

Formaldehyde-based Whole-Mount In Situ Hybridization Methodology for the Planarian *Schmidtea mediterranea*

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Animal Preparation

Note: Unless noted at particular steps, intact planarians may be nutated/rocked while early regenerating fragments (up to day 2) should be slowly rocked.

Day 1 (kill, remove mucus, fix, reduce/permeabilize, dehydrate, bleach)

- 1) Asexual planarians of a length between 2 and 4 mm were starved for one week and transferred either into 1.5ml Eppendorf-tubes (for processing up to 20 worms) or in 15ml Falcon tubes (for processing up to 200 worms).
- 2) Planarian water was replaced with **5% NAC solution**, 10min, room temperature (RT)

Notes:

* 1ml in 1.5ml Eppendorf tubes or 10ml in 15ml Falcon tubes

* NAC is a mucolytic reagent that both kills the worms and removes their mucus

- 3) NAC was replaced with **4% Fixative**, 15-20min, RT
- 4) Fixative was removed and worms were rinsed 2X 5min with **PBSTx**
- 5) PBSTx was replaced with preheated **Reduction solution**, 10min, 37°C

Notes:

* Reduction was carried out in a water bath with intermittent gentle agitation (specimens are fragile at this step)

* Reduction aids with permeabilization to allow probe penetration of neck region

- 6) After removal of Reduction solution, worms were rinsed 2X 5min with PBSTx
- 7) PBSTx was replaced with **50% Methanol solution**, 5min, RT

Note:

* Ethanol may be substituted for the Methanol solutions listed in Steps 7-12, yet this parameter was not systematically tested. We did find that long term storage in methanol may decrease overall signal detection.

- 8) 50% Methanol solution was replaced with 100% Methanol, 5min at RT, and then transferred to -20°C for ≥ 1 hour

Note:

* Specimens can be stored at -20°C in MeOH for up to several months

When ready (bleach)

- 9) Methanol was replaced with **6% Bleach solution**, under direct light, overnight, RT

Note: This step removes pigment from the animal and increases permeabilization to help with visualization of the signal

- 10) 6% Bleach solution was removed and specimens were rinsed twice with 100% Methanol

Note: Specimens were then returned to -20°C or used immediately

Day 2 (rehydrate, proteinase K, post-fix, hybridization)

- 11) 100% Methanol was replaced with 50% Methanol solution, 5min, RT
- 12) 50% Methanol solution as replaced with PBSTx, 5min, RT
- 13) PBSTx was replaced with **Proteinase K solution**, 10min, RT
- 14) Proteinase K solution was replaced with 4% fixative, 10min, RT
- 15) Fixative was removed and specimens were rinsed 2X with PBSTx

Hybridization:

Note: When using an in situ robot (Intavis), specimens were added to robot for the hybridization steps

- 16) Wash in 1:1 (PBSTx:**PreHyb**), 10min, RT

- 17) 1:1 mix was replaced with Prehyb, 2hrs, 56°C
- 18) Prehyb was replaced with **Riboprobe mix**, and hybridized >16hrs at 56°C

Note:

* Digoxigenin (DIG) labeled riboprobes were used for all specimens developed with NBT/BCIP

Day 3 (washing and antibody incubation)

Note: You do not need take precautions against RNase-contamination after hybridization because RNases from your hands and bacteria etc. are generally single-stranded RNases and will not cleave dsRNA. In fact, some WISH protocols treat with RNase post-hybridization to cleave unbound probe.

- 19) **Riboprobe mix** was removed (saved at -20°C) and animals were washed with the following times and solutions **preheated** to 56°C:
 - 2 x 30min 2xSSC + 0.1% Triton-X
 - 2 x 30min 0.2xSSC + 0.1% Triton-X
 - 2 x 30min 0.05xSSC + 0.1% Triton-X
- 20) Specimens were then allowed to return to RT and washed with **MABT** 2X 10min RT

Antibody incubation and development:

Note: If using an in situ robot, specimens were transferred to 24 well plates, each step was carried out with nutating or rocking

- 21) Specimens were transferred to 24-well plastic plates, and solution replaced with **Block solution**, 1-2 hrs. at RT
- 22) Block solution was replaced with **Antibody solution**, overnight at RT

Day 4 (antibody washes and development)

- 23) Antibody solution was removed and specimens were rinsed with MABT
Note: Antibody solution may be recovered and used at least 2 more times over the course of a couple weeks
- 24) Specimens were then rinsed at least 6 more times with MABT, 20min each
- 25) MABT was replaced with **AP buffer**, 10min, RT
- 26) AP buffer was replaced with **Development buffer** and placed in the dark
- 27) Rate and extent of probe development was monitored under a dissection microscope and stopped once an optimal signal-to-background ratio was reached
Notes:
 - * Time of development ranges from ~20min to >8hrs on a probe-by-probe basis
 - * Development taking longer than 3-5hrs was continued overnight at 4°C
- 28) Development was stopped by replacing Development buffer with PBSTx
- 29) Specimens were post-fixed with 4% Fixative, 10min, RT
- 30) 4% Fixative was removed and specimens were rinsed with PBSTx
- 31) PBSTx was replaced with 100 % Ethanol, ~20min, RT
Notes:
 - * This step removes non-specific background staining
 - * Specimens remained in ethanol until optimal signal to background ratio was reached and the NBT/BCIP precipitate had turned dark blue
- 32) 100% Ethanol was replaced with PBSTx, 5min, RT
Note: Wait until specimens sink before continuing. This can be aided by adding drops of PBSTx directly on top of floating specimens.
- 33) PBSTx was replaced by **80% Glycerol solution** and stored at 4°C
- 34) Cleared specimens (i.e., no longer floating) were transferred to a slide and mounted under a #1-weight coverslip.

35) Slides were stored at 4°C until viewing or imaging on a Lumar dissecting scope (Zeiss), equipped with an Axiocam digital camera (Zeiss).

Solutions

Animal preparation:

5% NAC solution: 5% N-acetyl cysteine (NAC; Sigma) dissolved in 1X PBS.

Note: 5% NAC solution should be made fresh, but is good at RT for ~1 month

PBSTx: 1X PBS + 0.5% Triton-X 100

4% Fixative: prepared fresh for each experiment by dilution of a 36.5 % formaldehyde stock solution (Formalin; Sigma) into PBSTx

Reduction solution: 50mM DTT, 1% NP-40, 0.5% SDS, in 1X PBS

50% Methanol solution: equal volumes of 1X PBSTx and 100 % Methanol

6 % Bleach solution: 6% H₂O₂ (30% stock; Sigma) in Methanol

Proteinase K solution: 2-5µg/ml Proteinase-K (Invitrogen), in 1X PBSTx

Hybridization:

Hyb: **50-55% De-ionized Formamide** (Roche)

5-10% Dextran Sulfate (Sigma, from 50% stock)

5x SSC

1x Denhardt's solution (Sigma)

100µg/ml Heparin (Sigma from porcine intestine)

1mg/ml yeast torula RNA (Sigma)

1% Tween-20 (Sigma, from 10% stock)

Notes:

* 1% SDS may be used instead of Tween-20, but tends to precipitate during storage at -20°C.

* Fresh de-ionization of formamide appears to affect staining differently in different organisms, but is critical for planarians. We de-ionize 1L of formamide (Roche) with 50g of Bio-Rad AG 501-X8 (D) Resin for 1hr at RT, then filter, aliquot, and store at -80°C.

Prehyb / Wash Hyb: Hyb without the dextran sulfate

Riboprobe mix: 400µl Hyb plus ~400ng (~4µl) of riboprobe

Note: Riboprobe mix was denatured at 72-90°C for 5 minutes, then placed at 56°C prior to use

MABT: 100mM maleic acid, 150mM NaCl, 0.1% Tween-20, pH to 7.5 with NaOH

SSC: 20X SSC stock (Sigma)

Antibody incubation and development:

Blocking solution: 5-10% horse serum in MABT

Note: Head-to-head comparisons indicate that bovine serum albumin (BSA) in the blocking solution is detrimental to signal detection

Antibody solution: Antibody diluted into Blocking solution

Note: anti-DIG-AP (Roche) was used at 1:4000 for all NBT/BCIP experiments

AP buffer: 100mM Tris, pH 9.5; 100mM NaCl; 50mM MgCl₂; 0.1 % Tween-20 brought up to volume with 10% polyvinylalcohol solution (PVA; Sigma P8136). This was prepared fresh prior to every experiment from stocks of 1M Tris, pH 9.5; 5M NaCl; 1M MgCl₂; 10% tween-20. The PVA solution is a 10% w/v stock in H₂O stored at RT.

Note: MgCl₂ is not necessary and may increase background

Development buffer: Freshly made AP buffer with 4.5µl/ml NBT (Roche) and 4.5µl/ml BCIP (Roche)

Note: PVA greatly increases AP activity and helps the most for weak probes.

80% Glycerol solution: 80% Glycerol; 10mM Tris, pH 7.5; 1mM EDTA

Riboprobe synthesis

Antisense-riboprobes were synthesized from PCR-templates using either T7 or T3 RNA-polymerase (Promega). Probe synthesis was carried out for 2 h at 37°C using digoxigenin (DIG¹⁵), fluorescein¹⁶, biotin¹⁶, or dinitrophenol (DNP¹⁷), - labeling mix and 400ng of DNA template. DNA was then degraded with DNase (Promega) for 15min at 37°C. Probes were hydrolyzed by adding 60µl of Carbonate Buffer (80mM Na₂CO₃, 53.3mM NaHCO₃; pH 10.2) and incubating at 65°C for 2-3min. The hydrolysis-reaction was quenched with 80µl of Stop solution (200 mM NaAc, pH to 6.0; adjusted with Acetic Acid). Hydrolyzed probes were precipitated by adding 40µg Glycogen (20mg/ml stock solution, Roche) and 400µl ice-cold 100% ethanol, and then centrifuged at high speed at 4°C for 40 minutes. The resulting pellet was dried briefly after complete aspiration of the supernatant and dissolved in 100µl of **Hyb solution**. Riboprobe stocks were stored at -80°C. For use, riboprobes stocks were generally diluted 1:100 or 1:200 into 400-500µl of Hyb solution.

Fluorescent multi-color probe development with antibody epitope detection

Animal preparation and hybridization was carried out as detailed above in the appropriate sections. The protocol only differs during the *Antibody incubation and development* steps and was adapted from the protocols on the weblinks below. Sequential rounds of Tyramide signal amplification were used for fluorescent probe development. FITC-Tyramide and Cy3-Tyramide were synthesized according to the protocols developed by Lance Davidson and Peter Vize, respectively (FITC-Tyramide:

http://www.engr.pitt.edu/ldavidson/fluor_insitu/fluorescent_in_situ.html; Cy3-Tyramide: http://www.xenbase.org/other/static/methods/FISH/Cy3_tyramide.jsp).

Riboprobe-antibodies included anti-DIG-POD (1:500, Roche), anti-fluorescein-POD (1:300, Roche), streptavidin-POD (Roche), or anti-DNP-HRP (1:100, Perkin-Elmer). After the initial riboprobe-antibody incubation, specimens were washed 6x for 20min in MABT and subsequently 1x 30min in PBST1 (PBSTx + 10mM Imidazole). Specimens were pre-incubated for 30 minutes in 400µl of the appropriate Tyramide dilution (FITC-Tyramide: ~1:1000; Cy3-Tyramide: ~1:500; dilutions in PBST1). Development was initiated by adding H₂O₂ to a final concentration of 0.002-0.015 % (H₂O₂ should be freshly diluted in PBST1 from a 30% stock) and allowed to proceed for 45 minutes under constant agitation on a shaker in the dark. Specimens were rinsed 2x 5 minutes in PBSTx, remaining peroxidase-activity was quenched by incubating for 45 minutes in 1% H₂O₂ in PBSTx, followed by 4 rinses with MABT. Incubation with the second riboprobe-antibody (directed against the second riboprobe) and subsequent detection were carried out exactly as above, except that the blocking step was omitted.

Non-riboprobe antibody staining was performed during the riboprobe-antibody steps above. Specifically, after incubating specimens with the first riboprobe-antibody (anti-DIG-POD) for 4 hours at RT, washes were performed with Blocking solution. Specimens were incubated with VC-1 antibody (diluted 1:10,000 in **Antibody solution**) overnight at RT. Following washes with MABT, the first riboprobe was developed and the reaction quenched. After incubation with the second riboprobe-antibody for 4hr at RT, specimens were washed in Blocking solution and then incubated overnight at RT with secondary antibody

(anti-mouse-Alexa Fluor 647) for VC-1. After the appropriate washes, the second riboprobe was developed with tyramide signal amplification. Following at least 6x 20 minutes washes with MABT, specimens were mounted in 80 % **Glycerol solution** under #1-weight coverslips and imaged on a Zeiss 510 Live confocal microscope.

WISH on large animals

Disclaimer: This portion of the protocol has not been extensively optimized, yet is based solely on observations made by Otto Guedelhofer performing WISH on specimens longer than 1 cm and wider than 1mm for transplantation experiments. All WISH on larger animals are to be performed by hand. Additionally, for the fixation steps, it is better to have only a few animals per tube as the big animals tend to stick together. Listed below are the changes to specific steps in the optimized WISH protocol that seems to help signal detection in larger animals. All other steps not mentioned remain the same.

Steps changed or modified from the optimized WISH protocol listed above:

Kill in 10% NAC solution for 5 minutes

Note: Higher concentration of NAC required for mucus removal and killing of bigger animals

Fixation in 4% FA for 20-25 minutes

Note: Slightly longer fixation time is required

Permeabilization with 1%SDS (in PBS) for 10 minutes

Note: Extra permeabilization step is required using a 1%SDS solution and is performed before reduction steps listed in step 5

Reduction Step for 20-30 minutes at RT

Note: Reduction step is incubated for a longer time period and at room temperature instead of 37°C

Bleach animals in 6% Bleach Solution for > than 18hrs, if having trouble with incomplete bleaching at this point, replace with fresh Bleach solution and continue incubation for an additional 12hrs.

Permeabilization with Proteinase K (2 or 20 ug/mL, depending animal size) for 20 minutes at RT

Note: Permeabilization step is incubated for a longer time period and with a higher concentration of Proteinase K

Development with NBT/BCIP usually takes 2-3 times as long as in smaller animals!