

1. Tyler Square
2. Host lab: Yu Lab
3. Dates of visit: 19-May through 1-June 2013
4. Raising *Branchiostoma floridae*
5. Rationale and background: to raise healthy amphioxus
6. Protocol:

PROTOCOL FOR GROWING ALGAE AND RAISING AMPHIOXUS

1) Culturing algae

The following steps are applicable to most algal genera, however *Rhodomonas* specifically tends to be quite variable in different environments, and requires some special practices (see below).

For all other cultures, all of the following steps can be carried out at room temperature (RT), at about 3000 to 3500 lux. When choosing light bulbs, it is best to use between 25% and 50% of bulbs designed specifically for growing plants (which have high emission in the 500-600nm range), while the rest should be standard “soft-white” fluorescent bulbs. Avoid bulbs with an orange or red hue, as these emit light that is mostly unusable to plants.

Rhodomonas requires special conditions. In our hands, cultures of this genera do best at 19 degrees C (they do not crash as quickly), though we do grow 10L cultures at RT, these typically are only useable (alive) at or near saturation for around 4 days. We also shade our cultures down to between $\frac{1}{3}$ and $\frac{1}{2}$ of the amount of light input as the other genera receive, as they seem to do better in lower light (for small cultures, we use a paper ‘veil,’ for large cultures we block $\frac{1}{2}$ of the light bulbs with a black piece of foam). This genera also does best with much higher aeration (for small cultures, we use a stir rig on a medium to high setting to create a small vortex in the solution), and its highest density is still far below the other strains outlined here. Although this strain tends to be more difficult to culture, it has a high nutritional value, and it is therefore a very healthy food source (especially for larvae). Linda Holland’s lab has had success growing their larvae without *Rhodomonas*, using *Isochrysis* as a substitute, while Jr Kai Yu’s lab never feeds larvae without adding *Rhodomonas*. We feed this stain to both larvae and adults, and we are currently collecting data on growth rates (of both larvae and adult gonads) to determine the level of its value.

Use sterile methods for all of the below steps.

1.1) Seawater

All seawater should have salinity concentration between 29 and 32 parts per thousand (ppt). If water from the ocean is available, be sure to filter it with at least a .22 micron filter and adjust salinity accordingly. If using artificial sea water (ASW), either use a .22 micron filter, or chemically sterilize the container which you will use for the algae, and keep the salt and DI H₂O

free of contaminants before/while adding. Chemical sterilization with bleach is an option, however the sodium thiosulfate used to inactivate the bleach can alter algal growth (and potentially the ASW buffering mechanism), so we do not use this procedure (for more information, see Creswell, 2010). Autoclaving seawater can also destroy the buffering system found in seawater, so we avoid this as well. It is best to allow large volumes of ASW to sit overnight on medium aeration before using them – this will completely dissolve the salt (especially important for amphioxus). Certain brands of salt are better than others – Instant Ocean (www.instantocean.com, Blacksburg, VA) is commonly thought to contain some sort of chelator (likely EDTA) to assist in its dissolution. This is not advantageous for algae culturing or amphioxus husbandry. We use Crystal Sea Marinemix (www.aquaneering.com, San Diego, CA), which is quite expensive but seems to be very high quality. This tends to leave behind a precipitate in our larger volume carboys, which is easily dissolved away with a 15 mM solution of HCl.

1.2) Algal medium

We use F/2 medium for all algal strains. A recipe for this growth medium can be found here: <https://ncma.bigelow.org/node/79>. As recommended on the website, silica is omitted unless we are growing diatoms.

To create F/2 medium plates, mix 9g of Bacto-Agar with 1L ASW, and heat until mostly dissolved (to avoid autoclaving, we use a microwave). Once cool, add F/2 ingredients, mix, pour into plates (preferably at least 1 cm thick), and let settle.

1.3) Algae Growth

Here I outline how to grow up to a 20L culture of algae, though this could be stepped up or down depending on your specific needs (i.e. how many animals need food). It is advised that this protocol be started from the beginning every 6 months to ensure healthy algae strains; the .5L or 2L stages can be used as a ‘reservoir’ to inoculate larger amounts more frequently.

To create a plate of algae, add 1 mL of liquid F/2 medium, spread, and let soak for 3-5 minutes. With your alga of choice, pick a colony from a plate, break it up with a spreader, and spread, or add one mL of liquid algae culture, and spread. Place face-up at around 3000 lux until the pool of liquid is soaked in/evaporated (usually two days), and flip upside down after this occurs. Algal growth should be apparent within 4 to 5 days after inoculation.

Using a sterilized loop, pick an algae colony and whisk into 50 mL of sterilized F/2 medium. Let sit in beaker (no aeration), covered with foil (or the like), swirling approx. daily until absorption reaches the minimum saturation for your given genera specified in table 1 at the end of this protocol (these respective absorptions will be referred to as ‘high density’ for the remainder of

this protocol). Once one is familiar with the color/density of these genera, it works fine to 'eyeball' their density before moving on to the next step (cultures top out and will remain stable for at least a few days).

Once the 50 mL culture reaches high density, use all of it to inoculate a .5L culture in a flask (a 1:10 dilution) covered with foil (or the like). Aerate lightly or use a stir bar on a medium to low setting to increase the evaporation/dissolution of gasses into the culture, but do not use so much aeration that the solution evaporates quickly. If the solution evaporates noticeably, add sterilized DI water to bring it back up to its original volume.

Once the .5L culture reaches high density, use 200 mL to inoculate a 2L culture (again, a 1:10 dilution). Aerate such that bubbles are constantly flowing, and the algae is circulated lightly (analogous to a simmer/light boil).

When this 2L culture reaches high density, you can use it to inoculate a large carboy (15-20L), again using a 1:10 dilution to inoculate. Aerate such that the solution is well circulated (analogous to a boil). Use as needed to feed animals. If a larger volume culture is desired, simply use a 1:10 inoculation volume, and aerate such that the solution is well-circulated.

Note that after the algal culture reaches its maximum density, it will die within the next 3 to 7 days (this depends on strain, temperature, locality, light, etc.). Adult amphioxus can eat dead algae, but we don't recommend feeding them dead algae regularly (or at all for larvae) as the dead algae will pollute the water and can lead to widespread death.

2) Amphioxus Husbandry

Amphioxus are filter feeders. With this in mind, the amount of algae added per animal, as well as the density of the algae, are both important factors. Changing the amount of water the animals are housed in can drastically increase or decrease how well they are able to feed on a given amount of algae.

2.1) Larval Husbandry

Culturing larvae at RT is sufficient, but faster development (up to about 50% faster) has been observed at 30° C (Yu and Holland, 2009).

The seawater used to keep amphioxus is the same as outlined in section 1.1 above, except for the addition of antibiotics. Adding antibiotics to seawater is recommended for amphioxus larvae. This prevents a biofilm from forming at the surface of the water (which greatly reduces the dissolution of oxygen into the water), and reduces the amount of effort needed to keep animals healthy. If desired, add .1mg/mL streptomycin, and .5 mg/mL penicillin G. If no

antibiotics are to be used, daily water changes are suggested to avoid suffocation and widespread death.

Since larvae swim towards light until they are a few weeks old, it is best to keep them in the dark, or with lighting beneath them to avoid their getting caught in the air/water interface and drying out (this is especially important for animals less than 20 days old). Animals can be kept in small bowls or petri dishes, initially at a maximum of 5 animals per mL of sea water. We typically keep 500 – 1000 young larvae in around .5L of ASW (1-2 animals per mL), though this volume changes between water changes (i.e. they start in 500 mL, but the volume increases with feeding every day until a water change, when they return to .5L again – see below for details on water changes). At 2-3 weeks of age, the volume of water per animal should be increased by 100-200%. At this point, we typically keep 500-1000 animals in 1 to 2 liters of ASW.

It is best to change the water and dish your larvae are kept in once every 2 days until one week of age, and once every 3-4 days until they are 40 days old (past 40 days we will consider them juveniles, although we have observed metamorphosis being delayed until as late as 70 days). The dead, uneaten algae will pollute the water rapidly if left alone. For small amounts of larvae, swirling them in a petri dish can concentrate them in the middle for removal with a pipettor/eyedropper, but for larger amounts, a filter made from a 50 mL tube and 20 micron mesh is suggested to concentrate them (later, you can use larger sizes of mesh when the animals are larger – by 2 weeks of age, you can switch to 70 micron mesh). When changing the water and dish, removal of deformed animals, unfertilized eggs, dead algae, and feces as much as possible is recommended. Swirling the dish also aids in this. To remove deformed animals, swirl everything to the center of the dish, apply light to the edges of the dish (which healthy animals will swim towards), and wait 5-10 minutes for the healthy animals to swim to the periphery. Remove the middle contents, (which will still have some healthy animals), place these into a new dish, and repeat as necessary to select for the deformed and/or dead animals for their disposal. Transferring some waste when doing a water change is not detrimental to their health, but removing as much as possible favors healthy development.

Once the majority of the larvae reach 7 gill slits (2-3 weeks of age), they can be put on sterilized sand. We have seen success using both commercially available silica sand, and fine grain beach sand collected from the ocean. Whether or not sand aids in amphioxus feeding is not yet known (Linda Holland's lab never uses sand, and has had successful spawnings).

2.1.1) Feeding Larvae

Amphioxus larvae need to eat live algae starting from the time their digestive systems begin working (about 36h at RT, about 28h at 30 degrees C). If their feeding is disrupted long enough

(as little as one day for very young larvae), their guts shut down, and the animal will be destined for death within a few weeks. Therefore, it is safest to constantly give them small amounts of food from the time their mouths form until metamorphosis.

The best feeding strategy is to check your larvae periodically to see how much food is in their guts, and how much uneaten food is in their water, and modulate their feeding based on these criteria. With this in mind, the following feeding schedule is approximate; if followed stringently, animals will likely be fed successfully, but the best way to be sure you are feeding adequately is to observe the contents of their stomachs under a microscope (a light source beneath the animals greatly aids in this). Their ilium (where the spinning contents of their hindgut are housed) should always have food in it, and it is best if there is also some food in their gut directly anterior to this (not spinning, and larger in diameter). If they are given more food than needed, their water can become easily polluted. If there is ever uneaten food 24 hours after a feeding, delay the next feeding and/or delay the next increase in feeding (see below). It may also be appropriate to do an immediate water change to remove the uneaten algae if there are very high amounts (i.e. the water is considerably brown). After one week of age, they are more resilient, and can go without eating for up to 2 days if necessary (although this is not encouraged). Be certain to look for the contents inside their guts, not the outer layer of pigment that builds up over time.

From the time their mouths open until one week of age, feed 1 mL of 20 micron filtered 1:1 *Rhodomonas/Isochrysis* (by cell count) per 200 animals (which we house in around 60 mL of ASW) daily. If your cultures behave as ours do, this should be about 4:1 *Rhodomonas/Isochrysis* by volume (again, *Rhodomonas* is the most dilute algae at saturation). This ratio is optimal for larvae, though feeding only *Rhodomonas* or *Isochrysis* seems sufficient as well. Starting at one week, increase the amount of feed by around 50% (rounding is fine) every 2 days until they are 40 days old. This should mean that at the 40 day mark, the animals are being fed about 3 mL of the above mix per animal (656 mL/200 larvae). This will obviously require a larger bowl, and the water they are in will be composed largely of the algae solution between water changes.

2.2) Juvenile and Adult Husbandry

All animals older than 40 days are housed in tubs with 8-12L of ASW, with 2-10 cm of sand (amount of sand is adjusted to the average length of the animals in a tank; again, Linda Hilland's lab does not use sand at all). Animals can be kept at RT, although preliminary data indicates that faster gonad growth in the adults occurs at 19 degrees C (Jr Kai Yu, personal communication). A full water change with sand rinsing is performed weekly (below).

2.2.1) Cleaning Tub

To do this, shake/swirl the tub to sift the animals from the sand, and pour them out with the water. Rinse with seawater and repeat until all animals are removed. Then rinse the sand out with DI water, stirring/agitating vigorously to remove waste. Once the rinse water no longer turns brown/has waste in it, rinse the sand with ASW two times, then replace 8-12 L of ASW into the tank, and catch the animals with a net (or the like) and put them back into the cleaned tank.

The number of animals housed in one tank described above depends on their size. For 40+ day old juveniles, up to 4000 per tank is ok. For every centimeter they grow, cut the number of animals in one tank down by about half until they reach 5 cm in length (which will be the maximum size of some individuals). So, assuming they are 1-2 cm in length when added to the tank, they should be at a density of 200-300 per tank by the time they reach 5 cm. This end density will be sufficient for the rest of their lives (which is thought to be up to 4 years).

If using silica sand, the fully grown adults are large enough to be filtered out with netting on an insert in the tank, which greatly reduces the amount of time needed to do a tank cleaning.

2.2.2) Juvenile and Adult Feeding

Feed each 8-12L tub twice daily with 1.5L of 20 micron filtered 3:1 *Isochrysis:Tetraselmis* (by cell count, which in our hands nearly corresponds to volume), spaced out by at least 6 hours. Jr Kai Yu's lab uses sometimes changes *Isochrysis* for a diatom, *Chaetoceros*, and *Tetraselmis* for another green alga, *Chorella*. Our lab has observed gonad growth on feeding regimens consisting of 3:1 *Isochrysis:Tetraselmis*, as well as 3:1 *Isochrysis:Rhodomonas* (by volume, not cell count; unpublished data). The food:animal ratio will increase when you reduce the number of animals per tank based on their growth. Participating labs are still collecting data on the relationship between the type of food and gonad growth, though the amount of food seems to be a bigger determining factor here (so long as their diet is composed mostly of either *Chaetoceros* or *Isochrysis*). We remove water from each tank daily to avoid overflow, which also aids in keeping their water clean.

Genera	Optical density at 750 nm for a "high density" culture
<i>Isochrysis</i>	.115
<i>Tetraselmis</i>	.125
<i>Rhodomonas</i>	.045

Table 1: Absorption at 740 nm of various algal genera at high density. These numbers are referred to as "high density" throughout this protocol. These measurements are taken in a spectrophotometer set to 750 nm, which measures diffracted light. These are not the absorption measurements at highest saturation – only those necessary to continue to the next step.

References

Yu JK, Holland LZ. Cephalochordates (amphioxus or lancelets): a model for understanding the evolution of chordate characters. *Cold Spring Harb Protoc.* 2009 Sep;2009(9):pdb.emo130. doi: 10.1101/pdb.emo130. PubMed PMID: 20147258.

Creswell, LeRoy. *Phytoplankton Culture for Aquaculture Feed.* [Stoneville, Miss.]: Southern Regional Aquaculture Center, 2010.