Naegleria minor:
Characterization of Its Differentiation and The Possibility of Genetics

Senior Thesis

Presented to
Faculty of the School of Arts and Sciences
Brandeis University
Undergraduate Program in Biology
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In partial fulfillment of the requirements for the degree of Bachelor of Science

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April 2014

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Abstract

Amoebae have long been thought of as asexual, partially because of their past phylogenetic grouping with “lower organisms” instead of nestled among eukaryotes, where they are today. Several species of amoeba have been shown to recombine sexually, however the sexual activity of *Naegleria*, an amoeboflagellate, remains in the dark. The Fulton lab has generally worked with the species *Naegleria gruberi*, studying its differentiation process, where it forms flagella and then reverts into an amoeba. Although no hint of mating has ever been observed in *N. gruberi*, the Fulton lab, in cooperation with other labs, sequenced and analyzed the genome of *N. gruberi* and found compelling evidence of sexuality. To understand what may encourage *Naegleria* to mate, we turned to a relative of *N. gruberi* called *Naegleria minor*. Two strains of *N. minor* were used, WTO43 (“Nami”) and PNG2 (“Ping”). The differentiation pattern of this species had not been extensively studied, but it was known to divide during differentiation. We tested conditions for the cells and dissected their differentiation. Using a low O$_2$-high CO$_2$ gaseous environment, cells maintained in suspension at 28°C produced a satisfactory differentiation at reasonable kinetics. The pattern was found to include at least two irregular divisions, one at 4.5 hours and one between 8 and 24 hours. At 3 hours the cells remain active but lose their culturability, which is only regained under certain conditions. We argue that the differentiation of *N. minor* is indeed gamete formation, followed under favorable conditions by zygote formation. Future studies include genetic tests of mating and recombination, initially using drug-resistant mutations as markers. From this we hope to establish a new genetic system and perhaps learn more about the ancient origins of sexuality.
Chapter 1: Introduction

To learn about the nature of an organism, the modern scientist takes advantage of its genetics. To learn about the eyes of *Drosophila melanogaster*, we cross mutants and wild-type flies. The amoeba *Naegleria*, however, has yet to offer itself to this method. *N. gruberi*’s karyotype shows about 23 chromosomes with 400 to 2,000 kilobase pairs per chromosome (Clark et al., 1993).

*Naegleria gruberi* is an amoeboflagellate: a single-celled organism that spends much of its life as an amoeba but if starved can undergo a drastic but rapid morphological change to a streamlined swimming flagellate [Figure 1-1] (Fulton, 1970 and 1977). The cells can also form cysts and remain dormant in this state for extended periods of time. *N. gruberi* in nature lives in the mud of most freshwater environments, but was isolated over 50 years ago and since then has lived as a cloned strain, strain NEG, in the Fulton lab. This lab and others have sought to understand the phenotypic changes that *N. gruberi* undergoes, using this model to study cell differentiation (Fulton, 1993). Unfortunately, never in the half-century that *N. gruberi* has been cooperating in experiments has it ever showed a hint of mating; only the life cycle in Figure 1-1 has been observed. Thus performing genetic experiments has not yet been feasible.

Amoeba and sexuality

Sexual mating in other amoebae has been observed; in *Chaos diffluens (Amoeba proteus)* mating occurs through haploid, flagellar gametes. After excystment, amoebae either mature or transform into gametes. Should the latter occur, cells grow a flagellum. The movement of the flagellum jerks the gamete around until it meets and fuses with a second gamete. The zygote then loses its flagella and encysts, beginning the cycle anew (Jones, 1928).
Another amoeba which undergoes sexual recombination is *Heteramoeba clara*, a relative of *Naegleria*. This species is thought to have two mating types: recombinant amoebas were only found in samples originally containing both mating types. This led Droop (1962) to postulate that the encysted amoebae are diploid and that some flagellates are haploid, allowing them to fuse as gametes and form a new diploid amoeba which encysts (Droop, 1962). Another possibility for *Naegleria* mating is that it only occurs occasionally or that it can only occur in the amoebae’s natural environment (Pernin, 1992). However, genetic exchange under experimental conditions has never been achieved, as with *Entamoeba histolytica* (Sargeaunt, 1985).

Although several cases of amoeboid mating have been observed, as seen above, amoebae are generally assumed to be asexual. This is an artifact from the old system of classification, where amoebae were grouped with lower protozoa (Lahr et al., 2011). This prohibited amoebae from being evaluated as the complex eukaryotes that they are. “Higher” eukaryotes, namely higher plants, animals, and fungi, are capable of sexual recombination. With new classification that places amoebae alongside sexual eukaryotes, in many different supergroups (Fritz-Laylin et al., 2010), theories on the origin of sexual mechanics should apply to amoeba as well. In Lahr et al. (2011) the authors argue that sexuality has long been present in the microbial eukaryote lineage and that it is asexuality which arose later and independently. Making this subject more revolutionary is the assertion that studying sexuality in amoeba may require us to reevaluate theories about how sexuality first arose. Due to its ancient nature, studying amoeboid mating also has the potential to reveal new fantastic and unusual ways of performing genetic exchange (Lahr, et al 2011).
Figure 1-1: A: Life cycle of *Naegleria gruberi* (Fulton, 1977). B: Differentiation of *Naegleria gruberi* (Fulton, 1970).
Genetic evidence for mating in *Naegleria*

The Fulton lab is actively in pursuit of establishing a *Naegleria* genetics system, spurred by recently found evidence of sexuality in the genetics of *N. gruberi*. In a major collaborative study involving Dr. Chandler Fulton, the genome of *N. gruberi* was sequenced and analyzed (Fritz-Laylin et al. 2010). Two clues given by its genome point to *N. gruberi* being a sexual organism.

First, the genome is diploid: this means that there are two sets of chromosomes inside the nucleus, and these are “heterozygous” in a way that indicates one set was given by each parent in a sexual interaction before the strain was isolated. Second, included in *N. gruberi*’s genome are functional genes for meiosis and gamete formation [Figure 1-2]. If these genes had not been used for millennia, their functionality would likely have disintegrated and disappeared long ago.

Excited by this information, the Fulton lab recently resumed looking for mating and genetic exchange through gametes in *N. gruberi*. However, so far only the life cycle in Figure 1-1 has been observed.
The common ancestor for both *Naegleria* and animals, including *Homo sapiens*, was capable of having sexual recombination (Fritz-Laylin et al., 2010).

**Figure 1-2:** The common ancestor for both *Naegleria* and animals, including *Homo sapiens*, was capable of having sexual recombination (Fritz-Laylin et al., 2010).
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**Naegleria minor as a new system for genetics**

Motivated by *N. gruberi*’s ancient genotypic ability to reproduce sexually but rebuffed by its phenotypic dry spell, we turned to relatives of *N. gruberi*. We reasoned that related species of amoeba would have conserved these genes and might demonstrate mating more readily [Figure 1-3]. The relative we focused on is *Naegleria minor*, or as it used to be known, *Willaertia minor*. *N. minor* is a unicellular eukaryote isolated by Bret Robinson of the State Water Laboratory in Southern Australia (De Jonckheere et al. 1995), (Dobson et al, 1993). “Nami,” as we have nicknamed the *N. minor* strain WT043, was chosen due to previous work done with this particular strain.

*N. minor* is similar to *N. gruberi* in phenotypic ways such as that when starved, they both differentiate to form flagella. Nami turns out to be different than *N. gruberi* in a very important respect, however: Nami performs cellular division during its differentiation [Figure 1-4] (Dobson et al. 1993). Previously, this division was said to be one that split the cell into two, each new cell bearing two to four flagella. The authors suggested that “the possibility that daughter flagellates of WT043 (“Nami”) may be haploid and act as gametes needs to be investigated” (Dobson et al, 1993). This could be the beginning of genetics of a *Naegleria* species.

Dobson et al. (1993) were unable to procure reliable differentiation of Nami amoebae in suspension. In 1970, Dr. Fulton found special conditions under which a related amoeboflagellate, *Tetramitus rostratus*, will differentiate (Fulton 1970). These conditions require a low oxygen level and a high carbon dioxide level. The lab found this approach also works for Nami (Karlan, 1995), and with this information, we have begun to study Nami’s life cycle.
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*N. minor*’s reported division (Dobson et al. 1993) is what attracted us to work with the species. Yet besides for the casual mention of division, no data was available on the nature of the amoeba’s division. Thus I began my careful study of the cells’ patterns during differentiation. Multiple “schemes” were drawn and redrawn as it was changed and refined. During this time, I conducted experiments to refine the process and optimize conditions for this species.
Figure 1-3: Phylogenetic relationship of some members of the *Vahlkampfia* and related amoeba based on SSUrRNA gene analysis (Clustal X), using *Entamoeba* as an outgroup. The *Heteramoeba clara* SSUrRNA gene sequence is from Silberman et al (2002).
**Figure 1-4:** Proposed differentiation and mating cycle of *Naegleria minor*, after Dobson *et al.* 1993.
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Alternate strain may enter meiosis more readily

Until this point, my early experiments focused on the “Nami” strain WT043. However, we were limited by the fact that these cells slowly vacuolated died during experiments under our laboratory conditions. Thus we shifted our focus to a new isolate of amoeba, PNG2, known in our lab as “Ping”. These cells appear to be a second isolate of *N. minor*, and behave in the same way as Nami and require the same conditions, yet the cells differentiate more uniformly and do not die during normal differentiation.

We next sought to better define the division process of Ping. This study took several forms. The first was a thorough quantification of cell body shape, number of flagella on the cell, and flagella position throughout PNG differentiation. In addition to using a light microscope for this delicate process, we quantified cell volume and density changes during differentiation using a Coulter Counter and MoxiZ Cell Analyzer. What struck us was the tendency of *N. minor* to divide unevenly. At 24 hours, samples of cells that had begun as amoeba were a mix of small flagellates of various sizes and with a variable number of flagella. This general phenomenon was noted by Robinson, et al. in 1989, and I further dissected the process to define two divisions during differentiation.

Gametogenesis?

Why do the cells divide unevenly, and why do they eject cell material in the process? We propose that the process we have observed is gametogenesis; we have shown loss of culturability as putative “haploid gametes” form and that the cells regain of culturability if putative “zygotes” reform. In ongoing studies, the Fulton lab is planning to cross drug-resistant mutants and select and characterize recombinants to demonstrate mating.
Chapter 2: Materials and Methods

Materials:

*N. minor* WT043 strain from ATCC and from Bret Robinson.

*N. minor* PNG2 isolated from a mud sample brought back from the highlands of Papua New Guinea by Dr. Fulton

Naegleria Medium (NM and PM) plates (Fulton and Dingle, 1967; Fulton, 1970)

*Klebsiella pneumonia* strain BS, overnight culture in Penassay broth (Fulton, 1970)

Water, demineralized (DMW)

30°C incubator

Tris buffer, 2 mM Tris·HCl, pH=7.2 (Fulton and Dingle, 1967)

40 ml IEC Autoclear centrifuge tube

Clinical centrifuge (IEC), set up as demonstrated by Fulton (1970)

125 ml screw top Erlenmyer flask, with silicone rubber septum

5 ml glass syringe

10 ml glass syringe

Glass syringe connector

Apparatus for gas exchange, described in Fulton (1970)

Nitrogen gas (N₂)

Carbon dioxide gas (CO₂)

Reciprocally shaking 28°C and 25°C water bath, 83 osc./min, 2.5 cm strokes

Lugol’s iodine (Fulton and Dingle, 1967)

1 ml plastic syringe

25 G 5/8 B-D needle
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12 x 75 mm Plastic test tubes

Methylene Green

Pipettes

MoxiZ Cell Analyzer (Orflo)

“S” type Cassettes for MoxiZ

Methods:

Edge plate (Fulton, 1970)

0.2 ml of Klebsiella was spread on one NM plate. One “loop full” of WT043 or PNG2 was placed on 1 mm$^2$ of the NM plate, 2 mm from one side. The plate was incubated at 30°C and inverted after 30 minutes.

Spread plate

When a WT043 or PNG edge was growing anywhere up to ¾ across the plate, an inch of the advancing edge was suspended in 0.5 ml DMW and vortexed lightly. 0.15 ml Klebsiella was then plated with 0.15 ml of the cell suspension, spread evenly and placed in a 30°C incubator overnight. The plate was flipped after 1 hour.

Harvesting

To begin a differentiation, cells were suspended in 10 ml Tris buffer and a stopwatch was started at time 0:00. Cells were transferred to an Autoclear tube and spun in the centrifuge for 45 seconds at setting 6. A brake brought the centrifuge to a stop (Fulton, 1970). Supernatant was discarded and pellet was resuspended in 10 ml Tris buffer, then vortexed. This step was repeated
two more times, then cells were distributed into screw top flasks. Tris buffer was added to increase the total volume in each flask to 10 ml. This process took about 9 minutes to complete. Typically each flask received the contents of one-third plate in a total of 10 ml Tris buffer.

Gas Exchange

All flasks were lined up next to the vacuum apparatus and processed in order from A to Z. Each flask was flushed with Nitrogen gas three times, reaching an internal vacuum of 71.5 mmHg during the evacuations. During the third refill with N\textsubscript{2}, a mixture of 0.8 ml air and 3.6 ml CO\textsubscript{2} were allowed to flow into the flask. As the flask reached positive pressure, the N\textsubscript{2} line was removed and the internal pressure of the flask was allowed to reach atmospheric pressure before the needle was then removed from the septum. When all flasks had been processed, they were placed into the 28°C shaking water bath. Water level in the bath was above where the liquid inside the flask reached during shaking (Fulton, 1970). The process of gassing took 3 minutes per flask.

Sampling and Fixing of Cells

At given times, one flask at a time was removed from the shaking water bath to take an aliquot of the cells. The flask was gently swirled (right-side up) with the intention of suspending all cells in the Tris, even those possibly adhering to the bottom of the flask. The flask was then turned upside-down and a 25 G\textsuperscript{5/8} B-D needle on a 1 mL plastic syringe was used to remove 0.3 mL of cells. This sample was added to 1 drop Lugol’s iodine in a 12 x 75 mm plastic test tube to fix them. The flask was then immediately returned to the water bath. The syringe was thoroughly rinsed with DMW before and after every use.
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*Observing Nuclei in Fixed Cells*

To observe nuclei in *Naegleria* becomes difficult once Lugol’s iodide is added. To observe flagella and nuclei at the same time, however, Lugol’s iodide should be added to the sample of cells to fix it. After fixation, a single drop of the fixed sample is placed on a glass slide with no cover and the slide is tilted back and forth in a process called “bleaching” as described in Fulton, et al. (1969).

Bleaching *N. minor* makes their nuclei fairly visible but can be enhanced with the addition of 15 uL 1/12 dilution of Methylene Green to 30 uL of bleached cells (Experiment NA51).

*Counting Cells with Moxi Z Cell analyzer*

Four routine cell counting, and approximate estimates of cell volume, cells were counted using an Orflo Moxi Z Cell Analyzer. Accuracy of cell counts were calibrated by comparing the Moxi Z and the Coulter Counter.

A concentrated stock solution, CSSI, was prepared in deionized water and brought to 500 ml, and contained NaCl 39.75g, KCl 2.0 g, NaH2PO4.H2O, 10.35 g, Na2EDTA 1.86 g, and NaF, 2.10 g. When diluted 1/10 this solution is equivalent to ~0.8% NaCl, which is suitable for counting mammalian cells. Since the electronics of the Moxi Z are quite sensitive to conductivity, we could not dilute the saline as low as the 0.4% NaCl used for the Counter Counter. We found we could use CSSI it at a dilution of 0.75/10. This "75S" solution is
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approximately 0.6% NaCl. If Naegleria were counted immediately after dilution, this diluent
gave accurate counts with only slight cell shrinkage.

Cells were diluted 1/10 in "75S", using 50 µL cells to 450 µL 75S in a 13 x 100 Pyrex tube,
which was mixed by gentle "thipping" of the tube. 70 µL of the sample was counted in a Type S
Moxi cassette.

*Counting Cells with Coulter Counter*

To examine cell number and volume changes during differentiation, we used a Coulter Coulter
Multisizer 4, Particle Analyzer, kindly loaned to Dr. Fulton by Beckman Coulter. The diluent for
cell counting was 0.40% NaCl ion deionized water, and cells were counting passing through a
100 µm aperture, as described (Fulton, 1970). The Analyzer was calibrated for accurate cell
diameters and volumes using 10 µm calibrator latex beads provided by Beckman Coulter, and
following the protocol provided with the Analyzer. At appropriately 1 hour intervals, 500 µL
samples of the cell in suspension were diluted in the saline and brought to a final volume of 25
ml. The diluted samples were mixed without vortexing by pouring back and forth between two
25 mm diameter tubes three times, and then immediately counted and sized.

*Plating for Culturability*

Dr. Fulton performed tests for recovery of culturability using several procedures, but a simple
protocol that gave the the most useful results so far was:

1. Any additives to be tested (e.g.,50 µL of an overnight Klebsiella pneumoniae culture in
Penassay broth) were put into 14 X 45 mm open-top vials. Vials with no additions were controls.
2. Differentiated cells were added to the vials (e.g., 0.5 ml/vial). The vials were left open to the atmosphere.

3. The vials were incubated in a shaker water bath with about 80 1-inch strokes per minute, typically for 3 hours at 28°C.

4. At the end of the incubation, samples (5, 10 or 20 µL) were added to 100mm PM plates with 0.15 ml of overnight *Klebsiella pneumoniae* and sterile deionized water to make a total volume of 0.35-0.7 ml. These plates were immediately spread, and incubated upright at 30°C until all the liquid was absorbed into the agar medium; then they were inverted. Incubation was continued at 30°C for about 24 hours, and then incubation was continued at room temperature until the plaques had developed and were counted (usually at about 48 hours).
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**Chapter 3: Revisiting and Refining the Differentiation Protocol for Nami**

As *N. minor*, strain WT043 (“Nami”) progress during differentiation, the cells form 2-to-8 flagella. Most flagellates have 6 flagella, however. In addition, most flagellates have an even number of flagella.

In 1995, the last time Nami was studied (Karlin, 1995), the following procedure was used to observe differentiation of the cells:

10 ml of Tris buffer and cells (2 X $10^6$ cells/ml) were placed into a 125 ml Screwtop flask with a septum. The flask was then evacuated twice with Nitrogen and after a third evacuation, 0.3 ml CO$_2$ and 1.9 ml air were added with the nitrogen at atmospheric pressure. The flask was then placed into a 30°C shaking water bath (Karlan, 1995). This method was derived from work with *Tetramitus rostrum* (Fulton, 1970).

Using these conditions, $T_{50}$, or time for 50% of cells to visibly have grown flagella was 200 minutes (3.33 hours) [Figure 3-1] (Karlan, 1995).

When I began differentiating Nami, I too used these conditions. However. I soon turned my efforts to refining the conditions under which Nami differentiates and potentially mates. Conditions I tested include cell volume in flasks, gas mixtures, water bath temperature, and agitation. These experiments served to find working conditions for my intended mating experiments; in the future they could be further defined and perfected.
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*Volume effects on differentiation success*

Volume was reexamined because the ability of cells to physically interact with each other may affect their differentiation or mating success rate. In addition, the differentiation of *N. minor* takes a number of hours and assessment of the sample involves taking a significant volume (0.3 ml) for each time point. Doubling the volume in the flask to 20 ml appears to be equivalent to the usual 10ml volume, but it resulted in a higher percent of cells forming flagella during differentiation [Figure 3-2].
Figure 3-1: *N. minor* strain WT043: appearance of flagella during differentiation as observed by Allison Kaplan in her 1995 thesis. $T_{50}$ is at 200 minutes (Karlan, 1995).
Figure 3-2: Volume variation’s effect on appearance of flagellates in Nami (Expt. NA5). Volume variation will be re-examined later in this investigation.
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Gas environment effects

Nami differentiation was best at high carbon dioxide, low air levels, as shown with *Tetramitus* (Fulton, 1970b) and with *N. minor* (Karlan, 1995). After further testing of varied combinations of carbon dioxide gas and air, it was determined that the highest percentage of flagellates are found using 0.8 mL air and 3.6 mL carbon dioxide. This mixture also led to the shortest time to $T_{50}$, or the highest rate of differentiation [Figure 3-3, Figure 3-4, Figure 3-5]. Carbon dioxide levels of 1.2 mL and higher was not found to make a significant difference in the success of differentiation [Figure 3-5]. 3.6 mL of carbon dioxide was chosen to use for future experiments for convenience of gas handling purposes. At the end of this project, it was found that 1.9 mL air may indeed be optimal, as described by Fulton in 1970 and Karlan in 1995.

Additives during differentiation

Several solutions were added to *N. minor* at the beginning of differentiation to observe their effect on differentiation rate, etc. Additives included calcium, MS-1 (contains compounds generally found in a pond environment) (Fulton, 1970), *Klebsiella* bacteria, fructose, dextrose, L-methionine, and sodium bicarbonate. None of these showed any improvement over Tris buffer (results not shown).

Temperature variation

The water bath in which the flasks containing Nami are placed is set to a certain temperature in order to regulate the rate of differentiates. Because Nami’s natural habitat is a freshwater body in Australia, and the temperature of this pond is unknown, it is possible that they could require warm water to perform various processes, such as differentiation. In previous experiments, the bath temperature was 30° Celsius. As noted by Fulton, however, while such high temperatures
Leah Naghi may sacrifice quality of differentiation (i.e., complex internal processes) to merely accelerate differentiation rate. After noticing that Nami gets “sick” later in differentiation under Karlan’s conditions, we reasoned that temperature may be a key factor and sought to find a lower temperature at which Nami would be differentiate more “healthily”. I further address the “sick” Nami issue in the latter part of Chapter 4. As seen in Figures 3-6 and 3-7, optimal temperature for Nami differentiation lie between 33 and 25 degrees. 28 degrees Celsius was chosen as it is closer to 25 degrees Celsius, the optimal differentiation temperature for NEG that still allows for a good differentiation of Nami (Fulton, 1970) [Figure 3-9].

Effect of agitation on differentiation success

When Nami was incubated at 28C in a stationary environment°, the cells did not differentiate and instead remained amoebae [Figure 3-8].

Defined differentiation conditions

10 mL of Nami under an atmosphere of nitrogen, 0.8 mL air and 3.6 mL carbon dioxide, in a shaking water bath at 28 degrees Celsius, showed reliable and consistent results [Figure 3-9].
Figure 3-3: Lower levels of air present in Nami’s differentiating environment lead to a higher percentage of differentiated cells (Expt. NA17).
Figure 3-4: In a first test of carbon dioxide levels, higher concentrations result in a greater rate of differentiation (Expt. NA18).

Figure 3-5: Testing higher levels of carbon dioxide with Nami (Expt. NA21).
Figure 3-6: Nami’s percent of cells which form flagella and its differentiation rate are both decreased when the cells are differentiated in a 25 degree Celsius environment (Expt. NA1).

Figure 3-7: Nami differentiation at 33 degrees Celsius yields a lower number of flagellates (Expt. NA13).
Figure 3-8: Failure to agitate Nami in its Tris buffer environment does not allow the cells to differentiate. (Expt. NA7).
**Figure 3-9:** Percentage of Nami flagellates during differentiation under conditions defined in this chapter. Standard error bars are from three identically treated samples (Expt. NA12).
Chapter 4: Nami Morphology

As I defined working conditions for *N. minor* WT043 (Nami), as described in Chapter 3, I simultaneously began to dissect the morphological pattern of differentiation.

**Shape change during differentiation**

By 2.5 hours after suspending Nami cells in Tris buffer and applying the gas mixture described in Chapter 3, the amoebae have rounded up into large flagellates and begun forming flagella. At 4 hours, the cells begin dividing into smaller flagellates and from that point, a mix of cell size and shape can be observed [Figure 4-1].

**First observations: Pairs**

From Dobson’s depiction of Nami’s differentiation, I expected to see the cells grow flagella and divide [Figure 1-4]. One of the first new observations I made was that before growing flagella, the amoeba paired up [Figure 4-2]. Because of our hypothesis that *N. minor* division is connected to sexual mating, we wondered if the pairs of amoebae might involve genetic exchange [Figure 4-3]. Based on the fact that other *Naegleria* species also have a similar stage where the amoeba act “sticky” and clump together before differentiating without any hint of genetic segregation or mating and due to the sheer complexity that would be involved in such an exchange between two diploid organisms that have not undergone meiosis, we tentatively dismissed the pairing of Nami as a significant step in its sexual process.
Figure 4-1: Morphology of Nami during differentiation. Amoeba grow flagellate ($T_{50}$ for flagella formation is 3.75 hours) and then become large flagellates, which divide at 5 hours to become smaller flagellates. Standard error bars are from three identically treated samples (Expt. NA12).
Figure 4-2: Nami amoeba form pairs between 1.5 and 3 hours. Standard error bars are from three identically treated samples (Expt. NA12).
**Figure 4-3:** Differentiation of Nami, my understanding after observing the amoeba form pairs before completing the rest of their differentiation cycle (Expt. NA2). The assumption/inference in this diagram is that the pairing of amoeba results in a joining of their nuclei. This theory was later discarded. There is no evidence that these pairs of amoebae fuse or become binucleate at this stage.
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*Number of flagella*

In a closer examination of Nami during differentiation, it was discovered that the cells do not form flagella in pairs, starting with two and adding another two pairs to each cell as described in Karlan [Figure 4-4]. Instead, Nami initially forms two to nine flagella. The $T_{50}$ for flagella formation, the time it takes for 50% of cells to form flagella, was 3.75 hours. Subsequent to their formation, the flagella then migrate in pairs or in groups of (usually) four to various points on the cell, and after divisions have been achieved cells with both two and four flagella can be observed [Figure 4-5].
Figure 4-4: Differentiation cycle of *Nam* after Karlan, 1995.
Figure 4-5: Histogram of Nami flagella growth (Expt. NA12).
Unequal divisions

The cells do not simply divide into two identical cells, contrary to what was anticipated, and was diagrammed in Karlan (1995) [Figure 4-4]. In fact, the daughter cells are not identical at all. After flagella migrate to various locations on the cell, the membrane divides the cell into two-to-four daughter cells. They do not all come apart at once, but cytokinesis shaping multiple daughter cells is clearly visible [Figure 4-6]. In addition, some of the cell material that comes off seems to be discarded cytoplasm or other cell material and does not have flagella or a nucleus.

Nami cells die during differentiation

Although Nami undergoes a cell differentiation which includes a cellular division, at 5.5 hours a large percentage (almost 40%) of cells, large and small alike, begin taking in water from their environment, forming vacuoles, and dying [Figure 4-7]. By 24 hours, practically 100% of cells were dead and appeared to have their cell bodies completely filled by a huge vacuole [Figure 4-8]. This presented a problem for intended mating experiments, because live cells are needed to mate with one another!

Factors were varied and additives were tested in hopes of fixing the “sick Nami” dilemma, however none showed any results. Luckily, this problem was soon resolved when we obtained a new strain of Naegleria minor. The second isolate of N. minor in the world, PNG2 (fondly known as Ping) was cultured from a mud sample taken in Papua New Guinea.
Figure 4-6: Differentiation pattern of *Nami* with unequal division (After expt. NA39)
Figure 4-7: Nami cell with large vacuole and 6 visible flagella (Expt. NA55).
Figure 4-8: Percentage of Nami cells with large vacuoles as differentiation progresses (Expt. NA54).
Chapter 5: *N. minor* strain PNG2 (“Ping”) may deliver genetic secrets more readily than Nami

**Similarities of Nami and Ping**

As mentioned previously, Ping (Strain PNG2) is the second known isolate of *N. minor* in the world. Like Nami, the cells begin differentiation by forming 2-8 flagella, the majority forming 6 flagella. The cells then divide in an unequal fashion.

**Differences**

Ping progresses through differentiation at a faster rate than Nami. At 28°C, the T$_{50}$ of flagellate formation in Nami is 3.75 hours, and Ping’s T$_{50}$ for flagellate formation is at 2.25 hours [Figure 5-1].

Ping’s enhanced rate of differentiation is apparent also through the appearance of dividing cells in Ping as opposed to Nami. Ping reaches its peak of observable dividing at 4.5 hours while Nami takes an additional hour to show a spike in dividing cells [Figure 5-2].

In addition, unlike Nami’s large vacuole formation at 5.5 hours, Ping does not take in excess water. Instead, Ping has a much greater success rate of smaller flagellate (daughter cell) formation. Ping’s cell population is nearly 60% small flagellates at 6 hours and is over 80% small flagellates at 23 hours. Nami, on the other hand, does not surpass 40% small flagellates at any point in this comparison [Figure 5-3].
Figure 5-1: Ping vs Nami % Flagellates vs. Time. Standard error bars are from three identically treated samples (Expts. NA12 and NA70).
Figure 5-2: Ping and Nami Cells in division vs. time (Expt. NA25)
Figure 5-3: Appearance of smaller flagellates vs. time (Expt. NA25).
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*Using the Coulter Counter for division analysis*

The Coulter Multisizer 4 allowed us to quantitate both the number and size of cells in successive samples. In Figure 5-4 the distinct changes in average size point to two major cellular divisions: Cells begin differentiation at an average diameter of 12 micrometers, and they first divide between 5 and 6 hours into cells with an average diameter of 8.9 micrometers. Their second division is between 8 and 24 hours, creating cells with an average diameter of 7.4 micrometers, as well as a huge number of tiny particles between 5 and 0 micrometers. Each successive division creates a new population with a wider range of particle diameters. There is likely a third division, but it is less clear because of heterogeneity in volume, including the production of many small anucleate cell fragments. The graph also shows clearly the consequence of increasing heterogeneity in size due to asymmetrical divisions of *N. minor* as well as the increasing asynchrony of the cell population as *N. minor* progress through differentiation [Figure 5-5]. We can also see some of these features using the much simpler Orflo Moxi Z Cell Analyzer (data not shown), but the Multisizer is necessary to get a full picture of the cell volume changes.

*N. minor nuclei*

When Methylene Green is applied to bleached cells, nuclei became fairly visible. (Attempts to stain nuclei with DAPI also worked, but we did not succeed in staining cells with DAPI while retaining the flagella.) Incorporating nuclear information into my analysis of Ping’s differentiation, I was able to get yet another, more specific picture of the process [Figure 5-6]. In this pattern, amoebae simultaneously form flagella and divide their nucleus into two. The $T_{50}$ for this process is 3.5 hours. At this point, the cells begin to divide. This division is uneven and produces particles such as small flagellates with no nuclei, small flagellates that contain one
nucleus, and small cell fragments that usually contain a number of vacuoles. The nucleus then seems to divide again at 6.5 hours and by 24 hours, tiny flagellates with one nucleus can be swimming rapidly in the sample.

Two sequential nuclear divisions with a reduction in cell size could be indicative of meiosis. In Chapter 6, I will address this possibility in greater detail.
Figure 5-4: Coulter Counter analysis of Ping cell size, measured by diameter. (Expt. NA28)
Figure 5-5: Asynchronous division in Ping, seen counterclockwise from lower right: an amoeba, a cell dividing into four daughter cells, and a large flagellate are all visible at once. Sample taken at 4.5 hours (Expt. NA55).
Figure 5-6: Hypothesized trend of cell shape and number of flagella and nuclei in Ping. Constructed from observations using nuclear dye under the microscope (Including expt. NA50).
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Ping analysis under the microscope

As time progressed, I reorganized and redefined stages of Ping’s differentiation cycle and now categorize the cells in any sample by number of flagella and by shape of the cell [Figure 5-7]. Number of flagella categories are: 0 flagella, 1-2 flagella, 3-4 flagella, 5-6 flagella, 7-8 flagella, and 9 or more flagella. As with Nami, Ping cells originally grow 2 to 8 flagella, with the median number of flagellates having 6 flagella [Figure 5-8].

Quantifying cells by their shape proceeds using the following categories:

Amoeba [Figure 5-9b,o]

Large Flagellate [Figure 5-9c-h]

Dividing Cell [Figure 5-9i-k]

Small Flagellate

Tiny Flagellate [Figure 5-9l-o]

Cytoplasm

Small Flagellates and Tiny Flagellates usually have two flagella, but can have up to 4 flagella. At the time this thesis was written, these two terms (Small and Tiny) are fairly arbitrary because divisions are asymmetric and cytoplasm is lost so there is a wide range of smaller flagellate sizes to cover. At the present, records are kept of these two types of flagellates separately, using my judgment to divide the sizes into two distinct categories. When it is difficult to assign a flagellate to one category or the other, I judge by comparing flagella size to the cell body: if the flagella are at least twice the length of the body, that cell is a Tiny Flagellate. As follows Figure 5-6, Figure 5-10 clearly illustrates the changing morphology of Ping during differentiation.
Figure 5-7: Typical layout of a data sheet for *N. minor* experiments. This page is taken from expt. NA70.
**Figure 5-8:** Histogram of Ping’s number of flagella during differentiation (Expt. NA70).
Figure 5-9: Digital photographs of Ping from expt. NA55, stained with Lugol’s iodide and bleached.  

**a.** A/O Scale. 1 division is 10 micrometers.  
**b.** Amoeba  
**c.** Large flagellate with one nucleus and 10 flagella  
**d.** Large flagellate with 6 flagella  
**e.** Large flagellate with groups of flagella separating  
**f.** Large flagellate with 6 flagella  
**g.** Large flagellate with flagella at two ends  
**h.** Large flagellate with flagella at three foci, cytoplasm appears to be grouping into three areas  
**i.** Lower cell appears to be dividing into three daughter cells  
**j.** dividing into two cells  
**k.** unequal division  
**l.** tiny flagellate  
**m.** tiny flagellate  
**n.** tiny flagellate  
**o.** tiny flagellate and amoeba
Figure 5-10: Ping morphology changes during differentiation. Standard error bars are from three identically treated samples (Expt. NA70).
Chapter 6: “Gamete formation” and “Mating” in *N. minor* PNG2

Much of the work in this chapter was done in collaboration with Dr. Chandler Fulton.

With the changes in morphology of *N. minor* PNG2 in mind, a conceptual description of its putative meiosis is now possible. An amoeba is a 2n organism. When starved in a warm, wet environment in some contact with other cells in a microaerobic, high carbon dioxide environment, i.e., under the conditions described in this thesis, the cells begin several meiosis processes, including replication of DNA and division of the nucleus. When a cell divides at 2.25 hours and then again at 10 hours (followed perhaps by a mitosis), we hypothesize that 1n cells are produced and that these 1n cells can be functional gametes.

Two characteristics of gametes are:

1. Their inability to grow on culture
2. Their ability to recombine and form a new 2n organism which is capable of growing on culture.

*Loss of culturability*

Many experiments performed with *N. minor* show loss of culturability with differentiation. The best example is Experiment NA60, using 0.5 plates of cells/10 ml Tris, shaking at 28°C.

Live Ping cells were sampled at various times during differentiation. These samples were then assessed by the MoxiZ for cell density and small amounts (10 μL of diluted samples) were plated on PM with Kp bacteria to assess the proportion that can form clones, as Fulton (1970). At 36 minutes, a first sample was drawn, counted, and plated when the cells were still all amoebae. *N.*
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Minor amoebae are defined to close to a 100% efficiency of plating (see Fulton, 1970): if the amoebae are plated on Kp bacteria, each will grow, feeding on the lawn of bacteria and forming a plaque (like bacteriophage). Three subsequent samples were taken and platted: at 2 hours and fifty-two minutes, at six hours, and at nine hours. As compared to the control sample from 36 minutes, the percentage of cells in a sample which were able to grow on culture dropped to less than 0.01% [Figure 6-1].

As differentiation progresses, cells change from amoeba into large flagellates and then divide: thus we learn that beginning the process of differentiation; i.e. growing flagella and starting the division process makes the cells unable to grow in culture. In other words, although the 2.8 hour sample showed no significant increase in cell number, its percentage of culturable cells decreased by ten-fold. While cell number nearly doubles in the next three hours, the percentage of culturable cells completes another 10-fold drop.

These results point to a meiosis-like event; if mitosis were occurring, cells should regain culturability after each division, but this is not the case.

The cells lose culturability quickly. In experiment NA28, we estimated that culturability dropped to an EOP (efficiency of plating) of ca. 0.1-0.3%. In experiment NA23, the viability of PNG2 at 22 hours was estimated as ≤2/1000 cells.

By comparison with other experiments PNG2 (and Nami) may lose culturability even before the occurrence of the first cell division, estimated to occur at roughly 6 h in experiment NA38 and 4.5 h in experiment NA56. (And we have data from the Coulter Counter analysis, NA38, which also suggests the second division by about 9 h (Experiment NA58) and probably a third by twenty-four hours.)
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The peak number of small flagellates was generally seen by 10.5 hours, at a plateau that lasted at least to 24 hours, and often was ~80% (e.g., NA56). (There were 56% small flagellates in experiment NA60. In NA55, there were 58% at 6 hours, and 97% at 24 hours.)
<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell density (Cells/mL)</th>
<th>% Culturable</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 minutes (0.51 hours)</td>
<td>$7.5 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>2 hours 52 minutes (2.8 hours)</td>
<td>$7.6 \times 10^5$</td>
<td>$\leq 10$</td>
</tr>
<tr>
<td>6 hours</td>
<td>$12.9 \times 10^5$</td>
<td>$\leq 1$</td>
</tr>
<tr>
<td>9 hours</td>
<td></td>
<td>$\leq 0.01$</td>
</tr>
</tbody>
</table>

Figure 6-1: Loss of culturability of Ping during differentiation, represented in table and graph form. On the graph, the solid line represents cell density (left y-axis) and the dotted line represents % growth of cells on culture (right y-axis). Cell density for 9 hour sample was not calculated. Numbers are rough estimates due to the low number of plaques on all plates except earliest plating (Expt. NA60).
Regain of Culturability

We conjectured that if our deductions were correct, after meiosis the gametes could regain culturability by fusing to form zygotes. (It is our working hypothesis that the diploid amoebae contain two mating types, so the “gametes” should contain a mixture of mating types.)

We explored various agents that we thought might induce the gametes to mate, as evaluated by regaining culturability. Samples of 0.5 ml of differentiated cells (usually at 28°) were transferred to open dram vials at the indicated time. The vials contained various supplements. The only one shown in the table is minus/plus an aliquot of an overnight culture of Klebsiella pneumonia, which has given our best success to date. The vials were incubated in a shaker (usually at 28°, but in some trials at 26 or 20°), open to the air. The differentiated cells, mostly small flagellates, were incubated in the presence or absence of bacteria for from about 3 hours to 18 hours.

Samples from the vials were subsequently plated to determine the number of culturable cells. For this purpose, 0.15 ml overnight Kp culture plus sterile water added to bring the total volume to 0.35-0.7 ml were put onto a PM plate, from 5 to 20 µL sample of the culture in the vial was added, and the mixture was immediately spread over the surface of the plate. Clones could be counted after 24 hours at 30° followed by another day at room temperature [Figure 6-2].
<table>
<thead>
<tr>
<th>Expt.</th>
<th>Cells to vials (hours)</th>
<th>+ bacteria (µL to 0.5 ml)</th>
<th>Cells plated (hours)</th>
<th>Plaques</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
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<td>6</td>
<td>0</td>
<td>24</td>
<td>0</td>
<td>≥99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA30</td>
<td>6</td>
<td>0</td>
<td>24</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td></td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>NA57</td>
<td>9</td>
<td>0</td>
<td>12.5</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td></td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>NA58</td>
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<td>0</td>
<td>13</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td></td>
<td>60.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>150</td>
<td></td>
<td>86</td>
<td></td>
</tr>
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<td>0</td>
<td>12</td>
<td>0</td>
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</tr>
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<td></td>
<td></td>
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<td>4</td>
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<tr>
<td></td>
<td></td>
<td>50</td>
<td></td>
<td>76</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6-2:** Table recording plaque growth during various experiments with variations in time of aliquot sample, time of cell plating, and amount of bacteria added.
If the differentiated cells were incubated too long before the “treatment”, few viable cells formed. E.g., after 29 hours in experiment NA70, the cells transferred to vials formed few culturable cells whether or not bacteria were added (data not shown).

Addition of bacteria during the incubation consistently increased the number of culturable cells in eight experiments, including the 6 tabulated above (and pilot experiments NA24 and NA26), but failed in two others (Experiments NA27, 63), for unknown reasons.

Other elements were tested including: substitution of \textit{E. coli} for \textit{K. pneumonia} (which gave a comparable stimulus), addition of stronger MOPs buffer, or addition of either fresh Penassay broth or Casamino acids. All of these supplements gave slight stimuli, but consistently the strongest stimulus came from the addition of overnight cultures of bacteria.

It is considered unlikely that the small amount of bacteria added would have supported much growth of PNG2 in the interval between the addition of bacteria and the assessment of viable cells by plating.

These results are promising, and fit the interpretation of loss of culturability caused by meiosis followed by restoration by zygote formation. The hypothesis needs direct testing with genetic markers, and these can also be used to determine and optimize the conditions for “mating.”
Chapter 7: Discussion

When Naegleria gruberi is grown in association with Klebsiella pneumonia, then washed and placed in a shaking water bath, the cells grow flagella in rapid synchrony beginning at 0.75 hours. 50% of cells have grown flagella at 0.95 hours, and nearly 100% of cells have by 1.083 hours. The cells then revert to amoeba and lose their flagella (Fulton, 1970).

During differentiation, Naegleria minor grows two to nine flagella (most form six) and divides unevenly. Nami’s T₅₀ for flagellate growth is 3.75 hours, and does not reach a 50% level of small flagellates due to intake of water and cell death. Ping’s T₅₀ for flagellate growth is 2.25 hours and for small flagellate formation is 6.25 hours. N. gruberi, Nami, and Ping differentiations are compared in Figure 7-1. Ping’s first division occurs between 4 and 5 hours, and its second between 8 and 24 hours. Nami’s first division takes place between 5 and 6 hours, and its second division has been difficult to observe due to the cells’ decline in viability.

N. minor differentiation proceeds best in a shaking water bath at 28 degrees Celsius, under a gas environment containing a small amount of oxygen and a high level of carbon dioxide. Using 0.8 mL air and 3.6 mL carbon dioxide inside 125 mL flasks produced a satisfactory differentiation at a reasonable rate. In the future, these settings should be further examined and fine-tuned to find the true optimal conditions.

In the pursuit of proving the presence of meiosis and mating and establishing a genetics system with N. minor, we switched from using Nami (strain WT043) to Ping (strain PNG2). This is because Nami cells die during differentiation by taking in water and bursting. Ping, however, does not have this barrier for us to overcome and also has a faster rate of differentiation, making practical usage of the cells easier.
Figure 7-1: *Naegleria gruberi* vs. Ping vs. Nami, % Flagellates vs. Time (*N. gruberi* from Fulton, 1970) (Expts. NA12, 70).
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Observing *N. minor*’s nuclei during differentiation gave more clues about the possible meiotic nature of the cells’ divisive differentiation: two nuclear divisions before the cells’ cytoplasmic divisions follows a classic meiotic pattern. Coupled with quantitative data from the Coulter Counter on cell volume, documenting the divisions, and with plating studies, showing the cells’ subsequent loss and regain of culturability, we believe that there is sufficient evidence to warrant further studies on *N. minor*’s sexual nature.

The next step in the Fulton lab’s study of *Naegleria* sexuality and genetics is to obtain various drug-resistant mutant amoebae (especially dominant mutations) and cross their gametes. Double-resistant mutants, or recombinants, will provide strong evidence for *Naegleria* sexuality. This can be further dissected by including other genetic markers in the cross. As discussed in general by Lahr, et al., we imagine that the sexuality and genetics of *N. minor* may be radically different from any previously observed system. It could offer new clarity to ancient genetic systems and about the origin of cellular sexuality (Lahr et al., 2011). We are especially interested in the irregular cell divisions we have observed. At this point, I have obtained a methanol-resistant mutant of Ping, but it proved insufficiently discriminating for clonal selection. Dr. Fulton is currently pursuing other potential poisons and resistant mutants.

Because of its relation to *N. gruberi*, we hope that the conditions which lead to successful mating in *N. minor* will give us clues to more speedily discover meiosis and mating in *N. gruberi* in the laboratory. As discussed by Fritz-Laylin, et al., this should be achievable, as strongly indicated by clues in *N. gruberi*’s genome (Fritz-Laylin, et al., 2010).


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Acknowledgements

To Chan and Elaine: You welcomed me, the girl who thought I could infect amoeba with my cold virus, with open arms and taught me so much more than I bargained for. And I couldn’t be more grateful. You taught me by both lecture and example how to be a happy, conscientious student and teacher. Moreover, you both taught me to enjoy my life through my work. Thank you.

To my Mama and Baba: Thank you for being the best parents in the world and sharing my enthusiasm for every accomplishment, no matter how big or small. You’ve been there for every down and up, keeping me positive through it all. Thank you for sending me to Brandeis, far away in Waltham so that I could have the most incredible four years of my life.

To Shauna, Gabriel, and Asher, and Mickey: You four inspire me constantly with your persistence, passion, talent, and accomplishments. I love you so much and miss you every day.

To my Turner Street clan/gang: Shani, Bronia, Sophia, Kosowsky, Kahnowitz, and Lehmann, my in-house support system: Thanks for listening to me, encouraging me, and visiting me in lab. You guys are the best.

Kosow: Thesis parties rock. Twinkle toes, tradition soup, nachos, doyouwannabuildasnowman, and funny shaped amoeba… I think that about covers it.

To Tova: I am so glad you joined me in Bassine Penthouse! Your smile is always welcome. Take good care of Chan and Elaine for me! (Don’t let them get away with shenanigans.)

To KC Hayes: Thank you for your guidance, lessons, beautiful photographs, and for keeping me company in lab. (And for not outwardly laughing at my singing.)

To Dr. Melissa Kosinski-Collins: I am so grateful to you for making me a part of your team (and of the original EL class!) and honestly caring about my development and well-being all through these years. You are an amazing teacher, role model, and a great friend.

To Brandeis University: Thank you for giving me the opportunity to work so closely with incredible professors and really feel like I was a contributing part of a thriving, intellectually curious community.