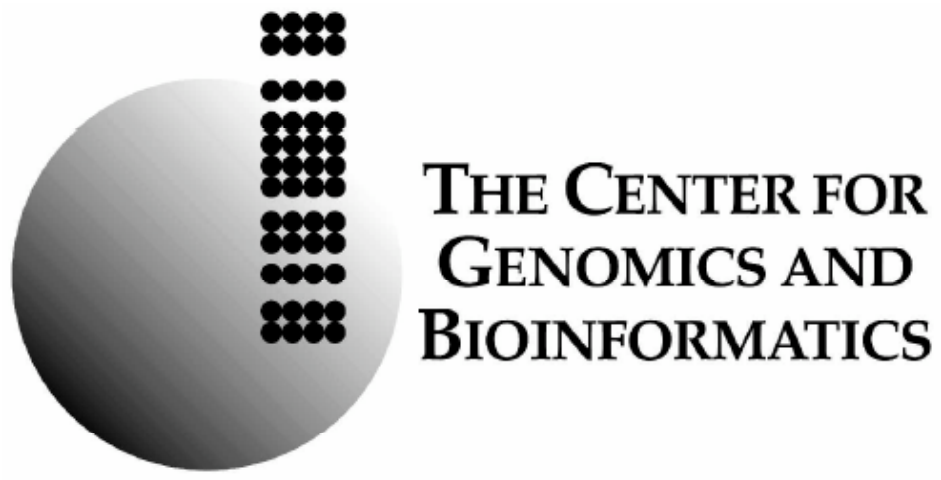

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Standard Protocol for Establishing Cell Lines from *Drosophila* Embryos

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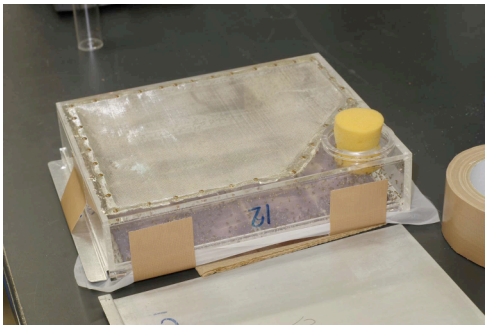
This protocol was prepared in conjunction with Dr. Ueda's visit to Indiana University, October, 2007. It is based on the procedures developed in the laboratory of Tadashi Miyake in the 1980s, and uses some of the original equipment from that laboratory.

I. Egg Collection plates

The following recipe, modified from Elgin and Miller (1978) is suitable for about 6-7 plates, using custom-made rectangular plates designed to fit the population cage. In the photo below, the plate occupies the bottom surface of the cage.



Materials for grape juice-agar plates:

Agar powder	22 g
Glucose	87 g
Dry yeast	18 g
Welch Grape Juice	455 ml
Water	543 ml

1.25N NaOH: 2.5 g NaOH dissolved in 50 ml H_2O

Acid Mix: 21 ml propionic acid + 2 ml 85% phosphoric acid + 27 ml H_2O

Procedure to prepare egg-lay plates:

Mix the agar, glucose, yeast, grape juice, and water. Add 22ml of 1.25 N NaOH, and heat (on a stirring hot-plate) to dissolve agar.

Cool down to about 60º C, then add 11ml of Acid Mix.

Pour into the egg collection plates, with a plastic wash bottle (ca 150ml/plate). An ordinary wash bottle will work if the dispensing tip is cut to permit dispensing of the viscous agar mix. The plates are custom-made rectangular plates designed to fit the cage (see photo above).

Store the plates in a Tupperware (or equivalent) container at 4º C.

II. Egg collection

Flies:

To populate the cage, you will need 10-15 bottles, each with around 200 flies 2-4 days past eclosion. In order to get good yields of eggs, the flies should be fat and healthy, i.e. they should come from rapidly passaged, uncrowded stocks. The genotype of the flies affects their ability to generate cell lines. Anecdotally, Canton S flies are poor for making cell lines, Oregon R flies are better. A $y^1\ v^1\ f^1\ mal^{F1}$ stock has been used extensively by the Miyake lab to make cell lines.

Yeast Paste:

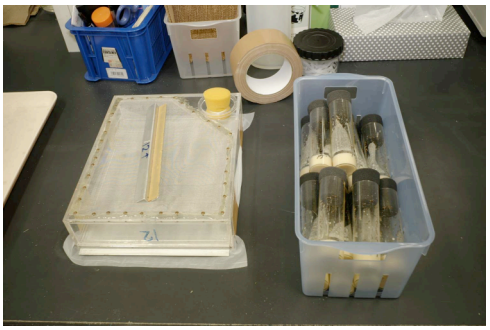
Ideally, use brewers yeast which has been passed through a 200-mesh screen. Coarser yeast may be used, but it is not effectively removed from the eggs by washing on a Nytex filter (see culture protocol) and is therefore more of a contamination hazard. Live yeast may be used, but it produces a lumpier paste, and the paste should be autoclaved before use to decrease the risk of contamination.

Mix dry yeast with sugar (approximately 2:1), add water and stir to make a smooth paste of a consistency between those of mayonnaise and creamy peanut butter. Autoclave if using live yeast. Store at 4º C.

Procedure:

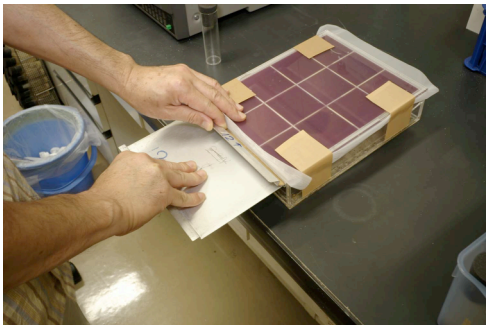
1. Bring an egg-lay plate and the yeast paste to room temperature.

2. Populate the fly cage. We use a custom-made cage in which the entire bottom surface of the cage can be converted into an egg-laying surface. Late in the afternoon, the day before you plan to prepare the cultures, collect about 20-30 ml of loosely packed flies, enough to produce a layer approximately 15-20 mm thick when tapped down in an otherwise empty standard fly bottle. Transfer the flies into the cage, using the stopped hole shown in the photo in the upper right corner of the cage. At this stage the bottom of the cage is covered by a sliding aluminum sheet.



3. Prepare the egg-lay plate. Lay a paper towel on top of the agar to remove excess moisture; it should be wet, but there should not be visible drops of liquid on the surface. Remove the paper towel, and replace it with a sheet of nylon mesh (200 gauge), and ease out excess bubbles. Using a spatula, apply a thin, smooth layer of yeast paste to the central portion of the plate (around two-thirds of the total surface area).

4. Insert the egg-lay plate into the cage. Invert the fly cage, so that the aluminum plate is at the top.



Carefully invert the plate with its mesh cover over the aluminum plate. It should be supported by plastic spacers on all sides so that it does not actually touch the metal. Tape the egg-lay plate to the cage so that it is securely fastened. Insert a short aluminum spacer between the metal plate and the egg-lay plate (held in the left hand in the photo). Tap the cage on a cushioned bench surface, and then quickly withdraw the aluminum plate, leaving the spacer behind. Add a strip of tape to hold the aluminum spacer in place, and any additional tape that may be necessary to keep the structure secure. Then invert the cage so that the egg-lay plate is at the bottom. Place the aluminum plate loosely on top, to decrease evaporation. Store the cage overnight undisturbed in the dark (an empty drawer works well).

5. Start the timed egg collection. In the morning, prepare a fresh egg-lay plate (see #3 above). Remove the cage from its dark location. Invert the cage, so that the egg-lay plate is at the top. Tap the cage a few times on the bench to knock as many flies as possible to the bottom of the cage (away from the egg-lay surface). Then quickly reinsert the aluminum plate, reversing the procedure in #4 above, trying to trap as few flies as possible between the aluminum plate and the egg-lay plate. Now loosen the tape, and remove the egg-lay plate. There should be a large number of white eggs visible on the yeasted portion and possibly on the rest of the grape-agar area, but these eggs will not be used for preparing cultures. Insert the fresh egg-lay plate, exactly as described in #4 above. Once the cage is set up, put it in a Tupperware box in a 25° incubator. Do not disturb (i.e. do not open the incubator) during the egg-lay period, typically 4 hr. The mesh from the overnight egg-lay should be washed for later re-use; the grape-agar can be discarded, and the plate washed for later re-use.

6. Stop the timed egg collection and age the embryos. A typical collection consists of a 4 hr egg-lay

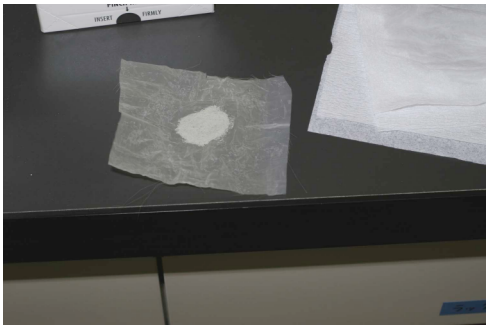
period, followed by a 2 or 4 hr aging period, giving embryos 4 or 6 hr ± 2 hr post-laying. Probably 4 hr egg-laying followed by 4 hr aging works best. At the end of the egg-lay period, recover the cage from the incubator, and remove the egg-lay plate, replacing it with the aluminum plate, as described in #5 above. Carefully remove the nylon mesh from the egg-lay plate, fold it (with the yeast paste and eggs on the inside), and place it on a moist paper towel in a Tupperware box. Close the box, and put it into the 25º incubator for the desired aging period.

7. (Optional) Prepare cage for egg collection the following day. If you wish to make more cultures from the same fly stock the next day, repeat step 4, re-using the 4 hr egg-lay plate with a fresh mesh smeared with yeast paste.

8. At the end of the aging period, proceed to the protocol for culturing embryonic cells.

III. Preparation of primary cultures

1. Recover embryos from the yeasted nylon mesh. Attach a rubber or plastic hose to a tap-water faucet, so that a strong stream of water can be generated by squeezing the hose. Recover the nylon mesh containing embryos of the appropriate age (egg collection protocol, step 6). Prop the mesh vertically against the wall of a 4 l Nalgene beaker, and wet it slightly so that it sticks to the wall of the beaker. Now wash the eggs (and accompanying yeast) off the mesh by directing a strong stream of cold tap water against the mesh (we use a stream of water flowing at about 4 l/min, focused by using a finger to partially block the tube); first wash the eggs off the top half of the mesh, and then raise the mesh a few inches and wash the bottom half. Remove the mesh (which can be washed for later re-use). Collect the eggs by pouring the liquid in the beaker through a two-tier funnel, shown in the photo to the right. The top part of this apparatus is a cut-off plastic bottle, with a coarse mesh inserted into a hole in its cap. The mesh is sufficient to trap flies and large fly parts. The bottom portion is a second cut-off plastic bottle, with a 200-gauge nylon mesh inserted into a hole in its cap; this mesh is sufficient to trap the eggs, but should allow the yeast paste to pass through. Pour the contents of the beaker through this apparatus, and briefly rinse any remaining eggs from the sides of the beaker into the funnel, followed by a few seconds of running water. At this point, the top (coarse) filter can be removed. The eggs should be easily visible on the bottom (nylon mesh) filter. They should be further rinsed, and the stream of water from the hose can be used to gather the eggs into the center of the nylon filter. The filter can then be removed from the filtration apparatus and placed on a dry paper towel to drain off excess water.



2. Surface-sterilize the embryos. Prepare a 50 ml disposable centrifuge tube containing 40 ml of 10% (v/v) saponated cresol. With a straight-ended spatula, scoop up the eggs from the nylon mesh and gently tap them into the cresol solution. Immediately swirl the tube, and bring it into a sterile laminar-flow hood. Pipet up and down with a serological pipet to dissociate any clumps of eggs, and continue to pipet or swirl to keep the eggs from settling to the bottom. 10 minutes from the time that the eggs were added to the cresol solution, remove them by passing the suspension through a nylon mesh filter, which consists of a cut-off top of a 50 ml disposable polypropylene centrifuge tube, in which 200-gauge nylon mesh has been placed in a hole in the cap. Wash with a large quantity (around 250 ml) of sterile water.

Note: Saponated cresol is a very old-fashioned disinfectant that is difficult to find in the U.S. It is, however, very effective for this purpose. The Miyake lab found it to be the best reagent for embryo sterilization, of the many disinfectants that they tested. It is likely that other reagents now exist that are more easily available and equally effective, but we do not know what they might be.

3. Dechorionate the embryos. Place the filter containing the sterilized embryos onto the bottom of a 100 mm Petri dish. Gently pour through the filter 40 ml of diluted bleach (final concentration 2.5% NaOCl). Gently pipet the bleach solution from the plate back over the eggs. It is important to monitor the dechorionation of the eggs by their change in color or by their appearance in a dissecting microscope. As the eggs lose their chorion, they lose their bright white color. Dechorionation should take approximately 4 minutes. At this point, wash the dechorionated embryos by pouring

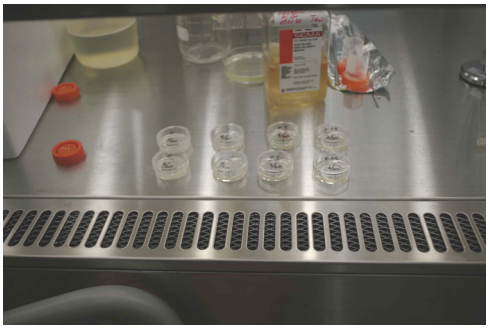
approximately 250 ml of sterile water through the filter, trying as much as possible to collect the embryos in the center of the mesh.

Note: Once the bottle has been opened, bleach slowly loses its effectiveness. It is therefore important to monitor the dechorionation by the change in appearance of the eggs. The dilution of the bleach may be adjusted to compensate for degradation in an old bottle.

4. Homogenize the embryos.

Using a sterile blunt-tipped spatula, gently collect the embryos and tap them into a homogenizer (see note below). Bear in mind that the dechorionated embryos are quite fragile. Add 5 ml of tissue culture medium (e.g. M3 + 10% FCS), making sure to wash all the embryos down from the side of

the homogenizer. Homogenize gently, using several full-length up-and-down strokes. Pour the homogenate through a sterile nylon mesh filter (exactly like the one used in step 3 above) into a sterile 50 ml centrifuge tube. This filtration is intended to remove fragments of vitelline membrane, any large clumps of cells, and unbroken embryos. Spin slowly (we use 500 rpm for 5 minutes) to pellet the cells, leaving most of the yolk granules in the supernatant. Handling the tube gently (the pellet is fragile), pour off the supernatant. Gently resuspend the pellet in an appropriate volume of tissue culture medium (probably between 2 ml and 8 ml), and plate in 35 mm Petri plates, 2 ml of culture per plate. It is advisable to do a series of dilutions, to make sure that some of the cultures



are at an appropriate concentration. Handle the cultures very gently; it is important to disturb the cells as little as possible during the first few weeks of culture. We find it useful to put up to about 6 35 mm plates containing primary cultures into a 150 ml Petri plate, so that they can be handled with less danger of jostling and contamination. The nested Petri plates are then placed into a sealed Tupperware container to maintain humidity, and left undisturbed in the incubator. They should be removed (carefully) only about once a week for observation.

Note: To ensure a correct concentration, it is helpful to remove an aliquot of the cell suspension just prior to

centrifugation, and to estimate the concentration of cells by measuring A_{660}. If a 1 to 10 dilution of the homogenate gives an OD of 0.1, then the pellet should be resuspended in 5 ml.

Note: Homogenization should be done with a 5 ml Potter homogenizer, with Teflon pestle. The pestle must be loose-fitting (0.2 mm clearance), so that the embryos are disrupted but the cells are not broken. Commercial Potter homogenizers have too tight a clearance; the homogenizers used in the Miyake laboratory were made from commercial homogenizers by machining the pestles to increase the clearance. A suitable homogenizer has the property that if the homogenizer containing the pestle is inverted, gravity will cause the pestle to drop slowly and smoothly.

IV. Care of primary cultures

The following text is a loose translation from Miyake (1984).

Cultures should be incubated at 25º C. At 19º differentiation and proliferation are very slow; at 30º, cells tend to lyse and it is difficult to establish cell lines. Nunc plastic plates are ideal, but glass or plastic from other manufacturers also work well. The primary cultures should be kept in plates, rather than in sealed containers.

Usually, the following successive changes can be observed: At inoculation, single cells and small aggregates are visible, usually floating. After 1-2 days, the cells attach to the substrate, and formation of large aggregates is observed. At 3-7 days, differentiation occurs; one can see neural fiber-like structures, oil droplet-containing fat body cells, and muscle contraction. At 7-14 days cell proliferation around the cell aggregates is observed, and the proliferating cells begin to form a monolayer.

It is generally not advisable to replenish the medium until the cultures are 3 weeks old. However, if cell proliferation proceeds vigorously, it may be necessary to add new medium before 3 weeks. The first medium changes consist of replacing ¼ or ½ of the volume with fresh medium. Repeat this procedure each week, until proliferating cells reach 2/3- 3/4 of the surface area. At that point, remove the cells from the substrate using a cell scraper, and dilute 2-fold into 2 plates. You should observe proliferation of the cells, and dilute 2-fold again; if they proliferate rapidly, you can use a higher dilution. After about 10 dilutions, the differentiated larval cells should be diluted out, and the culture should appear to consist only of proliferating cells; at this point it is time to name and freeze the line.

V. References

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VI. Acknowledgements

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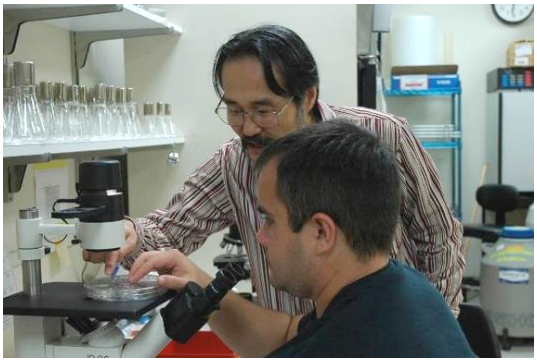
Appendix:

Miyake lab, 1989

R. Ueda and K. Ui-Tei, 2007

L. Cherbas and R. Ueda, 2007



R. Ueda and J. Roberts, 2007