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Oh! The Chaos the Chaos!

It is after all, an amoebae eat amoebae world!

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ABSTRACT

Phospholipid membranes of amoebae *Pelomyxa carolinensis* were stained with FM1-43 and examined using fluorescence microscopy. The stained membrane was shown to label the lipid membrane of post stained phagosomes within pelomyxa. The FM1- 43 was used in fluorescence microscopy to demonstrate the rate of formation of phagosomes in *Pelomyxa* under various fed states. Phagosomes were shown to be more numerous in the *Pelomyxa* that had been deprived of a food source for an extended period of time than the *Pelomyxa* with a consistent food source.

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INTRODUCTION

Pelomyxa carolinensis belong to the Protista kingdom. Protists are heterotrophs (Farabee, 2001). Heterotrophs consume external food sources to acquire energy for the processes of cell motility, mitosis, organelle production, and endocytosis. Because *Pelomyxa* depend on external food sources for energy, they are believed to respond to a deprived food environment by increasing the rate of phagocytosis when in the presence of food.

As unicellular organisms, the cytoplasms of Protists are confined by impermeable phospholipid bilayers (Farabee, 2001). During the process of endocytosis, the membrane of the Protist extends or contracts while engulfing particles from the cell's exterior. Endocytosis includes pinocytosis and phagocytosis. Pinocytosis and phagocytosis vary with respect to engulfed particle size; phagocytosis refers to cell "eating" while pinocytosis refers to cell "drinking" (Cooper, 2004). The membrane surrounding the phagosome consists of lipids and proteins from the lipid bilayer of the cell's plasma membrane. *Pelomyxa* are believed to be one of the largest members of the Protista kingdom. Because of the large size of *Pelomyxa*, and furthermore the enormity of its plasma membrane with increased surface area available for phagocytosis, the Protist *Pelomyxa* was chosen as the subject for the quantitative measurement of the rate of phagocytosis according to "fed" and "starved" states.

FM1-43 is a styryl dye that has been found to permeate the exterior of the lipid bilayer. FM1- 43 is essentially non-fluorescent in aqueous solution and upon binding to membranes it becomes internalized in the outer leaflet of the cell membrane where it is intensely fluorescent (Probes, 2003). The FM1-43 can be used in fluorescence microscopy to visualize the structures in the cell with which the stain has been associated. FM1- 43 has been used widely to monitor exocytosis and endocytosis (Betz, 1996). As a result of staining, subsequent membranes of the plasma membrane, including phagosomes, are thought to exhibit the fluorescence allocated to the FM1- 43 dye.

In this paper, I have developed a simple way of measuring the rate of phagocytosis for "starved" and "fed" *Pelomyxa* by tabulating the number of stained phagosomes present within the cytoplasm of each *Pelomyxa*. Under fluorescence microscopy, the FM1- 43 was found to be specific for phospholipid bilayers (Zweifachi, 2000). FM1- 43 was then used to stain the membranes of "starve" and "fed" *Pelomyxa*. This technique will allow me to count the number of labeled phagosomes. This technique will furthermore enable me to test the hypothesis that the rate of phagocytosis for *Pelomyxa* increases for Protists deprived of a food source in respect to the rate of phagocytosis for *Pelomyxa* in the presence of a constant food source.

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MATERIALS and METHODS

Isolation of Pelomyxa carolinensis

Pond water was filtered with a 200 mL syringe and a 0.22 µm gamma sterile filter unit and collected in a sterile vile. *Pelomyxa carolinenses* were removed from the sample and placed in filtered pond water. The *Pelomyxa* were then washed three consecutive times with filtered pond water and allowed to stand in the food deprived filtered pond water at 25˚C for 9 hours. *Pelomyxa* was chosen because it is a very large Protist with an extremely active plasma membrane that is involved in both cell motility and cell endocytosis.

FM1- 43 Preparation

FM1-43 was purchased from Molecular Probes (Eugene, OR, U.S.A.). Dr. Robert Morris prepared a 4 µM FM1- 43 dilution by adding Ringers solution to stock FM1- 43 (Cousin, 1999). The FM1- 43 solution was concealed in aluminum foil for the duration of the experiment to avoid chemical deterioration.

Staining of Cells

The negative control, *Pelomyxa* in the presence of a constant food source, was stained with 2.0 mL of 4µ M FM1- 43 solution and 2.0 mL of filtered pond water (Cochilla, 1999). The negative control was allowed to incubate for 20 minutes and viewed immediately after FM1- 43 was believed to have infiltrated the outer leaflet of the plasma membrane. 2.0 mL of 4 µM FM1- 43 solution was added to the isolated *Pelomyxa* in 1.0 mL of filtered pond water and was induced with 1 mL of *Paramecium bursaria*. The *Pelomyxa* were incubated under these conditions for 1 hour at 25˚C in the FM1- 43 solution. *Pelomyxa* in the presence of a constant food source were removed from the culture dish and placed in a 1.0 mL of Paramecium enriched solution. 2.0 mL of 4 µM FM1- 43 solution was then added (Cochilla, 1999). The *Pelomyxa* in the presence of a constant food source were incubated for 1 hour at 25˚C in the FM1- 43 solution. After incubation, the cells were removed from their respective solutions and washed 3 times in filtered pond water. Chip chambers were constructed from crushed glass cover slips and the stained Pelomyxa were placed within the chip chamber and covered with a glass cover slip.

Imaging with Florescence Microscopy

Unstained *Pelomyxa* in the presence of a constant food source were viewed with a Nikon Eclipse E400-brightfield Fluorescence Microscope located in the ICUC at Wheaton College in Norton, MA. Digital images were taken of the unstained Pelomyxa using a SPOT 2E digital camera and the SPOT Digital Imaging program. The cells were viewed with both 10X and 40X objective lenses, under Hoechst illumination and fluorescence illumination. After incubated, the *Pelomyxa* in the presence of food were viewed in a similar manner, as were the stained *Pelomyxa* in filtered pond water. The number of luminescent compartments meeting or exceeding 5 µm in width within the cytoplasm of each cell was counted for a comparative analysis.

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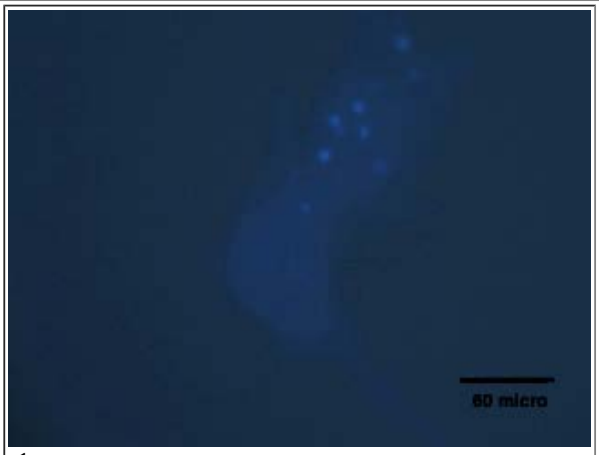
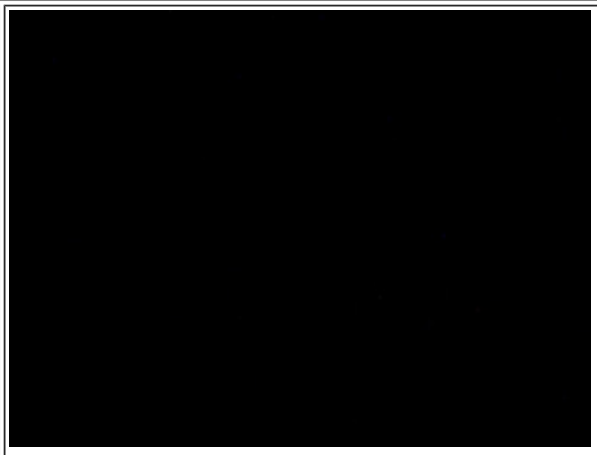
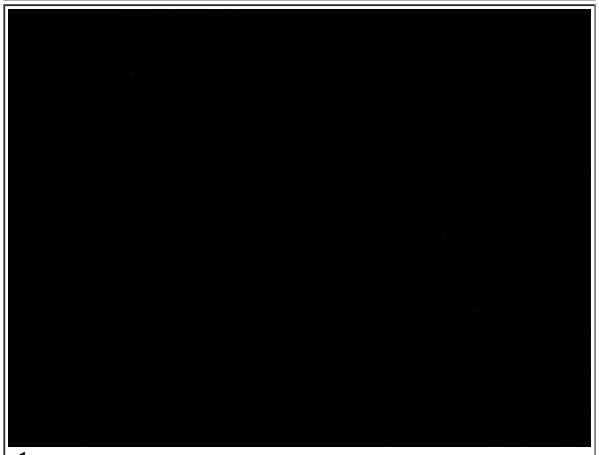
RESULTS



1a.



1b.

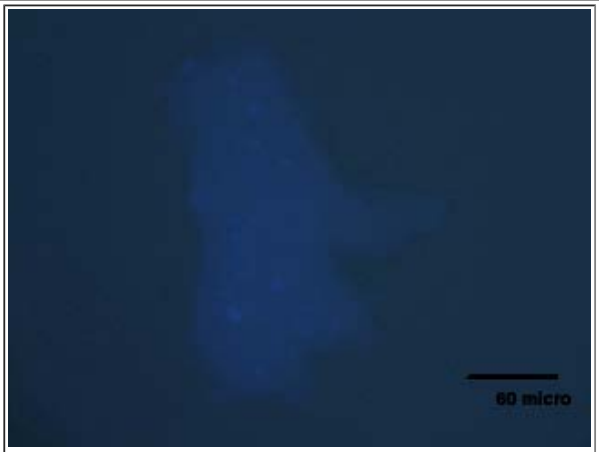


1c.

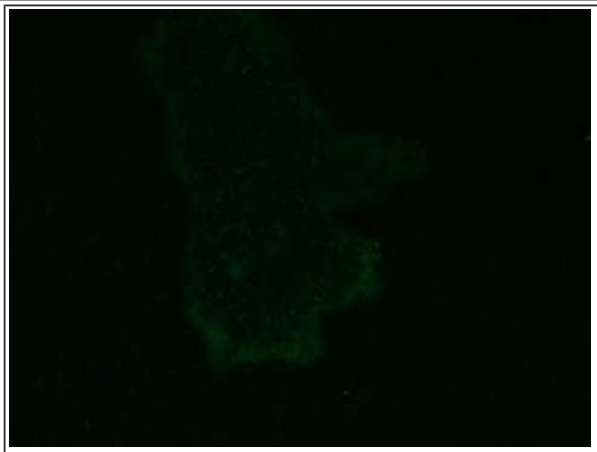
Fig 1a. UV illumination of unstained *Pelomyxa* with a 10X objective lens. The blue emission is indicative of auto-luminescent. Fig 1b. Blue excitation of unstained *Pelomyxa* with a 10X objective lens. No emission is evident. Fig 1c. Green excitation of unstained *Pelomyxa* with a 10X objective lens. No emission is visible.

Unstained Pelomyxa

Under fluorescent microscopy and UV illumination unstained *Pelomyxa* were luminescent (Fig. 1a). The plasma membrane surrounding the Protist, as well as some organelles within its cytoplasm, was identifiable by their contrasting coloration. Although the coloration was slight, the outline of the cell was apparent. The same *Pelomyxa* was then viewed under blue excitation which showed minimal luminescence (Fig. 1b). In addition to the blue excitation, the *Pelomyxa* was viewed with green excitation which revealed no luminescence of the plasma membrane or organelles (Fig. 1c).

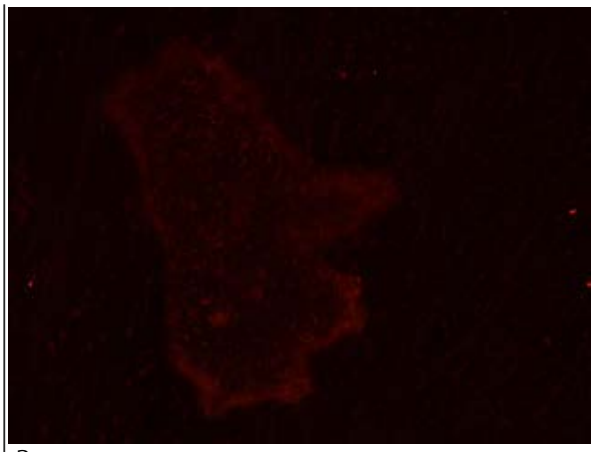


2a.



2b.

Fig 2a. UV illumination of stained *Pelomyxa* with 10X objective lens. The blue luminescent ring surrounding the cell indicates that the FM1-43 dye labeled the phospholipid membrane of the

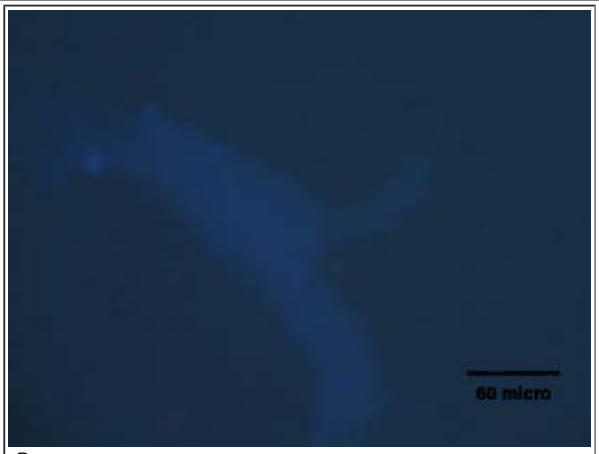


2c.

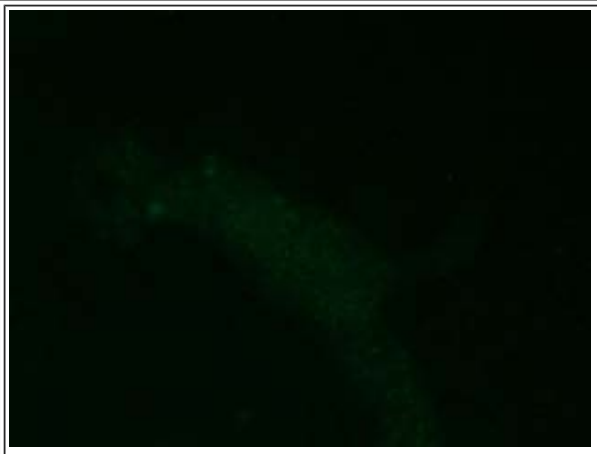
Pelomyxa. Fig 2b. Blue excitation of stained *Pelomyxa* with a 10X objective lens. The green outline suggests that the FM1-43 dye labeled the plasma membrane of the *Pelomyxa*. Fig 2c. Green excitation of stained *Pelomyxa* with a 10X objective lens. The red emission surrounding the cell is also indicative of the presence of FM1-43 in the outer leaflet of the membrane.

FM1- 43 Stained Pelomyxa (Control)

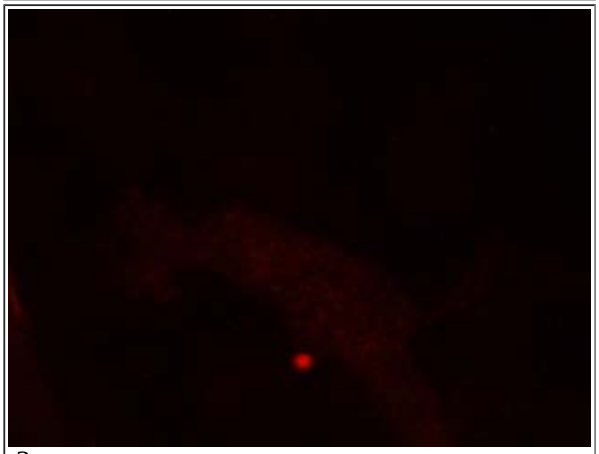
FM1- 43 stained *Pelomyxa* exhibited brilliant luminescence under UV illumination and fluorescence microscopy (Fig. 2a). Under UV illumination a brilliant blue concentrated ring surrounded the cell. Although the cytoplasm and its constituent organelles were also blue, the ring luminescence was brighter than the luminescence of the cytoplasm. Observing the same *Pelomyxa* under blue excitation revealed a bright green fluorescent emission surrounding the cell in the same location as the blue lighting viewed under UV illumination (Fig. 2b). Finally, the green excitation showed a similar red emission near the plasma membrane of the cell in the same location as the blue and green illuminations from the two previous cell viewings (Fig. 2c).



3a.



3b.



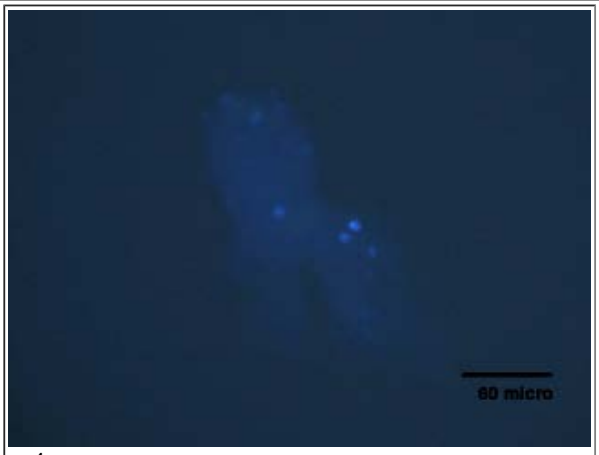
3c.

Fig 3a. UV illumination of stained *Pelomyxa* in the presence of a constant food source, with a 10X objective lens. No phagosomes are visible. Fig 3b. Blue excitation of stained *Pelomyxa* in the presence of a constant food source, with a 10X objective lens. No phagosomes are visible. Fig 3c. Green excitation of stained *Pelomyxa* in the presence of a constant food source, with a 10X objective lens. No phagosomes are visible. The brilliant red emission seen on the exterior of the *Pelomyxa* is a FM1 -43 stained *Paramecium*.

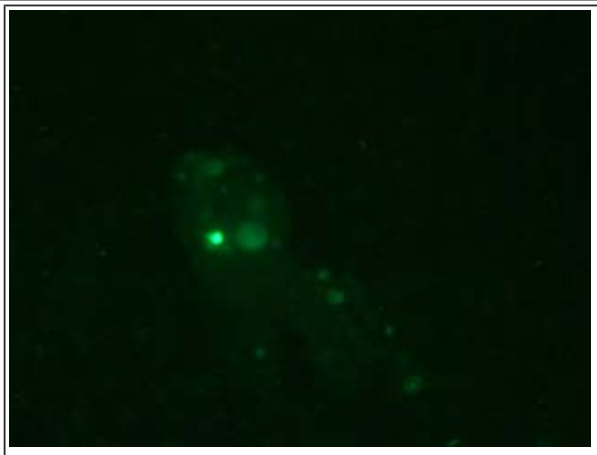
FM1-43 Stained Pelomyxa in the Presence of a Constant Food Source

The stained *Pelomyxa* in the presence of a constant food source were examined under the same conditions as those

viewed directly after staining and showed similar results (Fig. 3). Analogous to the UV illumination of the control *Pelomyxa*, the cytoplasm and the constituent organelles were labeled bright blue which mainly indicated the boundary of the cell (Fig. 3a). Under UV blue and green excitation, the boundary of the cell was clearly defined by green and red glowing emission rings respectively (Fig. 3b and 3c). The brightness of the rings surrounding the cells was not as brilliant as that seen in *Pelomyxa* viewed directly after staining. Upon viewing the interior of the cell, no luminescent vesicles that comparatively differed in size to those in the control were seen.



4a.



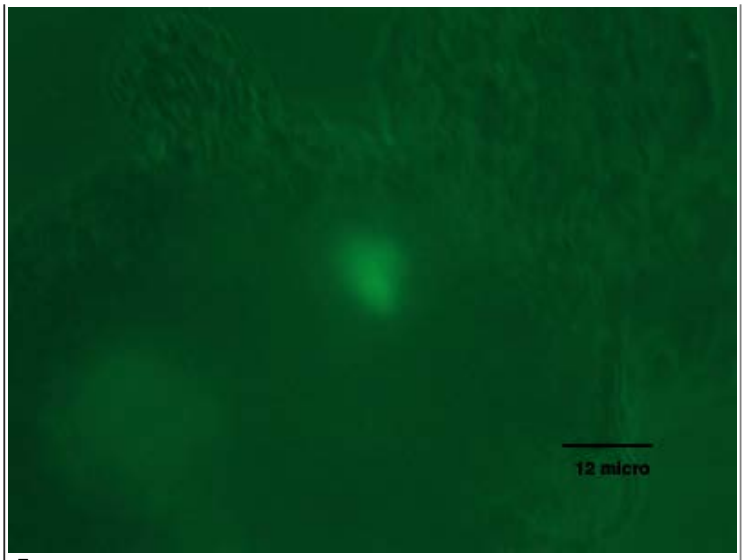
4b.

4c.

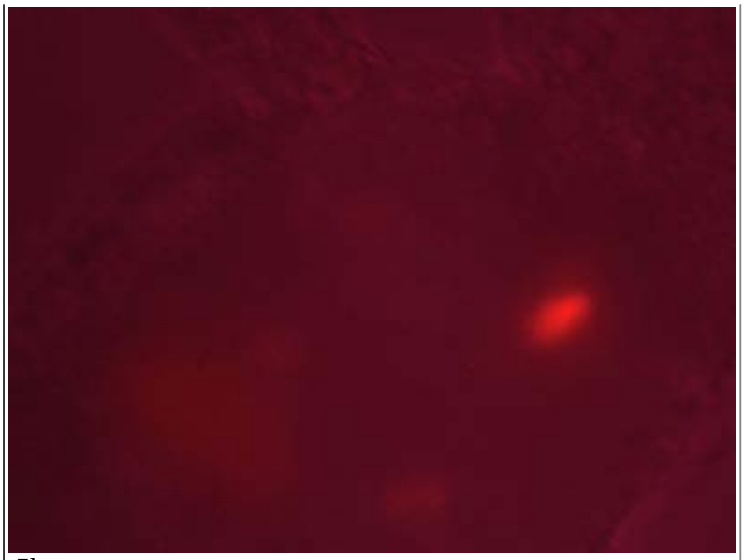
Fig 4a. UV illumination of stained, starved *Pelomyxa* with 10X objective lens. The brilliant blue spot seen within the cell wall of the *Pelomyxa*, meets the criteria of a phagosome. Fig 4b. Blue excitation of stained, starved *Pelomyxa* with a 10X objective lens. The bright green emission seen within the cell wall of the *Pelomyxa*, also meets the criteria of a phagosome. Fig 4c. Green excitation of stained, starved *Pelomyxa* with a 10X objective lens. The brightness of this image was decreased to further accentuate the brilliance of the spot. The red emission is in close proximity to the spots seen under UV and blue excitation and further indicates the presence of a phagosome.

FM1- 43 Stained, Isolated Pelomyxa in the Presence of a Food Source

Isolated *Pelomyxa* exhibited increased luminescence under UV illumination (Fig. 4b and 4c). Within the glowing green and red rings as seen under blue and green excitation, respectively, one brilliant spherical vesicle was seen. Because the UV illumination did not reveal a difference between stained and unstained portions of the cell, no evidence of cell activity or a spherical vesicle could be seen in such fluorescence (Fig. 4a). The magnification of the objective was increased to allow for a better visual of the phagosome. Under 40X magnification, with blue and green excitation, the small luminescent sphere appeared to twitch in a fixed location within the cell and with very little deviation in its movement (Fig. 5a and Fig. 5b).



5a.



5b.

Fig 5a. *Pelomyxa* viewed with a 40X objective lens under blue excitation. The bright green emission meets the criteria of a phagosome.

Fig 5b. *Pelomyxa* viewed with a 40X objective lens under green excitation. The bright red emission is in close proximity to that of the green emission seen in Fig 5a.

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CONCLUSION

In fluorescence microscopy, luminescence occurs when chemicals such as the FM1- 43 dye respond to fluorescent excitation. Under proper illumination, the chemicals responsible for luminescence react and are visible through microscopy. Although no dye was introduced to the media of the unstained *Pelomyxa*, the blue luminescence of portions of the cell under UV illumination indicates that *Pelomyxa* are auto fluorescent. Because the blue and green excitation showed no signs of cell auto fluorescence, the chemical responsible for blue glow under UV illumination was not affected by longer wavelength illumination.

In comparison to the unstained *Pelomyxa*, the stained *Pelomyxa* had an increased amount of luminescence, particularly in the area surrounding the cell. Because FM1- 43 dye is highly luminescent in the presence of a phospholipid membrane (10), the bright green and red glowing ring seen under blue and green fluorescence suggests that the phospholipid bilayer of the plasma membrane was in fact stained with the FM1- 43 dye. As a result of the successful staining of the plasma membrane, posthumous endocytosis would result in labeled endosomes, and subsequently, FM1- 43 labeled phagosomes visible under fluorescence illumination (11).

After 1 hour of incubation, the stained *Pelomyxa* in the presence of a constant food source exhibited no relative changes in illumination. The brilliant ring that was visible under the UV illumination marked the presence of FM1- 43 dye that was located within the phospholipid bilayer of the plasma membrane. Upon examining the size and brilliance of the spherical vesicles within the luminescent plasma membrane, no vesicles met the criteria to be considered a phagosome. The blue and green excitations showed no signs of endosome formation from the plasma membrane and therefore, no apparent phagocytosis. The blue coloration seen under UV illumination is attributed to the auto fluorescent quality of *Pelomyxa* as viewed in the unstained *Pelomyxa*.

In contrast to the *Pelomyxa* in a constant fed state, the deprived *Pelomyxa* exhibited signs of an increased rate of phagocytosis by an elevated number of luminescent vesicles seen within the cells. The result of UV illumination closely paralleled that of the stained *Pelomyxa* in the constant fed state, but differed in respect to an area of increased brilliance within the cell. One particularly bright blue sphere was seen under UV illumination which signifies the location of FM1- 43 dye and indicates the location of the plasma membrane and therefore the location of an endosome. This finding is further supported by the brilliant green and red spherical vesicle seen under UV illumination. The relative positions of the three emission in the images suggests that one source was responsible for the luminescent spot. In lieu to the determination of the brilliance of the spot, the width of the glow was measured and found to exceed the width requirement for a phagosome. According to the predetermined definition of a phagosome, the spot seen within the confines of the plasma membrane of the food deprived *Pelomyxa* was in fact a phagosome. The agitated movement

seen by the spot indicates that the contents of the phagosome was moving or that the membrane of the phagosome was distorting while the phagosome moved throughout the interior of the cell.

In a comparative analysis of the rate of phagosome formation for the food deprived *Pelomyxa* with respect to the *Pelomyxa* in the presence of a constant food source, the number of visible phagosomes in the deprived *Pelomyxa* exceeded those seen in the *Pelomyxa* with a constant food source. These data support the hypothesis that the rate of phagocytosis for *Pelomyxa carolinensis* is dependent on its fed state, and furthermore, these data suggest that *Pelomyxa* have an increased rate of phagocytosis after a deprivation period and react to an increase in food source.

Because the deprived *Pelomyxa* were placed in a *Paramecium bursaria* enriched environment, the increased rate of phagocytosis may not be *Pelomyxa*'s response to a deprived or fed state, but rather it may be a result of an increase in the abundance of food. The *Pelomyxa* in the presence of a constant food source were viewed after a one hour incubation period while remaining in the presence of a constant *Paramecium* food source (see methods above). However, the deprived *Pelomyxa* were viewed after a one hour incubation period in a food enriched environment. Again, this suggests the possibility that the phagocytosis seen was a result of food abundance and not necessarily increased phagocytosis. In future experiments concerning *Pelomyxa* phagocytosis in fed and deprived states, the food source must be controlled in a more appropriate manner to eliminate any questionable data.

Although the evidence supports the hypothesis that strongly. In order for this experiment to more strongly support the hypothesis that *Pelomyxa* respond to fed states, a larger experimental data pool must be constructed for the *Pelomyxa* in fed and deprived states. A larger data pool can more strongly support or rebut the hypothesis.

A collaborator, Diana Page, hypothesized that the rate of pseudopod formation decreases according to prolonged periods of starvation. Although the data did not support the hypothesis, the rate of pseudopod formation increased according to prolonged starvation. The data for the rate of pseudopod formation and for the rate of phagocytosis suggest that the rate of pseudopod formation may relate to the rate of phagocytosis. This hypothesis may be tested using a combination of the two data with additional research.

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COLLABORATORS

Initial digital images of *Pelomyxa* were taken in conjunction with Diana Page. The diluted FM1- 43 solutions were prepared by Dr. Robert Morris of Wheaton College in Norton Mass. Robert Borkowski and Danielle Kyes were gracious enough to assist in the digital imaging process completed in the ICUC at Wheaton College. In addition to assistance in fluorescence microscopy and digital imaging, Robert Borkowski was very helpful during the initial construction of this web page.

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USEFUL LINKS

Information about *Pelomyxa carolinensis*

Information about ICUC and Digital Imaging

Information about FM1 -43

Information about Phagocytosis

Information about Synaptic Vesicle Recycling

Motility Research of *Pelomyxa*