DHK's *Helobdella* multiplex fluorescence whole-mount *in situ* protocol (updated 7/2016)

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 by acetic anhydride may help reduce background.
   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(s) to 100 or 200 µL fresh **PreHyb-DS** buffer; the amount of probe is determined empirically for each probe and each stage. A higher probe concentration than standard AP substrate-based *in situ* hybridization protocol is often necessary (suggested starting point: 5 ng/µl or 5X probe concentration of the hybridization reaction using BM Purple or NBT/BCIP reaction; decrease concentration if the background is too high and increase concentration if the signal is too low).
   Place the probe solution in oven to reach hybridization temperature. Up to 4 different probes can be used in a single tube, provided that each probe is uniquely labeled with digoxigenin, DNP, biotin or fluorescein (priority: digoxigenin > DNP > biotin > fluorescein, the weaker probe gets higher priority).
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. Carrying out hybridization in DS-containing solution increases sensitivity, but high viscosity of DS solution makes handling of embryos harder. Be careful when removing probe solution at the end of hybridization step.

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).

Proceed to Antibody-TSA cycles (next page).

Antibody-TSA Cycle

Start with antibody labeling against the weakest probe first, followed by the next weakest (digoxigenin ⇒ DNP ⇒ biotin ⇒ fluoresceine). Complete a cycle of antibody labeling-antibody wash-TSA reaction-TSA wash-antibody stripping before moving onto the next. Skip the cycle for the type of probe you did not use in your hybridization reaction.

Cycle 1: digoxigenin

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 1-2 hrs. [Important: Blocking solution here is 10% Roche Western Blocking Solution in PBTw; **no serum** is added to avoid potential cross-immunoreactivity in multiplex labeling.]
2. Add 1/500 (1 µL) peroxidase-conjugated anti-dig antibody (Roche; diluted 1:1 from original stock with glycerol for cryopreservation) to blocking solution. Incubate overnight on a rocking table at 4°C (or 1 hour at r.t.).
3. 6X 20-minute washes with 500 µL PBT. Transfer embryos to a new 1.5 mL tube.
4. Wash twice in 1 mL borate buffer (100 mM borate pH 8.5 supplemented with 0.1% Tween-20), 5 minutes each.
5. Prepare TSA reaction buffer by adding 1 µL **0.3% H_2O_2** (made by adding 99 µL ddH_2O to 1 µL 30% H_2O_2) and 0.5 µL **100 mg/mL 4-iodophenol** stock solution (in EtOH) to 100 µL **DS-borate buffer**. Mix well.
6. Add 0.5–2 µL of TSA stock to 100 µL TSA reaction buffer. Mix by vortexing.
7. Incubate embryos in TSA+reaction buffer under dark at room temperature for 15-30 minutes.
8. 3X brief washes with 1 mL PBTw, followed by 4X 20 minute washes in PBT [go straight to step 10, if no additional labeling cycle will be performed].

Cycle 2/3/4: DNP/biotin/fluorescein

9. Strip antibody from the previous labeling round by 2X washes with 1 mL acid glycine buffer at room temperature, 5 minutes each, followed by 3X 5-minute washes in PBTw.

All subsequent steps are the same as the digoxigenin labeling cycle, except for different antibodies used.
DNP labeling cycle: 1:250 anti-DNP peroxidase conjugate (Perkin Elmer)
Biotin labeling cycle: 1:500 anti-biotin peroxidase conjugate (Invitrogen/Molecular Probes)
Fluorescein labeling cycle: 1:500 anti-fluorescein peroxidase conjugate (Invitrogen/Molecular Probes)

10. After the final labeling cycle, 1 overnight PBT wash, followed by 6X 20-minute washes with PBT.
11. Check under florescence microscope. If background is high, keep washing. Stop if the background is low enough or cannot be further reduced by washing.

Clearing and Mounting

Fluorescently labeled embryos are best mounted in anti-fading reagent containing buffered glycerol. Transfer the embryos to a new 1.5 mL tube. Remove as much washing solution as possible. Add ~200 µL 80% buffered glycerol with 4% n-propyl gallate. If fluorescein is used, it is recommended to use basic buffer (*e.g*. 5 mL 1 M Tris pH 9.5 + 15 mL ddH_2O is added to 80 mL glycerol, premixed with 4 g n-propyl gallate). Document the results by fluorescence microscopy or confocal microscopy as soon as possible.

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 (very viscous liquid, irritant)** to 2.95 mL ddH2O. Then, add 12 µL **HCl (concentrate, dangerous! protect yourself & careful with HCl vapor!)**, 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 0.5 mL **10% Tween-20** to 5 mL **Roche Western Block Solution**. Bring to 50 mL with PBS (do not add any normal serum to avoid cross-immunoreactivity in multiplex labeling)
7. Borate buffer (100 mM boric acid pH 8.5, 0.1% Tween-20):
   To make 100 mM boric acid solution, dissolve 6.2 g **boric acid** in 900 mL ddH_2O. Adjust pH to 8.5. Fill with ddH_2O to a total volume of 1 L. Add 0.5 mL **10% Tween-20** to 50 mL of 100 mM boric acid pH8.5 solution.
8. DS-Borate buffer (2% dextran sulfate in 100 mM borate pH 8.5, 0.1% Tween)
   Add 1 g **dextran sulfate** to 40 mL of 100 mM **boric acid** pH8.5 solution. Vortex to dissolve dextran sulfate; heat up the solution up to 60°C to help dissolving, if necessary. Add 0.5 ml 10% **Tween-20**. Bring to a final volume of 50 ml with 100 mM boric acid pH8.5 solution.
9. TSA reaction buffer
   Prepare immediately before use. Add 1 µL **0.3% H_2O_2** (made by adding 99 µL ddH_2O to 1 µL 30% H_2O_2), 0.5 µL **100 mg/mL 4-iodophenol** stock solution (in EtOH) to 100 µL **DS-borate buffer**. Mix well.
10. Acid glycine buffer (0.1 M glycine, pH2, 0.1% Tween-20)
    Dissolve 7.5 g **glycine** in 900 mL ddH_2O. Adjust pH to 2 with **HCl**. Bring to 1 L with ddH_2O. Add 1 mL **Tween-20**.
11. Buffered glycerol with anti-fading
    Stir 4g **n-propyl gallate** into 80 mL **glycerol** overnight at room temperature under dark. Add 5 mL **1M Tris pH 9.5**, 15 mL ddH_20 to make 100 mL buffered 80% glycerol.

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.

TSA Synthesis

Prepare stock solutions immediately before use. DO NOT STORE these solutions since humidity caught up during storage would hamper cross-linking reaction. Also make sure that N,N-dimethylformamide (DMF) is fresh from sealed container to avoid humidity contamination. Anhydrous DMF (Sigma 227056) is recommended for this purpose. For best results, carry out synthesis reaction in low humidity environment.

- 10 mg tyramine (Sigma T2879; MW 173.64) in 990 µL DMF + 10 µL triethylamine (Sigma T0886)
- 10 mg amine-relative fluorophore/hapten in 1 mL DMF

Some fluorophores/haptens that can be used for synthesizing labeled tyramide are given in Table 1 below; this list is non-exclusive.

Mix tyramine solution and amine-reactive fluorophore/hapten at a 1:1.1 molar ratio. Incubate the mixture in dark for at least 2 hours. See Table 1 for specific amounts used for each fluorophore/hapten.

Add an appropriate amount of ethanol to the mixture so the final concentration of fluorophore/hapten in the stock solution is 5 mg/mL.

Mix well, and aliquot. Store the aliquots in -20°C.

Table 1 Reaction setups for TSA synthesis

Fluorophore/hapten (M.W.)	Catalog #/pack size	DMF for fluorophore/hapten	Tyramine working solution	Final EtOH addition
NHS-Biotin (341.38)	Pierce 20217 / 100 mg	10 mL	5.6 mL	4.4 mL
NHS-Rhodamine (528)	Pierce 46406 / 25 mg	2.5 mL	0.9 mL	1.6 mL
NHS-Fluorescein (473.4)	Pierce 46410 / 100 mg	10 mL	4.0 mL	6 mL
DyLight 633 NHS Ester (1066)	Pierce 46414 / 1 mg	0.1 mL	0.018 mL	0.082 mL

Volume of tyramine working solution = (Volume of fluorophore/hapten solution) X 173.64 X 1.1 / (MW of florophore/hapten)