MeOH/Acetone fixation and staining of *C. elegans*
for anti-PGL-1 whole-mount staining

adapted by Erik Andersen from Mello Lab protocol by Daryl Conte, February, 2006

Things you will need:

Micro Slides, precleaned, frosted, 25 x 75 mm (VWR cat no. 48312-002)
Micro cover glasses, 25 mm square (VWR cat no. 48366-067)
Micro cover glasses, 18 mm square (VWR cat no. 48366-045)
PAP pen
Polylysine (Sigma)
Coplin jars
Block/wedge of dry ice in Styrofoam box with lid – dry ice must be flat as slides will be placed on top
100% Methanol (-20°C in Coplin jar)
100% Acetone (-20°C in Coplin jar)
Humid chamber – a plastic lidded box with moistened paper towels or whatmann paper in the bottom and a support to hold slides above paper
Gravid adult worms or Larvae grown at 23ºC for at least two generations
Mounting medium with antifade
DAPI or other counterstain
Glass Depression slide
Water in which to wash larvae
20 µL pipet and mouth-pipette tubing

Coating slides with polylysine – do this the day before. Many slides can be coated and stored for future use.

1. Prepare **0.01%** polylysine solution.

2. Pipette polylysine solution (about 10 µL) onto surface of slide.

3. Dry slides thoroughly at 65°C. I dry them overnight.

4. It seems best to label your slides with a diamond pen, because later alcohol-fixation steps dissolve most inks.

Fixing larvae to slides

1. Transfer >100 larvae of the same stage to 100 µL of water in a glass depression slide.

   It is very important to pick animals of the same stage, as larger worms, detritus or embryos will not allow for good crushing and subsequent fixation. For PGL-1 staining, I pick L1 or L2 larvae. Never have I seen staining in L1 larvae arrested

overnight after hypochlorite treatment. Bill Kelly said that staining of Z2/Z3 by H3K4monoMe antisera was absent after L1 arrest, so maybe it is best to pick from a non-arrested culture plate at 23ºC. The temperature made a big difference too. The higher the more ectopic PGL-1 was seen.

2. Wash the larvae to remove embryos and residual bacteria by mouth-pipetting from one 100 µL drop to at least two more drops. It is easiest to watch the transfer and washing under the dissecting scope. Let the worms settle and then remove them for a new wash.

3. After washing, mouth-pipette the animals to the polylysine-coated slide (on top of the dried polylysine) in as little liquid (10-20 *µ*L) as possible.

It is easier to transfer animals if the slide is on top of an inverted culture plate lid. This will allow you to go from your plate with worms to your slide while reducing the need to focus. It will also help with later manipulations because the slide will be off of the stage – you'll see what I mean.

4. Place a 25 mm square coverslip at a 45º angle on top of the animals. The corners of the coverslip should hang over the edges of the polylysine-coated slide.

Try to avoid trapping bubbles between the coverglass and slide. Bubbles will make it difficult to compress the larvae.

5. Tear small pieces/strips of Whatmann paper, and carefully lower coverglass onto worms by wicking liquid from between slide and coverglass.

Torn pieces work better than cut pieces. The cut pieces are too blunt.

6. If fixing embryos, as the adults are squeezed, embryos will burst out. For fixing larvae, wick away the liquid until you see the animals get pressed between the coverslip and the slide. I do this step until >90% of the larvae are stuck to the slide. Continue wicking until embryos are compressed to the point where you can distinctly see the nuclei of early embryos.

This step takes the most practice.

7. Place the slide onto flat dry-ice block. Try not to allow slide to move around. Incubate ~10 min or longer.

I prepare all of my samples and leave on the dry-ice block until I'm finished and have the fixation solutions out.

8. Remove slide from the dry ice and immediately crack off the coverglass by flicking from the exposed bottom of the coverglass.

   This freeze crack is important for getting through the eggshell of the embryo. Sufficient squeezing of the embryos is also important for efficient cracking. This step is also crucial for fixing of L1 larvae, perhaps caused by the difficulty of fixing through the cuticle.

9. Immediately place the slide into –20°C MeOH for 10 min.

   MeOH permeabilizes and fixes the sample.

   If time is limiting, I keep slides O/N in 100% MeOH at –20°C. This works fine for P-granule staining but might not be good for all epitopes.

10. Quickly transfer to –20°C Acetone for 5 min.

    Acetone precipitates proteins. I try to remove excess MeOH by touching the edge of the slide to a Kimwipe before putting into acetone.

11. Dry slides on a Kimwipe at room temperature on the bench.

    The Strome lab swears that efficient drying is crucial for proper epitope fixation.

12. Using a PAP pen, draw a circle around the embryos or larvae. Allow the PAP pen marks to dry before proceeding.

    Keep the circle as small as you can. This will allow you to use small volumes (~20 µL of antibody solution). But leave a little room between the sample and pen mark so that you can aspirate buffer without touching sample. The perimeter of the pen mark should also be small enough that it can be covered completely by a 18mm square coverglass.

13. Place a drop of PBS containing 0.1% Tween (PBST) over the embryos or larvae for ~5 min in humid chamber.

14. Carefully aspirate the PBST and replace with PBST + 1% BSA blocking soultion. Incubate for 15-30 min.

15. Prepare primary antibody dilution in blocking solution.

    I use the Strome rabbit polyclonal raised against PGL-1 at 1:20. There are two other sources of PGL-1 antibody (both monoclonals): K76 and 0IC104. Use K76 or OIC104 anti-PGL-1 monoclonal at the same concentration.

16. Replace the blocking solution with ~20 μL primary antibody dilution, and incubate overnight at room temperature.

    For K76, longer seems better for somatic staining of PGL-1 in lin-35 mutants. I replace block with antibody one at a time to prevent samples from drying out.

17. Remove the primary antibody and wash the embryos or larvae several times with PBST.

    Washes can be quick or up to five minutes. Using a loaded P1000 I typically drop PBST onto the inner edge of the of the pen mark. The buffer washes over the sample and then I aspirate the wash and drop again etc. I leave the last wash on the sample and move to the next slide. I rinse each slide at least three times and then do two five-minute washes.

18. Prepare a dilution of appropriate secondary antibody labeled with the fluorochrome of choice in PBST.

    Use a dilution recommended by the manufacturer. ***K76 is an IgM monoclonal – so you will need an anti-IgM secondary!*** *I use a secondary from Jackson ImmunoResearch F(ab)$_2$ fragment, because it has much lower background than the standard secondary antibodies. I use it at a 1:25 dilution.*

19. Replace wash with secondary antibody dilution, and incubate for no longer than one hour at room temperature.

    After washing all slides, I go through slides one-by-one aspirating wash and immediately replacing with secondary.

20. Remove the secondary antibody, and wash the embryos 1-2 times with PBST.

    After several washes, you can assess the staining by examining the sample briefly under fluorescence using a 10X dry lens. You should be able to see PGL-1 pattern of staining. Be brief and don't let the sample dry out or quench.

21. Wash once with PBST containing 1 μg/mL of DAPI for 10-20 min, followed by 2-3 washes with PBST.

    This DAPI wash is optional if you use a mounting medium containing DAPI or another counterstain.

22. Aspirate final wash, and add about 5 μL of mounting medium.

23. Carefully lower 18 mm square coverglass onto slide. Avoid bubbles. Carefully squeeze out, wick or aspirate excess mounting medium.

24. Seal coverglass with nail polish.

25. Examine using appropriate fluorescent channels.