# Chapter 2

# Obtaining Gametes and Embryos of Ascidians

## Charles C. Lambert

## Abstract

Ascidians are basal chordates that have become increasingly important for understanding chordate evolution. They comprise three orders. In the orders Phlebobranchia and Stolidobranchia, most species freely spawn eggs and sperm, whereas members of the order Aplousobranchia form colonies that brood their eggs and broadcast sperm. In the two free spawning orders, eggs and sperm are easily obtained for in vitro fertilizations. In the third order, slices of colonies yield gametes and embryos of all stages. Methods are described for obtaining gametes, performing fertilizations, and culturing embryos. Also included are methods for removing follicle cells and vitelline coats from oocytes.

**Key words** Ascidian, Tunicate, Aplousobranchia, Phlebobranchia, Stolidobranchia, Demembranation, Defolliculation

## 1 Introduction

The phylum Chordata includes the subphyla Vertebrata, Tunicata, and Cephalochordata. Tunicates are currently considered to be the sister group of the vertebrates [1]; they include the class Ascidiacea which are all sessile as adults and the pelagic Appendicularia and Thaliacea. Ascidians include both solitary and colonial forms. All tunicates are marine and require a salinity of at least 20 parts per thousand for development. All ascidians are hermaphroditic; some are protandric, but most are simultaneous hermaphrodites. Self-fertilization is possible in some species, but many are self-sterile [2]. The ascidians include three orders: Aplousobranchia, Phlebobranchia, and Stolidobranchia [3]. Aplousobranchs are all colonial with small zooids only a few mm in length embedded in a common tunic matrix. Each zooid in the colony is cloned from buds. Because of the small size of the zooids, only a few eggs are produced per zooid. The eggs are generally large and may take

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weeks to develop fully. Aplousobranchs spawn large complex sperm which fertilize brooded eggs. This type of reproduction has been termed spermcast reproduction [4]. Aplousobranch embryos are difficult to culture outside the colony, so most studies of their development come from embryos dissected from colonies. The location of embryos within the colony varies from family to family. Embryos may be within the zooid atrium, in brood pouches, or in the basal tunic of the colony.

Phlebobranch and stolidobranch ascidians are mostly solitary free spawners with eggs from 100 to 300 μm. Thousands of eggs are released at each spawning with fertilization occurring in the sea. These eggs are small compared to those of aplousobranchs, and the sperm is also much smaller and less complex than that of aplousobranchs. In the Stolidobranchia, the family Styelidae includes a number of colonial species. *Botrylloides violaceus* produces small eggs with a true placenta forming very large and complex larvae which may take several zooid generations to develop [5]. Only a few phlebobranchs are colonial. *Ciona intestinalis* is a large solitary ascidian that has been classified as either aplousobranch or phlebobranch in various publications. It has some properties of both groups. Based upon its small and simple sperm, its egg and tadpole larva, as well as its large size and solitary nature, it is considered a phlebobranch here.

## 2 Materials

Traces of detergents, soap, fixatives, and metal salts are toxic to gametes and embryos. Thus, it is best to use new glassware and plasticware whenever possible. Glassware can be washed with Micro cleaner and rinsed several times with very hot tap water followed by distilled water rinses; all glassware and instruments must be free of contaminants. If in doubt, wash.

### 2.1 Buffers and Seawater Solutions

1. Buffer A: 0.56 M NaCl 1 % EDTA (tetrasodium) pH 4.2.
2. pH 6 SW: with a pH meter measure pH and add 1 N HCl dropwise to clean SW to pH 6.
3. pH 9 Tris SW: with a pH meter add dropwise 1 N Tris to pH 9.

### 2.2 Solutions for Removing Vitelline Envelopes

1. Treat eggs with 0.1 % trypsin solution in SW for 2 h. Pipette to remove membranes. Wash thoroughly.
2. Buffer B: to 20 ml seawater (SW), add 0.15 g dithiothreitol, 0.72 g CHES, and 0.02 g pronase E = Sigma P6911 or P5147 pH 9.2 (make fresh).

## 3 Methods

### 3.1 Obtaining Gametes and Embryos from Aplousobranchs

Colonies are invariably asynchronous with regard to blastogenic and embryonic stages, making it necessary to collect and dissect several colonies to be assured of obtaining the required stages. Since aplousobranch eggs are brooded for extended periods and difficult to culture in the laboratory, it is usual to dissect colonies and isolate the required stages [6, 7]. Place the colony in a 60 mm Petri dish and slice it into 1–3 mm slices. Under a dissecting microscope, remove the required embryos. In *Distaplia* (family Polycitoridae), the embryos are embedded in the colony within a brood sac that may be independent of the zooids. In *Aplidium* [7] and other species from the family Polyclinidae, as well as other polycitorids, zooids brood their embryos in enlarged thoracic atria. Didemnid embryos are brooded in the tunic at the base of the colony below the zooids. Thus, it is important that the basal tunic is included when colonies are collected. This is also true for the colonial botryllid stolidobranch *B. violaceus*, though in other botryllids, the embryos are brooded in the zooids. Swimming tadpoles can be collected with a tadpole collector in response to light following darkness [8].

### 3.2 Obtaining Gametes and Embryos from Phlebobranchs

Store adults under continuous light to prevent light-induced spawning [9]. *Ciona* and *Corella* spawn when exposed to light for about 30 min after a 1 h dark period [9–11]. *Ciona* is self-sterile, so be sure to spawn or dissect at least two if you want fertilization [12]. Dissection is necessary for other phlebobranchs.

1. In the family Ascidiidae which includes *Ascidia*, *Ascidiella*, and *Phallusia*, remove the tunic to expose the gonoducts which are easy to see when full of gametes (*see* **Note 1**).
2. Dry the animal thoroughly with a Kimwipe, being especially thorough in the region of the gonoducts.
3. The oviduct overlies the sperm duct. Using a needle held parallel to the oviduct, carefully make an opening, and the eggs will stream out. The eggs are held together by a viscous mucus coating. Pick up the eggs on the *outside* of a dry Pasteur pipette. The mucus will stick the eggs to the outside of the dry pipette. Keeping the operating area dry prevents the eggs from streaming away (*see* **Note 2**).
4. Remove the tunic from *Ciona*, and open the atrium with fine scissors to expose the gonoducts. Dry the animal and remove eggs as for other phlebobranchs. After the eggs are removed, the sperm can be collected "dry" (i.e., in a concentrated mass) with a dry Pasteur pipette or a positive displacement pipette if sperm are scarce. Store sperm in an iced Eppendorf tube. Dry sperm may be stored up to a week in the refrigerator.

### 3.3 Obtaining Gametes and Embryos from Stolidobranchs

Store animals under continuous light to prevent unscheduled spawning [13] and to accumulate gametes. As is the case for *Ciona* and *Corella*, *Molgula* will spawn in response to a short light cycle [11]. Many species of *Styela* and sometimes other stolidobranchs will spawn if stored in the dark for 12 h or so and then exposed to light for 12 h. Many stolidobranchs are self-sterile, so it is necessary to use two or more animals to start a culture. If you wish to experimentally fertilize a culture, it is necessary to use a self-sterile species so that fertilization does not occur while dissecting eggs (*see* **Note 3**).

Many but not all species of the stolidobranchs including *Boltenia*, *Herdmania*, *Pyura*, *Styela,* and *Halocynthia* are self-sterile. *Molgula* spp. are generally self-fertile [2]. A few solitary stolidobranch and phlebobranch species are ovoviviparous. In this case, opening the atrium will release the brood.

1. To obtain gametes, clean epibionts from the tunic and bisect with a razor blade through the siphons. Remove from tunic and lay out the isolated half animals in a 60 mm Petri dish covered with SW. With forceps, remove and discard the branchial basket to expose the ovary and testis in the body wall. Oocytes with intact germinal vesicles are stored in the ovary. Germinal vesicle breakdown occurs within 30 min of dissection in pH 8 SW. If you want oocytes with intact germinal vesicles, dissect in low pH SW [14].
2. Remove the gonads and place in 35 mm Petri dish, chop with fine scissors, then pipette in and out of a Pasteur pipette. Be sure the scissors are clean and not contaminated with formaldehyde or detergents.
3. Pour through coarse (300 μm) Nytex. Squirt SW through tissue remaining on filter to remove more eggs and sperm. Pour egg/sperm suspension into 100 ml beaker with SW. Use a larger beaker if necessary.
4. Wash oocytes in a beaker by placing a 100 μm filter which is attached to the bottom end of a 38 mm Plexiglas tube 70 mm long into the beaker. Withdraw supernatant SW from the Plexiglas tube with a large-volume syringe or pipette to remove the supernatant. The filter allows removal of supernatant without loss of mature oocytes. Wash until supernatant is clear of sperm, adding more SW to the beaker as needed. This method also removes small immature oocytes that pass through the Nytex mesh. The Nytex screen prevents eggs from entering the interior of the tube where the supernatant is collected (*see* **Note 4**).
5. Eggs from colonial styelids are obtained by dissection as described for aplousobranchs. Embryos of *B. violaceus* are in the base of the colony. Thus, be sure to collect the whole

colony and not just the surface layers. The embryos of *Botryllus* spp., other *Botrylloides* spp., and other colonial styelids are brooded inside the atrial cavity of the individual zooids.

## 4 Fertilizations and Culture Methods

1. To insure synchronous fertilization, soak eggs for an hour in pH 6 SW. Wash 4× with normal pH 8 SW.
2. Dilute dry sperm 1:1,000 with clean normal SW or pH 9 Tris SW (*see* **Note 5**).
3. Add 10 ml diluted sperm to 100 ml of egg suspension. Most ascidians do not have a fertilization membrane. Thus, it can be difficult to know if fertilization has occurred before cleavage. However, if you examine the eggs with a 100× microscope 5 min and 15 min after fertilization, you will see that fertilized eggs are now quite elongate or pear shape [15]. About 10 min after fertilization wash off exogenous sperm with clean SW (*see* **Note 6**).
4. For short-term culture, you can leave the embryos in a monolayer in the bottom of the beaker where they will develop well to hatching without any aeration or agitation. Swimming tadpoles can be collected from the upper levels of the beaker; they are photopositive when first hatched but become photonegative when ready to settle.
5. For large cultures, place the embryo suspension in a 1 gallon jar and agitate with a plastic propeller at 30–60 RPM. Large quantities of tadpoles and newly metamorphosed tadpoles may be cultured in this manner.

### 4.1 Follicle Cell Removal and Isolation

1. To remove follicle cells of phlebobranch eggs, place eggs in $Ca^{2+}$-free SW containing EGTA and agitate vigorously [16]. Alternatively, rapidly squirt eggs in and out of a syringe with a 26 gauge needle (*see* **Note 7**).
2. For stolidobranchs like *Boltenia* and *Halocynthia*, removal of follicle cells is accomplished by a modification of a method by Fuke [17]. Oocytes are placed in a 15 ml centrifuge tube of Buffer A and shaken periodically for 30 min followed by 20 passages through a 163 μm Nytex filter. Follicle cells can then be separated from oocytes by passage through a 100 μm Nytex filter and pelleted in a clinical centrifuge at Ravg 1600 (Rmax 2683; 4,000 RPM) for 10 min and then re-suspended in SW [18].

### 4.2 Removal of Vitelline Coats

Originally vitelline coats were removed with needles, and this method is still in use when it is desirable to avoid the use of enzymes

[19]. To remove the vitelline coat, follicle cells, and test cells of *Phallusia*, agitate eggs in trypsin [20] (*see* **Note 8**).

The following method works on *Ascidia*, *Ciona*, and *Boltenia* eggs.

1. Add oocytes to freshly made Buffer B.
2. Rock gently 1.5–2 h (Nutator or other device that gently agitates gametes).
3. Place in tube, and shake fairly vigorously.
4. Wash by settling in clean SW.

## 5 Notes

1. Wear gloves when dissecting ascidiid tunicates as blood will leave a green stain on your fingers.
2. Do not suck up the eggs as they will stick to the inside of the pipette and are difficult to recover.
3. To avoid self-fertilization in self-fertile species, dissect eggs into pH 6 SW and wash the eggs with pH 6 SW until free of sperm. Return the eggs to pH 8 SW and observe at expected time of cleavage to be sure that no self-fertilization occurred [10].
4. Be sure to aspirate supernatant slowly enough that eggs are not forced through Nytex. Check with microscope to be certain that no eggs are being lost.
5. Check motility of diluted sperm without a coverslip using dark-field illumination and 40–100× magnification. To obtain dark-field illumination, shift the phase rings of a phase contrast microscope slightly off center. If sperm do not show vigorous translocational swimming, use pH 9.2 (Tris) SW to make another sperm preparation.
6. Left over sperm will rot and bacteria tend to kill embryos.
7. Be careful to examine *Ciona* eggs with a compound microscope as many treatments break the elongated *Ciona* follicle cells leaving the base of the cells still attached to the vitelline coat.
8. Another demembranation method utilizes higher pH and thioglycolic acid [21]. Demembranated eggs and embryos stick and lyse when they contact glass or plastic. To prevent this, coat glassware, plasticware, and cover slips with 1–2 % agar or as follows: soak briefly all glassware, plasticware, etc., in 0.1 % gelatin, 0.1 % formaldehyde in distilled $H_2O$. Rinse in tap then distilled water, drain thoroughly, air dry, and store until needed [22].

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