One Color Section In-Situ Hybridization for *Octopus bimaculoides*

Judit Pungor
Host Lab: Dr. Clifton Ragsdale, University of Chicago
Home Lab: Dr. Stuart Thompson, Hopkins Marine Station of Stanford University
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This protocol is a modification of one originally developed by the Ragsdale lab for in-situ hybridization (Rowell, 2010). While a number of changes have been made because of the challenges of octopus sections in-situ hybridization, protocol modification remains **in progress**. Further improvements will be published as they are established.

The goal of this protocol is the detection of *O. bimaculoides* specific gene products within the neural tissue of the animal.

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Prepare sections

1. Cut PFA fixed, sucrose-sunk tissue on a freezing microtome (25-50 µm). Dry sections well (2 hrs. or overnight at RT).

Day One: (RNase-free)

1. Place slides in RNase-free mailers.
2. Fix in 4% fresh paraformaldehyde (PFA) in PBS for 15 min.
3. Wash in DEPC-PBS at RT for 5 min. (X3)
4. Incubate in Proteinase K (1µg/ml in Proteinase K Buffer) at 37°C for 30 min.
5. Fix in 4% PFA in PBS for 15 min.
6. Wash in DEPC-PBS for 5 min. (X3)
7. Wash in TEA 10 min. (X1)
8. Add 125 uL Acetic Anhydride to 50uL of TEA. (Always add Acetic Anhydride to TEA fresh before each wash.) Wash slides in Acetic Anhydride in TEA 10 min. (X3)
9. Wash DEPC-PBS 5 min. (X1)
10. Hybridization: Add approximately 15 ml of prehybridization solution to each mailer. Slides may be stored at -20°C at this point or incubate slides, following warming to room temperature if stored at -20°C, in prehybridization solution in a 72°C water bath for at least 60 minutes.
11. Empty the warmed prehybridization solution into a fresh Falcon tube, add 100 µl of probe, mix well, and add to the appropriate slide mailer.
12. Incubate at 72°C overnight.

Day Two

1. Transfer slides to Day 2 mailers containing pre-heated Solution X. For the first Solution X Wash, add 5mg/mL of Yeast tRNA to the Solution X before adding it to the mailers. Mailers with hybridization solution and probe can be saved at -20°C and reused.
2. Stringent Wash: Solution X for 45 min. at 72°C. (X3)
3. Wash for 15 min. in TBST at RT. (X3)
4. While washing in TBST, prepare the preadsorbed antibody: Incubate 3-5 mg of octopus embryo powder in 0.5ml of TBST for 30 minutes at 72°C. Chill on ice for 15 minutes. Add 5μl of lamb serum and 3 μl anti-DIG alkaline phosphatase fab fragments. Incubate at 4°C for 60 minutes while rocking. Centrifuge for 10 minutes and remove supernatant containing preadsorbed antibody.
5. Block for 1 hr. at RT (or overnight at 4°C): 10% lamb serum in TBST.
6. Add preadsorbed Anti-DIG antibody to 15 ml of 1% lamb serum in TBST. Mix well and incubate at RT for 2 hrs. (or overnight at 4°C).
7. 15 min. TBST wash. (X3)
8. Wash for 10 min. in freshly prepared NTMT.
9. Color Reaction: Add 3.5 μl NBT and 3.5 μl BCIP per ml NTMT. Incubate at RT in the dark. Leave overnight in color reaction at RT or 4°C as appropriate. (To remove pink or light purple background, replace color reaction solution with plain NTMT and leave at 4°C overnight.)

Day Three:

1. Replace color reaction solution with fresh solution once a day or as appropriate to minimize background. When color has developed, rinse twice in Stop TE buffer, then wash in Stop TE for 1-2 hrs.
2. Wash in TBST overnight at RT.
3. Store in 10% Formalin in PBS overnight at 4°C.
4. Wash in PBS for 5 min. at RT. (X2)
5. Remove slides from mailers, place in slide holders and allow them to dry at RT.

Solutions

Hybridization Solution (500mL)

50% Formamide: 250 ml Formamide
5x SSC: 12.5 ml 20x SSC (pH 4.5)
1% SDS: 50 ml 10% SDS
.2 mg/mL heparin: .1 g
.5 mg/mL Acetylated BSA: 8.3 mL of ~30mg/mL stock
5 mg/mL Yeast tRNA: 2.5 g

.1M TEA pH 8 (for acetylation) (1L)

13.3 mL triethanolamine
983.7mL DEPC-H2O

3mL HCl (add to solution until pH is 8)

Solution X (50 ml)

50% Formamide: 25 ml Formamide
2x SSC: 5 ml 20x SSC (pH 4.5)
1% SDS: 5 ml 10% SDS

10X TBS (1 L)

250 mM TrisHCl (pH 7.5): 250 ml 1 M TrisHCl (pH 7.5)
1.36 M NaCl: 80 g NaCl
26.8 mM KCl: 2 g KCl
** 1% Tween 20: 100mL Tween 20

NTMT (1 L)

100 mM NaCl: 20 ml of 5 M NaCl
100 mM Tris HCl (pH 9.5): 100 ml of 1 M Tris HCl (pH 9.5)
50 mM MgCl2: 25 ml of 2M MgCl2
2 mM levamizole: 52 mg
** 1% Tween 20: 1 ml Tween 20

Proteinase K Buffer (500 ml)

100mM Tris-HCl (pH 8.0): 50 ml 1M Tris-HCl (pH 8.0)
50 mM EDTA (pH 8.0): 50 ml 0.5 M EDTA (pH 8.0)

TE Stop Buffer (500 ml)

10 mM Tris HCl (pH 7.5): 5 ml 1M Tris HCl (pH 7.5)
10 mM EDTA (pH 8.0): 10 ml 0.5 M EDTA (pH 8.0)

** Tween settles out of solution over time. It is best to make stock solutions of reagents without Tween, and add Tween fresh before use.

References

Rowell, J.J., Mallik, A.K., Dugas-Ford, J. and Ragsdale, C.W. (2010) Molecular analysis of neocortical layer structure in the ferret. *J. Comp. Neurol*. 518, 3272-3289.