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Practical lab: The planarian *Schmidtea mediterranea*

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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://smedgd.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 either 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 and see if you can visualize the results using a fluorescent scope. Follow **Protocol 3** for injections.

c. Fluorescence Imaging: We have also fixed and processed for immunohistology several samples for you to look at. In this case, *C. elegans* gene *unc-22* is used as a control. This is an opportunity to demonstrate your newly acquired expertise at capturing fluorescence/bright field images. You can try either confocal microscopy or a regular, mercury-lamp based microscope.

   Unc-22
   sec61 - HE.3.12b (H.22.10b, Accession# AY967531)
   cdc23 - NBE.4.10b (NB.33.12c, Accession# AY967619)

   The antibodies and reagents utilized were:
   primary - H3P (rabbit polyclonal from Upstate Biological)
   secondary - goat anti-rabbit conjugated to HRP tyramide alexa 568

   You can find a detailed fixation and immunohistology protocol in the enclosed paper ***Dev Cell.*** 5**: 635-49.**

d. 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 4**. Once finished, you should be able to look at the stem cells of *S. mediterranea* among other equally interesting planarian cell types.

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

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 shrap edge. Attach one of this to a holder.
2. Note: because planarians secrete quite a bit of mucuous, 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. Joya and I 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 5 volts.
3. Once cooled, place a planarian over the black filter paper and observe under the dissecting microscope.
4.

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.

Protocol 3: Microinjections

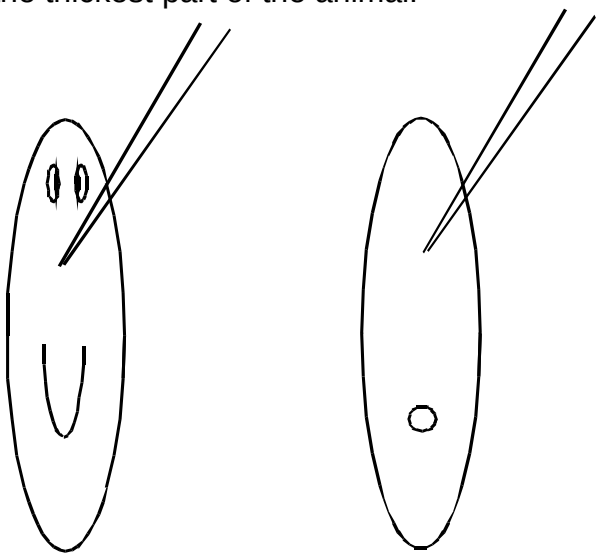
We will use a Drummond Nanoject to deliver fluid into either the parenchyma or the gastrovascular system of the animal. We will use ~33nl of fluid per injection.

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 neddle 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:

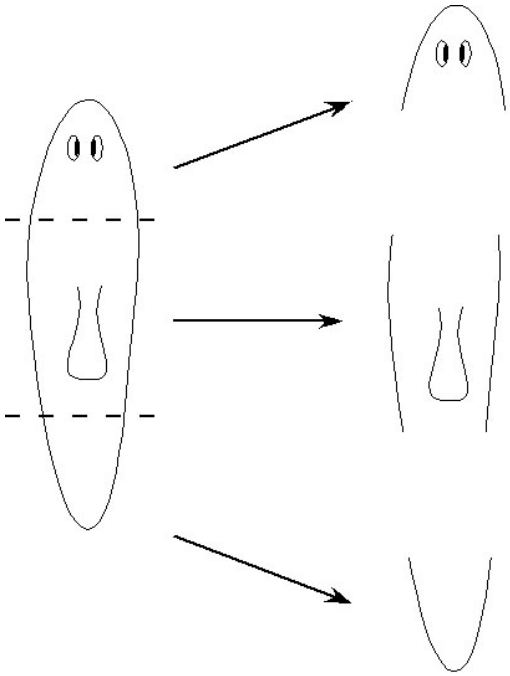
Sánchez Alvarado, A., and Newmark, P. A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci. USA* 96, 5049-5054.

Protocol 4: 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 5min with brake off. Remove supernatant and add fresh CMF to desired final volume.

11. Clean used filters

CMF (Ca/Mg-Free media)

NaH_2PO_4	400mg/L
NaCl	800mg/L
KCl	1200mg/L
$NaHCO_3$	800mg/L
Glucose	240mg/L
BSA	to 1%
HEPES	to 15mM

pH 7.3