

# Laboratory 10

## Factors Controlling Phagocytosis in *Tetrahymena*,

### I. Introduction

A characteristic feature of all eukaryotic cells is the ability to pinch off portions of a cellular membrane to create membrane vesicles or to fuse membrane vesicles with an existing cellular membrane. This can occur without destroying the integrity of the phospholipid bilayers. Membrane fusion processes are a key step in endocytosis, in which molecules are brought into a cell by creating an endocytic vesicle derived from the plasma membrane. They also occur in exocytosis, in which molecules are released to the extracellular environment by fusing a membrane vesicle with the plasma membrane, and in intracellular vesicle trafficking, in which molecules are moved between membranous compartments such as the endoplasmic reticulum and the Golgi complex in membrane vesicles. The purpose of this week's project is to explore these membrane fusion processes using the ciliated protist *Tetrahymena* as a model system. This microscopic organism is a chemoheterotroph that actively acquires nutrients by a type of endocytosis called phagocytosis. *Tetrahymena* take up bacteria and plant debris in membrane vesicles (food vacuoles or phagosomes) and eventually degrade them by fusing the vacuoles with lysosomes.

As part of this week's laboratory session, you will:

- Measure the amount of phagocytosis quantitatively using India Ink as an artificial substrate
- Determine the best of three possible India Ink concentrations for determination of phagocytic activity
- Determine whether a given food source influences phagocytic activity
- Use some simple statistical tests to determine if the Ink India concentrations tested give significantly different results.

### II. Pre-Lab Preparation

Read the Introduction, Background Information, and Experimental Procedures for this experiment. Also, please read the sections in your Cell Biology textbook which deal with endocytosis, phagocytosis, and cilia. You might also find it helpful to look at your General Biology textbook for more information about protists. Review the material in **Laboratory 2 (Introduction to Microscopy)** on light microscopy. After preparing for the lab, you should be able to answer the following questions.

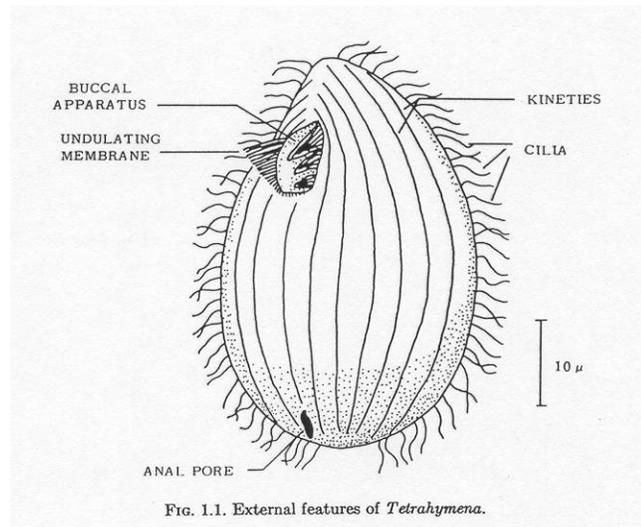
- A. How does *Tetrahymena* feed?
- B. Why is India Ink used in these experiments as an "artificial food source?"
- C. Why do you think feeding on India Ink results in many small ink filled vacuoles rather than one large ink filled vacuole?
- D. What is the purpose of fixing the *Tetrahymena* before examining them microscopically?
- E. Which fixative will be used in these experiments?
- F. What is the definition of a standard deviation?

- G. What is meant by the standard deviation of the difference of the means?
- H. What is the purpose of performing a Student's *t* test?
- I. What are the main components of the cytoskeleton?

### III. Background Information

#### A. Biology of *Tetrahymena*

*Tetrahymena* is a unicellular eukaryotic microorganism that has been used for studies of genetics and cell biology since the 1950s. Although it is naturally found in aquatic habitats like lakes and streams, it can be easily grown in the laboratory. The commonly-used laboratory strains were originally designated *Tetrahymena pyriformis*, but the organism is now called *Tetrahymena thermophila*. *T. thermophila* is a relatively-large actively-motile organism (Figure 10.1). The typical cell is about 50  $\mu\text{m}$  long and about 20  $\mu\text{m}$  wide, with a somewhat pointed anterior end and a more rounded posterior end. There are multiple bands of cilia on the organism's surface, which are involved both in movement and the acquisition of food. *Tetrahymena* obtains nutrients by a process of phagocytosis: the cilia on the surface sweep bacteria and other small particles into the buccal apparatus, where they are then taken into the cells in membrane vacuoles.



**Figure 10.1. External features of *Tetrahymena*.**

#### B. Phagocytosis

Phagocytosis is the engulfment of microscopic particles including microorganisms by eukaryotic cells. *Tetrahymena*, which is 30,000 times the size of most bacteria, can obtain all of its nutrients in this way. It has been reported to take up as many as 20,000 bacteria per hour. During the process of phagocytosis, a microscopic particle is encapsulated by a portion of the plasma membrane. The resulting food vacuole or phagosome then can fuse with a lysosome in the cytoplasm. The degradative enzymes in the lysosome break down the food materials into small molecules, which are then released into the cytoplasm as a source of nutrients. These nutrients are

degraded by specific metabolic pathways, leading to the formation of ATP in the mitochondria by aerobic respiration. Any residual material that cannot be digested is then ejected from the cells. (Figure 10.2)

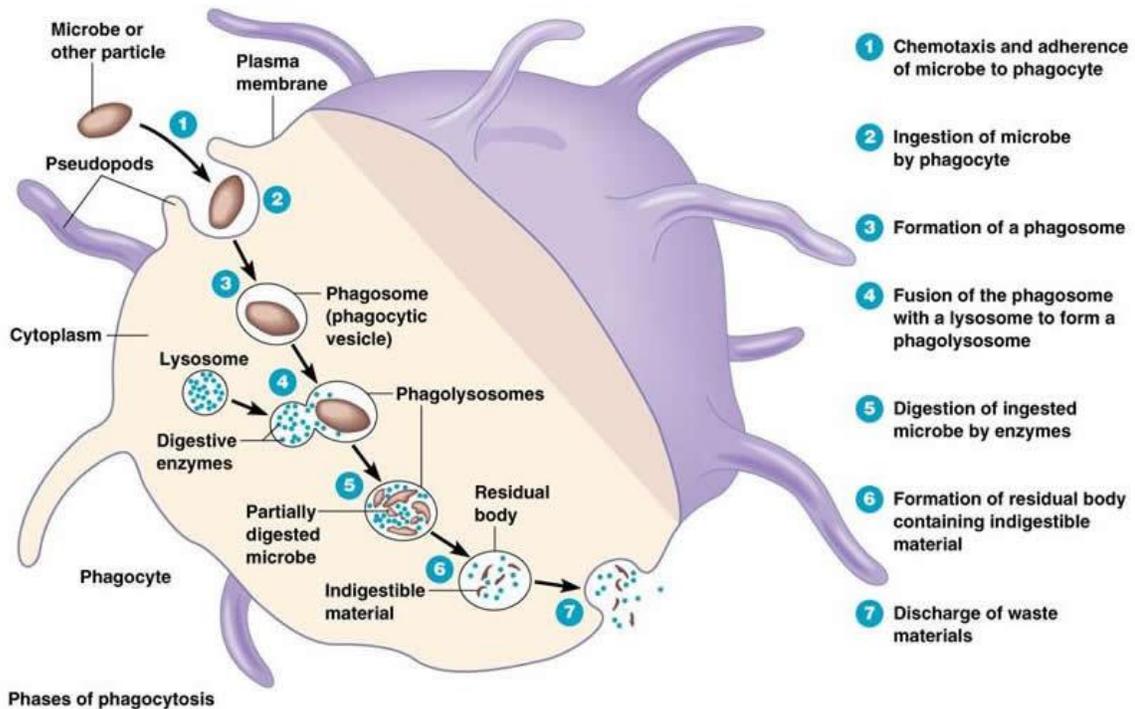


Figure 10.2. Location and descriptions of the phases of phagocytosis. (classes.midlandstech.edu)

The process of phagocytosis in *Tetrahymena* can be followed microscopically by using India Ink as non-nutrient food source. India Ink is a suspension of fine carbon particles in water. When it is taken up by *Tetrahymena*, the phagosomes are easily visible because they are black. (Figure 10.3)

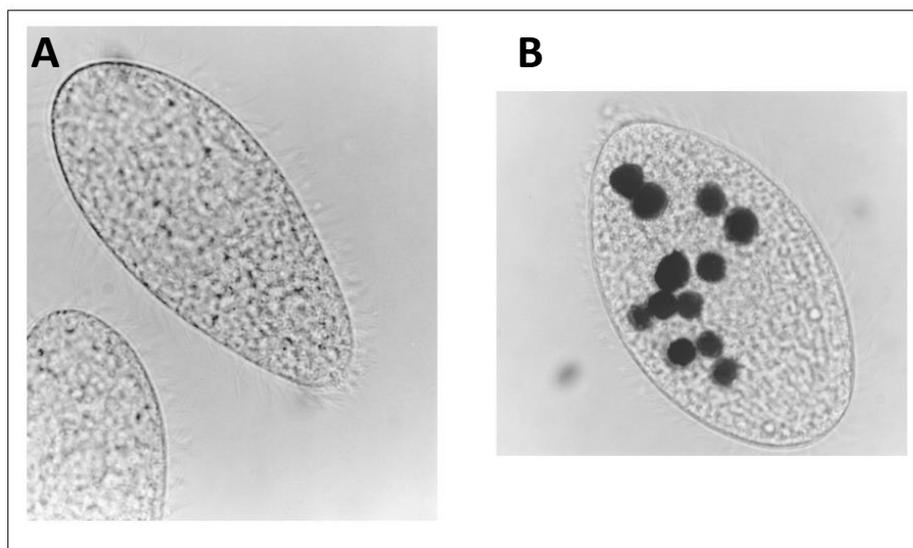


Figure 10.3 Photographs taken through a microscope of *Tetrahymena* (A) without vacuoles containing India ink (gray color is an artifact) (B) and with food vacuoles containing India ink.

## C. Basic Statistics

The process of phagocytosis in *Tetrahymena*, like most biological processes, is variable. Some cells may take up the India Ink more efficiently than others, and so even cells within the same sample may contain varying numbers of ink-filled vacuoles. How can we deal with this variation quantitatively? How can we tell if two samples, which might be taken at different times or after exposure to different treatments, differ significantly from one another? The answer to these questions is to use statistical tools. These tools can help us determine the amount of variation in a set of data and to assess the likelihood that differences between two or more sets of data are meaningful.

Suppose you examine 50 *Tetrahymena* cells by light microscopy and count the number of India Ink filled vacuoles in each cell. As shown in **Laboratory 1 (Scientific Calculations and Basic Lab Techniques)**, you can then calculate the mean number of vacuoles per cell, the variance, and the standard deviation. This will give you both an average value for the 50 replicates and a sense of the amount of variation among them. The numerical values for a mean and standard deviation can be calculated using either a scientific calculator or Excel.

Table 10.1 shows another example of this type of calculation, using a simple measurement of the stem length in mm of 10 plant samples.

**Table 10.1. Sample calculation of the standard deviation**

Observation	Individual measurement ( $x_i$ ) of stem length (mm)	Deviation from mean ( $x_i - \bar{X}$ )	$(x_i - \bar{X})^2$
$x_1$	20	-2	4
$x_2$	24	+2	4
$x_3$	22	0	0
$x_4$	19	-3	9
$x_5$	26	+4	16
$x_6$	22	0	0
$x_7$	24	+2	4
$x_8$	20	-2	4
$x_9$	22	0	0
$x_{10}$	21	-1	1
	TOTAL = 220 = $\Sigma x_i$	0	42 = $\Sigma(x_i - \bar{X})^2$

The total of all of the stem lengths for the 10 replicate samples was 220, so the mean or average was  $220/10 = 22$ . The deviation of each observation from this mean then was calculated, and from this, the sum of the squares of the deviations from the mean was determined to be 42. Recall that the standard deviation is the square root of the sum of the square of the deviations divided by  $N - 1$ .

$$s = \sqrt{\frac{\Sigma(x_i - \bar{X})^2}{N - 1}}$$

$$= \sqrt{\frac{42}{9}}$$

$$= \sqrt{4.66}$$

$$= 2.16.$$

So the standard deviation for these measurements is 2.16. In summary, the mean and standard deviation for this data set is therefore  $22 \pm 2.16$  mm.

There are several ways to compare two sets of data for which you have determined the means and standard deviations. One is to calculate the **standard error for the difference of the means**. This is given by the equation:

$$\text{S.E. } \bar{X}_1 - \bar{X}_2 = \sqrt{\frac{s_1}{N_1} + \frac{s_2}{N_2}}$$

where  $X_1$  and  $X_2$  are the two means,  $s_1$  and  $s_2$  are two standard deviations, and  $N_1$  and  $N_2$  are the two sample sizes.

The way this formula is used is first to calculate the standard error for the difference of the mean. If the actual difference between the two means ( $X_1 - X_2$ ) is two times this value, one can conclude that the difference between the two groups is not due to chance but rather due to some difference in how they were treated. In other words, the difference is statistically significant.

Another way to compare two sets of data for which you have determined the means and standard deviations is to do a **Student's t test**. This again will allow you to determine if the two sets of data are significantly different. The formula for this test is:

$$t = \frac{(\bar{X}_1 - \bar{X}_2) \left( \sqrt{\frac{N_1 N_2}{N_1 + N_2}} \right)}{\sqrt{\frac{(N_1 - 1)(s_1^2) + (N_2 - 1)(s_2^2)}{N_1 + N_2 - 2}}}$$

where again  $X_1$  and  $X_2$  are the two means,  $s_1$  and  $s_2$  are two standard deviations, and  $N_1$  and  $N_2$  are the two sample sizes.

The way this formula is used is first to calculate the  $t$  test value. You can then use the following chart to determine the significance of this value. The total number of degrees of freedom ( $n$ ) is  $(N_1 + N_2 - 2)$ . A confidence level of 0.05 is usually used, which means that if the  $t$  test number is less than the number given in the chart for that total number of degrees of freedom, the two samples are not significantly different 95% of the time. If the  $t$  test number is greater than the number given in the chart for that total number of degrees of freedom, the two samples are significantly different 95% of the time. (This assumes that the assumptions that underlie these tests are met)

Table 10.2. Significance limits of Student's t distribution

(n) degrees of freedom	Confidence levels		
	0.10	0.05	0.01
1	6.314	12.706	63.657
2	2.920	4.303	9.925
3	2.353	3.182	5.841
4	2.132	2.776	4.604
5	2.015	2.571	4.032
6	1.943	2.447	3.707
7	1.895	2.365	3.499
8	1.860	2.306	3.355
9	1.833	2.262	3.250
10	1.812	2.228	3.169
11	1.796	2.201	3.106
12	1.782	2.179	3.055
13	1.771	2.160	3.012
14	1.761	2.145	2.977
15	1.753	2.131	2.947
16	1.746	2.120	2.921
17	1.740	2.110	2.898
18	1.734	2.101	2.878
19	1.729	2.093	2.861
20	1.725	2.086	2.845
21	1.721	2.080	2.831
22	1.717	2.074	2.819
23	1.714	2.069	2.807
24	1.711	2.064	2.797
25	1.708	2.060	2.797
26	1.706	2.056	2.779
27	1.703	2.052	2.771
28	1.701	2.048	2.763
29	1.699	2.045	2.756
30	1.697	2.042	2.750
40	1.684	2.021	2.704
60	1.671	2.000	2.660
120	1.658	1.980	2.617
$\infty$	1.645	1.960	2.576

Source: Adapted from Table III of Fisher and Yates, *Statistical Tables for Biological, Agricultural, and Medical Research*, Oliver & Boyd, Edinburgh.

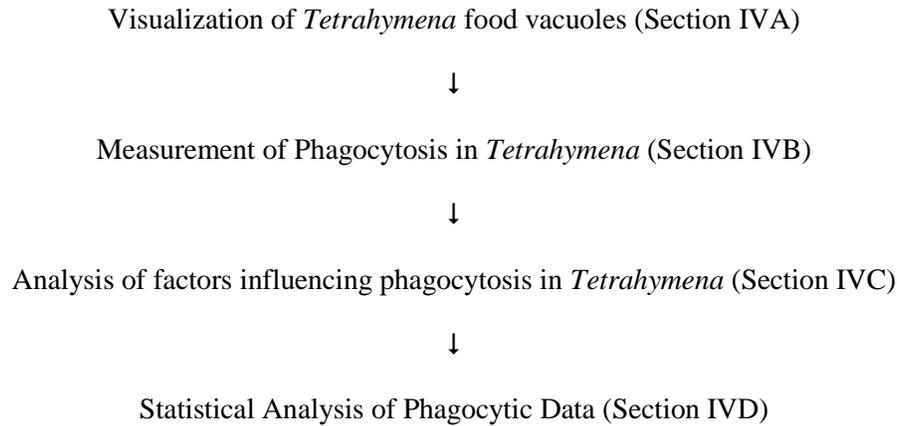
In order to use this information to analyze your data first you would compute the mean, and standard deviation. Assuming that your values are not equal to each other, you then compute the SE or the t-statistic and determine whether they are significantly different from each other based on the information given here. The formula given above for the t-statistic will yield a negative number if the second mean is larger than the first and a positive number if the second mean is smaller than the first. Do not confuse this with a negative number when looking it up in Table 10.2 above. The negative value is still the value you look up in the table, it does not suggest that the number is less than one, it just tells you the direction of the deviation

A word about controls, there may be times when you need to use more than one control, such as the time when you must include an additional chemical to dissolve the one you are testing. When this is needed you would compare the two controls to each other to see if there is a significant difference between them. Then if there were a significant difference between them you would need to use the control containing the chemical to compare to the other treatment groups since you would have shown that it did have an effect on the organism.

## IV. Experimental Procedures

This experiment has several parts that must be done sequentially. To save time, however, each person can use a separate microscope to study the uptake of different concentrations of India Ink by phagocytosis.

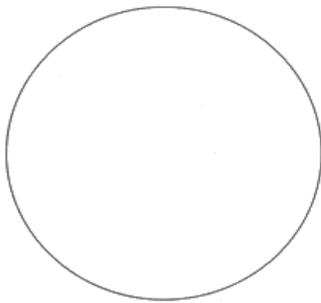
The following is a flow chart for this laboratory session:



### A. Visualization of *Tetrahymena* food vacuoles

The objective of this part of the lab is to learn to recognize ink-filled vacuoles in *Tetrahymena*. You will use the same Leica DME microscopes you used in **Laboratory 2 (Introduction to Microscopy)** so review the material from that experiment.

1. A **demonstration slide** with *Tetrahymena* under the 100X oil immersion objective will be available with ink filled vacuoles. Look at the slide carefully and note the appearance of the cilia and the intracellular organelles. Draw what you see and record any observations you might have.



## B. Measurement of Phagocytosis in *Tetrahymena*

Phagocytosis is the process by which large food particles or microscopic organisms are transported into the cell encapsulated in membrane vesicles. The objective of this part of the experiment is to determine the best of three possible India Ink concentrations for determination of phagocytic activity.

1. Obtain 4 0.65 mL microcentrifuge tubes and label them 0, 10, 20, and 30 minutes for each of the types of ink to be tested. Using a P-100 micropipetter and a small tip, add **20  $\mu$ l** of 0.3% formaldehyde to each tube. Formaldehyde is a fixative, which will both stop phagocytosis and kill the cells. Once the cells have been fixed, you can look at them microscopically at a more leisurely pace.

**Carry out the processes for 1%, 3% and 10% ink at the same time.**

2. With a P-1000 micropipetter and a large tip, add 0.5 ml (500  $\mu$ L) of the *Tetrahymena* suspension to a test tube. Then, using a separate tip, add 0.5 mL (500  $\mu$ l) of a 1% solution of India Ink. Carry out this same procedure for 3%, and 10% India Ink. Mix the solution gently by "thumping" the tube on the side (**do not vortex this solution!**). At 0, 10, 20, and 30 minutes, carefully mix the suspension again and remove a **40  $\mu$ l** portion of the *Tetrahymena*/India Ink mixture and add it to the corresponding microcentrifuge tube. Carefully mix the cell sample with the formaldehyde by drawing the liquid up and down (gently). The "0" sample should be taken as soon as you can after preparing the ciliate/India Ink suspension. Use a new pipet tip for each sample.
3. To examine the cells, place a small drop (15  $\mu$ l) of each of the fixed suspensions on a clean glass slide and cover it with a coverslip. Examine the cells with the 20X or 40X objective lens and count the number of ink-filled vacuoles per cell. Try to do this for at least 20 cells in each time point sample. If you cannot find 20 cells, use as many as you can. Record all of the results in a table using the following general headings. Also spend some time determining the best way to view the cells, for example if you do not see many cells you may want to look at the drop without a coverslip to determine if cells are present. If they are and you are not seeing them you may want to consider increasing the size of your drop or reducing the size of your coverslip in order to view them more clearly. In addition if the cells clearly do not show any vacuoles in any of the samples do not worry about it in the 0 min samples. However, if by 15 minutes you are not seeing any vacuoles you may want to talk to your instructor about why this might be the case and work to improve your processes.

### Uptake of 1% India Ink

Time	Individual counts of vacuoles/cell
0	
10	
20	
30	

**Uptake of 3% India Ink**

Time	Individual counts of vacuoles/cell
0	
10	
20	
30	

**Uptake of 10% India Ink**

Time	Individual counts of vacuoles/cell
0	
10	
20	
30	

**C. Analysis of Factors influencing *Tetrahymena* Phagocytosis**

Last week you prepared a food extract which was tested for its ability to stimulate a chemotactic response. This week, you will use the same food source to examine if it influences phagocytosis.

1. Obtain 4 0.65 mL microcentrifuge tubes and label them 0, 10, 20, and 30 minutes for each of the food extract dilutions (undiluted, 1/10, 1/100, and 1/1000) to be tested. Using a P-100 micropipetter and a small tip, add **20  $\mu$ l** of 0.3% formaldehyde to each tube. Formaldehyde is a fixative, which will both stop phagocytosis and kill the cells. Once the cells have been fixed, you can look at them microscopically at a more leisurely pace.

**Carry out the processes for all food extract dilutions at the same time.**

2. With a P-1000 micropipetter and a large tip, add 0.5 ml (500  $\mu$ L) of the *Tetrahymena* suspension to a test tube. Then, using a separate tip, add 0.5 mL (500  $\mu$ l) of the 10% solution of India Ink.
3. With a P-100 micropipetter and the appropriate tip, add 0.05 ml (50  $\mu$ L) of each food extract dilution to the corresponding tube. Mix the solution gently by "thumping" the tube on the side (**do not vortex this solution!**). At 0, 10, 20, and 30 minutes, carefully mix the suspension again and remove a **40  $\mu$ l** portion of the *Tetrahymena*/India Ink mixture and add it to the corresponding microcentrifuge tube. Carefully mix the cell sample with the formaldehyde by drawing the liquid up and down (gently). The "0" sample should be taken as soon as you can after preparing the ciliate/India Ink/food extract suspension. Use a new pipet tip for each sample.
4. Fix and examine the cells as you did in Part B. Count the number of ink-filled vacuoles per cell. Try to do this for at least 20 cells in each time point sample. If you cannot find 20 cells, use as many as you can. Record all of the results in a table using the following general headings.

**Undiluted Food Extract**

Time	Individual counts of vacuoles/cell
0	
10	
20	
30	

**Food Extract 1/10 Dilution**

Time	Individual counts of vacuoles/cell
0	
10	
20	
30	

**Food Extract 1/100 Dilution**

Time	Individual counts of vacuoles/cell
0	
10	
20	
30	

**Food Extract 1/1000 Dilution**

Time	Individual counts of vacuoles/cell
0	
10	
20	
30	

**D. Analysis of Phagocytic Data**

1. To analyze the data obtained using different concentrations of india ink, calculate the mean number of vacuoles/cell in each sample. Then calculate the standard deviation of the mean as described in the section on Basic Statistics. You can do this manually, but most standard scientific calculators have functions that will allow you to do this automatically. You can also do it with Excel. Summarize your data in the following table. Note: if all values are zero your standard deviation and average will also be zero.

**Table \_\_\_\_\_ Summary of data on India ink at various concentrations n=20**

Time (min)	India Ink Concentrations					
	1%		3%		10%	
	Mean	SD	Mean	SD	Mean	SD
0						
10						
20						
30						

- Now prepare a graph in which you plot the mean number of vacuoles/cell as a function of time for each of the India Ink concentrations. Include error bars of +/- 1 standard deviation above and below each data point. (See lab 1 if you do not recall how to make these graphs) Draw the best curve that you can through each set of points. **What conclusion can you draw from these data? (Include this graph in your lab manual.)**
- To determine if the India Ink concentration results in a significantly different rate of phagocytosis, use the standard deviations and the means, you have calculated from the 30 minute samples to determine the **t-statistic**, the formula is given in the Background Information, to compare the 3% sample to the 1% sample. Repeat the process to compare the 10% sample to the 1% sample. Summarize your results in the following table.

Comparison	t-statistic (or p-value if using Excel)	Are the samples significantly different? (Y/N)
1% vs 3%		
1% vs 10%		

Use a probability value of 0.05 and an n (20+20 or 40 -2) = 38. **Are the samples significantly different?**

Note that if you use Excel to compute a t-statistic you should use a two tailed test and the value it gives you is the p-value rather than the t-statistic.

- To determine if the India Ink concentration results in a significantly different rate of phagocytosis, use the standard deviations and the means, you have calculated from the 30 minute samples to determine the **standard error for the difference of the means (SE( $\bar{X}_1 - \bar{X}_2$ ))**, the formula is given in the Background Information, to compare the 3% sample to the 1% sample. Repeat the process to compare the 10% sample to the 1% sample. Summarize your results in the following table.

Comparison	SE( $\bar{X}_1 - \bar{X}_2$ )	Are the samples significantly different? (Y/N)
1% vs 3%		
1% vs 10%		

- To analyze the data obtained when exposing cells to different concentrations of a food extract, calculate the mean number of vacuoles/cell in each sample. Then calculate the standard deviation of the mean as described in the section on Basic Statistics. You can do this manually, but most standard scientific calculators have functions that will allow you to do this automatically. You can also do it with Excel. Summarize your data in the following table. Note: if all values are zero your standard deviation and average will also be zero.

**Table \_\_\_\_\_ Summary of data on food extract at various concentrations n=20**

Time (min)	Food Extract Concentrations							
	Undiluted		1/10 dilution		1/100 dilution		1/1000 dilution	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0								
10								
20								
30								

- Now prepare a graph in which you plot the mean number of vacuoles/cell as a function of time for each of the food extract concentrations. Include error bars of +/- 1 standard deviation above and below each data point. (See lab 1 if you do not recall how to make these graphs) Draw the best curve that you can through each set of points. **What conclusion can you draw from these data? (Include this graph in your lab manual.)**
- To determine if the food extract results in a significantly different rate of phagocytosis, use the standard deviations and the means, you have calculated from the 30 minute samples to determine the **t-statistic**, the formula is given in the Background Information, to compare each food extract sample to the 10% india ink sample from Part B. Summarize your results in the following table.

Comparison	t-statistic (or p-value if using Excel)	Are the samples significantly different? (Y/N)
Undiluted extract vs 10% ink		
1/10 dilution vs 10% ink		
1/100 dilution vs 10% ink		
1/1000 dilution vs 10% ink		

Use a probability value of 0.05 and an n (20+20 or 40 -2) = 38. **Are the samples significantly different?**

8. To determine if food extract results in a significantly different rate of phagocytosis, use the standard deviations and the means, you have calculated from the 30 minute samples to determine the **standard error for the difference of the means** ( $SE(\bar{X}_1 - \bar{X}_2)$ ), the formula is given in the Background Information, , to compare each food extract sample to the 10% india ink sample from Part B. Summarize your results in the following table.

Comparison	$SE(\bar{X}_1 - \bar{X}_2)$	Are the samples significantly different? (Y/N)
Undiluted extract vs 10% ink		
1/10 dilution vs 10% ink		
1/100 dilution vs 10% ink		
1/1000 dilution vs 10% ink		