DHK's *Helobdella* whole-mount *in situ* protocol (updated 08/2016)

Day 0: Fixation

1. Fix embryos of stages 1 & 6-11 with 4% formaldehyde in 0.5X PBS (16% formadehyde:ddH_2O:1X PBS = 1:1:2) for 1hr at room temperature (r.t.) or overnight at 4°C. For late stage 9 and older, manually remove vitelline membrane with tweezers and relax the embryo in ice-cold relaxation solution before fixation.
   Fix embryos of stages 2-5 with 4% formaldehyde in 0.25X PBS (16% formadehyde:ddH_2O:1X PBS = 1:2:1) for 1hr at room temperature (r.t.) or overnight at 4°C.
2. After fixation, wash embryos with 1X PBS several times.
3. For embryos of stages 1-early 9, remove vitelline membrane using broken pipette method.
4. 1 brief wash in PBS:MeOH (1:1) and 3 brief washes in 100% MeOH. Store embryos in MeOH at -20°C for at least overnight. Embryos can be stored in MeOH at -20°C for years.

Day 1: Pretreatment (for stage 1-8, ok to skip steps 3-4 for most probes)

1. Place embryos in 1.5 mL tube.
2. Wash embryos with PBS:MeOH(1:1), then 3X brief washes with PBS + 0.1% Tween-20 (PBTw).
3. (*¡only necessary for late stage 9-juvenile specimens!*)
   Proteinase K treatment reduces background by facilitating rapid probe removal during stringency wash. Not required for embryos of stages 1-8, but may reduce background in some cases. For treatment, incubate embryos with 20 µg/mL Proteinase K solution (20 mg/mL Proteinase K (NEB) : PBTw = 1:1000). Mix the Proteinase K solution by vortexing before add to the specimen.
   For stage 1-8 and early stage 9, treatment is optional. Treat for 1 minute at r.t. only if necessary.
   Treatment is mandatory for older embryos (late stage 9-stage 11) and juveniles. For late stage 9, 5 minute at r.t. is recommended. For stage 10, 15 minutes at r.t.; for stage 11 and juvenile, 30 minutes at r.t..
   Remove Proteinase K solution, and wash twice with PBTw.
4. (*¡only for protease-treated specimens!*)
   Post-fix embryos with 4% formaldehyde in 0.75X PBTw (16% formadehyde : PBTw = 1:3) for 20-30 min at room temperature. 5X washes with PBTw.
5. (*¡optional, may reduce background!*)
   Acetylation of reactive amine residues on the specimen may help to reduce background. But for most probes, this step makes no difference. Only try this if you encounter high background problem.
   2X brief wash with 0.1 M triethanolamine (pH 8.0). Incubate embryos in 1 mL of 0.1 M triethanolamine (pH 8.0) for 5 minutes at r.t.. Add 2.5 µL acetic anhydride and mix by gentle rocking for 5 minutes. Add another 2.5 µL acetic anhydride and incubate for another 5 minutes with gentle rocking.3X wash with PBTw.
6. Transfer embryos into new 1.5 mL tube(s). Up to 50 embryos allowed in a single tube.
7. Add 50 µL of PreHyb:PBTw (1:1) to the embryos and wait until embryos settling down.
8. Wash embryos twice with 100 µl PreHyb.
9. Replace the solution with 200 µl PreHyb, and incubate at hybridization temperature (65-70°C) for overnight.

Day 2: Hybridization

1. Add probe to 200 µL fresh PreHyb buffer; the amount of probe is determined empirically for each probe and each stage.
   Start with 1 ng/µl for stages 1-9 and 0.25 ng/µl for stages 10-11.
   (*optional*)
   Carrying out hybridization in PreHyb-DS instead of PreHyb increases sensitivity. But high viscosity of PreHyb-DS may make handling of embryos harder.
   Place the probe solution in oven to reach hybridization temperature.
2. Remove PreHyb solution from the embryo and save it in a separate tube for the following washing step. Add the pre-warmed probe solution and hybridize for 24-48 hrs.

Day 3/4: Probe Removal

1. Wash with warm PreHyb (~200 µL each, saved from the blocking step) in oven for 10 min.
2. Wash the embryos with warm 2XSSC (0.5-1 mL each tube) in oven for 20 min.
3. Wash the embryos twice with warm 0.2XSSC (0.5-1 mL each tube) in oven for 20 min each.
4. Wash the embryos twice with warm 0.1XSSC (0.5-1 mL each tube) in oven for 20 min each.
5. Remove as much liquid as possible, and then allow the tube and embryos to return to r.t. Rinse embryos in 0.1% PBTw twice (0.5-1 mL each tube), followed by a 5 min wash in PBTw (0.5-1 mL each tube).

Day 3/4: Antibody Labeling

1. Transfer the embryos into 0.6 mL tube using flamed glass pipet. Remove as much liquid as possible; then add 500 µL of Ab blocking solution. Block the embryos in room temperature on a rocking table for 2 hrs.
2. Add 1/2500 (0.2 µL) AP-conjugated anti-dig antibody (Roche; diluted 1:1 from original stock with glycerol for cryopreservation) to blocking solution.
3. Incubate overnight on a rocking table at 4°C.

Day 4/5: Antibody Wash

1. Remove the Ab solution; rinse embryos 3X with 0.5 mL PBTw; 1 min each. Wash the embryos 6 times with PBTw; 20 min each wash.

Days 4/5: Color reaction (Option 1: BM Purple)

1. Transfer embryos into a new 1.5 mL tube.
2. Remove as much liquid from the PBTw wash as possible. Add 100 µL BM Purple to the embryos. *Important! Mix BM Purple solution by shaking immediately before use*.
   For stages 8 and 9, overnight color reaction at r.t. generally gets good results. If see 'background staining' it is better to reduce probe concentration than to shorten color reaction time. If darker staining is desired, continue color reaction at 37°C for hours or r.t. for days. For other stages and abundant transcripts, start with color reaction at 37°C. Monitor color development hourly. If stain does not reach desired intensity in 3 hours, it is ok to continue at r.t. overnight.
3. Wash the cloudy deposit from the BM Purple reaction by rinsing the embryos several times in PBTw.
4. Passing the embryos through an EtOH series (50%, 70%, 80%, 90%, 95%, 100%, 100%). Embryos can be stored in EtOH at r.t. permanently. From EtOH, embryos can be cleared and mounted/embedded in BB:BA, EPON or PEG methacrylate. Alternatively, embryos can be rehydrated and cleared with glycerol or other aqueous clearing agents. For long-term storage, keep embryos in >95% EtOH.

Days 4/5: Color reaction (Option 2: NBT/BCIP)

1. Prepare fresh AP buffer. [Note: always prepare AP buffer immediately before use; precipitation forms within days after AP buffer is made]
2. Wash embryos with 1 mL AP buffer trice at room temperature, 1 minute each.
3. Incubate the embryos in AP buffer at room temperature for 5 minutes.
4. Prepare NBT/BCIP solution by adding 4 µL 25 mg/mL NBT stock and 1 µL 50 mg/mL BCIP stock to 250 µL AP buffer. Mix by vortex and spin down.
5. Remove AP buffer and add NBT/BCIP solution. Place the reaction in 25 °C incubator. Monitor the progress of color reaction by checking the embryos under dissecting microscope after 5, 10, 15, 30, 60, 120, and 180 minutes. Stop the color reaction by washing 3X with PBTw if the staining has reached the desired intensity.
6. Passing the embryos through an EtOH series (50%, 70%, 80%, 90%, 95%, 100%, 100%). From EtOH, embryos can be cleared and mounted/embedded in EPON or PEG methacrylate. Alternatively, embryos can be rehydrated and cleared with glycerol or other aqueous clearing agents. For long-term storage, keep embryos in >95% EtOH.

Appendix: Clearing and mounting with PEG methacrylate

1. From EtOH, embryos are placed into glycol methacrylate (or HEMA) and incubate for at least 1 hour.
2. Embryos are transferred into PEG methacrylate and incubate for overnight at 4°C.
3. Mount embryos in slide, with PEG methacrylate as mounting medium, for photographing.
4. If you want to keep the specimens for future use, return the embryos back into EtOH. Wash the embryos 3X with 100% EtOH. Embryos can be stored in EtOH indefinitely.

Recipes

1. PBTw:
   Add 0.5 mL of **10% Tween-20** stock to 50 mL PBS.
2. 0.1 M triethanolamine (pH 8.0):
   For each sample, ~ 3 mL triethanolamine buffer is required. To make 3 mL, add 40 µL **triethanolamine** to 2.95 mL ddH2O. Then, add 12 µL **HCl (concentrate)**, mix well. Always made fresh!
3. PreHyb:
   mix 25 mg **torula RNA type VI** (Sigma) into 25 mL **deionized formamide**; add 12.5 mL **20X SSC**, 0.25 mL 10 mg/mL **heparin**, 0.5 mL 100X **Denhardt's Solution**, 0.5 mL **10% Tween-20**, 0.46 mL 1M **citric acid**; bring to 50 mL with ddH_2O
4. PreHyb-DS (PreHyb + 5% dextran sulfate):
   mix 25 mg **torula RNA type VI** (Sigma) into 25 mL **deionized formamide**; add 12.5 mL **20X SSC**, 0.25 mL 10 mg/mL **heparin**, 0.5 mL 100X **Denhardt's Solution**, 0.5 mL **10% Tween-20**, 0.46 mL 1M **citric acid**; add 2.5g **dextran sulfate**, allow dextran sulfate to dissolve by putting the solution in warm water bath and mixing; bring to 50 mL with ddH_2O.
5. 2X SSC, 0.2X SSC and 0.1X SSC
   Take 5 mL (for 2X), 0.5 mL (for 0.2X) or 0.25 mL (for 0.1X) **20X SSC** (3 M NaCl, 0.3 M $Na_3C_6H_5O_7$ sodium citrate). Add 0.5 mL **10% Tween-20** or 0.5 mL **5% CHAP**. Bring to 50 ml with ddH2O.
6. Ab block solution:
   Add 1 mL **sheep serum** and 0.5 mL **10% Tween-20** to 5 mL **Roche Western Block Solution**. Bring to 50 mL with PBS.
7. AP buffer (100 mM NaCl, 100 mM Tris (pH 9.5), 50 mM $MgCl_2$, 0.1% Tween):
   For 10 mL, combine 200 µL **5M NaCl**, 1 mL **1M Tris (pH 9.5)**, 500 µL **1M $MgCl_2$**, and 100 µL **10% Tween-20**; bring to 10 mL with ddH_2O.

Probe Synthesis

1. From the plasmid map and sequence data, determine the species of RNA polymerase (SP6, T3 or T7) that will be used to synthesize antisense (or sense) riboprobe, and design your DNA template processing procedure accordingly.
2. Preparing linear DNA template by PCR or by restriction enzyme digestion of plasmid DNA.
   Setting up a 50 µL PCR reaction if using PCR method to prepare linear template. PCR template is 0.5 µL of 100X dilution of plasmid DNA miniprep.
   After reaction, take a small amount of PCR product or restriction enzyme reaction to run a gel. Make sure that only a single band of the expected size is observed. Purify linear DNA template by the Zymo-Spin I column (minimum elution = 6 µL); do gel extraction, if necessary. Elute linear DNA template in ~15 µL nuclease-free ddH_2O.
3. Quantify DNA template by A_{260}. No more than 1 µg template should be introduced into the probe synthesis reaction.
4. Assemble 20 µl reactions at **room temperature** (spermidine in the buffer precipitates DNA on ice!!!)
   For NEB T7 or SP6 RNA Pol

X µL	0.5-1 µg linear template DNA
13-X µL	RNase-free water (total volume = 20 µL)
2 µL	10X buffer
2 µL	10X Dig/biotin/fluorescein-NTP mix (Roche) or 10X DNP-NTP mix (Perkin Elmer)
1 µL	RNase inhibitor
2 µL	RNA Polymerase (SP6 or T7; 20U/µl)

   For Thermo T7, SP6, or T3 RNA Pol

X µL	0.5-1 µg linear template DNA
11-X µL	RNase-free water (total volume = 20 µL)
4 µL	5X buffer
2 µL	10X Dig/biotin/fluorescein-NTP mix (Roche) or 10X DNP-NTP mix (Perkin Elmer)
1 µL	RNase inhibitor
2 µL	RNA Polymerase (SP6, T7 or T3; 20U/µL)

5. Mix well, spin down and then incubate at 37°C for 4-24 hours.
6. (*optional*) Add 1 µL of RNase-free DNase, and incubate at 37°C for 30 mins.
7. Precipitate RNA by EtOH. Add 1 µL 0.5M EDTA pH 8, 4 µL 6M lithium chloride, and (*optional*) 0.5 µL 20mg/mL glycogen to the reaction, mix well and spin down, add 100 µL cold 100% EtOH, mix well, and store at -20°C for at least 1hr (up to one overnight). Centrifuge at maximal speed in 4°C for 15 min to pellet the RNA.
8. Wash pellet with cold 75% EtOH. Centrifuge at maximal speed at r.t. for 5 minute to re-collect the RNA precipitation. For a 20 µL reaction, the RNA pellet should be easily visible.
9. Remove all liquid carefully. Dry the RNA pellet with cap open in room temperature for 15 minutes, and resuspend RNA in 12 µL of RNase-free water.
10. Quantify RNA concentration by A_{260} (for single-strand RNA 1A=40 µg/mL). Run gel to determine the quality of probe, if necessary. Add appropriate amount of PreHyb to bring the final concentration to 100 ng/µL. Store the stock at -20°C.