Total Cytoplasmic Volumes of *Lytechinus pictus* Embryos Remain Constant During Progressive Embryo Stages

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Introduction:

In this experiment embryonic development of *Lytechinus pictus* (sea urchin) were studied using methanol fixation and immunofluorescent staining technique. The purpose of using immunofluorescent staining is to label antibodies or antigens with fluorescent dyes. This technique can be used to label specific molecules of interest within a cell. Immunofluorescent-labeled tissue sections can then be studied using a fluorescence microscope (Gratzner & Leif, 2005). The hypothesis tested in this experiment was that the *Lytechinus pictus* embryo starts as a solid single cell and proceeds to become a hollow multicellular embryo without any change in total cytoplasmic volume overtime. This hypothesis was tested by examining immunofluorescent labeled images of *Lytechinus pictus* embryos at different stages of development. Total tubulin was labeled in this experiment to determine the locations of the cytoplasm in the images (Salmon et al., 1984). Sea urchins serve as useful model organisms for studying early development. In the past, sea urchin research has been used to uncover a variety of classic developmental mysteries such as the mechanisms of fertilization, egg activation, cleavage, gastrulation, and the regulation of differentiation in the early embryo (Hardin, 2002). Early studies of the molecular basis of early development were also carried out using sea urchin models (Hardin, 2002). The stage in which a sea urchin embryos begins to divide is called the cleavage stage. Some of the distinct cleavage stages are 2-cell, 4-cell, 8-cell, 16-cell, 32-cell, the blastula stage, and the gastrula stage (Hardin, 2002). Many of these progressive embryo stages

were captured in the images used in this experiment. It is important to obtain information about early cleavage patterns, because abnormalities in cleavage patterns could have great impacts on further development in the sea urchin as well as the other organisms that the sea urchin embryo models (Salmon et al., 1984).

Materials and Methods:

The materials and methods used to carry out this experiment are described in the "Immunofluoresecent staining of sea urchin embryos, MeOH fixation (for classroom use, adherent embryo technique)" protocol designed and written by Prof. Morris in consultation with Drs. J. Henson and B. Shuster, MBL, in the Summer of 2008 (Morris, 2008). Methanol fixations of the samples of *Lytechinus pictus* used in this experiment were fixed prior to this experiment in August of 2008. One of the changes to the protocol in this experiment from Prof. Morris' protocol was that protamine sulfate solution was used instead of polylysine solution for the purpose of making coverslips cationic for cell sticking. Another change to the protocol was that incubations in primary and secondary antibody were only done for one-hour as opposed to the 24-hour incubation period that the protocol recommends.

Images of these embryos were acquired on a Nikon E400 epifluorescence microscope with Hoescht, FITC, and Rhodamine filter sets, using Spot Advanced software on a spot insight camera from Diagnostic Instruments. The 40X plan fluorescence objective was used for all images.

In order to collect data from these images, cell diameters and cell numbers of the embryos were used to calculate total cytoplasmic volumes of each of the embryos. Cell diameter was determined by locating the total tubulin in the images and measuring across this area. Total tubulin was labeled green by the FITC dye in these images. The formula used to calculate the cytoplasmic volumes of each embryo at various stages was Total Volume= $4/3\ \pi\ r^3$ X n when r = average radius of the cells in the image and n= number of cells depicted in the specified stage. In the stages in which the embryos were hollow, the calculated volume of the blastocoel was subtracted from the total volume of the embryo. The calculated cytoplasmic volumes were then compared through creation and analysis of a graph in order to determine whether or not cytoplasmic volumes of the embryos remained constant through different embryo stages.

Results:

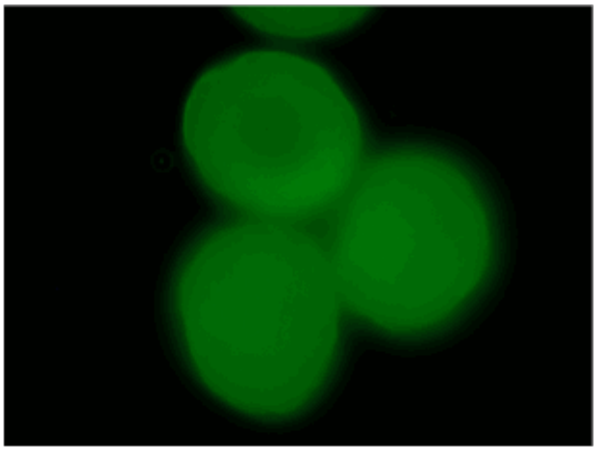

Figure 1: Image of Egg

The average diameter of these cells were used to calculate total cytoplasmic volume for the one-cell stage. This image measures 287 micrometers across the horizontal length.

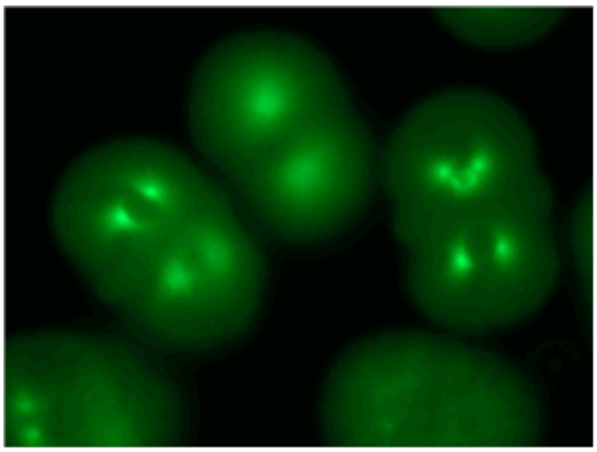

Figure 2: Image of two-cell stage.

The average diameters of these cells were used to calculate total cytoplasmic volume for the two-cell stage. This image measures 287 micrometers across the horizontal length.

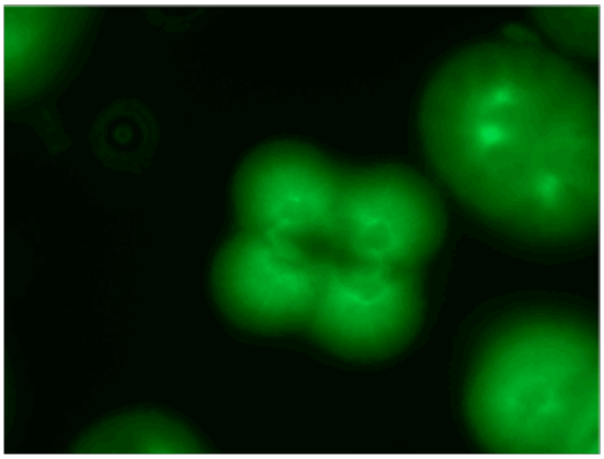

Figure 3: Image of four-cell stage.

The average diameters of these cells were used to calculate total cytoplasmic volume for the four-cell stage. This image measures 287 micrometers across the horizontal length.

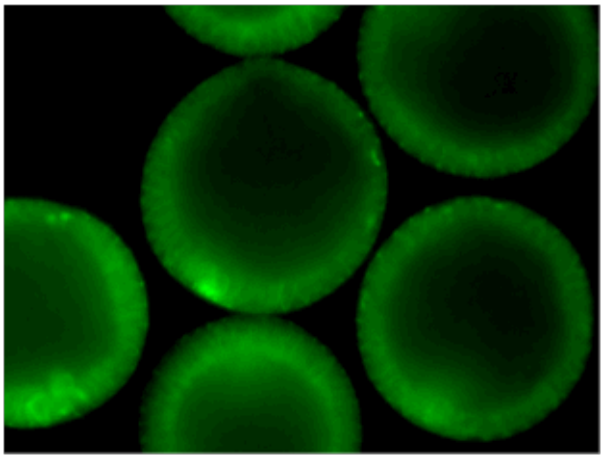

Figure 4: Image of the blastula stage.

The average volumes of the blastocoels were subtracted from the average total volume of the embryos in order to calculate total cytoplasmic volume of the blastula stage. This image measures 287 micrometers across the horizontal length.

Embryo Measurements at Different Stages					
Stage	**Average Diameter**	**Average Radius**	**Average Cytoplasmic Volume of Single Cell**	**Cell number**	**Average Total Cytoplasmic Volume**
Egg	80	40	267,946	1	267,946
2 -cell	65	32.5	143,720	2	287,440
4 -cell	50	25	65,416	4	261,666
Blastula	95 OD,70 ID	47.5 OR, 30 IR	2,103	Blastula (128)	269,189

Table 1: Embryo Measurements at Different Stages

This table shows the cell measurements that were used to calculate total cytoplasmic volume of each of the embryos depicted in the fluoresecent images. There are both inner and outer diameter and radius measurements for the blastula in order to account for the hollow blastocoels inside of the blastuas. The average cytoplasmic volume of a single cell figure for the blastula was not calculated using the formula. It was derived by dividing the total cytoplasmic volume by 128, a typical cell number for the blastula stage (Campbell & Reece, 2006). Cell number and average cytoplasmic volume of single-cell for the blastula stage are estimates.

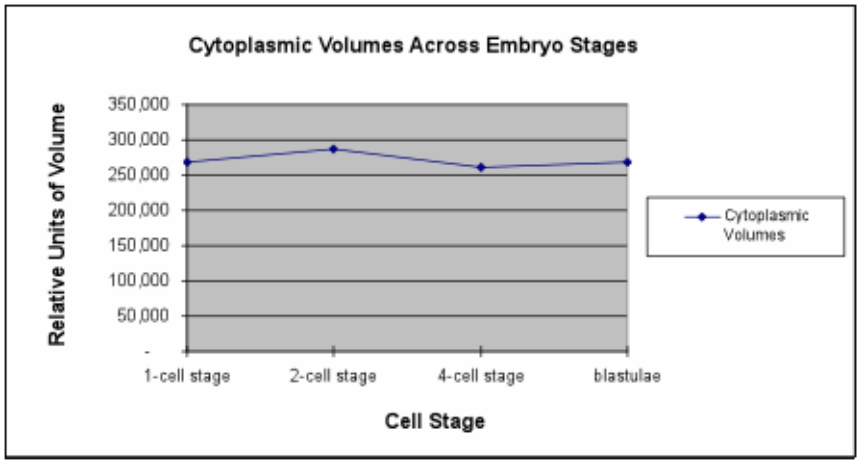

Figure 5: Cytoplasmic Volumes Across Embryo Stages

This graph shows the average total cytoplasmic volumes of the embryos from the images at different stages relative to each other.

Discussions and Conclusion:

The evidence uncovered from this experiment supports the hypothesis that the *Lytechinus pictus* embryo starts as a solid single cell and proceeds to become a hollow multicellular embryo without any change in total cytoplasmic volume overtime. The hypothesis is supported by the fact that total tubulin could be viewed in the center of the epithelium during the egg stage and but could only be found in a thin layer surrounding the embryo, no longer in the center of the embryo, during the later cell stages such as the blastula stage. Location of total tubulin indicates the general location of the cytoplasm (Salmon et al., 1984). Therefore, absence of total tublulin in the blastocoel of the blastula indicates that cytoplasm was absent in this area and that the area was hollow. The hypothesis is also supported by the fact that general size of the embryos seemed to remain constant in images showing a multitude of stages. Measured cell diameters used to calculate cytoplasmic volumes of different embryo stages demonstrated that embryo volumes stay constant even though cell number increases. Figure 5 demonstrates that total cytoplasmic volume of the embryos studied in this experiment remained relatively constant across progressive cell stages.

The idea that the embryo becomes hollow overtime is supported by Figure 4 in which the blastula image is show have its total tubulin in a halo formation around the outer region of the embryo. Papers by Dan, Wolpert, and Gustasfson,

Ettensohn, and Ingersoll all cited that during the blastula stage of sea urchin development cells form a hollow sphere surrounding a central cavity called the blastocoel (Gilbert, 2006). The blastocoele is depicted in Figure 4 as the black area in the center of the embryo. No cytoplasm is present in this area, and this area is therefore hollow.

The idea that the embryo did not change its total cytoplasmic volume overtime even though the number of cells increased as time progressed is supported by the fact the size of the embryos appeared to remain relatively the same through progressive cell stages. The magnification of the images was kept constant through out the image creation process. The total cytoplasmic volume figures presented in Table 1 lend further support to this initial observation. The cytoplasmic volumes of each of the progressive embryo stages were very similar relative to eachother. Many authors have offered explanation for why total cytoplasmic volume remains constant during progressive stages from the egg stage to the blastula stage.

Many previous studies have reported that upon fertilization of the egg, the secretion of cortical granules, development of the previtelline space, and formation of the fertilization membrane from the Zona pelucida spread over the entire egg surface within 20 seconds. The zona pelucida prevents nourishment from being taken up from the exterior of the egg. For this reason, cleavage progresses without increase in size of the total embryo (Drews, 1995). The embryo increases its cell number during progressive stages because it is cleaving and dividing, but the embryo cannot grow in size because it is not receiving enough nourishment.

Other studies support the idea that a modified cell cycle is responsible for the embryo retaining its total cytoplasmic volume of during cleavage in progressive cell stages. These studies support the idea that early cleavage divisions in sea urchin embryos are reductive. During cleavage, the cytoplasm of the embryos are divided into smaller and smaller cells. The total cellular volume of the embryo stays the same, but the number of cells within the embryo increases. This ability of the embryo to retain its total cellular volume over time is the result of cleaving cells having a modified cell cycle, in which the two gap phases, G1 and G2, are completely omitted. The cells cycle rapidly between M and S phases (Marcey, 2004). Therefore, the embryo retains in total cytoplasmic volume because it does not go through any growth phases in the cell cycle. These growth phases are completely skipped over during reductive cleavage.

One of the ways in which this experiment could be improved would be by using more accurate measurement techniques. Because the measurements used in the data for this experiment were taken by measuring images on paper print outs, an accurate measurement could not be taken, and the total cytoplasmic volumes had to be compared relative to one another. The measurements used in this experiment could be made more accurate if a scale was used to compare actual cell size to the size of the cells in the image or if the cells were measured using a very small ruler while the cell was being observed underneath the microscope. The experiment could also be improved if the exact same embryo could somehow be measured across progressive embryo stages. Because a multitude of different cells were examined in this

experiment, averages had to be taken in the place of comparing the same cells. The sources of error in this experiment were most likely related to human error in measuring the diameters of the cells and in comparing different embryos while making generalizations about similar embryos.

Future experiment that would be useful in extending the results of this experiment in new directions would be to focus on the zona pelucida or the absent growth phases that allow the sea urchin embryo to retain its cytoplasmic volume. The researcher involved in these experiments could compare differences in the zona pellucidas and absent growth phases to determine if there are certain defects in these features that would allow cytoplasmic growth of the embryo during cleavage. If researchers gained new insights to these features they might also gain new insights about causes of birth defects among organisms with developmental pathways similar to the sea urchin. This suggestion is based on literature that states that sea urchin embryo research as already taught biologist much about the molecular mechanisms of early development (Hardin, 2002).

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