Chapter 18

Isolation and Maintenance of In Vitro Cell Cultures from the Ctenophore *Mnemiopsis leidyi*

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Abstract

The ability to isolate, monitor, and examine specific cells of interest enables targeted experimental manipulations that would otherwise be difficult to perform and interpret in the context of the whole organism. In vitro primary cell cultures derived from ctenophores thus serve as an important tool for understanding complex cellular and molecular interactions that take place both within and between various ctenophore cell types. Here we describe methods for reliably generating and maintaining primary cell cultures derived from the lobate ctenophore *Mnemiopsis leidyi* that can be used for a wide variety of experimental applications.

**Key words** Ctenophore, *Mnemiopsis leidyi*, Suspension culture, Serum, Nonbilaterian

1 Introduction

Ctenophora, also known as comb jellies, are gelatinous invertebrates that inhabit marine ecosystems and represent one of the earliest diverging branches of metazoans [1–3]. The unique rotationally symmetric body plan of ctenophores is composed of two germ layers—an outer ectodermal layer and an inner endodermal layer—separated by a thick collagenous mesoglea populated with a variety of cell types, including muscle and motile stellate cells (Fig. 1) [4]. Recent studies in ctenophores have begun to characterize the range of cell types identifiable by both morphological and functional criteria [5–8] as well as gene expression criteria [9].

Across the metazoan tree of life, the extent to which organisms can heal and regenerate varies dramatically. All animals retain some capacity to repair and replace damaged cells, and the ability to restore injured tissues and organs is widespread among metazoan lineages [10]. Ctenophores have remarkable wound healing and regenerative capabilities (Fig. 2) [11–13]. Among ctenophores, *Mnemiopsis leidyi* has become a model system for understanding a variety of cellular, molecular, and developmental phenomena. *Mnemiopsis* can regenerate wounded tissues, restore entire organ
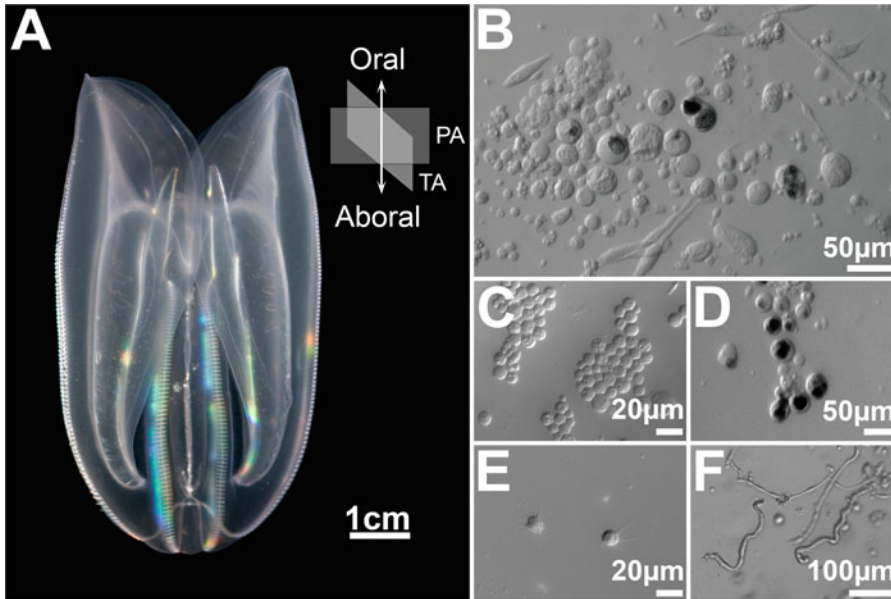
Fig. 1 *Mnemiopsis leidyi*. (**a**) Adult *M. leidyi*, axes labelled on the right, oral oriented up. (**b**) Representative field from primary cell culture 96 h postisolation. (**c**) Isolated proliferating ectodermal cells. (**d**) Isolated proliferating endodermal cells. (**e**) Isolated motile stellate cells. (**f**) Isolated giant smooth muscle cells
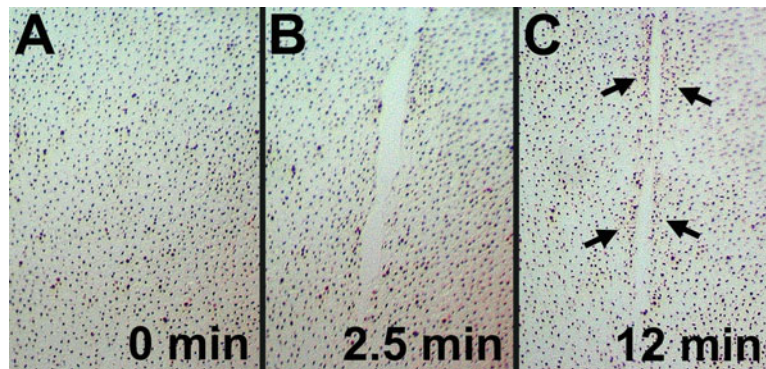
Fig. 2 *Mnemiopsis leidyi* ectoderm epithelium stained with neutral red vital dye. (**a**) Area of contiguous ectodermal epithelium prewounding. (**b**) 2.5 min postwounding with scalpel blade. (**c**) 12 min postwounding. Arrows indicate sites of cell aggregations along edges of the healing wound

systems, and recover large scale deletions of their body plan via whole-body regeneration (WBR) [10, 14]. WBR encompasses a complex set of context dependent cellular activities that includes wound healing, immune response, signaling, proliferation, and differentiation that ultimately result in tissue growth and reorganization of the affected region [14–18]. The phylogenetic position of the ctenophore lineage suggests that an improved understanding of WBR in ctenophores will offer unique insight into the evolution of metazoan regeneration [13, 14, 19].

Primary cell cultures provide a useful tool for the study of ctenophore cell biology. Reliable methods for generating and maintaining primary cell cultures from the model lobate ctenophore *Mnemiopsis leidyi* opens the door for assaying cell biological attributes in specific cell types of interest. In this chapter, we detail cell culture techniques for the selection and preparation of cell sources, the preparation of tissue explants, the dissociation of cells for small and large scale preparations, and cell culture maintenance. These protocols provide simple robust techniques to generate in vitro cell cultures (e.g., Fig. 1b) that can be used for a wide variety of downstream applications including live cell imaging, gene expression profiling, pharmacological assays, flow cytometry, and next-generation sequencing applications.

2   Materials

Store all solutions at 4 °C unless indicated otherwise.

1. Dounce homogenizer with tight and loose fitting pestles (15 mL).

2. Cell culture dishes (30 mm, 60 mm diameter).

3. Cell strainers with nylon mesh pore size of 300 μm, 100 μm, and 70 μm.

4. Filter sterilized artificial seawater (FSW): 35.9 g/L of commercial sea salt in deionized water, 0.2 μm filter-sterilized.

5. 3.125 mg/mL penicillin.

6. 5 mg/mL streptomycin

7. FSW P/S: 1% penicillin, 1% streptomycin in FSW.

8. Minimal Media: 2% (v/v) fetal bovine serum (FBS) in FSW P/S.

9. $10\times$ Phosphate Buffer Solution (PBS): 18.6 mM NaH_2PO_4, 84.1 mM Na_2HPO_4, 1750.0 mM NaCl. Mix phosphates in deionized H_2O and adjust pH to 7.4 with either NaOH or HCl. Store at room temperature (RT).

10. Flow Cytometry Buffer Media: 10 mL FBS, 2.5 mL penicillin, 2.5 mL streptomycin, 2 mL 0.5 M ethylenediaminetetraacetic acid (EDTA), 50 mL 4 M NaCl, fill to 500 mL final volume with 0.2 m filter-sterilized $1 \times$ PBS.

11. $10\times$ dissociation solution: 25 g trypsin, 2 g EDTA • 4Na, 8.5 g NaCl in 1 L of dH_2O.

12. Dissociation media: 9 mL FSW P/S, 1 mL $10\times$ dissociation solution.

3   Methods

Carry out all procedures at RT unless otherwise specified.

3.1   Ctenophore Mesogleal Serum (CMS)

This serum is used to supplement media in which ctenophore cells are maintained in culture.

1. Transfer animal tissue for CMS production to glass Dounce.
2. Dissociate tissue thoroughly using the Dounce homogenizer with 15–20 strokes of a tightly fitted pestle.
3. Spin homogenate at 7000 rcf for 30 min.
4. Carefully transfer the supernatant to appropriately sized conical tubes, leaving sedimented cellular debris behind.
5. Heat-inactivate the collected supernatant for 30 min on low rotation at 56 °C.
6. Mix heat-inactivated supernatant 1:2 with FSW P/S.
7. Allow the CMS to cool to room temperature.
8. Briefly vortex CMS before use.
9. CMS can be stored at 4 °C for 72 h.

3.2   Animal Preparation

1. Screen selected animals for visible parasites, ectosymbionts, and endosymbionts under a light microscope (*see* **Note 1**).
2. Rinse selected animals three times with FSW P/S.
3. Isolate washed animals in fresh FSW P/S overnight (*see* **Note 2**).
4. Perform 4 additional washes with FSW P/S immediately prior to use (*see* **Note 3**).
5. Prepare cell extracts using one of the approaches presented in Subheadings 3.2 to 3.6.

### 3.3   Tissue Explant Preparation for Small-Scale Cultures

1. Shear the bottom 2–3 mm off a microcentrifuge tube using a razor blade to create a jagged coring instrument (*see* **Note 4**).
2. Make a shallow cut along the surface of the ctenophore's epidermis with a scalpel under a light microscope (*see* **Note 5**).
3. Use the cut microcentrifuge tube to core out a small tissue explant at the wound site (*see* **Note 6**).
4. Place and incubate tissue explants in culture media supplemented with CMS at 16 °C (*see* **Notes 7–10**).
5. Remove explant remnants from cultures 24–48 h postplating with a pipette (*see* **Note 11**).
6. Maintain cell cultures as detailed in Subheading 3.7.

3.4 Mechanical Cell Dissociation for Small-to-Medium Scale Cultures

1. Dissect the tissue of interest under a light microscope using a scalpel or razor blade into small fragments that can be easily loaded into microcentrifuge tubes (*see* **Note 12**).

2. Divide the excised tissue among microcentrifuge tubes, filling no more than half of the total microfuge tube volume (*see* **Notes 13** and **14**).

3. Gently homogenize collected excised tissue until the consistency becomes uniformly smooth. Ensure that the pestle does not fully lift out of the sample to prevent introduction of air bubbles into the homogenate (*see* **Note 15**).

4. Spin resulting homogenate at 800 rcf for 10 min.

5. Carefully collect the loosely sedimented cell pellet using a trimmed pipette tip (*see* **Note 16**).

6. Transfer the cell pellet to 30 mm cell culture dishes with 2.5 mL of culture media supplemented with CMS (*see* **Notes 7–9** and **12**).

7. Gently swirl dishes to disperse the cell pellets.

8. Maintain cell cultures as detailed in Subheading 3.7 (*see* **Note 10**).

3.5 Mechanical Cell Dissociation for Large-Scale Cultures

1. Subdivide the prepared animal into tissue fragments using a clean razor blade (*see* **Note 17**).

2. Place prepared tissues in a Dounce homogenizer using a clean trimmed pipette.

3. Gently homogenize with 10–15 strokes of a loosely fitted pestle.

4. Funnel the resulting homogenate through a series of stacked mesh filters (300 μm, 100 μm, and 70 μm) arranged in descending order on a low pressure ring adapter with Luer-Lok port into a 50 mL tube (Fig. 3).

5. Use an appropriately sized syringe mounted to the Luer-Lok port to gently "pull" the viscous homogenate through the filter stack (*see* **Note 18**).

6. Distribute the filtered homogenate into 4 mL aliquots in 15 mL conical tubes for centrifugation.

7. Bring the volume of each conical tube to 8 mL with FSW P/S (*see* **Note 19**).

8. Gently swirl the contents of the tube with a sterile P1000 (or similar) pipette tip to resuspend cells.

9. Spin the tubes at 800 rcf at 16 °C for 8 min to sediment cells. (*see* **Note 20**).
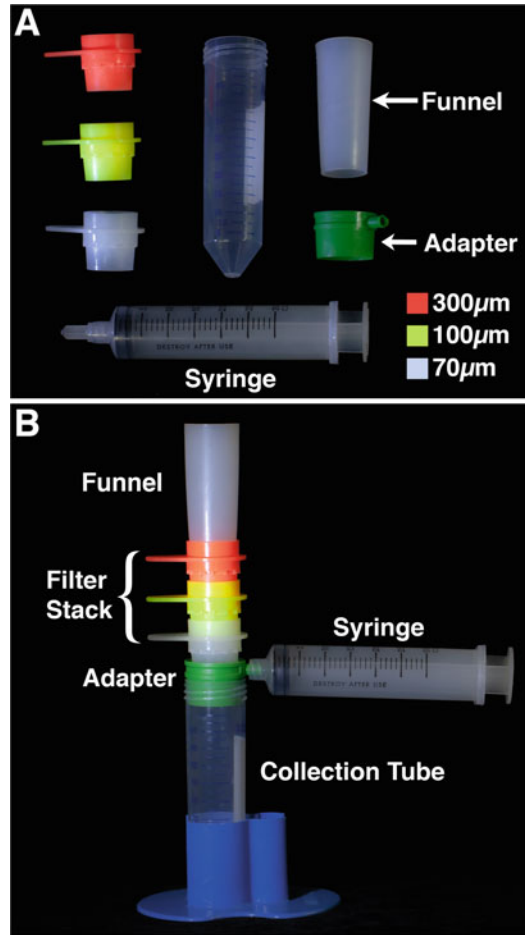
Fig. 3 Filter stack used for cell suspension size selection. (**a**) Filter stack components. The adapter includes a plug for syringe attachment. Legend at lower right indicates filter screen sizes. (**b**) Assembled filter stack with descending 300 μm, 100 μm and terminal 70 μm screens for cell size selection. Mounted syringe allows for the application of light suction below the filter stack to "pull" the initial viscous cell homogenate through the filter screens

10. Remove and discard the upper 4 mL of supernatant, retain the lower 4 mL containing a loose cushion of concentrated cells (*see* **Note 21**).

11. Repeat **steps 7** to **9**.

12. Carefully remove the upper 7 mL of supernatant, making sure not to disturb the lower 1 mL containing the loosely compacted cell pellet (*see* **Note 22**).

13. Carefully add 3 mL of culture media supplemented with CMS to the tube (*see* **Notes 7–9**).

14. Gently resuspend cells using a sterile P1000 (or similar) pipette tip (*see* **Note 23**).

15. Plate the cells in 30 mm cell culture dishes (*see* **Note 12**).

16. Maintain cell cultures as detailed in Subheading 3.7.

3.6 Preparing Enzymatically Dissociated Cells

1. Prepare glass slides for cell adherence and subsequent imaging by incubating slides with any desired collagenous matrices, according to manufacturers' instructions (*see* **Note 24**).

2. Excise tissue fragments using a razor blade.

3. Transfer the excised tissue to a glass Dounce.

4. Add 500 μL of FSW P/S.

5. Gently homogenize with 10–15 strokes of a loosely fitted pestle.

6. Centrifuge the resulting homogenate for 10 min at 350 rcf at RT to pellet cells.

7. Remove and discard the supernatant.

8. Add 10 mL of dissociation media.

9. Pipet up and down gently to break up cell pellet.

10. Transfer homogenate to a 15 mL tube.

11. Place tube on its side on an orbital shaker and agitate at 75 rpm for 10–15 min at RT.

12. Pellet cells by centrifuging for 10 min at 350 rcf at 16 °C.

13. Inactivate residual trypsin by resuspending the cell pellet in media (*see* **Note 12**; for example in FSW P/S + 10% CMS or 10% FBS).

14. Centrifuge for 10 min at 350 rcf at 16 °C.

15. Resuspended the enzymatically dissociated cells in culture media supplemented with CMS and plate as desired for downstream assays (*see* **Notes 7–9** and **12**).

16. Maintain cell cultures as detailed in Subheading 3.7 (*see* **Note 10**).

3.7 Primary Cell Culture Maintenance

1. Incubate cell cultures in humidified chambers at 12–16 °C (*see* **Note 10**).

2. Perform a ~50% media exchange every 48–72 h. To retain loosely attached and/or unattached cells, allow cell culture dishes to rest at a slight angle for at least an hour prior to performing media exchange (*see* **Note 25**).

3. Ctenophore primary cell and tissue cultures can be maintained under a wide range of CMS concentrations (*see* **Notes 6**, **26** and **27**). CMS with high mesogleal serum content will yield a higher-viscosity media.

4   Notes

1. Cultured animals are the preferred source of tissue for generating primary cell cultures in part due to decreased parasite loads. When the use of wild-caught animals is necessary, screening for visible parasites, epibionts, and/or endobionts is recommended. The translucent tissue of *Mnemiopsis* allows for rapid screening by light microscope for areas of discoloration and/or melanization that can often accompany infestations. Tissues that appear to be affected and heavily parasitized animals should be avoided.

2. Isolating selected animals overnight in fresh FSW will allow time for gut clearance and will reduce food debris contaminants in cell cultures.

3. After overnight incubation in FSW, additional washing of animals selected for cell preparations helps remove excess mucus and any loosely attached epibionts from the surface of the ctenophore.

4. Using a crude coring instrument generated from a cut microcentrifuge tube is advantageous for generating explants as the resulting explants have uneven/rough edges that slow wound healing. Delaying wound closure allows time for cells to migrate into the culture media before wound sites seal.

5. The shallow cut made in the epidermis serves to mark the site from which the explant will be taken and helps to keep the coring instrument in place over the desired explant tissue.

6. Align the cut end of the microcentrifuge tube over the wound site. Forceps may be used to hold the ctenophore in place. Press down and twist to tear the tissue generating a small explant.

7. CMS is an undefined media that partially recapitulates the in vivo environment and supports both cell growth and survival. CMS can be diluted with FSW P/S over a wide range of concentrations ($1\times$–$6\times$) without significant adverse effects on primary cell cultures. CMS is typically generated from the entire animal. CMS aliquots stored at 4 °C remain useful for 72 h.

8. Minimal media is highly reduced, has a significantly lower viscosity than CMS and aliquots stored at 4 °C remain useful for 2 weeks.

9. The flow cytometry buffer media is ideal for short term exposure in cell cultures that are explicitly prepared for flow cytometry processing. This media is pH and salinity buffered and also reduces cell clumping.

10. Cell cultures are incubated at approximately 16 °C in humidified chambers to prevent rapid changes in media osmolarity due to evaporation. This temperature was found to be optimal for long term cell culture maintenance. Low incubation temperatures also reduce metabolic load and slow bacterial and fungal growth in cultures.

11. In cell cultures prepared from tissue explants, after 24–48 h the remaining explant tissue should be removed to reduce metabolic load and potential cell crowding. Explant remnants are most easily removed from the culture dish using a pipette.

12. Specific tissues can be targeted for dissection and placed in culture. The tissue type, amount, as well as the destination plate/well size will depend on the assay or downstream application being performed. Thus cell density seeding should be optimized prior to performing downstream experiments.

13. A trimmed plastic transfer pipette, with an opening large enough to mitigate shearing effects that can lyse cells, can be used to transfer tissue segments.

14. Dissected tissues are divided across multiple tubes so that no more than half of a tube volume is filled prior to homogenization to prevent overflow/spilling.

15. Manual homogenization using a pestle and microcentrifuge tube takes approximately 2 min to produce a uniform smooth homogenate. As the homogenate becomes more uniform in consistency, its relative viscosity/stickiness should reduce along with the absence of visible tissue fragments.

16. Many ctenophores are relatively transparent. Thus, the loose cell pellet is often not clearly visible. Retaining the lower portion of the microcentrifuge volume will ensure the recovery of loosely sedimented cells.

17. Subdividing an animal into several tissue fragments makes it easier to load into the Dounce. For generating cultures other than from the whole organism or pharyngeal specific cultures, exclude the pharynx region. The proximal third of the pharynx produces digestive enzymes and is a relatively low pH environment. In small cultures the inclusion of high numbers of pharyngeal cells may alter the pH environment and introduce proteolytic enzymes that can have a negative effect on cell culture maintenance.

18. The initial ctenophore tissue homogenate is typically very viscous. Using a ring adapter with Luer-Lok port and a syringe to provide light suction significantly reduces the time required to filter viscous homogenates. Stacking progressively restrictive filter meshes allows for efficient size selection. This filtering method can be optimized to collect desired cell fractions based on a fixed upper cell size limit.

19. Ctenophore cell cultures are vulnerable to bacterial contamination. To inhibit bacterial growth within cultures, remove small noncellular particulates, and reduce the viscosity of filtrated cell homogenate, cell suspension homogenates are mixed with FSW P/S.

20. Centrifugation at 800 rcf results in a loose cell pellet at the bottom of the tube. When a whole animal is used, the final cell suspension after washing will yield approximately one million cells/mL.

21. Discarding the upper supernatant removes lysed cell material and small particulates. The bottom half of the initial homogenate volume is retained to ensure the concentrated cells will be collected.

22. The cells will be concentrated in a loose pellet. Often this cell pellet is not clearly visible. After two spin steps, the bottom 1 mL of the initial homogenate volume will contain a loose cell cushion.

23. Carefully resuspend cells in media by using a cut P1000 tip or wide tip transfer pipet. Carefully disperse the cells by slowly and gently pipetting up and down, high shear forces will lyse cells.

24. To promote cell attachment, glass slides can be coated with a collagenous matrix diluted in FSW P/S following manufacturer's instructions.

25. For media exchanges, set culture dishes on a ramp with a slight angle (we use a small 3D printed wedge with a 4° slope) and leave undisturbed for 1 h to allow nonadherent cells to settle and collect at the lowest point of the dish. Carefully and slowly remove ~50% of the culture media and replace with fresh media.

26. Long-term cell culture health is visually assessed during 50% media exchanges. When incubated at 12–16 °C with 50% media exchanges every 2–3 days, *M. leidyi* primary cell cultures can be maintained for >20 days. Primary cell cultures represent complex mixtures of cell types, including those competent for proliferation and also terminally differentiated cell types that will senesce [6]. Thus, the composition of *M. leidyi* primary cell cultures maintained over extended time periods is dynamic.

27. The described tissue explant, cell preparation, and cell culture maintenance protocols have been successfully applied to additional ctenophore species; *Bolinopsis infundibulum*, *Bolinopsis vitrea*, and *Pleurobrachia bachei*.

Acknowledgments

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