Bio219 Home

Barrier Formation in Developing Sea Urchin Embryos

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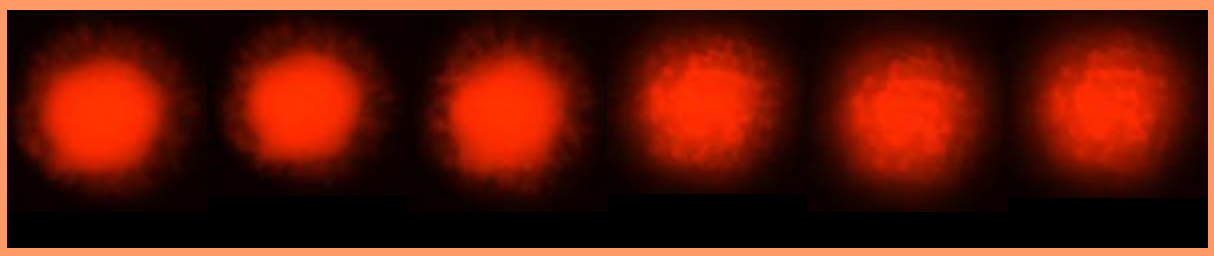


In this experiment, fluorescent seawater has been used to look at barrier formations in the white sea urchin *Lytechinus pictus.* This species is ideal for laboratory research because of the short maturation period of their eggs, they will divide within an hour of their fertilization and over the course of 7-8 hours, the fertilized eggs will mature into blastulae (Sidwell Research, 2003). A blastula is the common name for an embryo once it has developed into a liquid-filled sphere with the thickness of single cell (Kimball, 2003). The epithelial cells of the blastula form a sheet between the outside seawater and the blastocoel, the space in the middle of the embryo. Beyond the separation provided by each cells membrane bilayer, intercellular junctions form the primary barrier to ion and molecule diffusion.

Tight junctions exist as riveted networks of claudin and occludin protein strands between the plasma membranes of adjacent cells. The tight junction acts as the primary barrier against the diffusion of large molecules between cells (Cooper, 2004). Other barriers that exist between cells are the adherens junctions. These are formed as the actin cytoskeleton anchors to the membrane to create an adhesion between adjacent cells (Cooper, 2004). This study tests the hypothesis that a barrier to diffusion is formed between the blastocoel space and the exterior seawater during the development of a sea urchin embryo.

The dye used in this experiment is rhodamine that has been affixed to dextran molecules. The polysaccharide dextran contains a link that is unbreakable by many organic systems . Because of this inability to be cleaved, dextran will remain in an organism for a period of time, thus dextran will act as a long-term tracer. The dextran can be dissolved in seawater and viewed with fluorescence microscopy. During this process, light is produced at a specific wavelength by the microscope. The affixed dye is excited by the absorption of this light and reflects it at a different wavelength.

Special filters within the microscope will then detect the light at new wavelengths and make them visible to the viewer (Cooper, 2004).

To begin the experiment the urchin sperm and eggs were combined in filtered natural seawater to attain fertilization. To test the hypothesis, they were divided into three experimental groups. In the negative control group the embryos were left to develop naturally with no outside interference. The non-washed fluorescent group were incubated in labeled seawater and imaged. The washed fluorescent group were also incubated in the dye, they were then washed in the FNSW and imaged. The embryos were then incubated at 20-22 degrees Celsius in until they reached a desired stage in their development, both prior and post blastulae formation.

MATERIALS

Sea urchin embryos - *Lytechinus pictus*
Filtered natural seawater (FNSW)
Natural seawater (NSW)
FNSW-PABA:
10mM 0.5% p-aminobenzoic acid in FNSW
Nitex mesh: 80µm
Rhodamine dextran (r-dextran):
1 ml of 100µM dissolved in methanol
1 ml of 50µM dissolved in methanol
Highly concentrated seawater:
2.9g NaCl dissolved in 50ml FNSW (Stephens, 1986)
Glass slides and cover slips
Double stick tape with a thickness of 90µm
Spot Insight Camera with Mac G4 based program
Nikon Eclipse E200 microscope
Nikon Eclipse E400 fluorescence microscope
Adobe Photoshop 7

METHODS

Fertilization: The sperm and eggs were collected by a 4-5% body volume injection of 0.5 M KCl into the mouth region of the urchins. The female urchin was placed mouth side up onto a jar of FNSW. The released eggs settled in a pellet at the bottom of the jar. The water was then aspirated off the eggs and they were resuspended in fresh FNSW. This process was repeated twice to provide a thorough cleaning of the eggs. To collect the sperm, a male urchin was placed mouth side up on dry parafilm. The sperm were then activated in a 1:1000 dilution of FNSW. Fertilization occurred with the addition of one drop of the sperm solution to one ml of FNSW egg suspension. Within two minutes the fertilized eggs were imaged to verify the presence of fertilization envelopes. Three batches of eggs were fertilized over the

course of two weeks. This shedding procedure followed the research of F.J. Dufort (2000) with modifications made by Robert L. Morris (2003).

Fertilization Envelope Removal: The fertilized eggs were washed twice in the FNSW-PABA solution and filtered through 80µm mesh, following the edited procedure of Dufort. The PABA wash removed the excess sperm from solution and prevented the fertilization envelope from hardening. The 80µm mesh was slightly larger than the diameter of the eggs to strip the fertilization envelope. The stripped eggs were washed in FNSW to separate them from the envelope suspension and imaged to verify a complete removal of the fertilization envelopes.

Experiment: The fertilized eggs were collected in 0.1-0.5ml drops and separated into epindorph tubes. As the eggs settled to the bottom of the tubes, the excess FNSW was removed and 2-3 drops of 50-100µM rhodamine dextran (r-dextran) were added to the experimental groups. The fertilized eggs were incubated in a dark humidity chamber at 20-22 degrees Celsius for 20 minutes to several hours until imaging. After 4-7 hours of development, one group of fluorescent embryos was diluted with 20-50 ml NSW in a large test tube. The tube was lightly centrifuged for one minute, until the embryos were pelted at the bottom tip of the tube. This process was repeated up to three times to ensure the complete removal of excess r-dextran from the seawater surrounding the embryos. At this point the embryos were imaged along with a sample of both the non-washed and control embryos. The remaining non-imaged embryos were stored overnight in a humidity chamber. At the 17—25 hour post-fertilization mark the blastulae were deciliated. A deciliation solution was made from 2.9g NaCl dissolved in 5ml FNSW (Stephens, 1986). This mixture was then added at a 50:50 ratio with the FNSW containing the blastulae to be washed and with the FNSW containing the control blastulae. Since the deciliation procedure also acted as a wash of the r-dextran, the blastulae to be imaged in fluorescence did not go through this step. The two experimental groups were soaked for 2-4 minutes in the highly concentrated solution then centrifuged for one minute to form a pellet. The excess NaCl in FNSW was aspirated off and both blastulae groups were suspended in excess FNSW/NSW for imaging along with the non-wash blastulae.

Viewing: As per suggestion of Robert L. Morris to ease in the viewing of ciliated blastulae, special chambers were constructed. Two slices of 90µm thick, double stick tape were affixed onto parallel edges of a glass slide. A cover slip was then fastened to the tape creating a thin chamber between the cover slip and slide surfaces. The liquid samples were drawn into the chamber by touching a pipette tip to one side of the cover slip. The images were then viewed in a dark room by a Nikon Eclipse E400 fluorescent bulb microscope. The camera was connected to the Spot Insight program made for the Macintosh G4 processor with OSX operating system. The slides were viewed under green light and the images were captured at a 40x magnification.

Quantitate: In Figures 1, 2a, and 3 as seen below, eight points were randomly selected for a quantitated analysis on mean luminosity. The images were opened in Adobe Photoshop where choosing the box tool made it possible to draw a square around any area of the picture. The luminosity within the box was then analyzed using the histogram tool under image on the task bar. An average was taken of four different points chosen from the fluid outside the embryo or blastocoel, and one average was taken from four points within the embryonic or blastocoel fluids. The averages of each image were put into a ratio of mean luminosity, of arbitrary units, to be compared with the ratios of the other images.

RESULTS

In each fluorescent excitation setting the unlabeled negative control group produced a black field. Under normal exposure in the green light, the embryos were indistinguishable from the FNSW. A significant increase in the exposure time was necessary to make out the edges of the embryos and the cells within. The same black field was presented in the images of the unlabeled blastulae in the green light. Again, with a slight alteration of the exposure time the blastulae were slightly visible against the dark FNSW.

The image of the non-washed r-dextran labeled embryos after 4-6 hours revealed a vibrantly orange field. The presence and location of the fluorescence was considerably noticeable as seen in the Figure 1. The color of the exterior FNSW was the same as the color of the water surrounding the cells in the embryo. Under normal exposure in green light the entire slide shone orange. It was almost not possible to see an outline of the cells. The embryo was barely distinguishable from the labeled water.

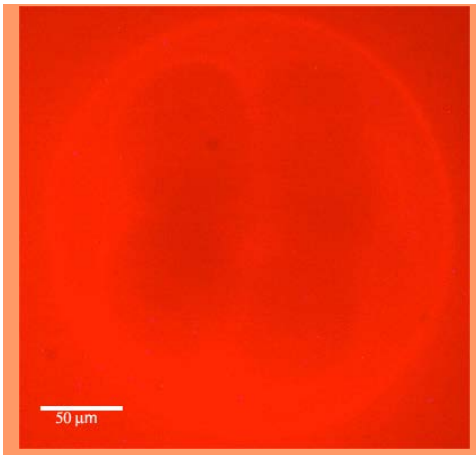
A wash of the r-dextran labeled embryos after 4-6 hours revealed that little to no color remained. Some embryos maintained a brighter tint than was observed in the negative control. The fluorescence that did exist in the cell was not present uniformly across the cell. The faint orange coloring appeared in dots scattered around the cells of the embryo. This amount was insignificant. The embryos did not contain a strong enough fluorescence to be visible in a captured image.

Twenty-five hours post-fertilization, the embryos had developed into blastulae. They had grown cilia and rotated in and out of the plane of focus. At this time all remaining r-dextran labeled blastulae were deciliated. This process recovered a single washed and decilliated blastula, seen in Figure 3. The blastocoel of the blastula retained a bright and vividly orange color in comparison to the surrounding dark black FNSW. The fluorescence radiated from the blastocoel into the finger-like spaces between the cells, giving the blastula the appearance of a sun. There was no obvious fluorescence located elsewhere on the slide.

Figure 4 represents a cross-sectional change in focus of the same blastula seen in Figure 3. Frame A, to the furthest left, is an equatorial cross-section of the blastula. Frames B-E represent what is viewed as the focus moves through the blastula. Frame F, to the furthest right, is a cross-section at the top of the blastula.

Figure 1 is a non-washed 4-hour embryo imaged under green light.

Exterior of embryo with rhodamine dextran	Mean Luminosity	Embryonic space between cells	Mean Luminosity
random point 1	89.8	random	93.8



		point 1	
random point 2	88.7	random point 2	90.2
random point 3	95.7	random point 3	90.8
random point 4	87.8	random point 4	90.0
average	90.5	average	91.2

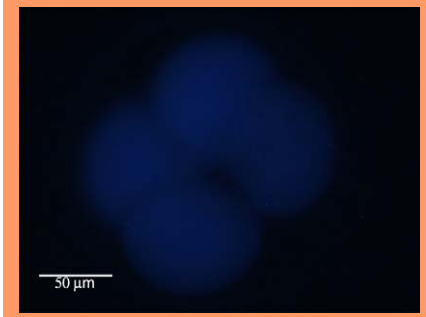
1:1 ratio exterior to interior

Figure 2a is a washed 4-hour embryo imaged under green light. Figure 2b is the same embryo under violet light to illustrate the location of the cells.

2a.

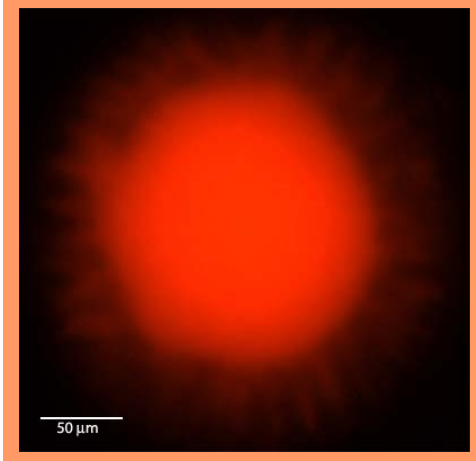
Exterior of Washed Cell	Mean Luminosity	Interior of Washed Cell	Mean Luminosity
random point 1	0.00	random point 1	0.00
random point 2	0.00	random point 2	0.00
random point 3	0.00	random point 3	0.00
random point 4	0.00	random point 4	0.00
average	0.00	average	0.00

2b.



1:1 ratio exterior to interior

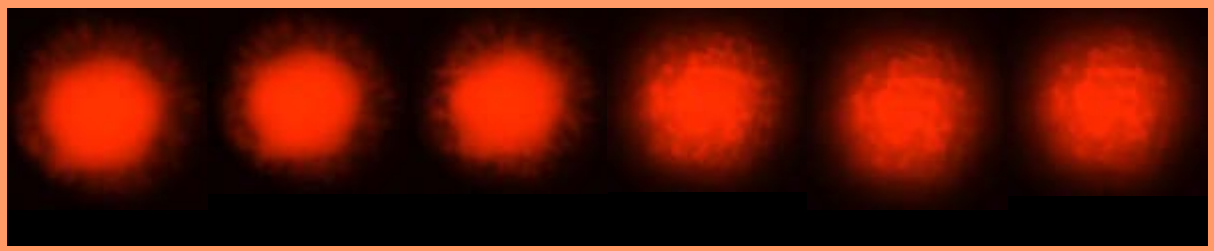
Figure 3 is a washed blastula imaged at 25 hours under green light.



Exterior of the Washed Blastula	Mean Luminosity	Interior of the Blastocoel	Mean Luminosity
random point 1	4.2	random point 1	106.4
random point 2	3.4	random point 2	91.7
random point 3	3.0	random point 3	100.6
random point 4	4.4	random point 4	104.2
average	3.8	average	100.7

1:25 ratio exterior to interior

Figure 4 is a series of the blastula from Figure 3 in different focus.



A B C D E F

DISCUSSION

After a multitude of washes there remained a perfectly orange blastocoel in the center of Figure 3. There is no obvious fluorescence elsewhere on the slide. The fluorescence is visible in the spaces where the cells meet and a continuously visible path of fluorescence from the exterior to the interior of the blastula does not exist. Figure 2a

shows that fluorescence was not retained within the perimeter of the cells. Figure 1 shows an r-dextran flooded embryo. There were no barriers restricting the fluorescence from saturating these cells. This would then imply that there were no barriers preventing the fluorescence from being washed out of the embryo seen in Figure 2a either. One would also expect that if the embryos were bringing fluorescence into their cells via endocytosis, they would also be bringing in the unlabeled seawater during the wash as well. This could keep the concentration of the fluorescence down in the early stage embryos seen in Figure 1 and 2a. The controls and the washed groups were a great comparison. The fluorescence found in the washed 4-hour embryos was barely visible, like that of the control. This is despite the fact that the controls were not immersed in glowing seawater while the experimental were. These data suggest that at the time Figure 1 and 2a were captured, a barrier had not yet formed. There is little explanation as to why the r-dextran labeled seawater would remain only in the blastocoel after washing if it were not for some type of barrier formation. The evidence gathered by this experiment supports the hypothesis that the separation of the blastocoel from the exterior seawater is a direct result of an intercellular barrier formation.

In Figure 1 and 2, the color of the fluid on the exterior of the embryo compared with that found on the interior is approximately at a 1:1 ratio. In Figure 3 the fluid located on the interior is 25 times brighter than the exterior fluid. It is not possible to compare the mean luminescence of the three images to one another as they were taken at different exposures. It can be concluded that this histogram ratio value indicates a difference of 25 times more fluorescence retained the blastula post-barrier formation.

All of this evidence does not directly test for the presence of tight junctions, or any other specific barrier for the matter. There is no direct proof that they are the reason for the limited diffusion. The formation of the tight junctions is however the best explanation for the observations.

The data in this experiment were collected at two points during the development of the embryo. Tight junctions, adherens junctions, and other barriers do not suddenly appear in an embryo. Their structures develop with the embryo. A larger collection of data over the entire developmental range of an embryo would narrow down the time at which certain barriers are formed. The more data that is available for analysis, the more specific the conclusions could be. An interesting follow-up experiment would be to look at r-dextran retention in a blastula if added post-barrier formation. Following the hypothesis, it is logical to expect that this fluorescence would not be retained in the same manner as it had been in the blastocoel formed with the r-dextran. The presence of fluorescence would also depend on how developed the urchin was at the time of the addition. According to studies such as that of my collaborator Jenna Gustafson*, the process of endocytosis would carry some of the fluorescence into the cells. Since the barrier would be present, the fluorescence would not have the ability to diffuse into the blastocoel. If the blastula had developed into a plutii and a gut had been formed before the fluorescence was added, it would be possible to observe the urchin ingesting the r-dextran in the seawater.

If I were to do this experiment again, I would be more careful and consistent with my procedures. As it is now, the experiment was run three times and each time the embryos were incubated over different intervals in the fluorescence. I would have a more efficient timing method for the addition of the fluorescence and a better imaging schedule of the embryos. Another inconsistency with the experiment is that I was unable to deciliate the fluorescent blastulae that were in the no-wash group. The deciliation process acts a wash of the sample. I would like to find a way to stop the

blastulae from moving without compromising essential portions of the experiment.

A change in the viewing chamber protocol would also increase the probability of obtaining more data. The idea of the cover slip chamber was to squish the blastulae enough that they would be unable to swim out of focus without damaging them. The chambers as they are now, are not thick enough. It turned out that many of the blastulae did not fit into the chamber and they became trapped under the edges of the coverslip. This is despite the removal of the cilia that reduced the diameter of the blastulae. It seems that a procedure in a future experiment that involved a thicker slice of tape, or perhaps a double layer of tape, would fix this problem.

*Jenna Gustafson focused her experiment around the rates of endocytosis in unfertilized and fertilized sea urchin eggs. Her primary procedures for the collection of the materials and specimen were identical to this experiment, however the final data on endocytosis is not pertinent to the substantiation of my barrier hypothesis. Some of the early developmental images of the embryos provided insight with the result of the interaction between the r-dextran and the cells.

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