The Establishment and Characterization of Primary Cell Cultures Derived from the Ctenophore Mnemiopsis Leidyi

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THE ESTABLISHMENT AND CHARACTERIZATION OF PRIMARY CELL CULTURES DERIVED FROM THE CTENOPHORE MNEMIOPSIS LEIDYI

By

Kaitlyn J. Warren

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THE ESTABLISHMENT AND CHARACTERIZATION OF PRIMARY CELL CULTURES DERIVED FROM THE CTENOPHORE MNEMIOPSIS LEIDYI

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We have developed a primary cell culture system that can be utilized to isolate adult somatic cell types from *Mnemiopsis leidyi*, a lobate Ctenophore. Our primary cell cultures are derived from tissue explants and maintained in a complex undefined media, which is generated from tissue isolated from Ctenophore lobes. Approximately 24 hours after explant removal, cultures are screened for the presence of desired cell types. These primary cell cultures can be reliably maintained and visually monitored for a variety of parameters including proliferation, changes in cell morphology and/or differentiation. Exemplar cell types that are easily isolated from primary cultures include cells derived from endoderm containing large pigmented vacuoles and giant smooth muscle cells exhibiting inducible contractile properties. In parallel we have also derived ‘tissue envelopes’ which contain sections of endodermal canal that are used to monitor targeted cell types in an *in vivo* context. Experiments further characterizing these primary cell cultures will facilitate the analysis of ctenophore development from a cell biological perspective.
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Chapter 1: Introduction

The goal of my thesis project was to develop an easy and reliable method for establishing a primary cell culture system to isolate adult somatic cell types from *Mnemiopsis leidyi*, a lobate Ctenophore (Ryan et al. 2013; Pang and Martindale 2008). Currently no methodologies for producing Ctenophore primary cell culture systems have been published. The protocols presented here will aid in the *in vitro* isolation and characterization of explicit cell types associated with this enigmatic group of metazoans. The primary cell cultures, resulting from the methods detailed in this thesis, can be reliably maintained and visually monitored for a variety of parameters including cell proliferation, changes in cell morphology and/or differentiation. Thus these primary cell cultures can be used to study aspects of Ctenophore cell physiology and biochemistry.

**Primary Cell Cultures:**

Primary cell cultures are often derived from tissue explants removed from an organism and maintained in an artificial environment (Li et al., 2014). These *in vitro* cell cultures can be used to approximate the *in vivo* cellular environment (Mishell and Dutton 1967). Primary cell cultures generally have a limited lifespan, unlike immortalized cell lines which are often genetically manipulated to proliferate indefinitely (Schneider, 1972; Todaro and Green, 1963). Primary cell cultures also typically produce a variety of cell types, each of which can be isolated for further study (Li et al., 2014; Tominaga et al., 2013). These and other characteristics of primary cells is why they can often provide a better representation of normal cell biology than immortalized cell lines. The use of primary cell cultures to monitor normal physiology make them the system of choice for the study of many cellular phenomena including, but not limited to: response to viral
infection, drug and vaccine candidates, and responses to potentially toxic compounds

Primary cell cultures are also excellent subjects for studying cellular metabolism, cellular
immune response, and even cell biological aspects of learning and memory (Yina et al.,
2016; Nargeot and Simmers, 2011).

In the next section, I will provide background on the Ctenophore body plan, tissue
types, and their evolutionary position to highlight the significance of these studies.

Ctenophores are gelatinous marine invertebrates named for their eight comb rows of cilia
(Dunn et al., 2008) (Figure 1), thus/which are also the source of their common name,
‘comb jellies’. These comb rows are made up of individual comb plates, which in turn are
made up of thousands of fused cilia. The coordinated beating of these comb plates
propels the Ctenophore through the water, making them the largest animals to use cilia as
their primary means of locomotion (Pang and Martindale, 2008). These comb plates also
act as diffraction gratings, which is why Ctenophores appear to have an array of colors.

Ctenophores are also bioluminescent and have light emitting organs called photocytes
located under the comb rows. These comb rows radiate from a single point at their most
aboral end at an apical organ, which is used in orientation (Figure 1). This apical organ
contains a statocyst with a single statolith. This statolith is composed of cells called
lithocytes, and is supported by balancer cilia, which control the beat of the eight comb
rows that radiate orally from the apical organ (Tamm, 2014).

Ctenophore Body Plan:

*Mnemiopsis leidyi*, the species used in this study, is a lobate Ctenophore. These
ctenophores have two lobes, as shown in Figure 1, that function in feeding. The tentacles
are greatly modified in *Mnemiopsis*; they branch at the tentacle sheath and spread along feeding grooves lining the inside of the lobes. Lobate Ctenophores also have two pairs of auricles on either side of the mouth, which move water containing planktonic prey towards the feeding grooves (Figure 1)

![Figure 1: Mnemiopsis leidyi anatomy shown from both the lateral and oral view.](image)

*Mnemiopsis leidyi* is native to Atlantic coastal waters. It is also considered an invasive species in Black, Caspian, and North Seas. This latter invasion has caused major ecological damage to native marine species in these areas (Shiganova 1998). *Mnemiopsis* has been used to study evolution and development, regeneration, and bioluminescence (Philippe et al., 2009; Henry and Martindale, 2000; Coonfield, 1936; Anctil, 1985; Freeman and Reynolds, 1973).

Ctenophores are often confused with Cnidarians because of superficial morphological similarities, however, unlike Cnidarians which have stinging cells called nematocysts, Ctenophores have sticky cells called colloblasts which cover their tentacles.
Ctenophores use their cilia and definitive muscle cells to move, whereas Cnidarians use pulsing sheets of myoepithelial cells to move; as Cnidarians lack individual muscle cells and instead possess epitheliomuscle (Martindale and Henry, 1999). The Cnidarian body plan is radially symmetric, whereas Ctenophores have a body plan based on biradial symmetry: the two orthogonal planes that run along the oral-aboral axis, the sagittal plane and the tentacular plane, are not mirror images of each other and exhibit rotational symmetry because of the two anal pores located at the aboral pole of the animal (Pang and Martindale, 2008).

**Ctenophore Tissue Layers:**

Ctenophores are classically described as having two germ layers; an outer ectoderm and an inner endoderm. These germ layers are separated from one another by a jelly-like mesoglea layer mostly composed of water and extracellular matrix (ECM). This middle mesoglea layer, however, is not completely acellular. The mesogleal space contains substantial numbers of muscle, nerve, mesenchymal and amebocyte-like cells. The principal organs associated with the endoderm are the branched gastrovascular through-gut responsible for distributing nutrients throughout the organism. Ctenophores have giant smooth muscle cells; however striated muscle cells have also been found in a single species (Mackie et al., 1988). Their nervous system is composed of a subepithelial nerve net, mesoglial nerve cells, and tentacular nerves (Jager et al., 2011); however, this nervous system lacks several of the neurotransmitters common among the Bilateria, such as dopamine and serotonin (Ryan et al., 2013; Moroz et al., 2014). Recent genome sequencing and analysis suggest that early animal evolution may have involved major losses and/or gains of canonical nerve and muscle cell types (Ryan et al., 2013).
Evolutionary Position of Ctenophores:

*Figure 2: Phylogenetic trees depicting (A) ctenophores as the sister group to all other animals and (B) sponges as the sister group to all other animals.*

The phylogenetic position of Ctenophores and their relation to other animals has been the subject of a long running debate for years. They have been assigned numerous positions on the animal phylogenetic tree. Because of their morphological similarities to
Cnidarians, ctenophores were once placed together with Cnidarians in a clade called Coelenterata, sister group to Bilateria (Ryan et al., 2013; Dunn et al., 2015). They have also been placed sister to a clade that includes all animals besides Porifera and as sister to all other metazoans, shown in Figure 2a whereas before, sponges were thought to be sister to all other metazoans (Figure 2b) (Dunn et al., 2015). This shuffling of the phylogenetic position of Ctenophores proved problematic for studies of early animal evolution until the Mnemiopsis genome was sequenced (Ryan et al., 2013). The Ctenophore genome sequence supports the Ctenophores as the likely the sister group to other extant metazoans.
Chapter 2: Establishing and Characterizing *Mnemiopsis* Primary Cell Cultures

Our goal was to develop an easy and reliable method for the establishment of a primary cell culture system that can be utilized to isolate representative adult somatic cell types from *Mnemiopsis leidy*, a lobate Ctenophore (Ryan et al., 2013; Pang and Martindale, 2013). Currently no methodologies for producing Ctenophore primary cell culture systems have been published. The protocols presented here will aid in the *in vitro* isolation and characterization of explicit cell types associated with this enigmatic group of metazoans.

Despite the absence of a distinct mesodermal germ layer, Ctenophore specific giant smooth muscle cells are distributed throughout the animal, and in a single species striated muscle cells have been documented in the tentacles (Hernandez-Nicaise et al., 1984; Mackie et al., 1988). The Ctenophore nervous system is comprised of a subepithelial nerve net containing both mesoglial nerves and tentacular nerves (Jager et al., 2011; Dunn et al., 2008). Recent genome sequencing and analysis suggest that early animal evolution may have involved major losses and/or gains of canonical nerve and muscle cell types (Ryan et al., 2013).

Ctenophores have an extensive range of unique cell types little-studied, contributing to their misrepresentation as ‘simple’ organisms. The unique attributes of the phylum are, in part, due to the long separation of the Ctenophore lineage from other metazoans (Dunn et al., 2008). The availability of an efficient and reliable primary cell culturing system will continue to play an important role in understanding the phylum’s basic biology.

The following methodology for primary cell cultures utilizes tissue explants rather
than bulk enzymatic digestion of tissue. Animals were selected based on size and relative health: animals with large lobes generate more Ctenophore mesoglea serum (CMS), the media in which the cells are cultured. Healthier, well-fed animals are less fragile and liable to tear, thus allowing for easier dissection and robust, cell-rich explants. Preparing glassware, dissecting the animal, and assembling the cell cultures all take place under a fume hood.

**Selecting the Culture Dishes:**

I tried culturing the cells in several different dishes. Initially, I used plastic 35x10 mm petri dishes. Holes were punched out of the bottoms of these dishes, which were then covered with 25x25 mm glass coverslips. Preparing these dishes requires two days. While the dishes worked well for cell culture, they were discarded in favor of a less labor intensive preparation. Four-well sterile cell culturing dishes were selected. However, they were too large for convenient high magnification microscopy using a compound microscope. Finally, I settled on using glass microscope slides fitted with a silicon gasket to culture the cells. These are easily prepared and work well for compound microscope imaging. Protocols for preparing both the petri dishes and the slides can be found below.

**Preparing the Petri Dishes:**

1.) Punch holes in the bottom of the plastic 35x10 mm petri dishes.

2.) Use a burr to file the edges of the holes in each petri dish. Try to remove all of the pieces being filed off before rinsing.

3.) Place dishes in a bucket and rinse the dishes with hot sterile water 3 times.

4.) Rinse the dishes with cold D.I. water 3 times.

5.) Place dishes upside down to dry on a tray lined with parchment paper.
6.) Use forceps to dip 25x25 mm glass cover slips in 200 proof ethanol and wipe off excess with lint free chem wipes.

7.) Place coverslips on a tray lined with parchment paper to dry.

8.) Once dishes are dry, apply medical adhesive using a 5ml syringe around the hole on the bottom of a dish.

9.) Place coverslip carefully onto the bottom of the dish, making sure the adhesive is in contact with the coverslip on all sides. Repeat for all dishes.

10.) Place dishes upside down on a tray lined with parchment paper to dry for 24 hours.

11.) UV treat the dry dishes for 1 hour.

**Generating Ctenophore Mesoglea Serum (CMS) Media:**

The methods for producing Ctenophore Mesoglea Serum (CMS) underwent several refinements during optimization for use with primary cell cultures. In order to generate the CMS, one of the dissected lobes is divided among 4-6 microcentrifuge tubes and then homogenized with an appropriate pestle for 5 minutes (a shorter period of homogenization results in high viscosity CMS, which will prevent isolated cells from settling on the bottom of the dish). Heat treating the CMS post homogenization further reduces viscosity, resulting in the majority of the explant isolated cells settling on the bottom. Reducing the viscosity of the CMS dramatically improved the retention of explant isolated cells during CMS exchanges.

Importantly, exchanging the 50 percent of the CMS dramatically increases the longevity of the primary cell cultures. I have succeeded in maintaining primary cell cultures for as long as 3 months with periodic exchanges with fresh CMS. If the CMS is not exchanged, cultures will typically last only 1-2 weeks. The optimal time to exchange
the CMS seems to be approximately 72 hour intervals. CMS exchange intervals greater than 96 hours reduce cell culture longevity, most likely due to an accumulation of metabolic waste products and dead cell debris.

**Solid Matrix:**

Early attempts at maintaining primary cell cultures included an effort to provide the cells with a solid matrix for support and/or adhesion. I generated a ‘solid phase’ from sedimented lobe tissue. After centrifuging homogenized lobe tissue, the supernatant was removed for CMS and the remaining sedimented pellet was used to generate the solid matrix. The pellet was resuspended with a small amount of CMS, and then spread on the bottom of the culture dishes and dried before adding tissue explants and CMS. Initially I tried covering the entire bottom of the dishes with matrix, which required a drying time of at least an hour. I also tried using a smaller amount (5µl), which allowed for a faster drying time of 5-10 minutes. Once the CMS was added however, the dried solid matrix would rehydrate, swell, and clump in the cultures dishes. Too much solid matrix resulted in extensive cell death. Comparing cell cultures with 5µl of solid matrix with cultures not containing any solid matrix clearly demonstrated that the addition of this solid matrix neither promoted cell growth nor aided in cell adhesion.

**Preparing the Microscope Slides:**

1.) Clean microscope slides with 70% ethanol.

2.) Use a lint free chem wipe soaked in 70% ethanol to clean the silicon gaskets.

3.) Use a 10µL pipette tip to spread a small amount of gasket grease on the silicon gaskets and place the clean slides on top. Press gently to remove any bubbles (Figure 3).
4.) Draw a small 5mm diameter circle on the bottom of the slide.

NOTE: Drawing circles aids in counting the cells. Cells inside the circle were counted to track proliferation and monitor changes in cell morphology and/or differentiation.

![Figure 3: Microscope slide fitted with a silicone gasket 0.5mm in depth.](image)

NOTE: Using too much gasket grease leads to leakage from under the gasket when placing the slide on top. While no adverse effects on the cells were observed, excess grease around the edges of the well makes for poor images. If pressed too hard, the silicone gasket will deform.

**Final Protocol:**

1.) Preparing the animal for dissection

1.1) Place the animal in a suitably sized glass dish and wash with 0.2µm filter sterilized artificial sea water 3 times before moving to work under a sterile hood.

NOTE: Washing steps are critical for preventing debris and particles that may be adhering to the animal from contaminating cell cultures. Using gloves, very gently rub the animal down with each rinse, being particularly careful not to tear the delicate oral
lobes. A disposable pipette works well to remove large particles and/or mucus strands that come off of the animal.

1.2) Let the animal recover undisturbed for 30-60 minutes before beginning dissection. NOTE: The length of time for recovery depends on how badly damaged the animal is after washing. If the animal is badly damaged, allow for more than 60 minutes to heal or use another animal. Healthy, well-fed animals tend to do extremely well when washed and require less time to recover and/or heal.

2.) Dissecting the animal

2.1) Using a scalpel and forceps, cut off both lobes just oral of the opening of the mouth and place the dissected lobe tissue in a sterile 100 x 15mm petri dish filled with 1X 0.2µm filtered sea water spiked with penicillin + streptomycin (pen/strep) solution (See Figure 4: Set up for animal dissection. Bottom left: 6 microcentrifuge tubes. Top left: two petri dishes for dissected lobes (I use one 100x15mm petri dish instead these two smaller ones). Center: large glass dish containing nearly transparent adult ctenophore. Bottom right: pestle, forceps, and scalpel. Top right: 1X 0.2µm filtered sea water + pen/strep solution)
note below). See Figure 4 for setup and materials.

NOTE: Prepare in advance a 5mL 100X pen/strep stock solution at 10,000 u/mL penicillin and 10,000 µg/mL streptomycin and store at 4°C. To prepare sea water spiked with pen/strep, add 2.5mL of the 100X stock solution to 250mL of 0.µ2µm filtered sea water.

NOTE: Excised lobe tissue is fragile and will tear easily if not handled with care. Wearing clean gloves, carefully place each excised lobe in the petri dish. Alternatively, use a suitable receptacle such as a disposable weigh-boat to move the excised lobes to the petri dish.

2.2) Let the excised lobe tissue sit for 5 minutes before removing seawater + pen/strep. Replace with fresh seawater + pen/strep via graduated pipette. Repeat this wash step 3 times.

NOTE: It is critical to avoid, as much as possible, additional tearing of the excised lobe tissue. One lobe needs to remain relatively intact for generating the tissue explants.

3.) Preparing the Ctenophore Mesoglea Serum (CMS).

3.1) Select one of the excised lobes and use a scalpel to evenly divide the tissue among several 1.5ml microcentrifuge tubes. The number of tubes will depend on the size of the excised lobe. Ideally, each tube should be approximately half full with tissue.

NOTE: Use the most damaged excised lobe for preparing the CMS. Use the other lobe for tissue explants as tissue for generating explants require intact lobe tissue.

3.2) Use a 1.5µL microfuge tube pestle to homogenize the lobe tissue. Manually disrupt the tissue in each tube for 5 minutes.

3.3) Heat inactivate the homogenized tissue by incubation at 56°C for 30 minutes to
reduce CMS viscosity, lyse remaining intact cells, and eliminate the introduction of low heat tolerant microbes that may be resistant to pen/strep. Aliquots of CMS can also be rotated at 300rpm during the heat inactivation step if desired.

3.4) Spin the heat treated microfuge tubes in a microcentrifuge for 30 minutes at maximum speed at room temperature to sediment cellular debris and ECM aggregates.

3.5) Collect and consolidate the supernatant from each microfuge tube to a 15mL conical tube and mix 1:1 with 1X 0.2μm filtered sea water + pen/strep solution. Discard microfuge tubes containing debris pellets.

NOTE: After centrifugation and prior to collection, visually check for debris floating in the tubes. It is best to avoid any debris for imaging purposes. If necessary respin CMS to remove remaining debris.

3.6) Prepared CMS should be stored at 4°C and can be used as needed for 72 hours.

NOTE: ECM material may continue to precipitate during cold storage. If necessary respin prior to use.

4) Preparing the tissue explants.

4.1) Using a scalpel, ‘wound’ the remaining intact excised lobe tissue via a unidirectional shallow cut along the lobe tissues ectodermal surface along with the ectodermal surface of the tissue.

NOTE: Only cut the thin surface layer epithelium, taking care not to slice completely through the lobe tissue. The lobe tissue should remain in one piece during the wounding step. Let the wounded lobe recover for 25 minutes to allow cells to begin recruiting to the wound site.

4.2) Cut the bottom fourth off of a new sterile 1.5mL microcentrifuge tube to use as a
stamping/coring tool for generating your tissue explants. Remove the microcentrifuge cap to make a convenient aperture for viewing the targeted tissue as it is being cored. Target areas for the desired explants under a dissecting microscope. The lobe tissue can be held in place with forceps while the coring tool is laterally twisted to release individual tissue explants.

NOTE: Using a microcentrifuge tube as a coring tool is an easy way to generate consistent tissue explant replicates across an experiment. The blunt edge of the coring tool creates explants with jagged edges, inhibiting rapid healing of the explant, thus allowing more time for cells to migrate out of, or slough off, the prepared explant during the subsequent steps.

5.) Establishing the Primary Cell Cultures

5.1) Use blunt tipped forceps to place each tissue explant on glass microscope slides fitted with 0.5mm deep silicone isolators. Cover each explant with 300µL of CMS that has been brought to ambient room temperature.

NOTE: Primary cell cultures can also be established and maintained on plastic cell culture dishes with no significant visible changes in cell viability.

5.2) Place prepared slides in a large covered dish (e.g. a 145 x 20mm petri dish) that will serve as an incubation chamber. Add a few clean lint free chemwipes soaked with sterile distilled water to maintain high humidity in the incubation chamber.

5.3) Incubate the cell cultures at 14-16°C.

5.4) Approximately 24 hours after seeding slides with explants, each individual culture slide can be screened for the presence of desired cell types that have either detached or migrated away from the tissue explant.
6.) Maintaining the Primary Cell Cultures

6.1) In order to maintain the primary cell cultures, the tissue explant must be removed to reduce cell crowding, which dramatically improve the longevity of the cell cultures. After approximately 48 hours carefully remove any remaining tissue explant by removing the tip of a sterile 200µL pipette to increase the aperture as needed. If the explant has disintegrated into multiple small fragments, trimming the pipette tip may not be necessary. Performing the explant removal step under a microscope is highly recommended.

6.2) After 72 hours, remove 50% of the CMS (~150µL) and replace with fresh CMS prepared as in step 3. Remove CMS from primary cell cultures slowly to avoid disturbing and/or removing cells of interest. It is strongly recommended that CMS exchanges be performed under a microscope.

NOTE: Exchanging the CMS improves the longevity of the cell cultures by replenishing nutrients, reducing waste products from cellular metabolism and removing cell debris.

6.3) Repeat 50% CMS exchanges as needed or until cells expire at ~72 hour intervals.

7.) Preparing Tissue Envelopes

NOTE: Tissue Envelopes can be used to study cells of interest in an in vivo context and can be used in conjunction with primary cell cultures. The following steps are used to generate tissue envelopes that contain endodermal canal tissue.

7.1) If necessary repeat steps 1-3 to generate sufficient CMS for Tissue Envelope maintenance. Each tissue envelope requires 300µL of CMS.

7.2) Use a scalpel to remove an approximately 5mm X 3mm portion of the oral-most edge of lobe tissue that also contains a small section of endodermal canal. To facilitate
rapid sealing of the cut site, use a scalpel to create a clean cut.

NOTE: Use forceps to hold the excised lobe in place during cutting. If the cut site closely parallels the endodermal canal, the Tissue Envelope may extrude the endodermal canal during wound sealing/repair. If primary cell cultures are also being generated, the same lobe used for cell culture explants can also be used for preparing Tissue Envelopes as long as the oral-most edge remains intact.

7.3) Use blunt tipped forceps to place each freshly cut Tissue Envelope on a glass microscope slide fitted with 0.5mm deep silicone isolator and cover with 300µL of CMS that has been brought to ambient room temperature.

NOTE: Tissue Envelopes can also be maintained on plastic cell culture dishes.

7.4) Place prepared Tissue Envelope slides in a large covered dish (e.g. a 145 x 20mm petri dish) that will serve as an incubation chamber. Add a few clean lint free chemwipes soaked with sterile distilled water to maintain high humidity in the incubation chamber.

7.5) Incubate Tissue Envelopes at 14-16°C.

NOTE: Tissue Envelopes typically seal within 4-6 hours. The amount of time it takes for the tissue envelopes to seal depends on how cleanly the edges were cut and on how healthy the original animal was. Tissue Envelopes typically last 72-120 hours before beginning to disintegrate.

8.) Loading Vital Dyes into the Cell Cultures

8.1) Add the vital dye(s) at their appropriate concentrations to CMS brought to ambient room temperature.

NOTE: The concentrations used here can be found in the Representative Results section.

NOTE: If necessary repeat steps 1-3 to generate sufficient additional CMS to both load
dye into the cell cultures and to wash out excess dye after loading the cell cultures. Ideally, this will be at least 6ml of CMS, which may require two animals.

8.2) Exchange 50% of the culture CMS with fresh CMS spiked with dye(s).

8.3) Allow dye(s) to load for 45 minutes in the incubator at 14-16°C.

8.4) Once loaded, perform 2-3 rapid 50% CMS exchanges.

9.) Loading Vital Dyes into the Tissue Envelopes

9.1) Add the vital dye(s) at their appropriate concentrations to CMS brought to ambient room temperature.

NOTE: If necessary repeat steps 1-3 to generate sufficient additional CMS.

9.2) Use a syringe to inject the dye-spiked CMS into the endodermal canal.

NOTE: To efficiently stain endodermal tissues deep within the Tissue Envelope may require the use of forceps or a sharp needle to poke a small hole into the endodermal canal followed by injection of the dye-spiked CMS via small gauge needle.

9.3) Allow vital dye(s) to load for 45 minutes in the incubator at 14-16°C.

9.4) Once loaded, perform 2-3 rapid 50% CMS exchanges to wash out residual dye surrounding the Tissue Envelope.

NOTE: It is difficult to exchange dye-spiked CMS within the endodermal canal without severely damaging the Tissue Envelope. Depending on the properties of the individual dyes used this may or may not present a problem for downstream visualization.

Representative Results:

Once the cell cultures have been established, the next step is to characterize the cell types present. This can include a variety of simple visual assays. Morphological analyses can include assessments of cell mobility, contractile properties, proliferation,
and differentiation. Morphological characterization can be combined with vital dyes to assess the distribution of specific intracellular organelles. Where possible we have compared results from our protocol to the unpublished thesis of Dodson, the only other experimental work on Cnenophore primary cell culture of which we are aware (Dodson, 2010). Importantly, unlike Dodson’s protocol, our protocol uses a heat-treated CMS media and includes 50% media exchanges at 72 hour intervals for the long-term viability of individual primary cell cultures. Our protocol also integrates the production of companion Tissue Envelopes that contain cell types of interest in an in vivo context intermediate between cell culture and the whole organism.

Figure 5: (A) Proliferative round cells (B) Multipolar cells (C) Bipolar cell (D) Hyper elongated cells. (E) Typical field of cells at 48 hours post-isolation (F) Typical field of cells at 96 hours post-isolation. Some cell types, such as the proliferative round cells (both the ectodermal and pigmented endodermal round cells) become more abundant over time.
We typically screen our primary cell cultures at 24 hours post explant isolation to confirm that desired cell types, such as muscle and pigmented endodermal cells, are detached/independent of the explant. The explant and explant remnants are then removed at 48 hours to prevent overcrowding on individual culture slides. During the development of this protocol we also performed total cell counts at several time points during the establishment and maintenance of the primary cell cultures (Table 1, Figure 10). Several distinct cell morphologies similar to those described by Dodson are consistently observed at 72 hours (Dodson, 2010). We have loosely classified them by morphology as hyper elongated, bipolar, multipolar, and round cells (Figure 5).

We monitored cell proliferation, differentiation, and changes in cell morphologies across independently prepared individual primary cell cultures. Briefly, a circle approximately 5mm in diameter, was drawn on the bottom of culture slides and cells inside the circles were counted beginning at 48 hours post isolation (Table 1, Figure 10). Cells within the perimeter of the circle were subsequently observed at ~24 hour intervals for visible changes in cell morphology. Pictures of the cells inside the circles were taken in order to aid in counting and monitoring changes in cell morphologies (Figure 5F and 5E). Using this strategy, we were able to track proliferation and differentiation in our cultures.

We tested contractile properties of individual cells by mechanical stimulation using a glass needle. Where possible, different regions of elongated cells were stimulated by the glass needle including medially, at the extreme ends, and any prominent processes. If the cell exhibited a direct response to stimulation it was considered to have contractile properties. In general, medial mechanical stimulation with a glass needle did not elicit a
contractile response from any of the cell types observed including giant smooth muscle cells whereas stimulation of extreme ends and prominent processes very often elicited contractile responses. Not surprisingly, putative muscle cells, bi-polar, and multipolar cells exhibited strong contractile properties when simultaneously exposed to Hoechst and shortwave length blue light (Figure 6).

![Figure 6: Examples of muscle cell contraction in giant smooth muscle cells after being exposed to Hoechst and shortwave length blue light. (A) and (C) Muscle cells pre-contraction. (B) and (D) Muscle cells post-contraction.](image)

**Vital Dyes:**

Primary cell cultures were exposed to several fluorescent vital dyes including MitoTracker, LysoTracker, and Hoechst in order to detect the presence of mitochondria, lysosomes, and nuclei respectively (Figure 7). MitoTracker (Molecular Probes) was used at a final concentration of 250nM. LysoTracker (Molecular Probes) was used at a final concentration of 33.33nM. Hoechst 33342 (Molecular Probes) was used at a final
concentration of $1.25\mu g/mL$. Individual vital dyes or combinations of vital dyes were introduced into the primary cell cultures during the CMS exchange at 72 hours (Figure 7).

**Ectodermal Round Cells:**

The ectodermally derived round cells, shown in Figure 5A, were the most abundant cell type. These cells proliferate in culture at the rate of ~1 division per 24 hours producing clonally related chains. Our analyses suggest that they are non-motile and possess a high nuclear/cytoplasmic volume ratio. Morphologically these cells resemble the highly abundant round cells also detected by Dodson (Dodson, 2010). These cells are included in the total cell counts in Table 1, however they do not have their own distinguishable category, along with other cell types such as neurons and epithelial cells. The vast majority of cells isolated from explant tissues are included in this proliferative

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**Figure 7:** Cells exposed to fluorescent vital dyes in order to detect the presence of specific organelles. The blue dye, Hoechst, highlights nuclei. The yellow dye, LysoTracker, highlights acidic lysosomal vacuoles. The red dye, MitoTracker, highlights mitochondria. (A) and (B) Muscle cells showing multiple nuclei and mitochondria. (C) and (D) Endodermally derived round cells showing two nuclei and large acidic lysosomal vacuoles.
ectodermal class, as indicated by the total cell count data.

**Bipolar and Multipolar Cells:**

Bipolar and multipolar cell morphologies, shown in Figure 5B and C use highly dynamic processes for mobility. Individual cells were classified as multipolar if they had more than two prominent processes. Most cell types with these prominent processes also demonstrated contractile properties when exposed to Hoechst and short wavelength blue light. The bipolar and multipolar class of cells described here also contain the ‘spindle’ and ‘stellate’ classes described by Dodson (Dodson, 2010).

**Muscle Cells:**

Our protocol facilitates the isolation of large numbers of intact giant smooth muscle cells through the use of tissue explants. In general, these cells are hyperelongated and multinucleated (Figure 5D and Figure 7A and B). During the first ~72 hours in culture, isolated giant smooth muscle cells retain a relatively consistent morphology exhibiting a largely constant diameter along their long axis as shown in Figure 5D. Isolated giant smooth muscle cells typically range from ~300-1000µm in length. Though non-motile they exhibit dramatic contractile properties. Mechanical stimulation with a needle at either end of these cells typically initiates a dramatic contraction. However, the same mechanical stimulation of the medial region of these cells notably does not result in strong contraction. Within a few hours a contracted giant smooth muscle cell will return to its relaxed elongated state. After ~72 hours, isolated giant smooth muscle cells begin to undergo significant morphological changes and develop multiple unorganized, large and increasingly chaotic processes along their entire length as shown in Figure 6A and C. This atypical branched morphology is not observed among giant smooth muscle cells
Another distinct cell type, which are not pictured, with properties suggestive of muscle or muscle-like fate appears frequently in our cultures, however they are much smaller than giant smooth muscle cells. These smaller muscle-like cells are ~50-100µm in length. They exhibit several properties analogous to giant smooth muscle cells including containing multiple nuclei and exhibiting dramatic contractile properties.

**Endodermally Derived Cells:**

Our primary cell cultures derived from tissue explants containing endodermal canals included endodermally derived digestive cells that typically line regions of the endodermal canal lumen. Often these large digestive cells could be uniquely identified visually based on the presence of pigmented organelles. LysoTracker co-localizes to these organelles suggesting that they are acidic lysosomal vacuoles (Figure 6C and D). These cells are heavily ciliated and highly motile, in addition they are typically binucleated (Figure 6C and D) suggesting they are terminally differentiated. However, our cell count data indicated that these cells both persist for extended periods of time and can increase in numbers over time (Table 1, 2, and 3; Figure 10 and 11). Further observation revealed a class of small proliferative endodermal cells commonly present in our primary cultures that are competent to differentiate into mature digestive cells containing large acidic vacuoles (Figure 6C and D).

**Sperm Cells:**

In ~30% of our primary cell cultures, active mature sperm cells are found in high numbers (Figure 8). Explants taken from both ectodermal and endodermal tissues are competent to produce sperm cells in culture. These active mature sperm cells typically
appear 72-96 hours post isolation. It is surprising to find these cells in cultures several days after initial primary cell isolation. This phenomenon is also observed in cultures derived from explants containing ectoderm and mesoglea only. We interpret this as evidence for cell types competent to differentiate into sperm cells being widespread and therefore perhaps not preferentially restricted to a particular germ layer.

**Figure 8:** Example sperm cell found in culture 96 hours post-isolation.

**Tissue Envelopes:**

In order to more closely visualize interactions between cell types of interest we also developed Tissue Envelopes suitable for detailed microscopy. We focused on the morphologically distinct endodermally derived digestive cells. Thus Tissue Envelopes containing small sections of endodermal canal were prepared as seen in **Figure 9.** Tissue Envelope preparations facilitated the study these cells in an *in vivo* context. For example, the large digestive cells that are commonly isolated in culture from explants containing endoderm, line the inner wall of the endodermal canals in these tissue envelopes. These large digestive cells are frequently sloughed from the inner wall and into the lumen of the
canals, suggesting a high turn-over rate for this particular cell type. Based on the intersection of observations from both primary cell culture and Tissue Envelopes, we are confident that the primary function of these readily isolatable and identifiable endodermal cells is closely allied with digestion.

![Figure 9: Tissue envelopes (A) Whole tissue envelope with section of endodermal canal. (B) Section of endodermal canal lined with pigmented endodermal cells. (C) Endodermal cells consisting of the large, fully mature vacuolated cells towards the outside, smaller immature endodermal cells towards the inside, and a group of proliferating cells in the middle.](image)

**Discussion:**

Understanding the basic biology of Ctenophores, the animal that is the sister clade to all other metazoan animals, will provide insights into the origins and evolution of diverse cellular properties. A critical component of this protocol is the generation of the CMS. Dissected lobe tissue must be thoroughly homogenized; failing to do so will result in CMS with high viscosity which impedes the settlement of cells. Subsequent heat treatment substantially reduces the viscosity of the homogenized tissue, and has the added benefit of reducing the likelihood of microbial contamination. Exchanging ~50% of the culture CMS at 72 hour intervals is critical to the long-term viability of the primary
cell cultures.

Generating tissue explants with a blunt edged tool is critical for preventing the explant from sealing before individual cells are able to detach/slough off the explant tissue. We used a microcentrifuge tube modified by removing the bottom fourth as a convenient tool for ‘ stamping/coring’ tissue explants. This method slows or prevents the explants from sealing. It also allows for control of consistent explant sizes across an experiment.

There are a few limitations associated with this technique. Explants from different locations on the animal will produce different ranges of cell types. For example, endodermal cells have only been found in cultures with explants containing endodermal canal. Additionally, using tissue explants alone will not recapitulate all cell types in the cultures, only cells that readily slough from the edges of the explant or are competent to migrate out of the explant will be represented. However, we have found this technique to be extremely effective for the isolation of a diverse range of ectodermal, endodermal and mesogleal cell types.

This technique was developed to aid the investigation of unique aspects of cellular differentiation associated with Ctenophore development and evolutionary history from a cell biological perspective. Primary cell cultures derived from Ctenophores will not only facilitate the characterization of cell types unique to Ctenophores but also importantly contribute a broader understanding of animal cell type diversity.
Table 1: Cell Type per slide at 48 hours post-isolation. The number of cells for each cell types, as well as the total cells, are recorded here for each slide (S1-S5).
Figure 10: Bar graph comparing the number of cells for each cell, as well as the sum of cells, across each slide (S1-S5) during the first 48 hours.
### Table 2: Cell Type per slide at 72 hours post-isolation.
The number of cells for each cell types, as well as the total cells, are recorded for each slide (S1-S5).

<table>
<thead>
<tr>
<th>SLIDE</th>
<th>MUSCLE</th>
<th>BIPOLAR</th>
<th>MULTIPOLAR</th>
<th>ENDODERMAL</th>
<th>OTHER</th>
<th>SUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>7</td>
<td>22</td>
<td>9</td>
<td>139</td>
<td>1,093</td>
<td>1,270</td>
</tr>
<tr>
<td>S2</td>
<td>61</td>
<td>118</td>
<td>73</td>
<td>520</td>
<td>3,832</td>
<td>4,604</td>
</tr>
<tr>
<td>S3</td>
<td>21</td>
<td>49</td>
<td>25</td>
<td>775</td>
<td>2,630</td>
<td>3,500</td>
</tr>
<tr>
<td>S4</td>
<td>47</td>
<td>42</td>
<td>20</td>
<td>635</td>
<td>1,901</td>
<td>2,645</td>
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<tr>
<td>S5</td>
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<td>65</td>
<td>15</td>
<td>570</td>
<td>2,644</td>
<td>3,381</td>
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<tr>
<td>Ave</td>
<td>45</td>
<td>59</td>
<td>28</td>
<td>528</td>
<td>2,420</td>
<td>3,080</td>
</tr>
</tbody>
</table>

![Pie charts showing cell types distribution for each slide and average.](image)

**Table 2**: Cell Type per slide at 72 hours post-isolation. The number of cells for each cell types, as well as the total cells, are recorded for each slide (S1-S5).
Figure 11: Bar graph comparing the number of cells for each cell type, as well as the sum of cells across each slide (S1-S5), during the first 72 hours.
### Table 3: Comparison of abundance of cell types found at 48 hours post-isolation and cell types found at 72 hours post-isolation.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUSCLE</td>
<td>108</td>
<td>45</td>
</tr>
<tr>
<td>BIPOLAR</td>
<td>107</td>
<td>59</td>
</tr>
<tr>
<td>MULTIPOLAR</td>
<td>85</td>
<td>28</td>
</tr>
<tr>
<td>ENDODERMAL</td>
<td>493</td>
<td>528</td>
</tr>
<tr>
<td>OTHER CELLS</td>
<td>1625</td>
<td>2420</td>
</tr>
</tbody>
</table>

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**Average 48hr vs 72hr**

![Pie charts showing cell type distribution at 48hr and 72hr.]

- **48hr**: 
  - Muscle: 67% (4%)  
  - Bipolar: 4% (4%)  
  - Multipolar: 20% (4%)  
  - Endodermal: 20% (4%)  
  - Other cells: 4% (4%)

- **72hr**: 
  - Muscle: 79% (17%)  
  - Bipolar: 1% (1%)  
  - Multipolar: 1% (1%)  
  - Endodermal: 2% (1%)  
  - Other cells: 2% (1%)
Figure 12: Bar graph comparing the average number of cells per cell type at 48 and 72 hours.
Chapter 3: Discussion and Conclusions

The majority of the time spent on this project involved establishing the protocol for the primary cell cultures. After which, I focused on characterizing two cell types: the muscle and the endodermally derived cells. While time constraints prevented me from exploring additional uses for this protocol, primary cell cultures are invaluable tools that can be used for numerous experiments: some of which I will touch on in this chapter. First, I will address some of the principal limitation of this protocol and how it may be confronted.

Further work with this protocol would involve overcoming some of the limitations outlined in Chapter Two. For example, using enzymatic digestion with trypsin, may result in more cell types per culture. However, high concentrations of trypsin may harm cell membranes and result in lower viability of the cell cultures. Successfully getting the cell to adhere to the slides may require the use of Matrigel or agar. Once the cells are adherent, it will be much easier to fix and stain them, which will allow for the use of phalliodin and other stains. Fixing the cells will also allow for the use of in situ hybridization to characterize the timing and expression of mRNAs.

Another topic for further research would be the establishment of an immortal cell line. A monotypic cell line was in the works for both the ectodermally derived and the endodermally derived round cells; however, this project did not reach completion. This process involved isolating these proliferative round cells by passaging. Because both cell types are proliferative, it could be possible to derive a Ctenophore cell line using either type. It may also be possible to derive an infinite cell line using Mnemiopsis embryos.

There are also cell types in culture that have yet to be characterized as extensively
as the muscle and endodermally derived cells, some of which fall into the
undistinguishable “other” category in Tables 1, 2, 3 and Figures 10, 11,1 2. For
characterizing the nerve cells, this process could begin by taking explants from the four
known neural cell populations in Ctenophores: the subepithelial nerve net, the
intramesogleal neural nets, the tentacles, and the aboral organ (Moroz, 2015). The
resulting cultures could then be compared with one another. Cells isolated from these
cultures could be used to generate gene expression profiles, possibly via single-cell RNA
sequencing. Some of these neurons and their synapses have been described via electron
microscopy (Hernandez-Nicaise, 1991). It would be interesting to study the interactions
of these neural types cells with themselves as well as with other cell types.

The additional class of muscle-like cells require further investigation. These cells
can contract on their own without requiring any stimulation via a glass needle or Hoechst
staining and blue light. These may be similar to striated muscle cells. To investigate this,
it would be beneficial to establish cultures with tissue explants containing tentacles.
However, the tentacles in lobate Ctenophores such as Mnemiopsis are reduced in size,
making it difficult to take explants containing these tentacles. It may be easier to establish
cultures using several Mnemiopsis cydipid larvae, which have relatively long tentacles,
rather than using the adult Mnemiopsis.

One of the most interesting observations during this project was the presence of
sperm cells in somatic tissue. Actively swimming mature sperm cells have been found in
cultures from 72 hours to 144 hours post isolation. A reasonable hypothesis for this
observation may involve the pluripotent stem cell populations. We suspect that the
pluripotent stem cells are differentiating into sperm cells in culture. It would be beneficial
to monitor isolated stem cell populations in parallel with stem cells mixed with other cell
types to observe any differences in morphology and differentiation over time. It would be
interesting to uncover the origin/source of the cells responsible for triggering sperm cell
differentiation and maturation.

One of the initial goals of this project was to generate gene expression profiles for
each isolated cell type. Our first experiments used slides containing several cell types,
rather than an isolated cell type. We tried extracting mRNA to build a cDNA library;
however, there were not enough cells to extract a quantifiable amount of mRNA. Larger
explants can produce an abundance of cells and leaving the explants in culture longer also
produces more cells; these could be solutions to overcoming this problem. With enough
mRNA, a gene expression profile could perhaps be generated for a ‘typical’ cell culture
containing multiple cell types. The next step would be to isolate enough cells for each
defined cell type in order to generate cell type specific gene expression profiles.

Another approach is to use single-cell RNA sequencing to generate expression
profiles. This method may work especially well for the cell types that have a short
lifespan or have a low abundance in culture, such as muscle cells and multipolar cells.
While this technique can be used for any of the cell types mentioned, it may be especially
useful for targeting the giant smooth muscle cells and the additional muscle-like cells, as
the muscle cells often have the lowest cell count numbers (Table 1, Table 2). Between
48 and 72 hours, individual cells from both of these cell types are relatively easy to
isolate as single cells. Beyond 72 hours however, these cells typically exhibit reduced
representation in primary cell cultures and often appear to join in clusters of cells, making
it difficult to isolate them.
The potential research applications for primary cell cultures are infinite. Therefore, the availability of a robust protocol for generating primary cell cultures for the Ctenophore model system, *Mnemiopsis leidyi* is significant. Further characterization of these primary cell cultures will allow for the investigation of unique aspects of cellular differentiation associated with Ctenophore development and evolutionary history from a cell biological perspective.
References:


