Name: Elizabeth Willsey
Host Lab: Kewalo Marine Laboratory (Dr. Michael G. Hadfield)
Dates of Visit: May 2, 2012-September 5, 2012.

Protocols:

1. H. elegans Culture Protocol
2. Settlement Assay Protocol
2. TriReagent RNA Isolation Protocol

1. Protocol for *H. elegans* culture
Goal: to culture competent larvae for experiment

Spawning *Hydroides elegans*

1. Remove 25-30 worms from Vexar.
2. Place worms in medium sized dish.
3. Put dish under dissecting scope and carefully break secondary tube of the adult using forceps.

- This mechanical perturbation should cause release of gametes from individual worms.
- Eggs are reddish orange and should sink to the bottom of the dish.
- Male worms will release large clouds of milky white sperm. Remove males from dish after they have released a small amount of sperm (to minimize polyspermy).
- Continue breaking tubes of adults until all individuals have been examined.

4. Add water to dish and allow embryos to develop for 20-30 minutes.
5. While embryos are developing, determine the density of cells in the *Isocrysis* culture using a haemocytometer.

- Dilute a sub-sample of the culture by 10 before estimating cell density. Larvae of *H. elegans* are fed *Isocrysis* at a density of 60,000 algal cells/ml of larval culture (We have developed a short-cut for determining the volume of *Isocrysis* culture needed to add to the *H. elegans* cultures: **1200 / ave. cell count** from haemocytometer). This volume of algae will be added to the *H. elegans* cultures later.

6. Pass developing embryos of *H. elegans* and debris through a sieve with pore size larger than 74 μm into a 500 ml beaker. This sieve will allow embryos to pass through but should catch the debris. Bring volume of seawater up to 200 ml.
7. Determine the number of embryos in culture by taking four 100 μl samples from the beaker.

- Gently mix the culture by pipetting before the first aliquot is taken (mix just vigorously enough to suspend the sediment off the bottom) and continue to mix between samples.
- Count number of embryos in each aliquot. Get average for the aliquots. Multiple by 10 (embryos/ml) and then multiple by volume of seawater in container (200). This gives an estimate of the total number of embryos in the container.

- We want to add approx $2x10^4$ larvae to each culture beaker. We keep the larvae in 2000 ml of seawater and want to raise them at a density of 10 larvae/ml (2000 ml X 10 larvae/ml = 2 x 10^4 larvae in each culture beaker.)

8. Estimate the number of beakers containing 20 k larvae we can obtain from the 200 ml beaker containing the fertilized embryos. Short cut: divide average embryo count in 100 µl drops by 10. This estimates the number of 2L beakers that will be used.
9. Fill beakers with 1800 ml of seawater, add a portion of the embryos and add the appropriate volume of *Isocrysis* (from step 5).
10. Cover beakers with saran wrap and write date spawned as well as an estimate of time spawned

Larval Culture

Larvae of *H. elegans* attain competency 5-6 days after fertilization if fed *Isocrysis* at a density of 60K cells/ml daily.

Feeding Schedule for *H. elegans*

Day 0: Spawn larvae and add algae to culture
Day 1: Add algae to culture
Day 2: Change water in culture and change culture beaker. Add algae to culture.
Day 3: Change water in culture and change culture beaker. Add algae to culture.
Day 4: Change water in culture and change culture beaker. Add algae to culture.
Day 5: Change water in culture and change culture beaker. Add algae to culture.
Day 6 (if necessary): Change water in culture and change culture beaker.

Changing water and beakers of culture.

1. Fill a clean 2L beaker with 1500-1800 ml of seawater.
2. Add appropriate volume of *Iso*. So that the density of algal cells = 60k cells/ml.
3. Bring larval culture to sink.
4. Pass larvae through a 41 um sieve. The larvae will be trapped in the sieve.
5. Rinse larvae out of sieve and into new culture beaker using squirt bottles filled with seawater.
6. Put saran wrap back onto the culture beakers
7. Refill carboy with seawater (if necessary).

To reserve larvae for experiments: write initials on saran wrap.

2. Protocol for Performing *H. elegans* settlement assays

Goal: to determine the effect of a treatment compound on the settlement and metamorphosis of *H. elegans*.

Settlement Assay Protocol

1. Use stock solutions of chemicals made in distilled water and dilute by a factor of 1/10 in filtered seawater before using for experiment.
2. Use a positive control and a negative control for each experiment. In all experiments, the positive control is a dish of filtered seawater with a bacterial biofilm on a piece of Vexar (biofilm must have developed in seawater for at least 10 days). The negative control in this case is a dish of filtered seawater. For compounds predicted to inhibit metamorphosis, biofilms are added to each experimental treatment after the larvae has been exposed to the compound for one hour.
3. Treatment solutions are added to Petri dishes. Four replicates of each treatment are performed.
4. Larvae competent for experiment (day 6) are added. Approximately 20-150 larvae are added using a pipette to each dish.
5. After one hour, biofilms are added in the positive control treatment. In addition, in experiments where the compound is predicted to inhibit metamorphosis, biofilms are also added to each dish after one hour.
6. For chronic exposure experiments, larvae are incubated overnight in the treatment and after 24 hours, the percentage of larvae to undergo metamorphosis, abnormal metamorphosis, and still unsettled are counted.
7. For pulse experiments, larvae are left in the treatment for either one-, two-, or six-hours and then are washed twice using filtered seawater to remove the treatment compound. A filter on a pipette is used to withdraw the treatment without removing the larvae from the dish. Larvae are counted after 24 hours following the same method as the chronic exposure.

3. Protocol for TriReagent RNA Isolation

Goal: to perform an RNA extraction

Homogenization:

1. Homogenize tissue in 1ml (TriReagent) / 50-100mg of tissue. Add a small volume of Tri reagent first, homogenize, then add the remaider.
2. Incubate the homogenate for 5 minutes at room.

Phase Separation:

3. Add 100µl of BCP (bromochloropropane)/1ml Trireagent, cover samples and shake for 15 seconds.
4. Store mixture for 15 minutes and room temperature.
5. Centrifuge at 12,000g for 15 minutes at 4°C (RNA will be in the upper aqueous phase).

Precipitation:

6. Transfer upper aqueous phase to a clean tube.
7. Add 500µl of Isopropanol/1 ml of Trireagent used (in the beginning) and mix.
8. Store samples at room temperature for 10 minutes (or for higher yields, overnight at -20°C).

9. Centrifuge at 12,000g for eight minutes at 4°C (RNA will form gel-like or white pellet).
10. Perform phenol clean-up. Add phenol 1:1 with RNA solution, vortex, spin down at 12,000g for 15 minutes at 4°C.
11. Add appropriate ammonium acetate, plus isopropanol, precipitate overnight at -20°C

RNA Wash:

12. Remove supernatant and wash RNA pellet by vortexing in 75% ethanol/1 ml Trireagent used, then centrifuge for five minutes at 7,500g at 4°C. (If RNA is on the side of the tube or floats – resediment at 12,000g)
13. Remove ethanol and airdry pellet for three to five minutes.
14. Solubilize in RNASE free water.

DNAse Treatment – (Qiagen RNAse free DNAse set)

1. Assemble Reactions to 1X:
   87.5µl of RNA solution (Dilution of Total RNA)
   10µl of BufferRDD
   2.5µl of DNAse I
2. Incubate at room temperature for ten minutes.
3. Perform phenol clean up. Add phenol 1:1 with RNA solution, vortex, spin down at 12,000g for 15 minutes at 4°C.
4. Add appropriate ammonium acetate, plus isopropanol, precipitate overnight at -20°C
5. Spin down RNA at 12,000g for 15 minutes at 4°C, pull of isopropanol, add 1ml of 75% ethanol, votex, spin for 15 minutes at 4°C.
6. Pull off remaining ethanol, airdry, and solubilize in RNAse free water.

Advantage RT-for-PCR-Kit

Follow protocol guidelines and optimize cDNA synthesis using 1µg of Total RNA.