Insulin stimulation of Akt phosphorylation in cultured organs (Laura Musselman)

1. Rinse larvae in PBS, bisect, and invert the anterior 2/3 of the animal.

    For adults, pin the neck and posterior on Sylgard plate and cut D-V using micro scissors

2. Place 8-10 inverted larvae in 500 µl oxygenated room temperature Schneider's medium + 1 µM insulin or insulin dilution buffer (25 mM HEPES). (Insulin stock at 50 µM = 50x)

3. Incubate at room temperature for 15 minutes.

4. Optional: cut the tip off of a 1 ml pipette tip and pipette up and down to dissociate fat bodies from the rest of the organs (larvae).

    → If you are using whole animals, homogenize in 50 µl media and boil in 50 µl 2X SB.

    Skip to step #10

5. Spin the samples at ~10,000 rpm for 1 minute.

    For 1M larvae, the fat bodies will be floating.

    For 0.15M larvae, the fat bodies will sit on top of the pellet

6. Gently pipette fat bodies onto a glass slide. Inspect for trachea, testes, imaginal discs, etc and remove all non-fat body tissue with fine forceps.

7. Take the remaining tissue = fat body and pipette into a new tube. Spin again for ~30 sec.

    For 1M larvae, fat bodies will float. Insert 200 µl pipette tip to draw off medium from the bottom of the tube and leave fat bodies in ~40 µl medium.

    For 0.15M larvae, fat bodies will pellet. Draw off all but ~40 µl medium.

8. Add 40 µl 2x sample buffer (Maniatis recipe + 50 mM $NaOV_3$ + protease inhibitor cocktail)

9. Pipet up and down to resuspend fat bodies.

10. Boil 4 minutes; place immediately on ice. Can be frozen indefinitely at -20°C.

Western blotting for PO_4-Akt

1. Make SDS + 10% polyacrylamide gels, running buffer (Maniatis recipes).

2. Load 15 µl sample per well. Run at 150V for ~45 minutes. \

3. Set up transfer in transfer buffer (Maniatis recipe).

4. Block for 1 hour in TBS-T + 4% milk powder.

    TBS-T = TBS + 0.1% Tween-20

5. Probe overnight at 4°C using Cell Signaling antibodies against *Drosophila* PO_4-Akt (#4054) or pan-Akt (#4691) and anti-syntaxin (DSHB 8C-3, as a loading control).

    Dilute anti-Akt at 1:750. Dilute anti-syt at 1:10,000. Use TBS-T + milk as the diluent.

    Can be reused several times. Save some blocking soln. to use for secondary antibody.

6. Rinse, then wash 3x in TBS-T.

7. Dilute secondaries (Santa Cruz) 1:10,000 in TBS-T + milk.

    anti-Akt are raised in rabbit; anti-syntaxin are raised in mouse.

8. Incubate 1-1.5 hours shaking at room temp.

9. Rinse, then wash 3x in TBS-T.

10. Meanwhile, make up 2 ml per blot chemiluminescent substrate: 1 ml from each bottle

11. After blot is washed and substrate has come to room temperature, add substrate to blot and incubate for 5 minutes.

12. Drain off excess substrate and expose for 15 sec to 5 minutes on Chemi-Doc.