The Temporal Mapping of *Tetrahymena Thermophila's* Cell Life Cycle Via Nutritional Synchronization

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ABSTRACT

Tetrahymena thermophila serves as a model organism that is used to study various fields about biology, however, there is limited research on the specific timing of the cell cycle's stages. Therefore, the objective of this study was to provide a detailed temporal map of *T. thermophila*'s cell life cycle. Using a nutritional approach described by Cameron & Jeter (1970), a culture of *T. thermophila* was synchronized to the G1 stage of the cell life cycle via starvation and was then sampled every 46 minutes over 144 minutes. For each sample, a hemocytometer and nanodrop were used to assess cell and DNA concentrations respectively. Additionally, an unsynchronized control culture was used to assess the impact of the starvation technique on *T. thermophila.* Although this study did not obtain data significant enough to meet our primary objective of mapping *T. thermophila*'s cell life cycle, it provides ample information regarding the use of *T. thermophila* as a model organism and potential mistakes that may be avoided in future research when utilizing similar nutritional techniques. The results of this experiment suggest that, prior to incubation, cell densities of *T. thermophila* should be greater than a magnitude of 10^3 cells/mL ($\varrho_{pre\_inc} > 1134$ *cells/mL,)*. Likewise, cell densities should be greater than a magnitude of 10^2 cells/mL ($\varrho_{refeeding} > 267$ *cells/mL)* during refeeding if significant data is to be obtained regarding DNA concentrations. Moreover, this study implicates precise preparation and normalization of *T. thermophila* cultures in the procurement of adequate cell densities and the success of nutritional approaches involving incubation and starvation in mapping *T. thermophila's* cell cycle.

INTRODUCTION

Tetrahymena is a unicellular eukaryotic organism that has been used as a long-serving model organism to study various fields of cell biology and genetics due to its unique features and accessibility (Ruehle et al., 2016). While extensive research exists on mapping the cell life cycle of *Tetrahymena*, there is limited understanding of the specific timing of the cell cycle's stages (Ruehle et al., 2016). A temporal mapping of *Tetrahymena's* life cycle can provide specifics for the analysis and advancement of applications in cell biology and genetics. *Tetrahymena* stands

out not only for its simplicity but also for its dual-nucleus structure and versatile modes of reproduction, encompassing both asexual and sexual pathways (McDonald, 1962; Ruehle et al., 2016). The two distinct nuclei offer a comparative perspective for researchers to see how these nuclei coordinate and function throughout different life cycle phases (McDonald, 1962). Moreover, the versatile methods of reproduction, asexual binary fission and sexual conjugation provide genetically identical and varied offspring, allowing for an understanding of how genetic recombination can impact the cell cycle (Ruehle et al., 2016).

As previously mentioned, *Tetrahymena* are of particular interest as they possess two nuclei: the macronucleus (MAC) and the micronucleus (MIC). These nuclei both participate in asexual and sexual reproduction, termed the vegetative and sexual cycles respectively (Figure 1). During the vegetative cycle, the macronucleus undergoes amitosis, where it pinches in half without undergoing the typical phases of mitosis. The micronuclei follow a similar cell life cycle to most single-celled organisms and participate in mitosis and meiosis phases during conjugation. *Tetrahymena* goes through the gap 1 (G1), the synthesis (S) and gap 2 (G2) phases during their cell cycle. During G1, the cell grows in size and performs normal functions. During S, DNA replication in both the macro and micronuclei occurs. In the G2 phase, the micronucleus divides mitotically first and then the macronucleus divides amitotically. During the sexual stage of the life cycle, the micronucleus participates in conjugation, where two cells of opposite mating type are paired and establish cytoplasmic continuity; this connection between the two cells initiates the complex of nuclear events involved in the sexual processes (Wolfe, 1973). The time that *Tetrahymena* cells spend in each of the G1, S, G2, and M phases is poorly understood and presents a key knowledge gap in current literature.

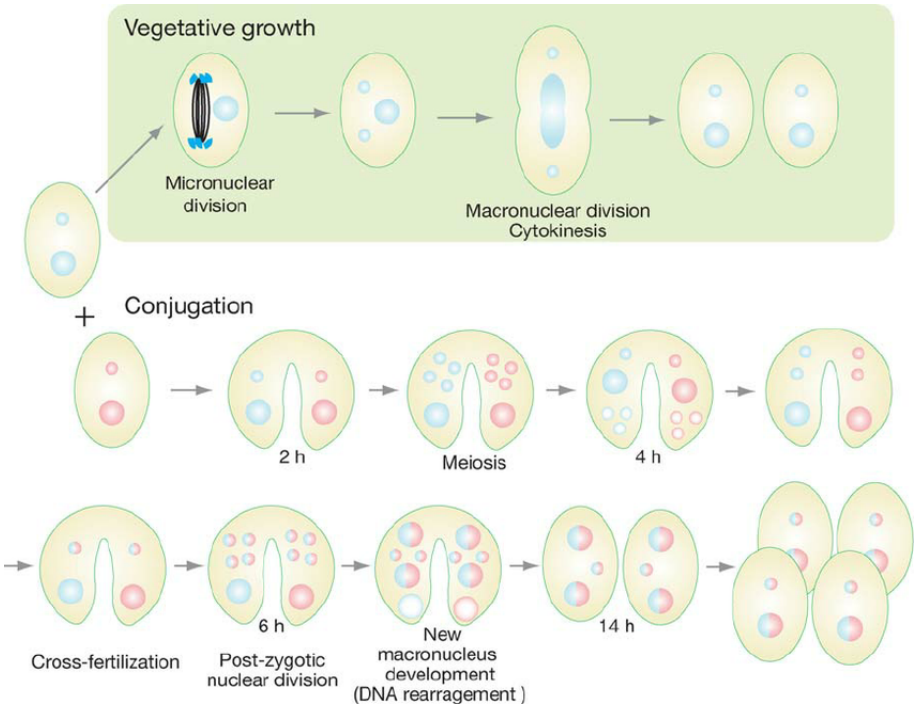

Figure 1: Asexual and Sexual reproduction of Tetrahymena (Yao & Chao, 2005).

An important factor to consider when studying cellular life cycles is synchronization (Ashihara & Baserga, 1979). Cell synchronization is essential for studying cell life cycles as it enables researchers to precisely control the progression of cells through specific phases, providing a uniform and controlled environment for analysis (Ashihara & Baserga, 1979). This synchronized approach allows for a detailed investigation of temporal events, isolation of distinct cell cycle stages, and exploration of regulatory mechanisms critical for understanding fundamental cellular processes and developing targeted therapies (Ashihara & Baserga, 1979). It is therefore essential for the successful synchronization of cells to accurately map out the temporal life cycle of *Tetrahymena* because it establishes a reliable starting point and all cells will progress at the same time, which contributes to an accurate and comprehensive analysis.

Many methods have been tested to synchronize cells but few have been successful at temporally mapping the cell life cycle of *Tetrahymena*. Past methods used heat-shock synchronization on *Tetrahymena thermophila* by exposing them to extreme heat impacting their cellular processes and inhibiting them from transitioning from the G1 to the S phase (Jeffery et al., 1970). However, a study done by Jeffery et al. (1973), indicated a supernumerary of S periods after heat shock was applied, which meant that DNA synthesis was still occurring beyond the normal and expected cycles, rendering the synchronization method ineffective. As such, an accurate mapping of *Tetrahymena's* cell cycle remains absent from the current literature.

Another study by Cameron & Jeter (1970) outlines a method that effectively synchronized the mass of cultures of *Tetrahymena pyriformis*. This nutritional strategy first exposed the mass cultures to a non-nutrient phosphate buffer so that these cells were starved. After 24 hours, the cells are refed with an enriched nutritional growth medium, which causes the mass culture to re-initiate their cell cycle. Cameron & Jeter provide radioautographic evidence that the starvation technique successfully stalled *T. pyriformis* in the G1 nuclear DNA stage of the cell cycle. What's more, Cameron & Jeter also reported that 240 minutes post-refeeding the first cells began to divide and that DNA concentrations increased shortly before this cell division began. Their findings did not, however, provide a more detailed temporal map of the cell life cycle and it remains unknown how long Tetrahymena remains in each phase of the cell cycle.

As such, the primary objective of this research is to provide a detailed temporal map of *T. thermophila's* cell life cycle. To this aim, we attempted to track the variations in DNA concentrations over time, utilizing a nutritional approach for cell synchronization. Given the background information provided on *T. thermophila's* cell cycle, it was believed that for the purpose of this study, the amount of time spent in each phase of the cell cycle could be marked

by an increase in DNA concentrations or a lack thereof. Additionally, the findings of this study are used to assess the efficacy of nutritional approaches of synchronization concerning the temporal mapping of the cell life cycle.

METHODOLOGY

Research Design

The cell life cycle of *T. thermophila* was temporally mapped using a technique described by Cameron & Jeter (1970). A 1 L culture containing *T. thermophila* was split into two groups: Both groups were incubated for 24 hours, one in a starvation media (S culture) and the other in regular growth media (R culture). In the S culture, the cell cycle of *T. thermophila* was to be synchronized to the G1 phase during incubation. In the R culture, the cell cycle of *T. thermophila* was not synchronized and all data collected served as a control for monitoring the potential effects of starving the S culture. After incubation, both cultures underwent refeeding, initializing synchronized cell replication in the S culture. Then the cell cycle was tracked, over 144 minutes, by sampling from each culture every 46 minutes to determine the cell count and DNA concentration (Scheme 1).

Preparing for Incubation

S Culture Preparation. To remove *T. thermophila* from the regular media, ~500 mL of culture media (50 mL x 8) was centrifuged at max speed for five minutes using a Sorvall Lynx 6000 centrifuge. As centrifuging did not produce a cell pellet, it was assumed that *T. thermophila* resided in the bottom portion of the media and the top portion (i.e. supernatant) was quickly removed to avoid re-dispersion and consequential cell loss. The bottom portion of each of the four centrifuge tubes was then recombined into two tubes where more culture was added and

re-centrifuged for another five minutes and supernatant removal was repeated. This process was repeated two times and the final portion of the culture media obtained was subsequently washed with 20 mL of starvation media to remove any residual regular-media proteins. After washing, *T. thermophila* was resuspended in 30 mL of starvation media (S culture).

R Culture Preparation. For intergroup consistency, the above procedure was replicated for the R culture using regular media instead of starvation media. The volumes of culture media and regular media used for centrifuging, washing, and resuspending the R culture are equivalent to the volumes of culture media and starvation media used for the S culture.

Cell Density Normalizing. To ensure that neither the S nor R cultures crashed during incubation, the cell densities were normalized between the two groups. Eight 100 μL samples of both S and R cultures were combined with 10 μL of fixative each and a cell count was performed using a hemocytometer. Moreover, based on the cell density data obtained the cultures were normalized to 1134 cells/mL (Table 7, Appendix). The S and R cultures were then split into triplicates with a minimum of 10 mL/tube and incubated at 35º C centigrade for 24 hours (Figure 1C).

Sampling

Refeeding & Cell Density Normalizing. After incubation, the S and R cultures were both removed from their respective media following a protocol similar to that described for S and R culture preparation. Both cultures were centrifuged two times at max speed for five minutes and when no pellet formed, it was assumed *T. thermophila* resided at the bottom and the supernatant was removed quickly. Washing was not required for either group as the succeeding resuspension in regular media during refeeding would introduce new proteins regardless. Before refeeding, however, the cell density of each S and R culture triplicate was determined via a hemocytometer.

Three 100 μL samples were taken from each triplicate of the S and R culture and mixed with 10μL fixative. After the cell densities of each triplicate were determined, they were normalized to 267 cells/mL during refeeding via resuspension in regular media (Table 8, Appendix).

DNA Quantification. Over 144 minutes, four 1mL samples were taken from each triplicate of the S and R cultures and the DNA was quantified. The samples were centrifuged (no pellet formed so previous assumptions carried), the supernatant was removed quickly, and 300 μL of Cell Lysis Solution with Proteinase K was added to each sample. They were then incubated at 65^{o} C in a hot bath for 15 minutes and vortexed every five minutes. Once the samples turned cloudy, they were stored on ice for an additional five minutes and 150μL of Protein Precipitate Reagent was added and vortexed for 10 seconds. The samples were then centrifuged again and when no pellet formed the DNA containing supernatant was collected by pipetting the top 950 μL of the sample into a new tube. Next, 500 μL of ice-cold isopropanol was added to every supernatant collected and after 30-40 inversions the sample was centrifuged again at max speed for ten minutes. Finally, the supernatant was removed carefully to avoid disturbing any pellets that formed during centrifugation and 20 μL of ethanol was added and removed from each pellet to aid in the drying process. Note that in many of the samples, no visible pellet formed so it was assumed that the DNA had adhered to the side of the falcon tube and was present. In the absence of a pellet, all liquid in the tube was discarded and ethanol was added and removed as previously described. All samples were left to dry overnight and were then resuspended in 20 μL of TE buffer for 30 minutes on ice. The concentration of DNA was then determined using a nanodrop.

Cell Count. For every sample taken during DNA quantification, an additional 100 μL sample of culture was taken from each S and R triplicate and was mixed with 10 μL of fixative to perform a

cell count with a hemocytometer and a compound microscope. During data analysis, the cell count of each sample was used to quantify the average amount of DNA per cell.

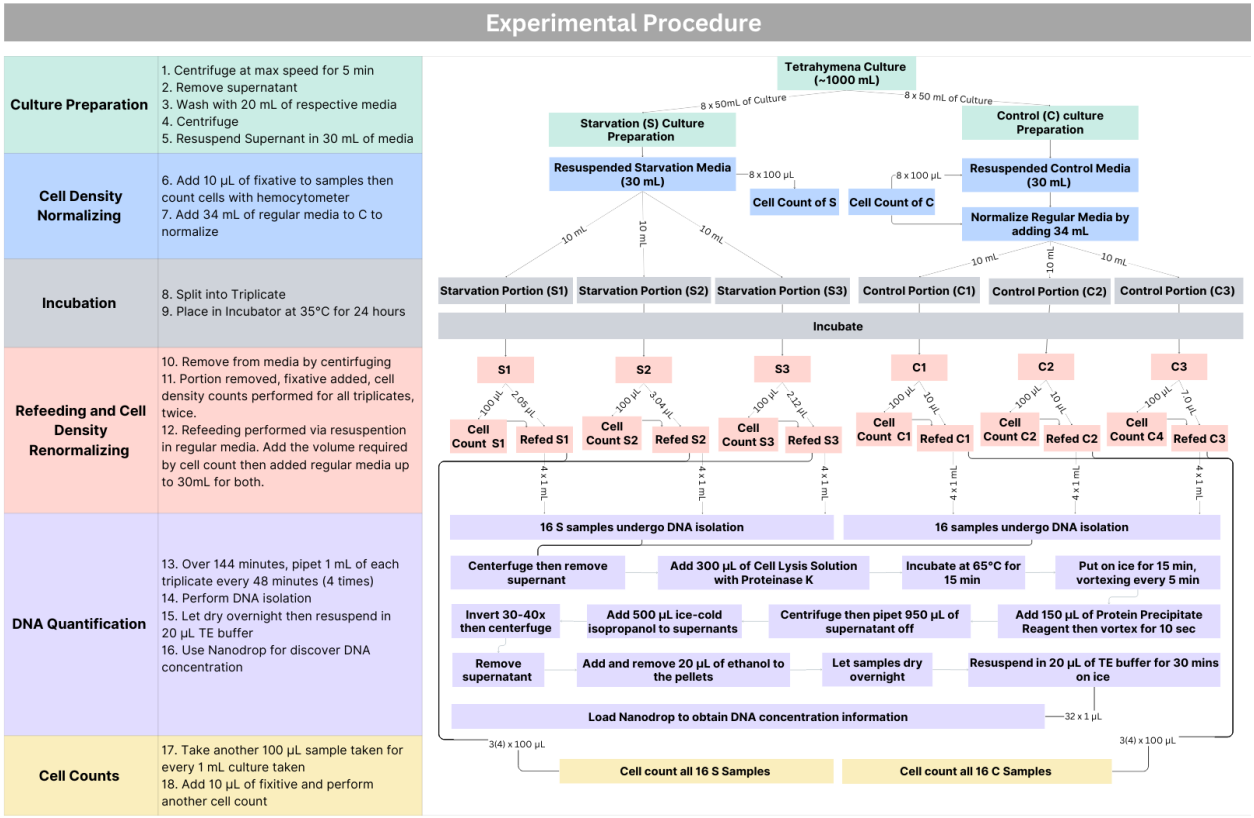

Figure 1. Procedural Flow Chart for *T. Thermophila's* Cell Life Cycle Tracking. This chart illustrates the step-by-step process for sample preparation, normalization, refeeding, DNA quantifications, and cell counts.

RESULTS

No apparent increase in DNA occurred during refeeding (Tables 1 and 2). Only the samples from S1 taken at times 0 and 96 minutes provided a DNA concentration high enough to accurately determine the purity of the sample (*113.3, 75.1 ng/µL > 50 ng/µL respectively*). However, given the nanodrop absorption measurements at 260 nm and 280 nm, the ratios of DNA to protein for the S1 samples at 0 minutes and 96 minutes suggest that the sample was contaminated, likely by RNA *(1.99, 1.99 > 1.8 respectively*). No samples from S2, S3 or R1-3 provided a high enough DNA concentration to determine the purity of the sample.

Table 1. DNA Concentration of Each S-Culture Triplicate Sampled (ng/μL)

	S1	S2	S3
Sample Time			
0 minutes	113.3*	42.5	4.3
48 minutes	47.1	38.8	2.4
96 minutes	75.1*	2.2	3.0
144 minutes	1.8	1.7	0.9

† The data presented was determined using a nanodrop (X ng/μL)
**DNA concentration is >50 ng/μL ∴ purity may be determined*

Table 2. DNA Concentration of Each R-Culture Triplicate Sampled (ng/μL)

	R1	***R2***	***R3***
Sample Time			
0 minutes	*7.2*	*7.7*	*35.2*
48 minutes	*6.1*	*1.0*	*2.7*
96 minutes	*9.6*	*0.7*	*1.3*
144 minutes	*1.2*	*3.4*	*1.1*

† The data presented was determined using a nanodrop (X ng/μL)

The amount of *T. thermophila* did not increase during refeeding for the S- or R-cultures (Tables 3-4). For each triplicate of the S/R-culture, the cell concentration was determined for each sample using Equation 1 (see sample calculation 1).

Equation 1. Hemocytometer Cell Concentration Formula

Total Cell Concentration = (Mean Number of Cells) / (Number of Squares) × (Hemocytometer Dilution Factor) × (Sample Dilution Factor) × (Fixative Correction)

Sample Calculation 1. Cell Concentration of S1 at 0 Minutes

$$(1.\overline{6} \,/\, 1) \times (3.125E2) \times (1) \times (1.1) = 572.91\overline{6}\ cells/mL$$

Table 3. Cell Concentration of Each S-Culture Triplicate Sampled (rounded to the nearest whole cell)

	S1	***S2***	***S3***
Sample Time			
0 minutes	*573*	*115*	*115*
48 minutes	*229*	*229*	*573*
96 minutes	*0*	*344*	*344*
144 minutes	*458*	*115*	*0*

† The data presented was determined using a hemocytometer (X cells/mL)

Table 4. Cell Concentration of Each R-Culture Triplicate Sampled (rounded to the nearest whole cell)

	R1	***R2***	***R3***
Sample Time			
0 minutes	*458*	*115*	*0*
48 minutes	*458*	*688*	*229*
96 minutes	*458*	*115*	*802*
144 minutes	*0*	*0*	*688*

† The data presented was determined using a hemocytometer (X cells/mL)

The average amount of DNA per *T. thermophila* did not appear to increase during the refeeding process (Tables 5-6). For each triplicate of the S/R-culture, the average amount of DNA per cell was determined by dividing each DNA concentration (Tables 1-2) by the respective cell concentrations (Tables 3-4) and normalizing the units of volume (see sample calculation 2). The overall amount of DNA per cell was then averaged between all S/R-triplicates for each sampling time (see sample calculation 3). To accommodate for the error of dividing any integer by zero, if the cell concentration of any sample was zero, the average DNA concentration was assumed to be zero as well. Given that the purity of the DNA concentration analyzed cannot be satisfactorily determined this assumption is safe to make for the purpose of this report as it cannot be known whether the reading obtained was of DNA or impurities.

Equation 2. DNA per Cell Formula

DNA per Cell (ng/cell) = (DNA Concentration (ng/μL)) × (Cell Concentration (cells/mL)) × (1000 μL) / (1 mL)

Sample Calculation 2. DNA per Cell of S1 at 0 minutes

$(113.3\ ng/\mu L)\ /\ (572.9167\ cells/mL)\ \times\ (1000\ \mu L)\ /\ (1\ mL)\ =\ 197.75999\ ng/cell$

Sample Calculation 3. Overall Average DNA per Cell at 0 minutes

$$(197.7 \times 370.9 \times 37.52) / 3 \times (1000\,\mu L) / (1\,mL) = 202.1\,ng/cell$$

Table 5. Average DNA per Cell of Each S-Culture Triplicate Sampled

	S1	***S2***	***S3***	***Overall Average***
Sample Time				
0 minutes	197.7^{5999}	370.9^{0920}	37.52^{7284}	202.0^{6549}
48 minutes	205.5^{2697}	169.3^{0884}	4.189^{0907}	126.3^{4163}
96 minutes	0^{*}	6.400^{0000}	8.727^{2727}	5.042^{4242}
144 minutes	3.927^{3013}	14.83^{6368}	0^{*}	6.254^{5564}

† The data presented in this table was calculated using equation 2 (ng/cell), see sample calculation
**No cells were present to average the amount of DNA over*

Table 6. Average DNA per Cell of Each R-Culture Triplicate Sampled

	R1	***R2***	***R3***	***Overall Average***
Sample Time				
0 minutes	15.70^{9219}	67.20^{0020}	0^{*}	27.63^{6408}
48 minutes	13.30^{9188}	1.454^{5455}	11.78^{1801}	8.848^{5114}
96 minutes	20.94^{5607}	6.109^{0927}	1.620^{7793}	9.558^{4930}
144 minutes	0^{*}	0^{*}	1.600^{0000}	0.5333^{3333}

† The data presented in this table was calculated using equation 2 (ng/cell), see sample calculation
**No cells present in the sample to average DNA over*

A timeline for *T. thermophila's* cell life cycle cannot successfully be mapped with the data procured in this experiment as the DNA readings were not high enough to determine purity. However, a temporal map of the data obtained shows that the average amount of DNA per cell in the S- and R-cultures decreased over the re-feeding period (Figure 2). This temporal map cannot be used as a representation of *T. thermophila's* life cycle timeline, but it may demonstrate for future studies what data trends can be expected if *T. thermophila* cultures are not prepared and normalized accurately.

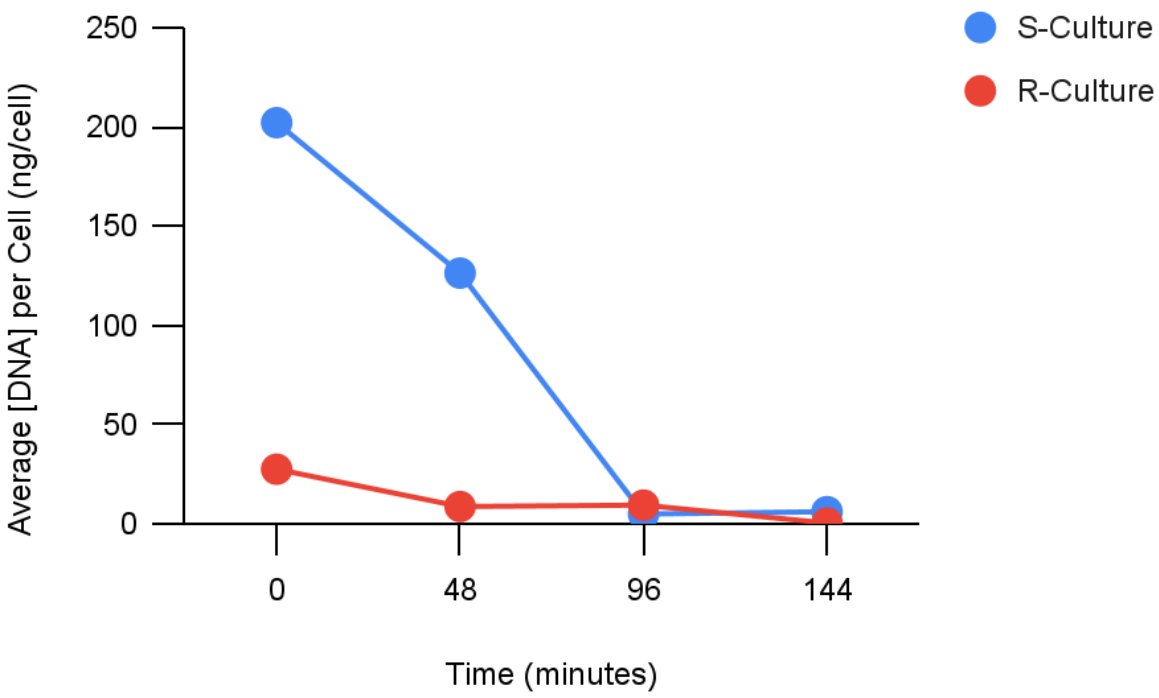

Figure 2. Insignificant Data Trends that May Arise During Refeeding as a Result of Improper *T. Thermophila* Culture Preparation & Normalization: Average DNA concentration over all R/S-culture triplicates.

DISCUSSION

The results of this experiment focused on the DNA concentration and cell density of *T. thermophila* during the refeeding of a synchronized starved culture and an unsynchronized control culture. The objective of this study was to track the changes in *T. thermophila's* DNA concentration and cell density over 144 minutes and use that data to create a temporal map of the cell life cycle. To this end, the experiment was unsuccessful, yet, the data collected provides valuable insights for future experiments utilizing similar nutritional approaches.

Ruehle et al. (2016) address *T. thermophila's* biology, and evolution while explaining their cell life cycle while modelling their two nuclei. Hoping to expand on the extensive research of *T. thermophila's* cell cycle progression completed by Ruehle et al. (2016), this study aimed to

discover the specific timing of when each cell stage hit. However, as the concentrations of DNA obtained are not significant, no conclusions can be reached about when the cells approached G1, S, and G2. To this end, there were no upturns in DNA amounts correlated to the macro- and micronucleus, first studied by McDonald (1962). Therefore the results of this study are inconclusive in explaining the timing of T. thermophila's asexual and sexual reproduction.

Although the data obtained during sampling provides no useful information, this absence of significant results, in conjunction with the data obtained during normalization provides useful insight into the potential limitations of nutritional synchronization techniques. During cell density normalization, prior to refeeding, the cultures were diluted to a common cell density of 267 cells/mL (see Appendix, Table 8) to have a universal environment. This reduced the concentration of *T. thermophila* immensely and may have contributed to our inability to obtain significant DNA data. Normalizing to such a low magnitude may have simply diluted the culture beyond the point of detection, and therefore the DNA concentrations obtained were insignificant. These notions would be supported by the data from the S3 triplicate in Table 3 and R1/R2 triplicates in Table 4. All of these cultures ended with a cell concentration of 0, suggesting the samples we took simply did not have enough cells for significant DNA extraction. Alternatively, it could be that the cells had ruptured and were no longer recognizable when doing the cell count.

Laboratory restraints resulted in the planned DNA quantification period being shortened from 240 minutes to 144 minutes. Although *T. thermophila* has been shown to have large variations in their duplication times, Cameron and Jeter have shown through their nutrient-based synchronization of *T. thermophila* that the cells act on a four-hour duplication period (1970). Therefore, our culture may not have reached the cycle stages where we would see large DNA concentration changes, as this would likely happen in the later S phase. However as there were

so few cells in all the samples we monitored, this likely had little impact on our results but should be considered in future research.

Another potential source of error in our experiment may include the preparation of our S and R cultures. *T. thermophilas* are fast swimmers and after centrifuging, likely moved quickly and diffused into the supernatant liquid being poured out (Cassidy-Hanley, 2012). This could be the reason we obtained such low cell densities during our sampling and consequently were unable to obtain significant DNA concentration data. Alternatively, during DNA extraction the DNA-containing pellet was never visible and it is possible that it was removed with the supernatant. This could also explain the insufficient concentrations of DNA obtained which resulted in an indeterminable degree of purity.

This experiment lays a foundational framework for further research aimed at elucidating the temporal dynamics of *T. thermophila's* cell life cycle. By continuing this line of investigation with a more extensive sampling strategy using cell density of sufficient magnitude to yield results, smaller time intervals over a longer sampling time, and microscopy techniques to observe the cells as they move through the life cycle, researchers could precisely track the transitions between different stages of the *T. thermophila* life cycle. Insights into the cell cycle of *T. thermophila* could potentially contribute to drug development and disease research, particularly in areas where understanding cell cycle regulation is critical, such as cancer therapeutics.

CONCLUSION

T. Thermophila's cell life cycle was not successfully temporally mapped and the effect of starvation treatment on the DNA concentration of *T. thermophila* was inconclusive. The amount

of DNA per *T. thermophila* was not found to increase over refeeding time for starved and regularly treated cultures, likely due to the low cell densities yielding undetectable DNA concentrations, of which the purity cannot be satisfactorily determined. Rather, when mapped, the data provides a template of what one can expect if a nutritional technique is not carried out with a high enough degree of precision. Our results also show the importance of having adequate cell densities when performing nutritional approaches like the one utilized in the present study. These findings highlight important limitations of nutritional approaches in cell synchronization that should be considered when interpreting the results of the current literature and when designing future studies. Doing so has the potential to aid in drug development and disease research.

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Appendix

APPENDIX DATA

Table 7. Pre-Incubation Cell Density Normalization (cells/mL)

	Initial Cell Concentration (C_1)	Initial S/R-Culture Volume (V_1)	S/R-Media Volume Added (V_{added})	Normalized Culture Volume (V_2)	Normalized Cell Concentration (C_2)
Starvation Media (S)	1134.375	30 mL	N/A	30 mL	1134.375
Regular Media (R)	2277.3425	34 mL	34.26	68.26 mL	1134.375

† The data presented was determined using a hemocytometer and the formula $C_1V_1 = C_2V_2$ where C is cells/mL and V is mL

Table 8. Post-Incubation Cell Density Normalization (cells/mL)

	Initial Cell Concentration (C1)	Initial S/R-Culture Volume (V_1)	Culture Volume Added (V_{C_added})	R-Media Volume Added ($V_{M\ added}$)	Normalized Culture Volume (V_2)	Normalized Cell Concentration (C_2)
S/R-Culture Triplicate						
S1	3895.53	10.00	2.05	27.95	30.00	267.36
S2	2635.33	10.00	3.04	26.96	30.00	267.36
S3	3781.25	10.00	2.12	27.88	30.00	267.36
R1	802.08	10.00	10.00	20.00	30.00	267.36
R2	802.08	10.00	10.00	20.00	30.00	267.36
R3	1145.75	10.00	7.00	23.00	30.00	267.36

† The data presented was determined using a hemocytometer and the formula $C_1V_1 = C_2V_2$ where C is cells/mL and V is mL