Leslie Slota

Cohn Laboratory

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Limulus Whole Mount *In Situ* Protocol

-To visualize gene expression in horseshoe crab embryos.

- This is the original protocol that was taken from Nadean Brown's Lab. This summer we used this original protocol and tried several modifications of this protocol.

Embryo Preparation

- Allow fertilized eggs to develop in a Petri dish of either real ocean water or instant ocean (Aquarium Systems). Change water daily or every other day.
- When embryos have reached the desired stage, remove them into a 50ml conical tube/ If they are older than stage 19 then remove chorion with forceps first.
- Wash the embryos in 10 ml of a 50% bleach solution for 5 minutes.
- Wash 3 times 1 minute with 10 ml of milliQ Water.
- Fix in 20 ml of 4% PFA and 20 ml of Heptane for 1 hour while rocking.
- Remove the 4% PFA (lower layer) and dispose of
- Add 15 ml of methanol and wash for 30 seconds shaking gently by hand.
- Remove all liquid, Heptane (top layer) and most methanol (bottom layer).
- Transfer embryos into a 1.5 ml microfuge tube and wash 3 times for 5 minutes with methanol.
- Store embryos in methanol at -20^0C. Embryos stored >1yr still work great.

Day 1

(All steps done under clean, RNase free conditions)

- Whole fixed embryos stored in 100 methanol should be used for whole mount *in situ.*

  - Re-hydrate embryos through Methanol/PBTX washes. (20 Minutes each wash)
    - 75% MeOH, 25% PBTX
    - 50% MeOH, 50% PBTX
    - 25% MeOH, 75% PBTX
  - Wash 3 times in PBTX for 10 minutes each.

- Incubate with 1:20 dilution of 5 U/ml chitinase (Sigma, Cat. No.), while rocking overnight

Day 2

Wash 3 times with PBTX for 10 minutes

Incubate with 1:1000 dilution of 10 mg/ml Proteinase K (made in dH20) for 25 minutes

Wash 3 times for 5 minutes with PBTX

Re-fix for 20 minutes with 0.2% Glutaraldehyde/4% PFA in PBTX

Wash 3 times in PBTX for 10 minutes each

Heat embryos for 30 minutes at 75^0C (This step eliminates endogenous Alkaline Phosphatase

Wash 2 times with PBTX for 10 minutes each, turn on hybridization incubator to 70^0C and heat prehybridization mix

Add 1ml of preheated mix to embryos and incubate at 70^0C for 2hrs

Preheat the diluted riboprobe in prehyb mix to 90^0C for 5 minutes

Remove the prehybridization and add the probes (1ml/tube of embryos)

Incubate overnight in the 70^0C oven.

Day 3

- Remove Probes and save (After this RNase free conditions are no longer necessary). Note: Diluted probe can be used up to 5 times, stored at -20^0C
- Wash through the following series, 20 minutes each, at 70^0C
    - 100% Solution 1
    - 75% Solution 1/ 25% 2xSSC
    - 50% Solution 1/ 50% 2xSSC
    - 25% Solution 1/ 75% 2xSSC
- Wash with 2xSSC, 0.1% CHAPS twice for 30 minutes at 70^0C
- Wash with 0.2xSSC, 0.1% CHAPS twice for 30 minutes at 70^0C
- Wash with TBTX twice for 10 minutes at room temp
- Pre-block embryos with block buffer for 2-3 hours at room temp
- Remove pre-block buffer and add pre absorbed and diluted antibody, rock over night at room temp. Preabsorb antibody at 1:500, use at 1:2000.

Day 4

- Remove antibody and save at 4^0C for reuse.
- Wash at least 5x one hour each with TBTX, while rocking
- Wash with NTMT (made fresh on day of use) 3x 10 minutes each, then leave on overnight, while rocking

Day 5

- Develop with fresh NTMT + 4.5µl NBT and 3.5µl BCIP per ml, in nine well glass dishes keep in the dark.

- Check progress every half hour on dissecting scope and change developer every hour.
- Wash 3x 20 minutes in PBS to stop development. Store at 4oC until destained or fixed, protect from the light.

- De-stain with PBS 1% Trition X-100 while rocking for about 6 hours to remove background staining, check progress of destaining visually every hour.
- Store the embryos in 4% PFA at 4^0C until embedded or photographed.

Preabsorption of Antibody

The antibody must be preabsorbed with embryo powder (the longer the better), and can be used after dilution up to four times.

- During the washing of the embryos on day three, weigh out 3mg of embryo powder into a microtube, add 0.5mL of block buffer and 1µl anti-Dig-AP Fab fragment.
- Rock gently at 4^0C for 3 hours or longer
- Spin in a Microfuge for 10 minutes at 4^0C
- Remove supernatant and place in new tube to save.
- Store at 4^0C until use and dilute jus prior to use.

Solutions

25% Glutaraldehyde: store in aliquot amounts at –20^0C and thaw just prior to use.

PBTX: PBS (pH 7.4) with 0.1% Trition X-100

Prehybridization Mix: 50% Formamide, 5x SSC, 2% Boehringer blocking powder (cat. No. 1096176, dissolve directly into the mix. DO THIS BEFORE YOU ADD FORMAMIDE), 0.1% Trition X-100, 0.5% CHAPS (Sigma C-3023), 1mg/ml yeast RNA (sigma R-6625), 5mM EDTA, 50µg/ml heparin. For hybridization, add probe to dilution determined in dot plot.

Solution 1: 50% formamide, 5x SSC, 0.1% Triton X-100, 0.5% CHAPS

TBTX: 50mM Tris (pH 7.5), 150mM NaCl, 0.1% Trition X-100

Crab Powder: Homogenize the soft body parts of 5-10 juvenile horseshoe crabs in a minimum volume of PBS. Add 4 volumes of ice-cold acetone, mix and incubate on ice for 30 minutes. Spin at 10,000 RPM for 10 minutes and remove supernatant. Wash the pellet with ice-cold acetone and spin again. Spread the pellet out and grind into a fine powder on a sheet of filter paper and allow it to air dry. Store in an airtight tube at 4^0C.

Block Buffer: 10% Sheep serum, 2% BSA and 0.2% Sodium Azide, in TBTX

NTMT: 100mM NaCl, 100mM Tris (pH 9.5), 50mM $MgCl_2$, 0.1% Tween-20. This must be made fresh on the day of use as the pH decreases during storage due to the absorption of CO_2.

NBT*: 75mg/ml in Dimethylformamide (store at -20^0C)

BCIP*: 50 mg/ml in dimethylformamide, 4-Tolouidine Salt (store at -20^0C)

*It is best to let these reagents warm to room temperature before use as it may decrease the amount of crystal formation in the color reaction.