Protocol: Maintaining *E. muscae in vivo* culture in a private residence

(aka keeping my experimental research system happy during the Covid-19 crisis)

Materials needed:

-Consistently-eclosing, inbred healthy flies to infect (I recommend CantonS - they're very susceptible to infection)

-Fly food to rear healthy flies

-An enclosed space that is well insulated against ambient light (e.g. a well-sealing cupboard, a poorly-sealing cupboard with blackout fabric taped to block out doors, a cardboard box).

-A low-heat lightbulb (I recommend a 60W equivalent LED, but CFL can also work if your enclosed space is sufficiently large and ventilated)

-A 24 hour outlet timer (e.g. this)

-Humid chamber (e.g. clean quart plastic yogurt tub with damp paper towels at bottom + lid)

-A fridge (4C) and freezer (-20C)

-Frozen ice pack (e.g. the ones that come with molecular biology reagents, or the ones you use for picnics, mine is a Nordic Ice pack that's 10 cm x 11.5 cm x 4 cm thick)

-Chilled aluminum block (mine is 10 cm x 7.7 cm x 2 cm thick)

-Small ice bucket or styrofoam container (mine is 14.5 cm x 12 cm)

-Paintbrushes (I prefer Princeton Artist Brush Co. #4350R-2 (2 round) or Loew-Cornell 795 (2 round))

-Aspirator (⅛" tubing with filter and tip at one end)

-Small dishes with lids, for collecting cadavers (such as these)

-Whatman paper or other absorbent paper

-Small dishes for pouring food (can be same as those for collecting cadavers)

-New wide-mouth Drosophila vials (e.g. these, any material is fine)

-Wide-mouth Droso-plugs (exactly these)

-Long (>=6") blunt-ended forceps

-Short (~3") semi-blunt ended forceps, such as these)

-Permissive solid media (e.g. 5AS MQ [5% sucrose, 1.5% agar in milliQ water] or chunks of organic banana)

-Small funnels (such as these)

-Morgue (small capable container containing >= 100 mL 70% ethanol, e.g. an empty fly bottle and lid)

-Understanding roommates

~2 weeks before beginning

-Seed 2x vials of healthy stock (e.g. CantonS) on your favorite fly diet.

-House these flies in an enclosed space under a 12:12 light cycle such that ZT14 (2 hours after light to dark transition) is a convenient time for you to manipulate flies. This is easy to achieve with a light cord, a light bulb (be careful to use one that will not emit a lot of heat!), and an outlet timer.

> -It's ideal to test how hot your bulb will get by plugging it in for 12 hours, housing in your enclosed space and checking heat output. If the space gets too hot, you need to find another space or find another bulb.

-Flip vials onto new food every 3 days until you have flies that have just started to eclose. **You will need to continue doing this as long as you want to keep growing *E. muscae*.**

> -Flies used for seeding vials should only be used for 2 weeks before replacing with a new batch of younger and hotter flies.

-Prepare permissive *E. muscae* diet, such as 5AS MQ.

> -The key to a permissive diet is to omit preservatives, e.g. tegosept or propionic acid. The inclusion of these chemicals can completely inhibit the passage of infection.
>
> -Adults only need sugar and water to survive (we don't care if the females can make eggs). Less is more.

Day 0 = Day of exposure, ZT14

1. Check exposure vials for cadavers. Flies that will die today should be dead by now.

2. Prepare cold anesthesia chamber - place frozen ice pack in styrofoam container then place chilled aluminum block on top. The aluminum block absolutely must be pre-chilled for this to be effective.

3. Prepare the cadaver dish by placing a small piece of Whatman paper in the dish and wetting with water. Top with lid.

    a. Trying to collect cadavers in a dry dish is a static nightmare. This cuts down on that problem substantially. You will still want to keep a lid on this container.

    b. You want the paper to be moist not sopping. It is possible to drown the fungus.

4. Take one exposure vial with cadavers and place tilted upside-down in freezer for 2 minutes to knockout flies.

5. Immediately place vial on its side on ice pack and gently tap the vial down sideways such that any flies that are on the side off of the ice pack fall to the side that's next to the ice pack.

6. In an area with good lighting, carefully transfer the knocked out flies to the aluminum block by removing the flug and gently tapping flies onto the block. If flies have died on the flug, there is no need to transfer them to the aluminum block.

7. Sort cadavers by eye using a paintbrush to move flies around.

8. Count cadavers and transfer them to the prepared cadaver dish using a paintbrush.

9. Count survivors and transfer them back into exposure vial.

    a. Place a small funnel in the vial.

    b. Pick up the chilled aluminum block and gently tilt.

    c. Use the brush to guide the flies into the funnel.

    d. Return aluminum block to freezer pack.

10. Repeat steps 4-9 until all cadavers are collected.

11. Melt permissive media (e.g. 5AS MQ) by microwaving.

    a. If you do not have access to this media, you can instead place chunks of organic banana into an empty vial, sprinkle on 6-8 cadavers and jump to step 18.

12. Pour an ~4 mm layer of media into a small dish (e.g. 60 mm petri dish) and wait until media has just set.

   a. You can tell the media has just set by the “jiggle test” - gently jiggle the dish - if the media jiggles, it’s not ready.

   b. The media will also become opaque as it cools.

13. Using dissecting forceps, plunge 6-8 of the plumpest cadavers headfirst into the hardening media, arranging them in a circle with their dorsal sides facing outward. Tucking the wings of the fly into the media will ensure they don’t get in the way of firing conidia.

14. Repeat step 13 until you have the number of desired “circles of death” (this will dictate the number of exposure vials you create).

15. Use a wide-mouth vial to make two cuts around each “circle of death”, such that you have cut out a football-shaped food disc with a diameter a bit less than that of a wide-mouthed vial.

16. Using long, blunt forceps, shimmy the forceps under the“circle of death” and carefully transfer into an empty wide-mouthed vial so the cadavers are face up.

17. Gently press the food to the base of the vial to close any gaps between the bottom of the vial and the food. Be careful not to squish or dislodge the cadavers.

   a. Flies are dumb and will go under the food and not get infected. That’s not what you want.

18. Use a ruler to mark 2 cm above the top of the food with a permanent marker. This will be the amount of space to which the healthy flies you add will be confined.

19. Place a new Droso-plug in each new exposure vial you have prepared.

20. Wipe the aluminum block (and gloves, if you’re wearing them) with 70% ethanol to remove any errant spores.

21. Retrieve 1-2 vials of young (0-5 days post-eclosion) healthy flies and place in freezer, tilted upside-down for 2 minutes.

22. Immediately transfer vials on their side to the freezer pack. Tap vials down so that all flies are on the side of the vial in contact with the freezer pack.

23. In an area with good lighting, carefully dump out the knocked out flies and spread out with a paintbrush.

24. Using a paintbrush, count out 50 flies and push them into a small pile away from the other flies.

25. Aspirate the entire pile of flies.

    a. It's okay if they start to wake up in the aspirator tip while you're doing this, just be sure to cover the tip when you're not applying negative pressure!

26. Deposit aspirator tip into the prepared exposure vial and eject all flies from tip.
27. Tap vial down to get all flies at the bottom of the vial, then use the blunt forceps to shimmy the Droso-plug down until the bottom of the plug is flush with the 2 cm line you drew earlier.
28. House the vial in a humid chamber for 24 hours under 12:12 L:D conditions.
29. Discard any extra healthy flies into the morgue, using the funnel to help target flies into morgue vessel.
30. Return freezer pack to -20C, return aluminum block to 4C.

Day 1 = Day after exposure, ~ZT14

1. Use the long forceps to pull the Droso-plug up to the top of the vial.
2. Move flies out of the humid chamber (i.e. to ambient conditions) and continue housing flies under 12:12 L:D conditions.

Day 4 = Four days after exposure, ZT14

1. Check for cadavers and start new exposure vials (see above).

Day 5 = Five days after exposure, ZT14

1. Check for cadavers and start new exposure vials (see above).
2. Vials can be discarded after extracting cadavers. Few flies will die on six and seven days after exposure.