\*\* Adapted from Brand 1999. Published in Drosophila Protocols (CSH) 2000

# Introduction/Notes

This protocol is adapted from Brand 1999, Drosophila Protocols (CSH) 2000, and from Colleen Hannon for Double staining. I have included notes from an example experiment for reference. The fixation protocol is three days long (not including the collection and fixation). Days 2 and 3 can be combined but that would be a very long day. All washes are at room temperature except overnight washes or incubations which are at 4˚C. This protocol includes a fix with paraformaldehyde in a solution containing Triton. The Triton makes it easier to use plastic tubes but experiments comparing paraformaldehyde, formaldehyde, and heat fixation showed little difference for both the anti-Zelda and anti-Bicoid antibody so any other preferred fixation method is fine.

If performing a double staining, make sure that primary antibodies are from different host species so that the secondary antibodies do not detect the wrong antibody. Do single stainings of each primary before or concurrently with the immunostaining.

# Solutions

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Fixing solution – 10mls** | Final | Stock | Add | For 1ml |
| PBS | 1x | 10x | 250ul | 100 ul |
| TritonX | 0.1% | 100% | 2.5ul | 1 ul |
| Paraformaldehyde | 4% | 16% | 0.625ml | 250 ul |
| H20 |  |  | 1.625ml | 640 ul |

|  |  |  |
| --- | --- | --- |
| **PBT – 500mls** | Final | Add |
| 10xPBS | 1xPBS | 50ml |
| 100% Triton-X | 0.1% Triton X | 500ul |
| Water |  | 450ml |

\*\*Make fresh each time and filter Sterilize before hand

|  |  |  |
| --- | --- | --- |
| **PBTB – 5mls** | Final | Add |
| PBT |  | 5ml |
| BSA | 1% | 50 mg |

\*\*Make fresh every time and Keep at 4dC when not using.

# Protocol

**Collection**.

1. Clear for **<30** minutes. Embryos collection for **2:00** and aged for **1:20**.

* These times are to collect mostly embryos before nuclear cycle 14.

1. Embryos are bleached for 2-3 minutes in 50% Bleach to dechorionate. Rinse well with water. Pat dry on towel.
2. Rinse with 1xPBS + 0.5% Triton.
3. Take a large brushful of embryos and place them in a 1.5ml tube filled with 1xPBS + 0.5% Triton.
4. Take off and wash with H20.
5. Take off supernatant and wash with 100% isopropanol.

**Fix**.

1. Add 500ul of Heptane + 500ul of fixative solution in an eppi
2. Fix for 30 minutes with gentle rocking on the rotator plate.
3. Remove the lower aqueous layer and replace with **750ul of methanol** and vortex for **exactly** 30 seconds to remove the vitelline membrane.

* Embryos that sink have their vitelline membrane removed; those that stay at the interface still have their vitelline membranes.

1. Remove as much heptane and methanol as possible. Replace with fresh methanol and wash rapidly (10 seconds).
2. Add 1ml of fresh methanol and keep O/N at 4dC.

**IF day 1.**

1. Remove methanol and rehydrate in PBT (PBS + 0.1 Triton).
2. Wash 1x with PBT
3. Wash 2x5 minutes with 1ml PBT at RT on a rocker
4. Wash1x30 minutes in 1ml PBT at RT on a rocker
5. Resuspend in 100ul of PBTB + Block for 30 minutes at room temperature on a rotor.
6. Divide into tubes per sample.
7. Add primary antibody(S) to each sample (see below). Rock O/N at 4dC.

**IF Day 2.**

1. Rinse embryos 3x with 1 ml PBT.

* the primary antibody used for the incubation can be recovered and saved for further use. Antibody can be used several times.

1. Wash 6x20 min in 1 ml PBT

**1 2 3 4 5 6**

1. Block for 30 min at room temperature in 100 μl PBTB
2. Add diluted secondary antibody- wrap tube with alumina foil, rock slowly at room temperature for 2 hrs.
3. wash embryos three times for 5 min each with PBT
4. Wash the embryos six times for 20 min (or 4x30 min) each with PBT. \* Usually I do the last wash O/N at 4˚C

**1 2 3 4 5 6**

**IF Day 3.**

1. Add 1ul of Dapi to the 1ml PBT.
2. Incubate for an 5 minutes.
3. Wash 6x in PBT

**1 2 3 4 5 6**

1. Add 100ul of Dabco and let incubate at RT (STANDING) for at least 30 minutes out of the light.
2. Mount slides. Keep at -20.
3. When ready to mount embryos, bring the embryos to room temperature. Transfer 25-30 ul of embryos to a clean slide, place two coverslips on both sides of drop, about 1 cm apart, place a third on top.

* Alternatively, to image with the 63x objective on the confocal simply place one coverslip over about 25ul of dabco/embryos and then seal the edges with clear quick-dry nail polish. Make sure to use an eyedropper to drop nailpolish around the edges (not brush) so that there is a thick seal.

1. To save the slide, completely fill the space underneath the coverslip with glycerol and seal all the edges of the coverslips with fingernail polish. The slide can be kept at 4oC in the dark for at least several days.

## Image

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample** | **Fly line** | **1°** | **2°** | **Imaging parameters** | **Notes** |
| 1.1 | ZldsfGFP | - anti- GFP chicken (AVES GFP-1010)  **1:1000** 🡪 1ul of 1:10    - anti-bcd (rabbit XY)  **1:500** 🡪 1ul of 1:5 | - Alexa Fluor® 488 AffiniPure Goat Anti-Chicken IgY (IgG) (H+L) 🡪1:1000 (1 ul of 1:10 added to 100ul BB)  - goat anti-rabbit IgG Alexa633  🡪 1:300 (20ul of 1:50 added to 100ul of BB) | |  |  |  |  | | --- | --- | --- | --- | | Color | B | G | R | | Laser % | 2.4 | 2.2 | 3.5 | | Gain | 530 | 530 | 530 | |  |