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Zoo lab: The planarian *Schmidtea mediterranea*

Alejandro Sánchez Alvarado
Howard Hughes Medical Institute
Dept. of Neurobiology & Anatomy
University of Utah School of Medicine
sanchez@neuro.utah.edu
http://planaria.neuro.utah.edu

The following is a brief description of the experimental system accompanied by several activities and protocols appropriate to the scope of this lab. Enclosed you will also find copies of the following papers:

- An under-appreciated classic:

  T.H. Morgan (1898). Experimental Studies of the Regeneration of *Planaria maculata*. *Arch. Entw. Mech. Org.* **7**: 364-397, 1898

- A review on the biological attributes and classical experimental results:

  Reddien, P. W., and Sánchez Alvarado, A. (2004). Fundamentals of planarian regeneration. *Annu Rev Cell Dev Biol* **20**: 725-57.

- A review on why use *S. mediteranea* to study regeneration:

  Alejandro Sánchez Alvarado (2006) Planarian Regeneration: Its End is Its Beginning. *Cell* **124**:241-5

- The first RNAi screen in *S. mediterranea*:

  Reddien PW, Bermange AL, Murfitt KJ, Jennings JR, Sánchez Alvarado A. (2005). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Dev Cell.* **5**: 635-49.

Table of Contents:

I. Overview: the planarian *Schmidtea mediterranea* as a model organism

Planarians are free-living representatives of the phylum Platyhelminthes, a group of some 50,000 species of flatworms. Flatworms are among the simplest bilaterally symmetric animals: they are acoelomates, yet they possess derivatives of all three germ layers organized into complex organ systems. Thus, Platyhelminthes have been thought to occupy an important position in Metazoan evolution. Current models place the Platyhelminthes in a large assemblage of protostome invertebrates, known as the Lophotrochozoa, a sister group to the Ecdysozoa (to which insects and nematodes belong).

Planarians are best known for their capacity to regenerate complete individuals from minuscule body parts, as well as for their ability to “de-grow” when starved. Such extraordinary plasticity in the adult is in direct contrast to the rigidity displayed by currently used invertebrate models such as *Caenorhabditis elegans* and *Drosophila melanogaster*. The difference lies in a population of adult somatic stem cells, called neoblasts, that are distributed throughout the planarian body. Neoblasts are the only mitotically active cells in planarians, and their division progeny generate the 30-40 different cell types found in these organisms. In intact planarians these stem cells replace cells lost to normal physiological turnover; whereas, in amputated animals, they give rise to the regeneration blastema, the structure in which missing tissues are regenerated.

Until the mid-20th Century, planarians were a key model for studying development and regeneration. Yet, as attention shifted towards animals amenable to classical genetic analysis, the use of planarians declined. Recently, however, the successful introduction of cell, molecular, and RNAi techniques in planarians, along with heightened interest in stem-cell biology and the plasticity of the differentiated

state, has re-kindled interest in these fascinating organisms. Part of this renaissance, includes an ongoing Genome sequencing project, being carried out by the University of Washington Genome Sequencing Center in St. Louis, MO (http://genome.wustl.edu/projects/planarian/). Sequencing the ~8 x 10^8 bp genome of the sexual, diploid planarian *S. mediterranea* will provide a vital resource for the development of a unique model to study metazoan evolution, regeneration, and the regulation of pluripotentiality. Mechanistic insights into these basic biological problems will have deep and obvious implications to our understanding on biology and perhaps for the improvement of human health.

II. Suitability of *Schmidtea mediterranea* for experimentation:

Table 1. Key Features of *S. mediterranea*

Biological Traits	
Haploid Chromosome #	4
Genome Size	~8x10^8
Adult Animal Size	
Asexual biotype	1-8 mm
Sexual biotype	1-3 cm
Cellular Organization	Multicellular
Germ layers	3 (triploblastic)
Ploidy	Stable diploid
Generation Time	
Asexual biotype	10 days
Sexual biotype	1 month
Cultivation	Freshwater, aquatic animal; easy to rear, expand, and breed in the lab
Genetic Resources/Tools	
cDNA/EST resources	~93,000
Gene Inactivation	RNAi
Gene Expression	Microarrays and whole-mount *in situ* hybridizations
Immunocytology	20 cell types distinguishable by antibodies
Loss-of-function phenotypes	Growing rapidly. Presently, over 240
Spontaneous mutants	3 thus far in sexual strain
Special Strengths	Asexual and sexual clonal lines developed Inbred line available "Self crossing" by amputation, regeneration, and breeding Regeneration and stem cell animal model Ease of transplantation and surgical manipulations Ability to carry out large-scale gene inactivation screens with RNAi Ability to study embryogenesis and adult regeneration in same species Purification of stem cells by FACS Tissue culture of neurons Extensive classical literature
Weaknesses	Transient transgenesis only; stable transgenesis expected in near future Stem cell culture poorly developed

Databases	EST resource, gene expression, RNAi data and antibody markers resource **http://planaria.neuro.utah.edu**
Number of labs	Ca. 30 in planarians; nearly 400 in platyhelminthes

III. Proposed activities:

a. Amputation of animals: Easiest of all the activities? Perhaps, but what is the smallest fragment you can cut that will survive the night? Follow **Protocol 2** for amputation instructions.

b. Injection of animals: Given the short duration of the lab, this activity aims at getting you to try your hand at injecting planarians. We can use food dye so you can assess your success (or failure thereof) at injecting the innards of planarians. You could also try to inject Hoechst dye or CellTracker Orange and see if you can visualize the results using a fluorescent scope. Follow **Protocol 3** for injections.

c. Observation of RNAi-induced phenotypes: We have prepared live animals for you with the following genes knocked down by feeding them bacteria producing dsRNA. The genes are:
β-catenin – (Accession# ABW97513)
adenomatous polyposis coli (APC) – (Accession# EU130785)
You can consult information on these phenotypes (sequence of gene, immunos, etc.) by going to http:/smedgd.neuro.utah.edu and typing the appropriate accession numbers. These animals can be used for phenotype observation and/or additional experimentation.

d. Tissue Transplantation: We have provided the necessary reagents and tools to perform tissue transplantation from one planaria to another. The method described in **Protocol 4** utilizes cylindrical shaped grafts that can be transplanted into a hole made in a host worm. Whereas correct placement and orientation of the graft in the host causes the tissue to be seamlessly

integrated, incorrect graft placement and/or orientation can induce abnormal organism morphology.

e. Whole mount RNA in situ hybridization (WISH): Planarians are very amendable to identifying the location of transcript expression in intact animals by WISH. We have provided the following ribo-probes that you can use with **Protocol 5** to detect cells expressing the corresponding gene:
smedwi-1 – piwi-1 (neoblast marker)
smed-porcn-1 –porcupine (gut marker)
smed-PC2 – prohormone convertase 2 (nervous system marker)
smed-slit – slit (midline marker)
laminin – laminin (pharynx marker)
smed-sfrp-1 – secreted frizzled receptor 1 (anterior marker)
smed-fz-4 – frizzled (posterior marker)
smed-wnt-5 – wnt 5 (edge marker)
You may *wish* to combine this staining with one of the above activities.

f. Cell Isolation: This one is for the dedicated and curious ones among you. The objective here is to isolate cells and label them with Hoechst such that they can be visualized under the microscope either under Nomarski or by fluorescence of both. Follow **Protocol 6**. Once finished, you should be able to look at the stem cells of *S. mediterranea* among other equally interesting planarian cell types.

IV. Protocol 1: Water Formulation

The *S. mediterranea* asexual clones are maintained in a 1X salt solution prepared in milliQ ddH2O. This solution was originally developed by myself at the Carnegie Institution of Washington, Department of Embryology, and later refined by Dr. Phil Newmark at the University of Illinois Urbana-Champaign (http://www.life.uiuc.edu/newmark/)

The salt concentrations listed below came from a salt analysis of the water in an abandoned fountain in the Park of Montjuic in Barcelona, Spain, where we first collected the animals in 1998. The 1X Montjuic salt solution is prepared as follows:

	1L of 1X stock	2L of 5X stock
1.6 mM NaCl	320 µl 5M stock/L	3.2 mL 5M
1.0 mM CaCl2	1 ml 1M stock/L	10 mL 1M
1.0 mM MgSO4	1 ml 1M stock/L	10 mL 1M
0.1 mM MgCl2	100 µl 1M stock/L	1 mL 1M
0.1 mM KCl	100 µl 1M stock/L	1 mL 1M
1.2 mM NaHCO3	0.1008 g/L	1.008 g
		pH to 7.0 with 2N HCl

V. Protocol 2: Amputation of animals

First, you will need to prepare amputation tools and learn how to set up and use the peltier cooling device (see below)

A. Tools:

1. Take a razor blade and with scissors cut these into small trapezoids with the base being the sharp edge. Attach one of this to a holder.
2. Note: because planarians secrete quite a bit of mucous, it is advisable that you clean your blade with a Kimwipe frequently after cutting.

B. Prepping cutting surface:

Traditionally, planarians have been amputated by cooling the animals down to 4ºC, or by placing them on a cooled surface. Unfortunately, this usually means that you have a limited time under which to do your work as the surfaces/animals eventually warm up to room temperature. To overcome this problem, I have devised a contraption using peltier and a direct current power source that allows one to keep surfaces cold for indefinite periods of time. We will show you how to use these.

1. Prepare cutting surface by cutting a 3x3 cm square of parafilm. Cover the surface area with Kimwipes (~6-10 layers) cut to the same dimensions. Lightly wet these with planarian water and place a black filter paper on top.
2. Place the entire assembly over the peltier cooler and turn on the power supply. Set it to 3-5 volts.
3. Once cooled, place a planarian over the black filter paper and observe under the dissecting microscope.

C. Amputations:

1. Once the planarian(s) are on the peltier, make sure they stop moving. If they are still moving, increase the voltage on the power supply making sure you DO NOT FREEZE THEM.
2. Once immobilized, use your amputation tool to cut the animal as you see fit.
3. Once done lift the black paper and place it over a Petri dish with planarian water. Using a transfer pipette, apply water to the filter until the fragments come off into the Petri dish.

Alejandro Sánchez Alvarado
http://planaria.neuro.utah.edu

VI. Protocol 3: Microinjections

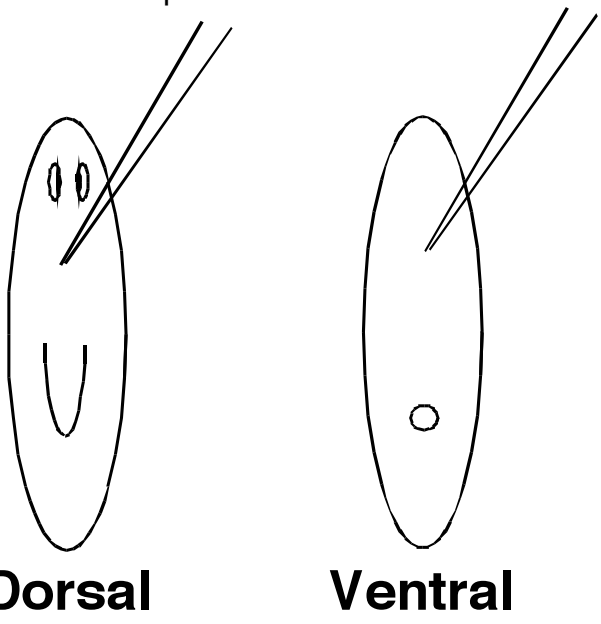
We will use a Drummond Nanoject to deliver fluid into either the parenchyma or the gastrovascular system of the animal. We will use ~33nl of fluid per injection.

Pulling Needles:

Normally, we use an old-fashioned Knopf needle puller. Here, we will use a Sutter puller. We will use PROGRAM 10. I will show you how to use the machine if needed. If you have any questions regarding the use of this machine, please come talk to me before you use it.

Injections:

1. Fill the needle with mineral oil using a syringe.
2. Insert the needle in the injector making sure it is tightly in place. Once secured, you should be able to pull the injector by the needle and the needle should not come off.
3. Place 2-5 µl of the solution to be injected in the center of a small Petri dish.
4. Fill the needle, making sure no bubbles are introduced.
5. Retract the injector using the micromanipulator, and remove the Petri dish.
6. Setup the peltier as described in **Protocol 2**.
7. Place the worm on the black filter paper and align the worm and needle such that the needle penetrates the animal somewhere along the prepharyngeal region. I prefer to inject dorsal side up (photoreceptors visible). Others in my lab prefer to inject the animal ventrally (pharyngeal opening visible). Irrespective of the D/V orientation you should aim for the prepharyngela region as this is the thickest part of the animal.



8. Slowly drive the needle into the body cavity, making sure you do not impale the planarian. Once inside, deliver three pulses of ~33nl each.

9. Remove the needle and transfer the animal to a Petri dish as described in **Protocol 2**, Section C2.
10. Allow the animals to heal for a few minutes. Observe under the dissecting microscope.

*dsRNA is injected in this fashion and a description of this method and the first experimental results obtained can be consulted in:

Sánchez Alvarado, A., and Newmark, P. A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci. USA* 96, 5049-5054.

VII. Protocol 4: Planarian Transplantation by Plug Graft

Otto Guedelhoefer, Alejandro Sánchez Alvarado Laboratory, Neurobiology & Anatomy, University of Utah, Salt Lake City, Utah – otto@neuro.utah.edu
Adapted from: Santos, F.V. (1931) Studies on transplantation in planaria. *Phys Zool* **4**, 111-164.
See also: Kato et. al., Development, 1999.
Updated: May 26, 2010

Materials:

Large (>1cm in length) planaria (*S. mediterranea* sexual strain, asexual strain grown at 10°C, or extremely well fed asexual worms)
Capillary tubes interior diameter (0.75mm) and exterior diameter (0.7mm) size matched and flame bent at 90° angle (FHC catalog #'s 30-30-0 and 30-50-8)
Observation scope
Peltier cooler plate
Parafilm
Kimwipe
Black filter paper (cut into rectangles ~2.5cm x 1.5cm) (Schleicher & Schuell, Ref. No. 10310809)
Whatman #2 filter paper (small disks ~6cm in diameter) (Cat. No. 1002 055)
Whatman #3 filter paper (cut into rectangles ~2cm x 0.5cm) (Cat. No. 1003 185)
Kimwipe (folded and cut into wads ~3cm x 0.5 cm x 4 ply) (Kimberly-Clark Cat. No. 34155)
Cigarette rolling paper (cut into rectangles ~3cm x 2cm) (Zig-Zag, original, with gum-strip removed)
10cm^2 (medium size) Petri dishes (Greiner bio-one Cat. No. 628161)
Forceps (FST by DUMONT, INOX #5, two pair)

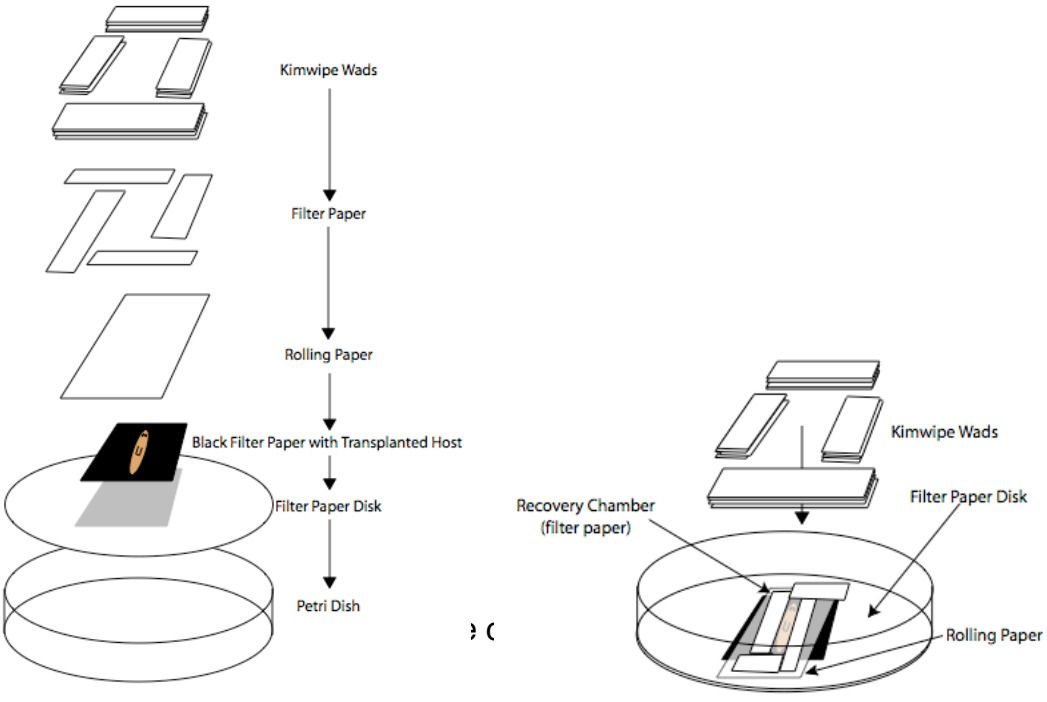
Solutions:

1X planarian water
Chloretone solution: 0.1 - 0.2% chloretone in planarian water
Holtfreter's solution (see attached sheet)

Procedure:

1) Cool all solutions on ice.
2) Cool peltier plate (at 3-5V).
3) Wet the filter paper that lines the recovery dish with ice cold Holtfleter's solution and place the dish on ice (you may wish to prepare all recovery dishes for this set of transplants in this way at this time).
4) Staple a folded Kimwipe to a square of Parafilm, place it on the cooled Peltier plate with the parafilm side down, and wet the Kimwipe with planarian water.
5) Place two small rectangles of black filter paper onto the damp Kimwipe.

6) Soak the donor and host worms in chilled to RT chloretone solution for 5-10 min or until the worms become motionless.
7) Remove the worms from the chloretone solution and rinse them briefly in Holtfreter's solution.
8) Remove the worms from the Holtfreter's solution and place each worm on a separate rectangle of black filter paper.
9) Using a 0.75mm inner diameter capillary tube remove the graft plug from the donor and place it on an out of the way portion of the host.[1]
10) Using a 0.7mm outer diameter capillary tube remove a plug from the host and using forceps position the graft into the hole that is left behind.
11) Transfer the transplanted host on its black filter paper rectangle into the recovery dish.
12) Wet a piece of rolling paper with casein saturated Holtfreter's solution and place it on top of the transplanted host as diagramed below.
13) Soak four pieces of filter paper in casein saturated Holtfleter's solution and encase the transplanted host as diagramed below.
14) Soak four wads of cut Kimwipe in casein saturated Holtfleter's solution and lay them over the filter paper from step 13. Replace lid and put the petri dish on ice.
15) Transfer the donor worm into planarian water to recover, heal, and regenerate.
16) When all transplants are completed, place the transplanted worms into a 10°C incubator overnight (14-20 hrs).
17) The following morning, taking care not to disturb the graft, uncover the worm and transfer it (on its black filter paper) to a petri dish filled with planaria water.
18) Either allow the worm to dislodge itself from the filter paper or gently remove it with forceps.
19) Change the planarian water once every 2-3 days.



Alejandro Sánchez Alvarado
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Modified Holtfreter's Solution
NaCl 3.5 g
$NaHCO_3$ 0.2 g
KCl 0.05 g
$MgSO_4$ stock solution 333 μl
$CaCl_2$ stock solution 333 μl
DI H_20 1 liter

Check pH; should be between 7 and 7.5.

$MgSO_4$ stock solution
$MgSO_4$ 300 g
DI H_20 500 ml

$CaCl_2$ stock solution
$CaCl_2$ 150 g
DI H_20 500 ml

Alejandro Sánchez Alvarado
http://planaria.neuro.utah.edu

VIII. Protocol 5: Formaldehyde-based Whole-Mount In Situ Hybridization

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Animal Preparation

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, 5-10 minutes, room temperature (RT)
3) NAC was replaced with 4% Fixative, 15-20 minutes, RT
4) Fixative was removed and worms were rinsed 1X with PBSTx
5) PBSTx was replaced with preheated Reduction solution, 5-10 minutes, 37°C
6) After removal of Reduction solution, worms were rinsed 1X with PBSTx
7) PBSTx was replaced with 50% Methanol solution, 5-10 minutes, RT
8) 50% Methanol solution was replaced with 100% Methanol, 5-10 minutes at RT, and then transferred to -20°C for ≥ 1 hour

When ready (bleach)

9) Ethanol was replaced with 6% Bleach solution, under direct light, overnight, RT
10) 6% Bleach solution was removed and specimens were rinsed twice with 100% Methanol

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

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

Hybridization:

16) Wash in 1:1 (PBSTx:PreHyb), 10 minutes, 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

Day 3 (washing and antibody incubation)

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 1:1 [Wash hyb: (2xSSC + 0.1% Triton-X)]
    2 x 30min 2xSSC + 0.1% Triton-X
    2 x 30min 0.2xSSC + 0.1% Triton-X
20) Specimens were then allowed to return to RT and washed with MABT 2X 10min RT

Antibody incubation and development:

21) Specimens were transferred to 24-well plastic plates, and solution replaced with Block solution, 1-2 hrs. at RT, or overnight at 4°C
22) Block solution was replaced with Antibody solution, 4hrs at RT, or overnight at 4°C

Day 4 (antibody washes and development)

23) Antibody solution was removed and specimens were rinsed with MABT
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

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
32) 100% Ethanol was replaced with PBSTx, 5min, RT
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 made fresh.
PBSTx: 1X PBS + 0.1-0.5% Triton-X 100
4% Fixative: prepared fresh, dilution of 36.5 % formaldehyde stock (Sigma) in 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% H_2O_2 (30% stock; Sigma) in Methanol
Proteinase K solution: 2µg/ml Proteinase-K (Invitrogen), 0.1% SDS, in 1X PBSTx

Hybridization:

Hyb: **50-55% De-ionized Formamide** (Roche)
5-10% Dextran Sulfate (Sigma, from 50% stock)
5x SSC
1mg/ml yeast torula RNA (Sigma)
1% Tween-20 (Sigma, from 10% stock)
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 priot 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
Antibody solution: Antibody diluted into Blocking solution (1:4000 anti-DIG-AP (Roche))
AP buffer: 100mM Tris, pH 9.5; 100mM NaCl; 50mM $MgCl_2$; 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 $MgCl_2$; 10% tween-20. The PVA solution is a 10% w/v stock in H_2O stored at RT.
Development buffer: Freshly made AP buffer with 4.5µl/ml NBT and 3.5µl/ml BCIP (Roche)
80% Glycerol solution: 80% Glycerol; 10mM Tris, pH 7.5; 1mM EDTA

WISH on large animals

Listed below are suggested changes to specific steps in the optimized WISH protocol that seems to help signal detection in larger animals (longer than 1 cm and wider than 1mm). All other steps not mentioned remain the same.

Kill in 10% NAC solution for 5 minutes

Fixation in 4% FA for 30 minutes

Permeabilization with 1%SDS (in PBS) for 10 minutes prior to reduction (step 5)

Reduction Step for 20 minutes at RT

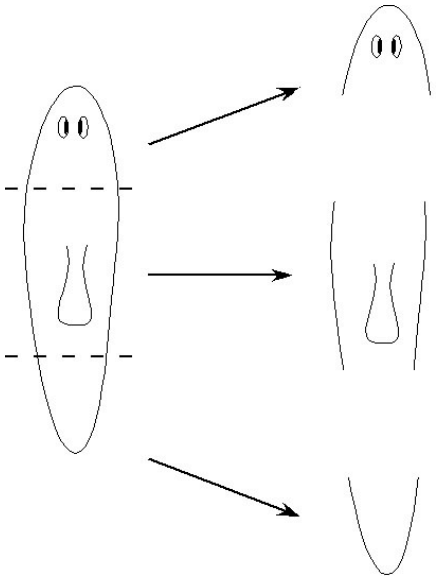
Bleach animals in 6% Bleach Solution for > than 18hrs. If animals are incompletely bleached at this point, replace with fresh Bleach solution and continue incubation

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

Alejandro Sánchez Alvarado
http://planaria.neuro.utah.edu

IX. Protocol 6: Cell Dissociation

1. Place worms in 60cc Petri dish on a cold plate. Remove all planarian H2O.
2. Wash 3x in cold CMF
3. Cut planarians post- and pre-pharyngeally.



4. Dice planaria into as small fragments as practical
5. Wash diced planaria into a 15ml centrifuge tube with 12mls of CMF plus trypsin at 10-20 units (2ul)
6. Rock at RT for 1 hour. Pipet up and down 6-8 times every 15 minutes with a transfer pipet. Pipet firmly, but without making bubbles.
7. After 1hour pipet with transfer pipet again, followed by a P1000 in the same manner.
8. Prepare in advance a 53 micron filter by washing in 10N NaOH, placing into a filter holder, and autoclaving.
9. Pour the planarian cell solution into a 5cc syringe attached to the 53micron filter container and collect the cells as they pass through in a tube. Let the cells drip through the tube rather than pushing them with a plunger. Wash filter with a few mls of CMF.
10. Preferred for tonight's sake: let large fragments sediment, remove supernatant and pass through serial filters.

    Optional: Pellet cells at 250g for 5mon with brake off. Remove supernatant and add fresh CMF to desired final volume.
11. Clean used filters

CMF (Ca/Mg-Free media)

NaH2PO4	400mg/L	KCl	1200mg/L	NaHCO3	800mg/L
NaCl	800mg/L	Glucose	240mg/L	HEPES to 15mM	
BSA to 1%				pH to 7.3	