Testing Novel Culturing Systems to Promote the Proliferation *in vitro* of a marine invertebrate species, *Botryllus schlosseri*

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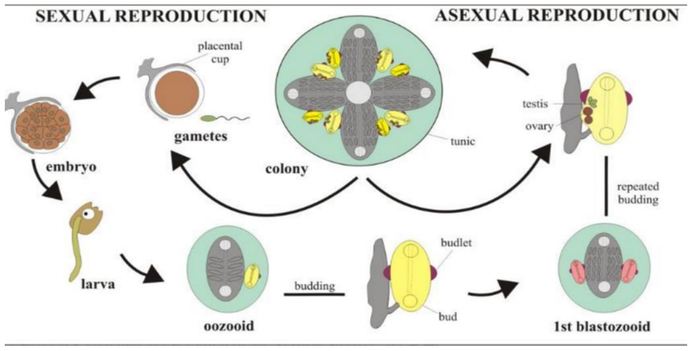
Introduction

Immortalized cell lines may be applicable to further research on stem cell regeneration, allopathic medicine and the beauty industry. Research to establish an immortalized marine invertebrate cell line has been under development since the 1970's. Marine invertebrates are a major source of biomaterials and bioactive natural products that can find applications in various areas of study

Botryllus schlosseri is of particular interest for its regenerative capabilities. *B. schlosseri* is a colonial ascidian tunicate, composed of several zooids that form colonies with shared vasculature. Each generation of primary and secondary zooids grows to replace the previous zooids in a process known as takeover, which occurs every two weeks, but may also perform whole-body regeneration in response to injury. Stem cells from a zooid or vasculature can regenerate an entire colony. Despite decades of effort, no immortalized cell lines for marine invertebrates like *B. schlosseri* have been created.

To better understand the conditions needed to promote the survival and continuous proliferation of *B. schlosseri* stem cells, we isolated zooids using microdissection techniques and cultured these cells in five different media types with varied composition. An optimized tunicate culture medium was supplemented with antibiotics like penicillin, streptomycin and amphotericin. Additionally, amino acids and vitamins were administered to the culture media. It was hypothesized that if *B. schlosseri* is placed in an optimized culture media, then this will support the growth of healthier and more successful colonies over time, while also promoting growth and colony proliferation.



Gasparini et al., 2015

Methods & Materials

- Colonies of *B. schlosseri* were collected from the floating docks from Breakwater Marina located in Tacoma, and then directly transferred to the seawater system at UWT. Colonies were washed with 70% ethanol and rinsed with artificial sea water containing penicillin, streptomycin and amphotericin-B (ASW+PSA) before micro-dissection.
- Cell content was isolated from the colony to generate two different cell contexts; individual zooids and a zooid cell suspension.
  - Cell suspension: Cell content was rinsed with ASW-PSA and mechanically extracted through a vial to dissociate individual cells from the tissue. The cells were then centrifuged and the pellet was used to seed culture flasks.
- Cell content was equally seeded in 5 unique culture media within culture dishes (500 & 250 uL of specific treatment medium) grown at 20°C and checked every 24 hours. New media was added if needed.

Different Culturing Medias Used:

1.) ASW+PSA
2.) ASW+PSA+AA (Amino Acids)
3.) Tunicate Culture Media (TCM=L-15 +FSW)+FBS+AA
4.) TCM –FBS +AA
5.) TCM

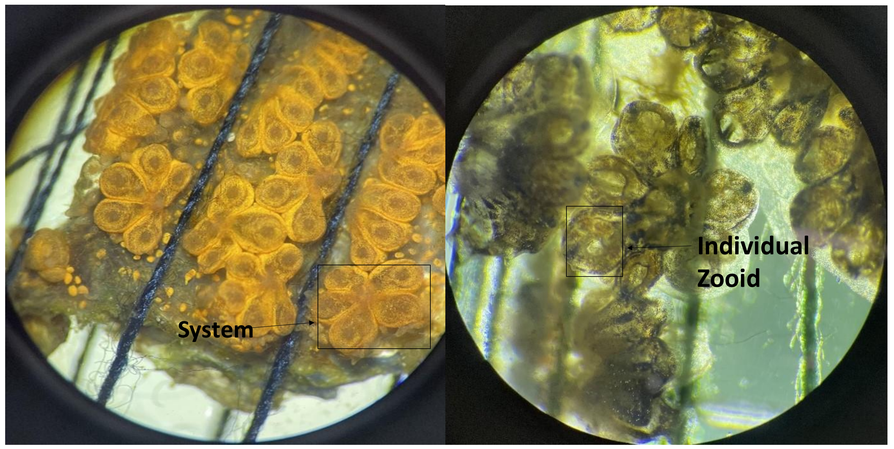


Figure 1. *Botryllus schlosseri* colonies used for microdissection. Colonies collected from the field were tied down to a glass slide and maintained in the seawater system prior to experimentation. This species has different color morphs, as seen by the bright orange colony (left image) and the dark green/black colony (right image). One system and one single adult zooid are indicated on the images.

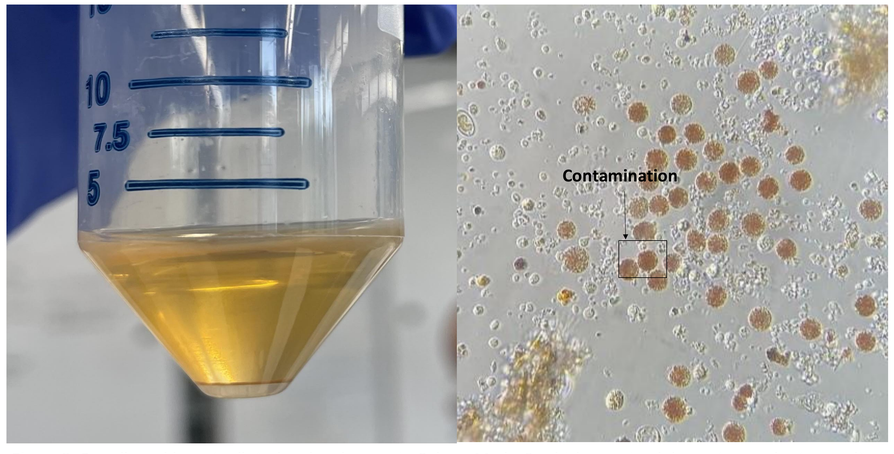


Figure 2. *Botryllus schlosseri* cells isolated and grown in Culture Media 3, which contained the tunicate culturing media (TCM) supplemented with amino acids and FBS. Brightfield images were taken at 60X total magnification 48 hours after seeding (right image). Some cells are translucent while others are dark in color under . All cells seem to be shriveled up and dead. The orange circles and the long green contaminants indicated are most likely different types of bacteria and fungi from the whole organism that contaminated the culture.

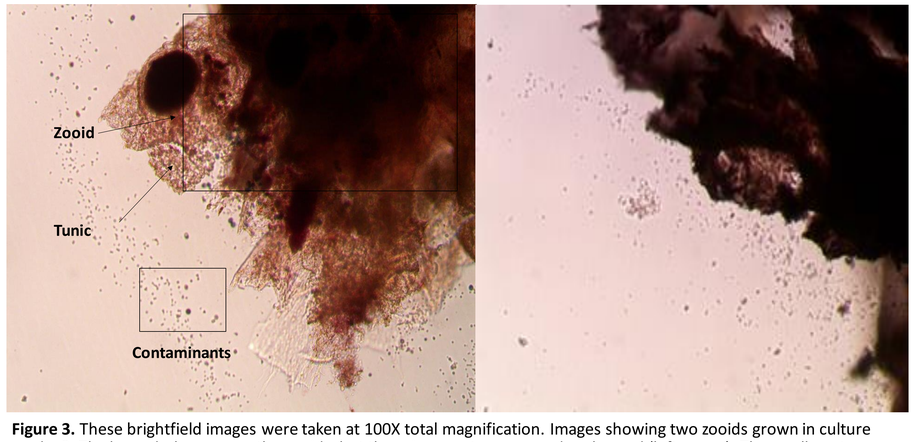


Figure 3. These brightfield images were taken at 100X total magnification. Images showing two zooids grown in culture media 3. The large dark areas are the zooids, but there is some tunic around each zooid (left image). The small spots floating on the sides are most likely fungal contaminants (both images). All other zooids in other experimental medias, also showed signs of contamination.

Results

- Cell Suspension: Cell layer was unobservable in all media types and cells expired before any outgrowth of new cells could be observed. Contamination was also observed in all wells from culture dishes. The media type did not appear to make a difference in terms of cell outgrowth and survival.
- Zooid: Seeded zooids were able to adhere in all media types, to varying degrees. Zooids that did adhere did not propagate any new cells, and slowly expired. Zooids retained their vibrant color, healthy tunic, vasculature and reactivity best in media types closely related to their natural habitat. In media types saturated with FBS and growth media, zooids did not appear to have visible contraction, responsiveness and had damaged or deflated vasculature. Zooids also responded less to growth serum with only L-Amino Acids (AA) added and they appeared to have a darker than usual color, little to no responsiveness to light or touch, delated and thin vasculature and less present. In the presence of growth media, AA and FBS, the zooids appeared to have less vasculature, less responsiveness, darker color, appeared bruised and showed no signs of movement.

Zooid Data

Table 1. Summary of qualitative characteristics that determine whether zooids were healthy, unhealthy, or dead/dying.

Zooid	ASW PSA	ASW PSA +AA	Media +FBS -AA	Media +FBS +AA	Media -FBS +AA
Trial #1	Color is darker orange than usual. Free floating of not adherent to the culture dish. Some zooids seem unresponsive. No visible contractions. No increase in size.	Free floating of not adherent to the culture dish. Vibrant orange color. Some zooids seem unresponsive. No visible contractions. No increase in size.	Free floating of not adherent to the culture dish. Color is darker and the zooid is small. No visible contractions. No increase in size. Deflated with thin vasculature.	Free floating of not adherent to the culture dish. Color is darker and the zooid is small. No visible contractions. No increase in size. Deflated with thin vasculature.	Free floating of not adherent to the culture dish. Color is darker and the zooid is small. No visible contractions. No increase in size. Deflated with thin vasculature.
Trial #2	Lighter zooid color and tunic color. All zooids adherent to the culture dish. No response to light or touch. No visible contractions or vascular circulation.	Vibrant zooid color and tunic color observed. All zooids adherent to the culture dish. No response to light or touch. No visible contractions or vascular circulation. Healthiest zooids in comparison to others.	Color is darker than usual. Less tunicate. All zooids adherent. No visible contractions or vascular circulation. Not responsive to touch or light.	Color is darker than usual. Less tunicate, color is dark. All zooids adherent. No visible contractions or vascular circulation. Not responsive to touch or light.	Increased amount of tunic present. All zooids adhered. Not responsive to light or touch. No movement noted. Zooids and tunic appear darker in color.

Future Research: Further understanding of optimal culturing conditions for *B. schlosseri* and preventative measures for contaminants still needs to be discovered for establishing and maintaining an immortalized cell line. Assuming this is successful, a mutagenic or carcinogenic agent can then be utilized to attempt to facilitate an immortalized cell line.

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References