Feeding the Masses: Colonial Transport in the Marine Bryozoan *Membranipora membranacea*

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Abstract

The transport system of cheilostome bryozoans is unusual among long-distance transport systems. In these colonial animals, a network of strands (the funicular system) carries nutrients to non-feeding individuals and to the growing edge of the colony. However a complex of cells appears to plug the pores that connect individuals. Focusing on the cheilostome, *Membranipora membranacea*, we used time lapse movies to test whether there were contractions/dilations of funicular strands, as expected if muscular pumping were to move material through the strands, and to test whether cells or large vesicles moved directionally along the strands, potentially carrying nutrients. Neither contractions/dilations of the funicular strands, nor persistent movement of particles or other features along the strands, were visible in time lapse videos (10 to 120 min at 4 to 10 sec per frame). The only visible movements were rare back-and-forth movements along the strands, or shaking of the strands. We injected materials that differed in molecular/particle size to investigate the specificity of transport at pore plates. Both fluorescein (367 Da; as sodium salt) and fluorescein-dextran (70,000 Da) moved between individuals; however 2.0μm fluorescent polystyrene beads did not. The fact that both fluorescein and fluorescein-dextran were transported suggests that transmembrane channel or transporter proteins are not required for transport; however there may be an upper size limit (<2μm) caused by something other than the pore itself. Our results are consistent with some transport mechanisms (e.g. paracellular diffusion or transcytosis at the pore plate) but inconsistent with others (muscularly-pumped flow along funicular strands, cell crawling, or transmembrane transport via transporter or channel proteins).

Introduction

Because all animals have to transport nutrients from their point of uptake to other tissues, different types of transport systems have evolved that vary in transport mechanism and complexity (Ruppert and Barnes, 2004; Ruppert and Carle, 1983). The two major types that exist in bilaterian animals are coelomic and blood-vascular (hemal) transport systems (Ruppert and Barnes, 2004; Ruppert and Carle, 1983). Coelomic transport systems are a series of chambers or a single chamber that transports material (e.g. nutrients, O_2) using ciliary movement or diffusion (Ruppert and Barnes, 2004). Blood-vascular (or hemal systems) use muscle contraction or pumping of either the vessel or muscle surrounding the vessel to transport material (Ruppert and Barnes, 2004). In both types of systems, nutrients and other materials are carried by flowing fluids for long distance transport. Similarly, in cnidarians and ctenophores, fluid flow in the gut cavity or gastrovascular system is involved in transport (Ruppert and Barnes, 2004). In small animals, diffusion may be the primary transport mechanism.

Although the transport system of cheilostome bryozoans appears homologous to the hemal system of other bilaterians (Carle and Ruppert, 1983; Ruppert and Carle, 1983), structural differences suggest it may not function in the same way. Bryozoans are colonial suspension feeders. The colonies grow by asexual budding. All the asexually produced individuals (zooids) within the colony remain anatomically and physiologically linked. Individual zooids catch and digest suspended food particles using a crown of ciliated tentacles (the

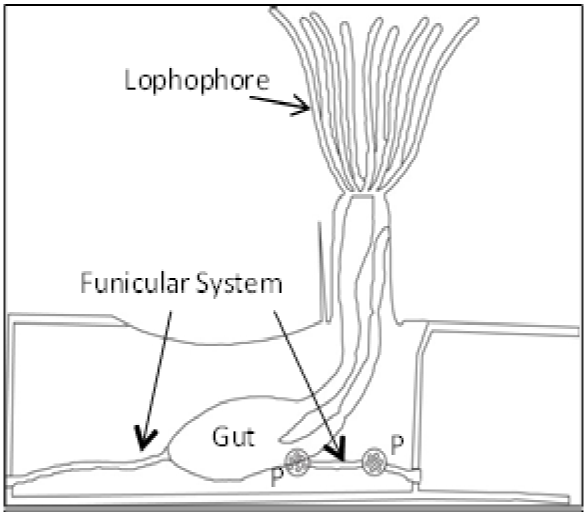

Figure 1 Diagram of a Cheilostome bryozoan P (pore plates)

lophophore; Fig. 1). These feeding zooids have to transfer nutrients to non-feeding individuals such as growing buds or specialized zooids (Best and Thorpe 1985; Harvell and Helling 1993; Miles et al.1995). However, in the cheilostome bryozoans, the zooids are separated by calcified walls (Mukai et al. 1997). The cheilostome bryozoan's transport system consists of a branched network of strands called a funicular system that connects between individuals of the colony at specialized regions of the walls called pore plates (Fig. 1) (Mukai et al., 1997; Bobin, 1979). What makes the cheilostome system so unusual is that at the pore plate there is actually a barrier of three layers of cells plugging the pore, and separating the individual transport systems of each zooid from each other (Mukai et al., 1997; Bobin, 1979).

According to several previous studies, despite this unusual breakup of the transport system, cheilostome bryozoans still achieve colonial transport, and there appears to be a trend of materials transporting towards the growing edge of the colony. In one study, Lutaud found that a colony fed ^{14}C labelled algae showed transport of ^{14}C to transport to the external wall of the individual zooid, and vitally stained food reaching the pores within four hours of ingestion (Lutaud, 1983). Two other studies using ^{14}C fed colonies that showed that ^{14}C was transported between individuals in the direction of the growing edge (Best & Thorpe 1985; Miles et al. 1995). .In an experiment exploring inducible reproduction, Harvell and Helling presented a theory that the growing edge acts as a carbon sink and pulls the transport in a unidirectional manner towards the growing edge (Harvell and Helling, 1993).

The purpose of this study was to test five hypotheses for how the funicular system and the pore plates transport material between zooids. The first hypothesis is that material is transported by pumping of fluid through the lumen of the funicular strand by muscle activity. Although there is no known heart in bryozoans, muscular activity in the zooids themselves could pump material through the strands. For example, pressure changes in the body cavity during lophophore extension and retraction could drive fluid flow through the funicular strands. Also, hypothetically, contraction and relaxation of the cells that make up the strands could drive fluid along the strands. In principle, the cells plugging the pores could open a channel to allow fluid flow between zooids. The second hypothesis is that vesicle transport along the strands is responsible for moving material along the strand and across the pore plate. The third hypothesis, related to the previous one, is that cell-crawling along the strands transports material. The fourth hypothesis is that transcytosis (i.e. endocytosis on one side of the cells plugging the pore, followed by exocytosis on the other side of the cell; Tuma and Hubbard, 2003) in the cells that plug the pores is responsible for the movement of material across the boundary of the pore plate. The fifth hypothesis is that transport at the pore plates is occurring due to transmembrane transport, such as by transporter or channel proteins, moving molecules across the membranes of pore cells (Hill et al., 2008). In principle, diffusion could also carry material across the pore, although this seems unlikely given the directionality of transport found in previous studies (Lutaud, 1983; Best and Thorpe, 1985; Miles et al., 1995).

These hypotheses were tested using time lapse videos and injections of materials with different molecular or particle sizes. The first hypothesis about muscular pumping or strand contraction driving fluid flow predicts that the strands will contract and dilate like blood vessels in the human body. The hypotheses that cell crawling or vesicle movement drives transport along the strands predicts that features should move directionally along the strands.

The size-selectivity of the plate was tested by injecting materials of differing molecular or particle sizes to determine what molecular sizes are transported, and if transport is visible across the pore. Because transmembrane transporters and channels are highly specific for particular molecules or types of molecules (Hill et al., 2008), the hypothesis that transmembrane transport is involved predicts that only specific small molecules would be carried across. Transcytosis is less specific for molecule types (Tuma and Hubbard, 2003), however we would not expect that very large particles (on the order of the size of the cells themselves) would be transported. Diffusion across cell membranes can only occur with small, uncharged molecules or non-polar molecules, however, both small and large molecules, and charged and uncharged molecules, could potentially diffuse between cells.

The cheilostome *Membranipora membranacea* was chosen for this study because it was a readily abundant species, and its single-layer encrusting growth pattern makes it easy to view the funicular system and the individual zooids.

Methods

Preliminary studies were performed in order to determine if transport between zooids could be visualized using a microscope, and to determine if injecting into the coelomic cavity was a viable option of inserting the desired material into the zooid and achieving transport between individuals, as opposed to inserting the material into the gut . Methylene Blue dissolved in sterile seawater, or 1% Sumi Ink in RO water was injected and used for the time lapse videos.

A preliminary test to this study determined that fluorescein sodium salt, fluorescein dextran, and fluorescent microbeads were ideal for testing the size-selective permeability of the pore plates because they could be easily seen under the microscope and could be injected with few problems using a Picospritzer III pressure microinjector.

The fluorescein sodium salt was made at 0.01% concentration. It was first mixed in a 2mL centrifuge tube using RO water to create a 1% solution, and then diluted to 0.01% in a PCR tube using sterilized seawater. The fluoresbrite beads were prepared by diluting by a factor of 100 in an eppendorf tube using RO water out of concern for organic matter in the seawater interfering with the properties of the beads or causing them to adhere to each other. The 70,000 Da (70 kDa) dextran was premade with 25mg/ml in double distilled water.

Size-Selective Permeability

In order to test the size-selectivity of the transport system, *Membranipora membranacea* colonies were collected on sea grass blades in False Bay, San Juan Island, WA. The colonies were then gently removed from the grass by bending the grass to peel the colony away from the blade. Colonies were collected and peeled off seagrass as described above free of any algae or detritus on the blades that might interfere with viewing the funicular system of the colony. After removal the colonies were placed in a mesh-sided divider box in a sea table for 18-24 hours to allow the colonies to recover from being peeled off of the grass.

Prior to injection the colonies were imaged using Motic 2.0 software and a Moticam 2500. The images were put into the program Paint and injected zooids were labeled in the image to help keep track of the location of injection sites between injection and viewing them under the microscope. The images were also used to keep a record of any transport that was seen during the trials by marking zooids where transport was seen at the 1, 5, and 10h time intervals.

The colonies were prepared for injection by flipping them upside down in just enough water to keep them wet, and then injected through the bottom of the colony where there was less resistance from the cuticle. The colonies were injected with one of three substances; 0.01% fluorescein sodium salt (367 Da), 2.5% fluorescein-dextran (70.000 Da; courtesy of Dr. Lance Davidson), 5% fluoresbrite plain YG 2.0 micron polystyrene microspheres from Polysciences

Inc (cat# 19096-2). The needle used was a *Xenopus* style needle from the Sutter Instrument Cookbook. For the 2μm bead injections, the colonies were also given 160 μL of 0.33M $MgCl_2$ to anesthetize the colony and prevent muscle movement from expelling the beads from the injected zooid. Three injections in each colony, with six colonies for each injection material, for a total of 18 injections per injection material. Each injection material was done on a different day with different colonies. The injection volumes ranged from 0.005 nL to 1.5 nL due to inconsistencies when breaking the needle tip to allow injection, especially for the microbeads.

After the colonies were injected they were placed in petri dishes of seawater which were then placed into dark plastic bags in a seatable to prevent photobleaching. Images were taken and observations of the injection sites and transport were recorded at 1, 5, and 10 hours.

Time-Lapse Videos

For time-lapse videos, colonies were placed into petri dishes with fresh seawater and a 48mmx65mm coverslip was placed over the colony to improve optics by holding the colony in place. The colony was placed onto a Nikon TE2000-U Inverted Microscope. The time-lapse was taken viewing through the bottom of the colony because it offered the best view of the funicular system. All time-lapses were taken using brightfield and the 20x objective.

In order to track if transport was occurring during the time-lapse, the descendent zooid at the growing edge was imaged before and after the time-lapse in 6 colonies, and the growth measured using the Image J program. Difference and percent change were calculated. After each time-lapse the colony was checked to make sure that the colony was still alive, and for colonies injected with methylene blue, that the injected zooid was still alive.

Time-lapses were left to run for varying amounts of time (10min-4hour) with the average length being two hours. The videos were focused on either the adult zooids (n=21) or on developing zooids at the edges (n=7) of the colony where the individuals had developed, but were not able to feed yet (Fig 2). Frame rates of 4 s/f to 10 s/f were used. Thirteen time-lapses were run with Methylene Blue injected to visualize transfer with the dye, and 15 videos were taken with no material injected. After completion of the time-lapses, the videos were observed for signs of cell-crawling or visible movement within the funicular system, and analyzed using kymographs for contraction or dilation the funicular strands. Kymographs are made from a series of frames of a time-lapse movie by taking a thin slice from each frame of the movie, and stacking those slices next to each other. This allows one to look at changes over time occurring along that slice (e.g. a single line of pixels) in a single two dimensional image.

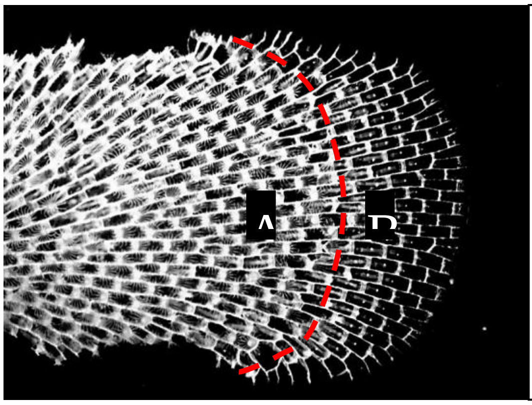

Figure 2 Location sites of time-lapse videos. Dotted line indicates where feeding zooids turn into developing (non-feeding) zooids
A: the area where adult zooids were observed
B: The area where

Results

Time lapse videos

The hypothesis that transportation occurs due to muscular contractions driving fluid flow through the lumen of the strands, such as in blood vessels, predicts that we would see changes in the diameter of the funicular strand over time during time-lapse videos. All twenty-eight time-lapse videos were observed for contractions/dilation. In both the stained and unstained videos there was no visible contraction or dilation in a crosswise manner of the individual strand seen. In two videos the strands were seen to be stretching towards the proximal end throughout the duration of the video, however in this case the zooid was found to have died during the video so the shrinkage is assumed to be the decay of the funicular system after death. Also in both the methylene blue and the non-stained zooids violent movement of the system was seen when the entire lophophore and gut reacted. This movement was likely driven by the gut pulling on the funicular system due to observations of gut movement see at the same time as the jerking motions. However it was not associated with obvious contraction or dilation of the funicular strands.

The hypotheses stating that vesicle transport along the strand and/or cell crawling was responsible for transport predicts that in the time-lapses we would observe features moving along the strands in a persistent, unidirectional manner. Such features could include cells or groups of cells, large vesicles or aggregates of vesicles, or the entire strand (if all the cells move in the same direction). However, the observations taken from all twenty-eight time-lapses show no persistent, linear movement of either vesicles or cells regardless of location within colony.

In the thirteen methylene blue injections, the injected dye formed into small spherical shapes that did not appear in the funicular system and did not move except in a few cases where a spherical blob was seen to move a slight distance over time in the zooid. However, there was no discernible or predictable pattern to their movement; they would move sometimes and not others, and only some blobs of dye would move, not all. There was one out of thirteen methylene blue injections where after four hours the methylene blue appeared in the funicular system, but another time-lapse immediately following showed no movement of the dye (see fig. 3) It is unknown if the dye stained cells in the system or if the system had absorbed vesicles of the dye.

A mass of cells was also seen to grow and disappear on the side of the funicular system, but it did not travel along the system (see figure 3, center of strand; arrow indicating it).

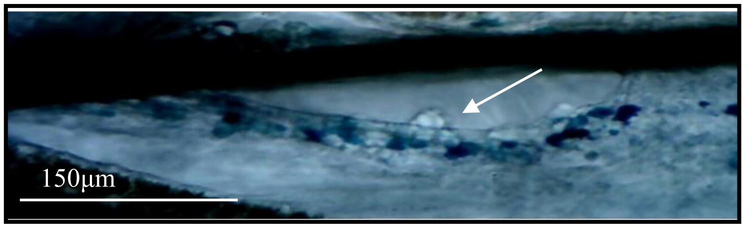

Figure 3, Methylene Blue lined up in the funicular system four hours after injection; Arrow indicates mass of cells that grew and disappeared

We did not see the predicted persistent movement of material along the funicular strand in the fifteen time-lapses with no staining. There was small random movement observed where unknown material could be seen moving in a back and forth linear motion along the strand, but with no net direction.

Kymographs were created (n=8) to determine if there was movement of material occurring along the strands. Kymographs display a line in space over time, so that one can see changes along that line over time in a single two-dimensional picture. If there was movement along the strands, we would expect to see an angled or curved trajectory of darker bands over time in transverse slice along the strand.

We saw no indication of persistent movement of material in a particular direction (Fig 4). Slight variations from a straight trajectory in the form of jitters or small jumps (i.e. lines appear wiggly) were seen in most kymographs. However, aside from the small jitters, lines remained approximately parallel to the time axis, indicating that features

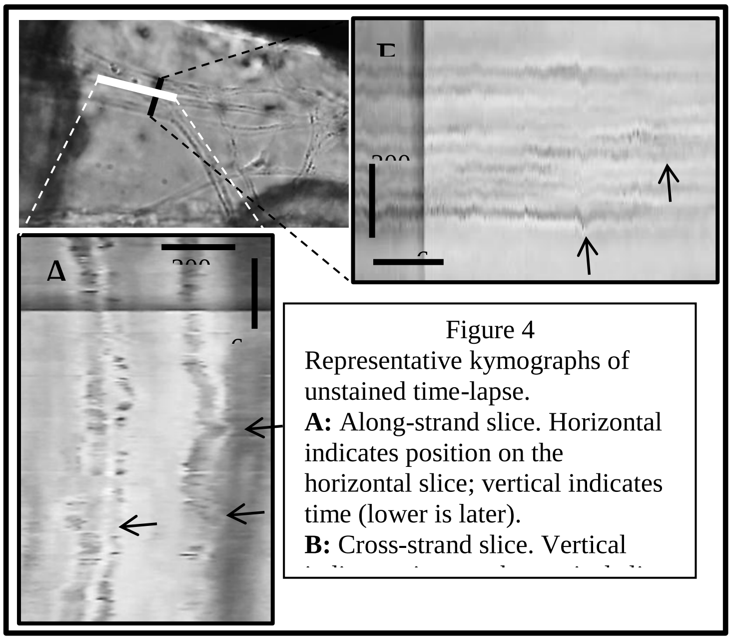

Figure 4
Representative kymographs of unstained time-lapse.
A: Along-strand slice. Horizontal indicates position on the horizontal slice; vertical indicates time (lower is later).
B: Cross-strand slice. Vertical

did not move substantially along the strands (Fig. 4 A.

If there were contractions or dilations of the strands, we would expect to see the spacing between and the width of dark and light bands change in kymographs taken crosswise to the strand. Although the lines jittered, they appeared to move in parallel with each other (Fig. 4, B). Their spacing and width appeared constant.

Growth of the growing edge of the colony did occur during the time-lapses, with an average elongation of the edge zooid measured at roughly 1.4 +/- 1.30% (n=6) growth over two hours. This was measured by taking the difference in length of a developing zooid at the growing edge before and after the time-lapse.

Size selective transport

To test the selectivity of transport, we injected fluorescent materials of different molecular sizes into the zooids and observed whether the fluorescence spread to neighboring zooids. Both fluorescein sodium salt (376 Da) and the fluorescein-dextran (70 kDa) were transported to multiple zooids within 10 hours after injection (Fig. 5); however, the 2.0 µm fluorescein microbeads did not transfer within the 10 hours (Fig. 5). None of the substances were seen at the growing edge within 10 hours of injection. Due to trials that failed to inject into the zooid and failed to label, all injection trials ended up with sixteen injections instead of eighteen injections. For colonies injected with fluorescein sodium salt the fluorescence was seen in the surrounding six zooids within 5h in all of the injected zooids except one trial, which transported to all surrounding six zooids in 10h. In eleven of the sixteen injections the fluorescein sodium salt also traveled an additional zooid laterally left or right, sometimes both. Also, in six of the sixteen the fluorescence was seen two zooids in the distal direction from the injection site. The

70 kDa fluorescein-dextran also transported to the surrounding six zooids for all injections. For ten of the sixteen colonies it took 5 hours to transport to the six surrounding zooids, however for six of the sixteen it took 10h. With the 2.0 µm beads, it was observed that there was minimal scatter of beads across the colony surface after injection which persisted throughout the 10h. Also, multiple zooids in the surrounding colony picked up loose beads that were seen in the stomach and gizzard. No beads were seen within the surrounding zooids (e.g. the coelomic cavity) other than within the guts and gizzards of varying numbers of individuals in the colony. For all colonies and all materials injected, the colonies and injected zooids remained alive for at least twelve hours after initial injection.

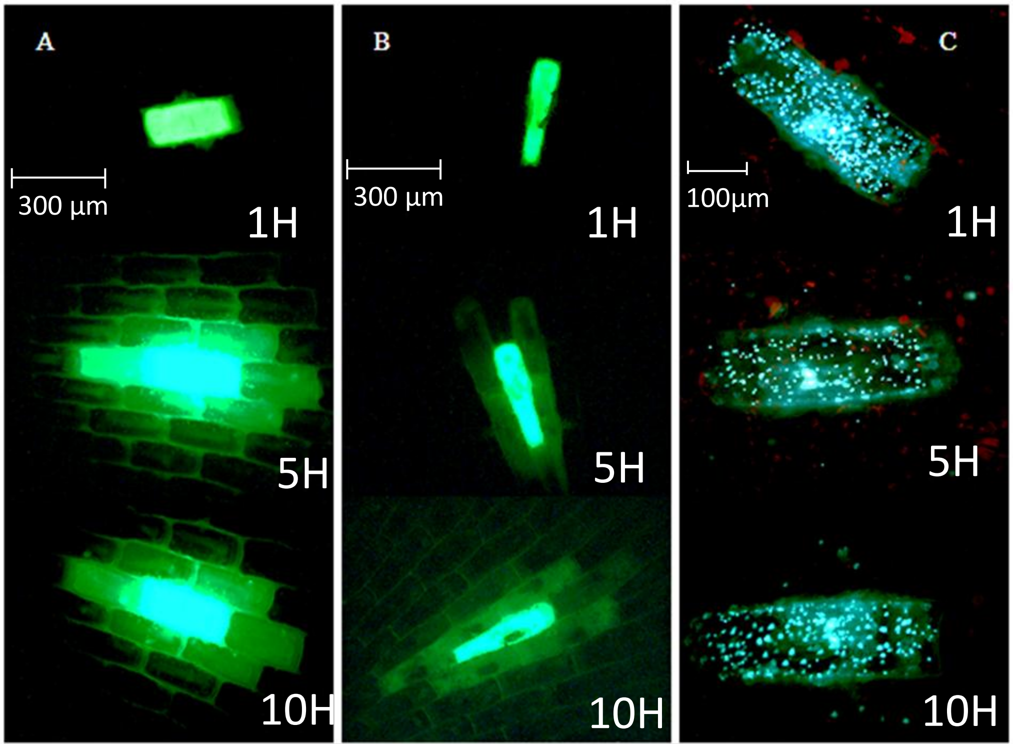

Figure 5, Representative transport of materials of different sizes; imaged at 1 hour, 5 hours, and 10 hours **A:** Fluorescein Sodium Salt trial **B:** 70KD fluorescein dextran **C:** 2.0µm fluorescent beads; blue dots are the beads, red is chlorophyll on surface of colony

Discussion

The results of our study allow us to draw several conclusions about the transport within the strands and across the pore plates of the cheilostome *Membranipora membranacea*.

Because the time-lapses did not show any noticeable contraction or dilation of the individual strands, the hypothesis that transport occurs via muscular pumping of fluid along the strand can be rejected. This result means that we can conclude that the funicular system does not function like a typical hemal system, such as the vertebrate circulatory system, because it lacks contraction/dilation of the vessels. Further analysis is necessary to confirm or refute this conclusion in case changes in strand diameter were too small to see, or to fast to observe at the frame rate used.

Because the time-lapses did not show any persistent or directional movement of features as predicted by the hypotheses that transport occurred by the movement of vesicles or by cell-crawling along the strand, both of those hypotheses can also be rejected as options for transport. However, we cannot rule out the possibility that vesicles were too small or cells were too low contrast to observe using bright-field and a 20x objective. Unfortunately, due to the thickness of the zooid, we were not able to improve the optics further.

We can also reject the hypothesis that material is transported across the cells at the pore plate using specific transporter or channel proteins because it is highly unlikely that the pore plate cells have receptors for both fluorescein sodium salt and fluorescein-dextran.

However, we cannot rule out transport by transcytosis (i.e. exocytosis of material on one side of the pore cells followed by endocytosis on the other) or diffusion. Transcytosis can non-specifically transport both small and large molecules because the cell takes up a volume of fluid, with all the molecules it contains, and carries it to the other side of the cell to be exocytosed.

This is consistent with the transport of both fluorescein and dextran, and – because the beads are quite large with respect to the size of the cells (Mukai et al., 1997) – it is also consistent with the failure of the beads to move between zooids. The hypothesis that molecules diffuse between the pore cells also predicts that small molecules will spread faster than large molecules. Because the beads are large (larger than the space between the cells; Mukai, et al. 1997), we would not expect to see diffusive transport of beads.

Another conclusion we are able to draw from the microinjection results is that transport is size selective. In the study, both the 367 Da fluorescein sodium salt and the 70 kDa fluorescein dextran transported with almost equal success across the pore plate barrier; however the 2.0 μm microspheres were unable to transport. In preliminary imaging of bleached pore plates, we determined that the pores of the *Membranipora membranacea* are typically 3 to 4 μm in diameter, leading to the conclusion that there is some property of the transport system excluding the larger microspheres. However, it is conceivable that this depends on either the chemistry of the beads (which would affect how cells interact with them) or whether they can get into the funicular strands.

Overall from this study, we are able to conclude that the cheilostome bryozoan does not function in the same way as typical hemal or coelomic systems. We were also able to determine that transport is size-selective. The hypotheses that transport at the pores is mediated by transcytosis or by diffusion could both explain the results we observed. However, in contrast to previous work using radio labeled food (Lutaud, 1983; Best and Thorpe, 1985; Miles et al., 1995) our results showed no directionality of transport of injected material. Injected zooids remained alive during our experiments, and the colony edge grew during time-lapses. Therefore we expect the normal transport processes were occurring during this period. However, it is possible that feeding the colony (as done in previous studies) causes a change in the regulation of transport so that it becomes more directional, that different transport mechanisms exist for different materials, or that the regulation of transport (directional vs non-directional) differs among materials.

In the future, a larger range of sizes, charges, and polarity of material need to be tested to determine exactly how selective the pores are. Their selectivity could narrow down the possibilities for transport mechanism. Also, more time-lapses need to be performed with longer durations and a larger variety of frame rates to more rigorously test the hypotheses of transport by muscle-driven contractions. Confocal time lapse microscopy of vitally stained zooids may show cell or vesicles movements not visible in bright-field microscopy.

By gaining a better understanding of the cheilostomes unusual transport system, we will gain a better understanding of how other long-distance transport systems, especially in colonial organisms may operate.

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