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Hydroides in-situ hybridization protocol
To visualize gene expression in different developmental stages of *Hydroides elegans.*

Hydroides in-situ **hybridization protocol**

**Credit to Dr. Elaine Seaver for developing this protocol for Capitella, and Dr. Brian Nedved and Dr. Audrey Asahina for modifying and adjusting it in Hydroides.*

Use RNase-free equipment and solutions through hybridization step. All washes are 500 µl for 5 min. at RT on nutator unless otherwise stated. To avoid losing or drying out larvae, leave 100-200 µl in well between washes. Make solutions fresh.

-DAY 1-

Pretreatment

- Look at larvae under scope to ensure integrity.
- Transfer larvae (>100 per well) to a 24 well dish (in MeOH)
- For washes, when always pipette gently on side of well.
- Rehydrate, each wash 500µl (5 min each): 60% MeOH/40% PTw
  30% MeOH/70% PTw
  4x PTw washes
- Look at larvae under scope to ensure integrity.
- Digest with Proteinase-K (0.01 mg/mL in PTw – dilute just prior to use) for 2-5 min (no rocker).
- Stop digestion by carefully pipetting 2 x PTw + 2 mg/mL glycine washes. Until re-fixation the animals can be fragile, so do not pipet liquid directly onto them.
- Look at larvae under scope to ensure integrity.
- Wash 1 x in 1% triethanolamine in PTw with 1.5 µl acetic anhydride added per 500µl. Make sure the acetic anhydride is in solution and work quickly since its efficacy goes down with time. Wash 1 x in 1% triethanolamine in PTw with 3.0 µl acetic anhydride per 500 µl added.
- Wash 2 x in PTw.
- Refix in 3.7% formaldehyde in PTw for 30-60 min at RT.
- Wash 5 x in PTw.
- Look at larvae under scope to ensure integrity. Heat embryos (still in PTw) at 80°C for 10 min to kill endogenous alkaline phosphatase activity.

Prehybe

- Remove as much liquid as possible, wash in 500 µl hybe buffer (store at –20°C, heat to dissolve) - for 10 min at RT.
- Remove liquid - add 500 µl pre-heated hybe buffer. Place at hybe temp (**hybe temp=65°C**) overnight in tupperware with diH2O dampened towels with lid on but cracked at corner and parafilm 24 well plate. To reduce stringency, use hybe temp=60°C

-DAY 2-

- Look at larvae under scope to ensure integrity.

Hybe

- Dilute probe to a final concentration of 0.05-3.0 ng/µl (usually 1.0 ng/µl)* in hybe solution (dig-labeled probe should be stored as a 50 ng/µl stock in hybe buffer at -20°C).
- For tubulin control, use 0.1ng/µl.
- Denature probe at 80-90°C max for 10 min.
- Make a master mix, remove prehybe, and add probe to each well. Parafilm 24 well plate. Place in tupperware with diH2O dampened towels with lid on but cracked at corner. Hybridize ≥48 hours at hybe temp (Day 3,4)

-DAY 4-

- Remove probe (Can be reused 4-5 times depending on probe. Store at –20°C.)
- Wash 1 x for 5 min and 1 x for 20 min with hybe buffer at hybe temp. (Do not forget to pre-warm hybe buffer, 2XSSC solutions and the 0.05xSSC – do not warm the 0.05xSSC + PTw solutions)

- Wash:
  - 10 min in 75% hybe + 25% 2X SSC at hybe temp (SSC pH 7)
  - 10 min in 50% hybe + 50% 2X SSC at hybe temp
  - 10 min in 25% hybe + 75% 2X SSC at hybe temp
  - 10 min in 100% 2X SSC at hybe temp

  - gloves no longer required -

  - 2x 30 min in 0.05X SSC at hybe temp (pre-warm 0.05X SSC-hybe temp)*
  - 5 min in 75% 0.05X SSC + 25% PTw at RT*
  - 5 min in 50% 0.05X SSC + 50% PTw at RT*
  - 5 min in 25% 0.05X SSC + 75% PTw at RT*
  - 5 min in 100% PTw at RT
  - *To reduce stringency, use 1-2X SSC.

Visualization of Probe

- Wash 5 x with PBT (Store at 4°C.)
- Block in Boehringer-Mannheim Blocking buffer for 1 hour at RT – make fresh! (dilute autoclaved 10X block buffer to 1X with filter sterilized or autoclaved maleic acid buffer: 100 mM maleic acid, 150 mM NaCl, pH 7.5).
- Incubate with Boehringer-Mannheim anti-Dig/AP (diluted in blocking buffer to 1:5000) at 4°C overnight on rocker.

-DAY 5-

- On white background, tilted, with light from sides, observe larvae by eye and under scope.
- Wash 7-8 x for 10 min in PBT. Before last wash, make up AP buffer (Make AP buffer for approx. 7 washes/well). (20% Tween is made fresh as well).
- Rinse 1 x quickly in 800µl AP buffer without $MgCl_2$. The first will be cloudy and clears over washes. (The initial wash is to prevent precipitation).
- Rinse 2 x quickly in 800µl AP buffer.
- Wash 2 x with 500µl AP buffer on rocker for 5'.
- Develop in AP substrate solution* (make fresh - add 4.4 µl of 75mg/mL NBT and then 8.25 µl of 20mg/mL BCIP per mL of AP buffer) at RT in dark (foil). Add 500µl per well. Place on rocker covered.
  *<u>Note</u>: Once AP solution is added, pipet up and down to break up clumps or use eyelash brush to make sure larvae don't stick to the bottom of the well.
- Monitor color development
  - -Record time started.
  - -Change AP substrate solution when it turns from yellow to pink to replace enzyme.
  - -Look for blue/purple staining. Pull out larvae already stained purple.
  - -Continue monitoring: Can also develop slower at 4°C or faster at 37°C.
- Stop color reaction by washing 5 x with PTw. Keep in PTw (at 4°C) for ≥ 1 day.

-DAY 6-

- Store in 80% glycerol in 1X PBS in the dark at 4°C wrapped in parafilm and covered in aluminum foil.
- Animals can be mounted in 80% glycerol in PBS

Solutions

Hybe Buffer (40 mL) Add in order!	ADD	[FINAL]
Formamide	20 mL	50%
20x SSC (pH 4.5)	10 mL	5x
20 mg/mL heparin	0.1 mL	50 μg/mL
20% Tween-20	0.2 mL	0.1%
10% SDS	4.0 mL	1.0%
10 mg/mL Salmon Sperm DNA (*boil!)	0.2 mL	50 μg/mL
dH_2O (DEPC)	5.5 mL	

*must heat at 100°C for ~5-10 min, then can ice-shock for ~2 min before adding to hybe → store at –20°C (Hybe buffer is stored at–20°C and should be heated to hybe temp for use)

10x PBS = 18.6 mM NaH_2PO_4 (2.23 g NaH_2PO_4 per liter dH_2O)
84.1 mM Na_2HPO_4 (11.94 g Na_2HPO_4 per liter dH_2O)
1,750 mM NaCl (102.2 g NaCl per liter dH_2O)

Mix phosphates in about 800 mL of dH_2O for a 1.0 L volume. Check pH. It should be 7.4 ± 0.4. If more than 0.4 off, start over. Otherwise adjust pH to 7.4 with NaOH or HCl. Add the NaCl and rest of dH_2O.

PTw = 1x PBS + 0.1% Tween-20 detergent - filter sterilize

PBT = 1x PBS + 0.2% Triton X-100 + 0.1% BSA - filter sterilize (store at 4°C)

20x SSC = 0.3 M Na citrate + 3 M NaCl
(for 1 L, add 175.3 g NaCl + 88.2 g Na citrate, pH to 7.0 with HCl, add DEPC, and autoclave)

Alkaline Phosphatase buffer (50mL)		ADD	[FINAL]
	dH_2O	36.25 mL	
1 M	NaCl	5.0 mL	100 mM
1 M	$MgCl_2$	2.5 mL	50 mM
1 M	Tris, pH 9.5	5.0 mL	100 mM
20%	Tween-20 (make fresh)	1.25 mL	0.5%

(Prepare AP buffer just prior to use. The solution will become cloudy after a few hours and will no longer work for the enzymatic reaction.)

AP Substrate Solution

To AP buffer, add 4.4 μl/ml NBT (stock: 75 mg/mL in 70% dimethyl formamide: 30% water) and then 8.25 μl/mL BCIP (stock: 20 mg/mL in dimethyl formamide). Keep this solution in the dark.

Maleic Acid Buffer (500 mL)

Bring 5.804g maleic acid ($C_4H_4O_4$) and 4.383g NaCl up to 500mL (100mM $C_4H_4O_4$, 150mM NaCl). Add concentrated NaOH until pH 7.5. Filter sterilize and store at 4°C.

Product Information

Product	Vendor	Catalog Number
anti-DIG/AP	Roche	11 093 274 910
BCIP	US Biological	B0800
Blocking Reagent	Roche	10057177103
NBT	US Biological	N2585
Proteinase-K	GibcoBRL (Invitrogen)	25530-049
Salmon Testes gDNA	Sigma	D 9156