based on this localization pattern and what you learned in Chapter 12 about constitutive heterochromatin surrounding centromeres, what protein do you think is recognized by the antibody? (#9)*
10. *Are there any other proteins (or covalently modified form of histone) you would expect to have a similar staining pattern? (#10)*
11. *Careful examination of the immunostaining pattern reveals a few minor sites of staining on the chromosome arms. Speculate what the constitutive heterochromatin protein is doing at these sites. Don't be afraid to speculate. Your hypothesis is as good as anyone else's. (#11)*
12. *Briefly describe one experiment you could use to test your hypothesis. (#12)*

Lab Exercise 4B. Live Visualization of Insulin Signaling through GFP-Tagging

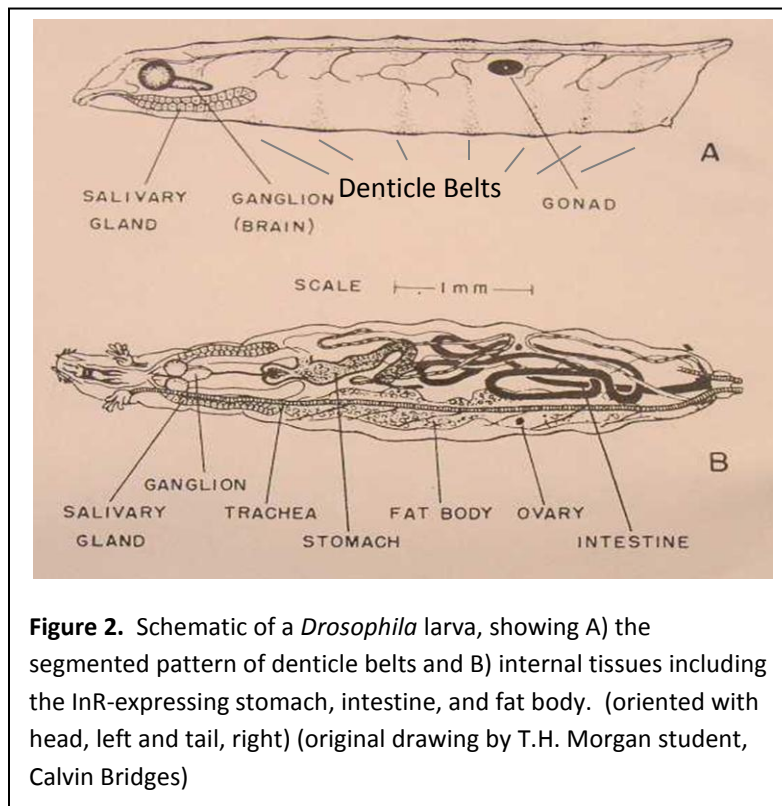
A response is required for each item marked: (#__). Your grade for the 1A/1B lab report will be the fraction of correct responses on a 50 point scale[(# correct/# total) x 50].

In this lab we will use GFP-tagging as a means to monitor activity of the insulin signaling pathway in *Drosophila* larvae. The insulin signaling pathway is a highly conserved pathway for sensing and responding to glucose levels in the blood stream or hemolymph of multi-cellular animals. It is essential for cells to maintain constant glucose levels, through organs that sense their levels in the bloodstream. In vertebrates, the pancreas is responsible for sensing these levels. Pancreatic alpha cells produce glucagon in response to low glucose levels in the bloodstream; beta cells produce insulin in response to high levels. Insulin is a small peptide that binds to a receptor on the surface of cells to trigger uptake of glucose from the

bloodstream, as well as storage of excess glucose in the form of glycogen and triglycerides and decreased gluconeogenesis (synthesis of glucose from complex macromolecules). A diagram of the pathway from your text (Figure 15.23) is shown in **Figure 1**. Binding of **insulin** (rectangle) to the **insulin receptor** (L-shaped molecule) stimulates it to phosphorylate itself, thus activating the pathway. The phosphorylated receptor allows insulin receptor substrates (purple) to bind, which in turn activate a series of downstream kinases to bring about the cellular response. One of these kinases (**PI3K**) phosphorylates a lipid in the plasma membrane, allowing recruitment of plekstrin homology domain (**PH-domain**) containing kinases to the plasma membrane for activation. The final PH-domain kinase activated in the pathway PKB brings about the cellular response. PKB stimulates fusion of membrane



vesicles for the glucose transporter with the plasma membrane to allow glucose uptake from the bloodstream. PI3K also receives signals from another pathway (mTOR) that monitors availability of amino acids and other nutrients and the general energy status of the cell (ATP/AMP ratio; NAD⁺/NADH ratio). When nutrient levels are high, the cell is stimulated to undergo anabolic pathways to grow through PKB activity. When nutrient levels are low, the cell is stimulated to stop growth and catabolize cellular macromolecules, such as glycogen, fats, and proteins, and to utilize glucose as efficiently as possible. This starvation response is an ancient mechanism used by cells to respond to any stressful condition and involves the induction of genes needed to remove damaging agents (such as superoxide free radicals) from the cell. A moderate chronic starvation response is probably responsible for the effects of caloric restriction on life span extension. Indeed, genetic studies in *C. elegans* and *Drosophila* have shown that mutations in PI3-Kinase and other components of the insulin signaling pathway also extend life span.

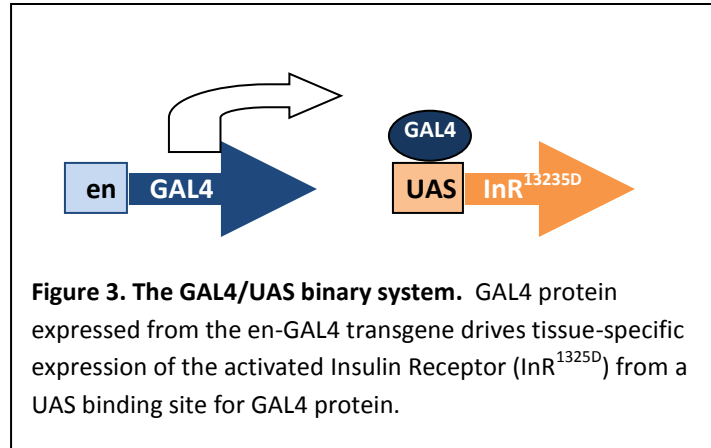


In today's lab, we will monitor the activity of the insulin signaling pathway in *Drosophila* through a **GFP-tagged version of the PH domain** the kinases in **Figure 1** that allows them to become membrane localized upon pathway activation. **The GFP-tagged PH domain indicator protein is called tGPH.** We will monitor membrane localization of the tGPH reporter in larval tissues that normally express the Insulin Receptor (InR) and respond to insulin signaling (stomach, intestines, and fat bodies)(**Figure 2**). We will also compare the membrane localization of the reporter in

cells of the cuticle epidermis expressing a constitutively (constantly) active form of the receptor (InR^{1325D}) to those expressing the normally regulated InR.

The GAL4/UAS binary system (**Figure 3**) will be used to express InR^{1325D} in the posterior (back) half of each body segment of the larval cuticle. GAL4 is a yeast transcription factor that activates transcription from its DNA binding sequence called the Upstream Activating Sequence

(UAS). When a tissue-specific promoter is used to express GAL4 protein in an animal, this will dictate which tissue will express a second gene of interest from a UAS promoter sequence (UAS-InR^{1325D} in our experiments). We will use two different tissue-specific promoters to drive GAL4 expression (and, thus, UAS-InR^{1325D}) in today's exercise. One will drive expression in the eye (GMR-GAL4); the other (en-GAL4) will drive expression in the back (posterior) half of each segment of the larval cuticle. In the larval cuticle, the cells in the neighboring anterior half of each segment will provide a negative control to those in the posterior half expressing UAS-InR^{1325D}.



Lab Exercise

You will use *Drosophila* stocks carrying foreign genes (transgenes) for the GFP-tagged PH domain protein (tGPH) and the activated Insulin Receptor (InR^{1325D}) under the control of either the eye-specific or posterior segment-specific GAL4 protein. Membrane localization of tGPH will provide one means for monitoring activation of the insulin signaling pathway in cells expressing the activated Insulin Receptor. You will also be able to monitor the effect of the activated Insulin Receptor on cell growth and division in the animals.

Reagents and Supplies

1. Eppendorf tubes of 1) wt and 2) GMR-InR^{Y1325D} flies
2. Bottle of en-GAL4, tGPH/Tb x UAS-InR^{Y1325D}
3. Dissecting microscope
4. Forceps
5. Saline
6. Mounting Medium
7. Microscope slides
8. Coverslips

Expression of activated InR through eye-specific GAL4 driver (GMR-GAL4)

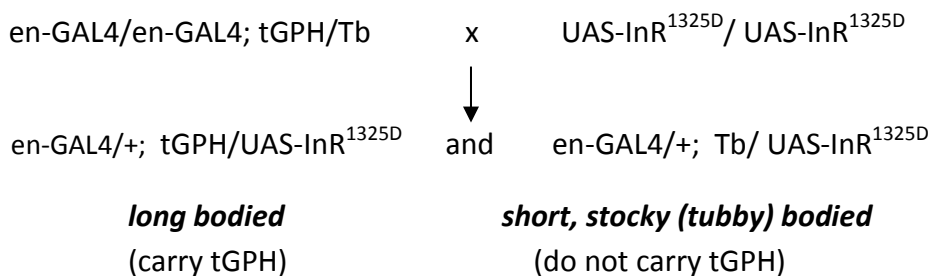
1. Empty the GMR-GAL4, UAS-InR^{Y1325D} flies onto the stage of your dissecting microscope. Empty the wt flies onto the stage next to them. Compare the size, color, shape, and number

of cells on the surface of the eyes in the two populations. *Describe (or sketch) any differences you see in the space below (on next page). (#13)*

Expression of activated InR through posterior-specific en-GAL4 driver

As described above, the en-promoter used in today's experiment to express GAL4 protein, and, thus, the activated InR (InR^{1325D}), is only active in the posterior half of each tissue of the *Drosophila* larva and in the posterior half of the larval abdomen. You will monitor the effects of expressing the activated InR in larvae through membrane localization of the tGPH reporter for insulin signaling.

1. The *Drosophila* culture bottle on your bench contains females of the genotype (en-GAL4/en-GAL4; tGPH/Tb) crossed to males of the genotype (UAS-InR^{1325D}). (en-GAL4 is located on the second chromosome; tGPH and UAS-InR^{1325D} are each located on the third chromosome.) The cross will yield two classes of larval progeny:



2. Use your forceps to remove several **long bodied** larvae (carrying tGPH) from the side of the bottle and place them in a small pile on a microscope slide. Put a drop of mounting medium on the slide, next to the pile of larvae. You will place one larva at a time in the drop of mounting medium for the dissections.

3. Use the tips of your forceps to pinch off the anterior (head) and posterior (tail) tips of the larva (**Figure 4**). Then use the long edge of one pair of forceps to gently press along the length of the larva (starting from the anterior end) and push the internal organs (through the opening at the posterior end). Use the schematic in **Figure 2** to identify the stomach, intestines and fat body tissues. Use your forceps to position these tissues away from where you are working. You will mount these next to a filet of the larval cuticle case (Figure 5).

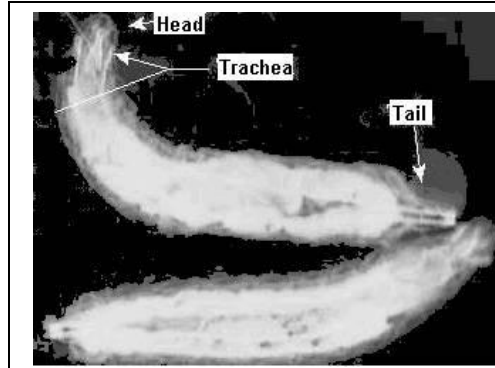


Figure 4. Photograph of *Drosophila* larvae. The head at the anterior end and tail at the posterior end and a pair of tracheal tubes running the full length of the body are indicated. The trachea are more widely spaced at the anterior end.

4. Starting with the cuticle case from above (**Step 1**), make a slit along one edge of the empty cuticle case by positioning the sharp edge of razor blade along the length of one edge and pressing down firmly (**Step 2**). Then use your forceps to open the cuticle case into a single layer (**Step 3**). **This is analogous to unzipping a sleeping bag and then flattening it out into a single layer.** Because only the cells lining the inside of the cuticle case express the en-GAL4 transgene needed to drive InR^{1325D} expression, it is important that the inside of the cuticle case be facing up on the slide when mounted. Position the larval cuticle filet with inside cells facing up next to the tissues dissected from it on the slide and place a coverslip over them. Remove any air bubbles from the mounting medium before mounting.

8. Now observe the specimen by fluorescence microscopy. Turn on the Mercury (Hg) lamp box sitting next to your microscope. **REMEMBER NOT TO TURN OFF UNTIL FINISHED.** First use standard light to bring the specimen into focus. All shutters between the Hg lamp and the specimen must be closed when using the standard light source (both levers in, dial turned towards C). As always, begin with the lowest resolution objective and work your way up to 10x. After you have brought the specimen into focus with standard light (*everything will appear uniformly non-fluorescent green, because the fluorescent filter cube is in place*), open all Hg lamp shutters. If the filter cube is not already in the green position (G), slide the lever out to bring it

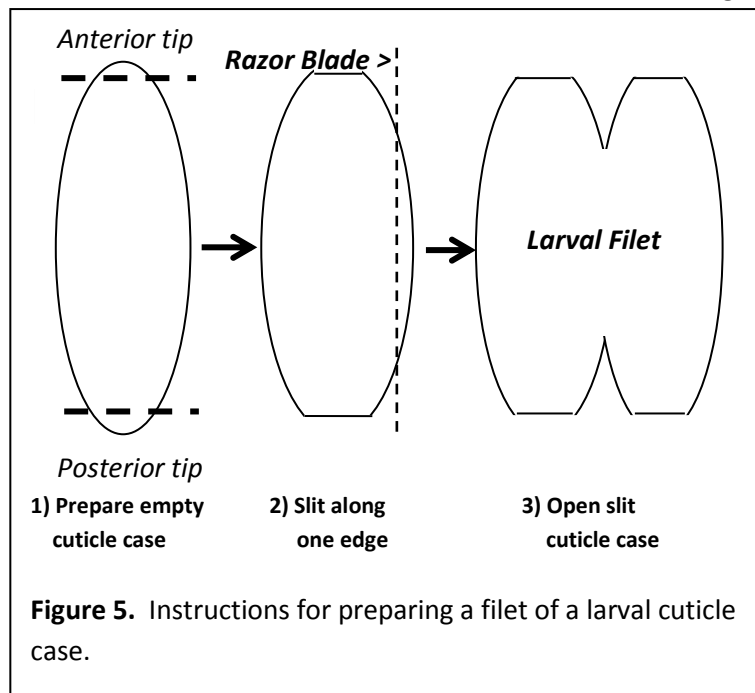


Figure 5. Instructions for preparing a filet of a larval cuticle case.

there.

9. Describe the tGPH localization pattern in the empty larval cuticle case and in the dissected gut tissue. In the cuticle case, you will observe rows of denticle cells expressing high levels of tGPH. These rows mark the segments in the cuticle case. The *en-GAL4* transgene will drive expression of the *InR^{1325D}* in the cells in the back half of each segment. Are you able to detect a striped pattern of cells with strong membrane localization of the tGPH? Do you observe any difference in the size of cells in the front and back half of each segment? In the space below, describe what you observe, commenting on these two points. Drawings may be helpful. **(#14) Report what YOU observe.**

Now describe the pattern of tGPH localization in the stomach, intestine, and fat body tissues. Is the tGPH strictly localized on the plasma membrane? Do you observe it in any other cellular compartments (cytosol, nucleus)? Diagrams may be helpful. **(#15) Report what YOU observe.**

Questions

1. *Why are the eyes of the animals expressing the activated InR in the eye larger than those of animals not expressing it? Examine the surface of the eyes in the two animals at high magnification. Does the increased eye size appear to be the result of an increase in the size of individual cells (cell growth) or an increase in the number of cells (cell division)? (#16)*

2. *Is the subcellular distribution of the tGPH different in the cells of the stomach, intestine, or fat body tissue dissected from the long-bodied (expressing activated InR) vs. the short bodied larvae (not expressing activated InR)? Give an explanation for any difference you observe. (#17)*

3. *Is the subcellular distribution of the tGPH uniform throughout the empty larval cuticle case, or are you able to discern a pattern of GFP expression in the cuticle case? Give an explanation for any pattern you observe. (#18)*

4. *Were the cells larger or smaller in the back half of each segment, where activated InR was expressed? How do you explain this? (It may be easier to see this in a filet of the larval cuticle case. (#19)*

5. *How do you explain the effect of the InR^{1325D} mutation on the activity of the Insulin Receptor? Assuming that Tyrosine is present at amino acid 1325 in the wild type InR, why does this mutation cause the receptor to be constantly (constitutively) active? (#20)*

6. *Mutation of another amino acid in the transmembrane domain of the InR to Aspartic Acid also results in a constitutively activated insulin signaling. How might you explain this effect? (#21)*

7. *How might the cell cycle regulation of eye cells expressing the activated InR differ from those not expressing it to cause them to continue to divide? Apply what you have learned in lecture about cell cycle regulatory proteins. What is needed to stimulate cells to enter S phase? Recall that mutations in one cell cycle regulatory protein results in giant animals with giant organs due to excessive cell divisions. (See pg 46 Exam III Material Slides pdf.) (#22)*

8. *How might the cell cycle regulation of the epidermal cells expressing the activated InR differ from those not expressing it to stimulate them to grow larger? Again apply what you have learned from lecture about cell cycle regulatory proteins. (#23)*