# *In-situ* + protein stain in D. melanogaster

## Reagents

**Nuclease Free H20**
Sigma W4502

**PBS packets with Tween 20**
Sigma P3563

**20X SSC**
Sigma S6639

**Formamide**
Spectrophotometric grade
Sigma 295876

**PBT + Tx (1L)**

| | |
|---|---|
| PBS + 0.2% Tween pH7.2 | 1 bag |
| 20% Triton X-100 | 10 mL |
| ddH20 | to 1L |

**1% w/v BSA in PBT + Tx**
BSA : Sigma A7888

**Hybe B (200mL)**

| | |
|---|---|
| ddH20 | 200 mL |
| 20X SSC | 50 mL |
| Formamide | 100 mL |
| 10% TritonX-100 | 4 mL |

**Anti-Histone H1**
Mouse monoclonal line AE-4
Santa Cruz Biotech sc8030

**Anti-DIG HRP**
Boehringer Mannheim 1207733

**Anti-DNP HRP**
Perkin Elmer kit NEL747 A001KT

**Streptavidin HRP**
Perkin Elmer

**Coumarin-tyramide**
TSA coumarin system NEL703 001KT

**Coverslips**
Fisher 12-548-B 22x22mm #1
Washed in 10% SDS, rinsed in ddH20 and 100%EtOH and stored in 100% EtOH.

**Slides**
Gold Seal, Becton Dickinson
Cat No. 3010 3x1" 0.93x1.05mm
Bellco Glass 5638-11013

**Mounting Media**
DEPEX
Electron Microscopy Services
Cat No. 13514

## Prepare heat blocks/water baths and probes

Day 1

Prepare an ice bucket and two heat blocks at 55-59ºC and 100ºC.

Boil probes for ≥3 min and snap cool on ice.

*Not all probes require boiling. Those that do not, can be reused 2-3 times. Snap cooling is not essential if you can pipette still boiling probe.*

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## Aliquot embryos

Day 1

Pipet embryos with 1mL blue tips into eppendorf tubes. For 20-50µl settled embryos, adjust with Hybe sol'n to 200µl. For 50-100µl settled embryos, adjust with Hybe sol'n to 300-500µl.

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## Add probes

Day 1

Warm the aliquoted embryos to 55-59ºC.

Add 1 of the probes below (either boiling hot or snap cooled):

2 µl/200µl for DIG probes

1-2 µl/200µl for DNP probes

3 µl/200µl for biotin probes

Mix by turning tubes over 2-3 times.

Hybridize overnight (up to 48 hours) at 55-59ºC.

*Too low a temperature will increase unspecific stain; too high a temperature will damage nuclear morphology.*

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## Preabsorb anti-DIG antibody (if needed)

Day 1

Rehydrate ~50µl of any embryos (good to use those not suitable for staining) by washing 3X with PBT.

Add 1 mL 1% BSA in PBT.

Add 20µl anti-DIG HRP to generate 1:50 stock solution.

Incubate overnight at 4ºC while nutating.

*Preabsorbed anti-DIG HRP can be stored at 4ºC for a couple of months.*

## Wash/block

Day 2

Collect probes for reuse (can be used 2-3 times unless they require boiling), or aspirate to formamide waste.

Wash with Hybe B at 55-59ºC

5 min 15 min 15 min 30 min 30 min

Wash 3X with PBT-Tx at room temp

Wash 4X 20 min with 1% BSA in PBT-Tx at room temp

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## Add secondary antibodies to RNA, primary antibodies for protein

Day 2

**2ºAb for RNA :**
for DIG probes : anti-DIG (1:500) 2 hours
for DNP probes : or anti-DNP (1:100) 2 hours
or for biotin probes : streptavidin-HRP 1:100 (1 hour)

*if using biotin probes, first incubate 1hr with 1ºAb for protein, then add streptavidin-HRP for 2nd hour*

**1ºAb for proteins :**
anti-mouse for H1 (1:50) 2 hours
other 2º Ab against protein of interest (1:10-1:10,000) 2 hours

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## Wash

Day 2

Wash 3X with PBT-Tx (quick)

Wash 6X 20 min with PBT-Tx

*The first 3 of these washes shouldn't be longer than 20 minutes or the background will increase.*

Wash with PBT-Tx overnight at 4ºC.

*Overnight wash is essential for anti-DIG HRP, optional for anti-DNP or streptavidin-HRP.*

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## Color reaction to detect mRNA

Day 3

Aspirate PBT-Tx, leaving 100µl embryos + buffer. If there are more than 100µl embryos, double all volumes.

For every 100µl embryos + PBT-Tx, add 100µl Tyramide amplification diluent.

For every 100µl volume in tube, add 1µl Coumarin-tyramide and mix well by pipetting up and down.

Nutate tubes at room temp.

Take an aliquot from each tube and place on microscope slide and cover with 22 x 22mm coverslip; one slide can hold 3 samples.

Observe the color reaction under the UV-filter on a fluorescence microscope. When a pattern becomes visible as bright grains, stop the reaction by adding 1mL PBT-Tx.

If there is not a pattern after 1 hour, the staining has probably failed. Stop the reaction at 1hour 15 minutes and continue to determine if it worked too weakly for the eye to detect.

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## Wash/block

Day 3

Wash 3X with PBT-Tx (quick)

Wash 4X 20 min with 1% BSA in PBT-Tx

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## Add secondary antibodies against proteins

Day 3

Aspirate PBT-Tx

Add 500µl PBT-Tx

Add 2º antibodies:

anti-mouse Alexa-488 or Alexa 555 (1:500) 2 hours

anti-protein of interest Alexa-488 or Alexa 555 (1:500) 2 hours

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## Wash

Day 3

Wash 3X with PBT-Tx (quick)

Wash 6X 20 min with PBT-Tx

Wash overnight at 4ºC in PBT-Tx

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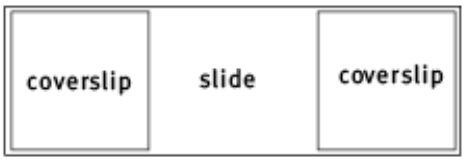
## Dehydration/mounting

### Day 4



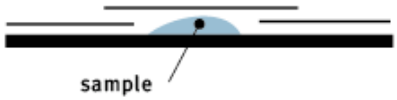

30% : 50% : 75% : 87.5% EtOH in H20, 10 min each

3X quick 100% EtOH

Aspirate EtOH, add 75µl xylene/slide to be mounted.

Wipe slides clean with EtOH, layout on paper towels.

Make bridges using #1 coverslips from the box.




Pipet embryos up and down to keep them moving, and add to the slide.

Cover embryos with 350µl DePeX using cut blue tips. Use a different tip for each slide.

Pick a clean coverslip from EtOH with forceps, dry it with lens paper and drop onto sample. Beware of bubbles.

Allow slides to dry 2-4 days in dark.

*Even after that, the slides should be kept flat for approximately one month, since the DePeX will flow slowly if the slides are sideways. The slides will be dry enough for staging the following week.*

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