NE 455L/481 Nanoscale Biosystems Lab Report

University of Waterloo
4B Nanotechnology Engineering
Group 7

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Lab 4 Introduction to Cell Culture Procedures and Techniques

Introduction and Objective

Cell culture refers to the technique of growing and maintaining animal cells *in vitro*. Cell culturing is useful because scientists and engineers can investigate cell physiology, biochemistry and immunological processes of cells in controlled lab environments. It is also useful to produce drugs, proteins and other disease-fighting vaccines on a massive-scale for the use in medical products.

The primary objective of this laboratory is to culture and maintain a human prostate cell line, PC3 for use in labs 2 and 3 in the following weeks. Stringent aseptic techniques for cell culturing will be learned and practiced. All safety protocols related to environmental control, personal hygiene, equipment and media sterilization will be observed.

Experimental Procedure

The procedure for this laboratory was obtained from the NE 455L/481L Nanoscale Biosystems Laboratory 4 part of the Nanotechnology Engineering Program 4B Lab Manuals, 2010. No deviations were observed.

Discussion and Analysis of Data

Question 1

Using the hemocytometer count, calculate the original cell concentration of your T-25 flask of cells. Calculate % viability. Show all work. What are the drawbacks of the Trypan Blue Exclusion Dye Assay?

Cell Concentration

From Table 2, the total cell count for all 9 cells is 207. With the coverslip on top, each of the smallest squares of the counter forms a counting chamber volume of 0.05 mm x 0.05 mm x 0.01 mm deep. The volume of the each square in the 9-square grid is therefore 0.05mm * 0.05mm * 0.01 mm * 16 * 25 = 0.01 mm^3, or 1E-5 cm^3 which is equal to 1E-5 mL (1 cm^3 = 1 mL).

The dilution factor is 2 since 50 μl of the cell suspension was mixed with 50 μl of Trypan Blue dye.

Thus the cell concentration in the *centrifuge* tube is:

$$\text{Cell Count} = \frac{\text{No. of cells counted in all 9 squares}}{\text{Number of squares (9 squares)}} \times \text{Dilution} \times \frac{1}{\text{Volume of each square}}$$
$$= \frac{207}{9} \times 2 \times \frac{1}{1 \times 10^{-5}\ \text{mL}}$$
$$= 4.6 \times 10^{6}\ \frac{\text{cells}}{\text{mL}}$$

The concentration in the T-25 flask was diluted again by a factor of 4 with 3 mL of F12K medium (steps 39 and 40 in the lab manual). This means our actual cell concentration in the *T-25 flask* is $\frac{4.6 \times 10^{6}}{4} = 1.15 \times 10^{6}\ \frac{\text{cells}}{\text{mL}}$

Percentage Viability

We only counted one dead cell in all the 9 squares, so the expected viability is $\frac{207-1}{207} \times 100\% = 99.5\%$.

Drawbacks of Trypan Blue Exclusion Dye Assay

The trypan blue dye exclusion dye assay, although a very good and proven method for determining cell viability (rapid blue staining on the cells is considered a sign of membrane damage and cell death), does not provide any exact information about how much the damaged cell is able to proliferate relative to a healthy cell in the same medium. In order to do so, we need to isolate the damaged/dead cells and culture them separately to determine how viable these damaged cells are. Therefore, results of a trypan blue test do not indicate the capacity of cells to grow in culture, only the number of cells whose cell membranes are damaged. This is a significant drawback. In general, the Trypan blue assay overestimates the number of viable cells.

The other drawback is that this test is subjective. There may be a small amount of dye taken up by injured cells that go unnoticed.

Question 2

Compare the medium that was in your flask with the fresh medium. Why did this change take place and what is the term for it?

The fresh medium looked pinkish in colour. In comparison, the medium in our flask look yellowish-brown. We think this change in colour was because the fresh medium was rich in glucose and other nutrients, whereas our flask contained a medium that was deficient in glucose but contained byproducts from cellular processes and other waste products.

The other reason for the colour change could've been the rich presence of CO_2 in our flask which was not present was only in moderate quantities in the fresh medium. The CO_2 in our flask and other by-products came about as a result of ***digestion*** of surrounding nutrients, ***aerobic respiration*** and ***reproduction*** by the living cells in the medium.

Mammalian cells are nearly continuously ingesting their surrounding fluids and molecules as part of the body's normal and defensive pathways. This is known as ***endocytosis*** and allows the cells to transport molecules that are unable to pass through the hydrophobic plasma membrane because of their size.

Question 3

What are the limitations of in vitro cultured cells when it comes to drawing conclusions about cell behaviour?

In vitro systems have inherent limitations in their ability to model the whole animal, which must be appropriately considered when drawing conclusions about cell behaviour.

In vivo systems have very specific behaviours because they are not in such a controlled environment. In an *in vivo* system, every cell's behaviour is regulated by every other cell that neighbors it. The growth environment of mammalian cells is highly important as changes in the environmental factors can easily induce growth arrest (apoptosis) or even stimulate proliferation or differentiation.

It is impossible for *in vitro* system to mimic these specific animal-cell behaviours due to their highly controlled nature. *In vivo* systems are significantly more complex, and thus drawing conclusions about cell behaviour based on an *in vitro* culture may not be entirely accurate.

As such, *in vitro* systems are only a *model*. And no model is 100% representative of the real system.

Summary and Conclusions

In this cell culture laboratory, a human prostate cell line, PC3, was cultured using newly-learned aseptic techniques. Working in a BioSafety Class II Type A2 laminar flow hood was learned. Cell counts using a hemocytometer were obtained. Distinguishing between dead/damaged and live cells was performed by means of a Trypan Blue Exclusion Dye Assay.

The original cell count/concentration in the T-25 flask was calculated to be 1.15 million cells per mL. This is a very reasonable number.

One of the few drawbacks of counting cells is that it can be labour intensive. In order to produce reproducible results when counting the cell number in a larger number of samples, it might be better to use an electronic counter. These not only take far less time, but also improve

the accuracy of the count. It also ensures overlapping cells that are in contact with each other are counted correspondingly.

Another possible improvement is to use a plastic hemocytometer over the glass one we used. Plastic hemocytometers are much safer.

In order to eliminate the subjective nature of the viability test, more accurate cell viability tests can be performed such as fluorescence microscopy after staining with a fluorescent dye. This determines the cell's light scatter characteristics using a flow cytometry.

In general, the laboratory was a success and the qualities of results are sufficiently adequate for an undergraduate-level lab experiment performed just once.

Lab 5 Polymeric Nanoparticle Drug Delivery to Human Cancer Cells

Introduction and Objective

The objective of this laboratory is to prepare two nanoparticle (NP) solutions made of PLGA-PEG synthesized using the nano-precipitation technique learned in Lab 1. One NP solution will contain nanoparticles that encapsulate the cancer drug Docetaxel, while the other NP solution will encapsulate no drug. These two nanoparticles solutions will be mixed with the live cells cultured in Lab 4 in a 96-well micro plate, and will be incubated in a CO_2 incubator for a week, ready for examination in Lab 6.

Experimental Procedure

The procedure for this laboratory was obtained from the NE 455L/481L Nanoscale Biosystems Laboratory 5 part of the Nanotechnology Engineering Program 4B Lab Manuals, 2010. Only two deviations were observed:

- The final volume in each 15 mL centrifuge tube was increased from 1.5 mL to 2.5 mL
- Instead of using a cell concentration range from 30,000-1,000,000 cells/mL, we decided to use a range from 15,000-500,000 cells/mL instead.

Discussion and Analysis of Data

Table 4 shows the cell counts after the one week incubation of our cells from Lab 4. The total number of cells counted was 975. Thus, our original cell stock concentration is

$$\frac{975}{9} \times 2 \times 10,000 = 2.17 \times 10^6 \ \frac{\text{cells}}{\text{mL}}$$

The following table shows the layout of our 96-well microplate. Concentrations are in units of **cells/mL. Blanks** are the medium/buffer in use to account for background noise whose values will be subtracted from all microplate readings. We had room for as much as 3 replicates for

each of our data points. A negative control was necessary to ensure the polymeric nanoparticles themselves weren't having an effect on the cells, and that any effect observed on the cells would be purely due to the docetaxel molecules.

96-Well Microplate Template													
	Conc.	1	2	3	4	5	6	7	8	9	10	11	12
A													
B	500,000		NON DCTX	NON DCTX	NON DCTX	DCTX	DCTX	DCTX	NEG	NEG	NEG	BLANK	
C	250,000		NON DCTX	NON DCTX	NON DCTX	DCTX	DCTX	DCTX	NEG	NEG	NEG	BLANK	
D	125,000		NON DCTX	NON DCTX	NON DCTX	DCTX	DCTX	DCTX	NEG	NEG	NEG	BLANK	
E	62500		NON DCTX	NON DCTX	NON DCTX	DCTX	DCTX	DCTX	NEG	NEG	NEG	BLANK	
F	31250		NON DCTX	NON DCTX	NON DCTX	DCTX	DCTX	DCTX	NEG	NEG	NEG	BLANK	
G	15625		NON DCTX	NON DCTX	NON DCTX	DCTX	DCTX	DCTX	NEG	NEG	NEG	BLANK	
H													

We then prepared 6 dilutions with the concentrations shown in the table above. The dilutions were prepared in a step-wise serial dilution order. A step-wise serial dilution order was preferred in order to keep the dilution process simple. It also avoids having to draw liquid from the concentrated stock solution all the time. This is preferred since even small percentage errors in a concentrated solution can lead to large absolute errors in the weak dilutions. The drawback of serial dilution was that it led to us throwing out 2.5 mL of medium from the last tube (Tube 6), but this was not considered to be a critical issue. More medium was provided to us when we ran out.

The following table shows how the 6 dilutions were prepared.

Tube	Initial Concentration of Cell Suspension (cells/mL)	Volume of Cell Suspension Needed (mL)	Volume of Medium Needed (mL)	Final Concentration (cells/mL)	Final Volume (mL)
1	2,170,000	0.9375	4.0625	500,000	2.5
2	500,000	2.5	2.5	250,000	2.5
3	250,000	2.5	2.5	125,000	2.5
4	125,000	2.5	2.5	62500	2.5
5	62500	2.5	2.5	31250	2.5
6	31250	2.5	2.5	15625	2.5

The volume of cell suspension and medium needed for Tube 1 were calculated as follows. Let *x* be the volume of cell suspension needed. Since the final volume required in the tube is 2.5 mL, we need the sum of the volumes of cell suspension and medium to be twice that amount, i.e. 5 mL. Thus the volume of medium needed is 5-*x* mL. The following equality of ratios must hold to obtain the desired dilution:

$$\frac{5-x}{x} = \frac{2{,}170{,}000}{500{,}000} = 4.33$$
$$\therefore 5 - x = 4.33x$$
$$\therefore x = \frac{5}{1+4.33} = 0.9375$$
$$\text{and, } 5 - x = 5 - 0.9375 = 4.0625$$

Once the dilution for Tube 1 is prepared, half (2.5 mL) of the solution is drawn from Tube 1 (resulting in a final volume of 5-2.5=2.5 mL) and mixed with another 2.5 mL of medium into Tube 2. This makes the concentration of Tube 2 half of that in Tube 1.

Summary and Conclusions

This experiment (Lab 5) was one of the few experiments that went without any glitches. Except for the fact that the lab was too long (~7 hours), everything in the procedure was followed correctly. The experiment should be shortened for future runs of the lab by perhaps having the nanoparticle solutions pre-prepared by the lab instructor for all students. This will save the students considerable amounts of time and will ensure the duration of the lab period does not spill beyond the allotted time for the experiment. Spill overs are difficult to handle since the lab space is taken up by the next class. Students are also unable to discuss the procedure during the experiment as the next class expects us to keep our voices down.

In the end, we were able to perform the experiment successfully without contamination. We were able to fill 54 cells in our 96-well microplate with nanoparticles encapsulated with docetaxel and nanoparticles with no docetaxel and also 18 cells with our negative control, which are just the live cells with no nanoparticles at all. The blanks will be added next week. The microplate was left for a 1-week incubation period. Results will be analyzed the following week in Lab 6.

Lab 6 MTT Proliferation Assay

Introduction and Objective

The objective of this laboratory experiment was to verify (or refute) the hypothesis from Lab 5. This hypothesis stated that nanoparticle-based drug carriers are *not* recognized as foreign objects and are therefore not quickly degraded or eliminated from circulation by macrophages in the liver and spleen. If these nanoparticles were indeed eliminated, they would have no therapeutic effect on the cancer cells cultured in Lab 4, and the number of live cells would be more or less the same as the cells treated without any drug-encapsulated nanoparticles. The hypothesis also stated that once the nanoparticle-based drug carrier reaches the cells, the nanoparticles degrade and the chemotherapeutic in question, i.e. docetaxel is able to exert its toxic effects. If the nanoparticle never degraded, the effect on the cells would be the same as non-docetaxel encapsulated nanoparticles.

The MTT Cell Proliferation Assay is a method used to measure the viability of a population of cells. In this lab, we treat our population of cells with a toxic reagent and see how the system responds and measure the degree to which MTT is toxic to the living cells, known as cytotoxicity. MTT is short for 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide. Viable, living cells can convert MTT into a molecule called formazan which is purple in colour. Thus, using absorbance spectroscopy, the more purple we see, the more number of living cells we have.

Experimental Procedure

The procedure for this laboratory was obtained from the NE 455L/481L Nanoscale Biosystems Laboratory 6 part of the Nanotechnology Engineering Program 4B Lab Manuals, 2010. Only 2 deviations were observed:

1. We added 100 μl of Acridine orange instead of 500 μl specified in the lab manual
2. Instead of washing both sides of the coverslip with water in step 6 of section 1.3 of the lab manual (Staining with Acridine Orange and Fluorescence Imaging), we only washed the bottom side of the slide with water.

Discussion and Analysis of Data

The following images show how our florescence scans observed under the microscope after post processing.

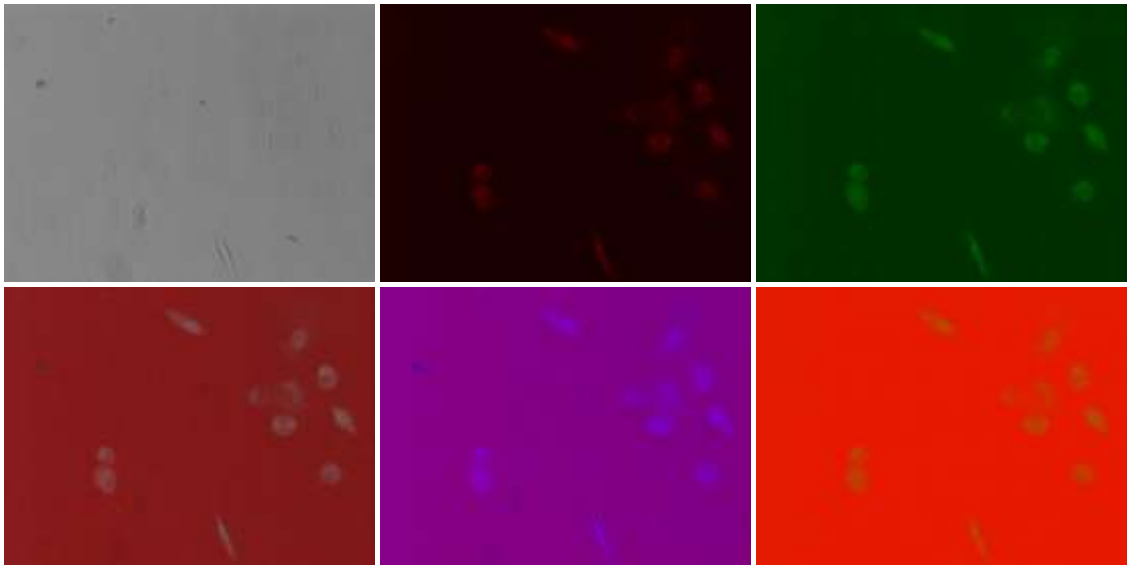


Figure 1 Florescence Images observed under microscope after post-processing. Different colour channels were applied to the images to obtain different contrasts.

Table 1 shows absorbance readings for our microplate *after* subtracting out the blank values.

Table 1 Microplate Absorbance Readings at 540nm (Background Removed)

	1	2	3	4	5	6	7	8	9	10	11
A											
B		0.249	0.372	0.369	0.194	0.131	0.296	0.262	0.542	0.415	0.004
C		0.392	0.447	0.471	0.093	0.160	0.047	0.219	0.417	0.318	-0.007
D		0.256	0.285	0.215	0.026	0.089	0.058	0.287	0.298	0.294	-0.006
E		0.204	0.269	0.243	0.027	-0.003	0.010	0.188	0.248	0.293	-0.006
F		0.154	0.212	0.108	0.012	0.045	0.029	0.130	0.127	0.111	0.006
G		0.012	0.047	0.089	0.012	0.013	0.001	0.085	0.091	0.048	0.007
H											

Question 1

State the hypothesis of Lab 5. Create an Analysis of Variance table (ANOVA) for the MTT data for NPs with Docetaxel. Using $\alpha = 0.05$, test the hypothesis stated for Lab 5.

The hypothesis stated in Lab 4 was that the nanoparticles will not be treated as foreign particles and will therefore be able to be uptaken by passive diffusion (endocytosis) by the cells. Once in the cells, the nanoparticles will then degrade releasing any encapsulated therapeutic drugs which will inhibit cell growth by promoting cell death of cancerous cells.

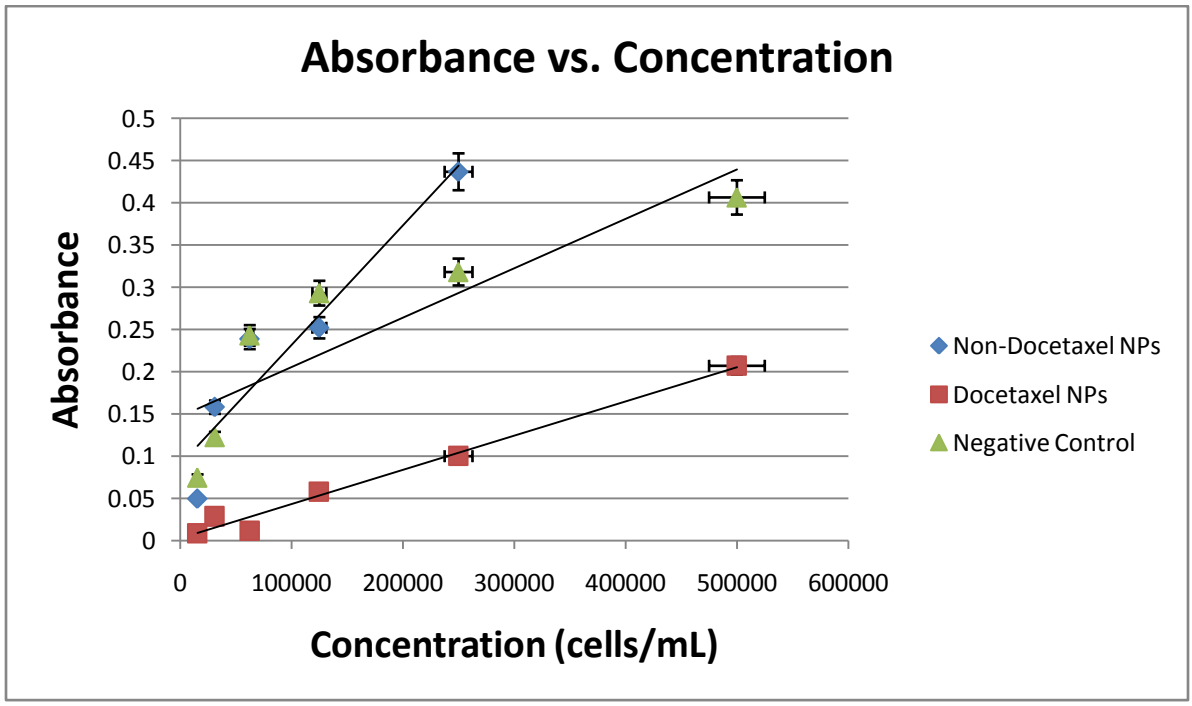
The ANOVA results were obtained using the "ANOVA: Two-factor Method With Replication" data analysis tool in Microsoft Excel. We used an alpha (confidence) value of $\alpha = 0.05$. The results are attached in Appendix B.

The ANOVA results for NPs with docetaxel indicate that the variance (uncertainty) in our data *reduces* with concentration. It also indicates that the 500,000 cells/mL data point for NPs without docetaxel is a clear outlier in the data since its average is significantly lower than that of the 250,000 cells/mL absorbance values. This data point should therefore be rejected from the analysis.

Question 2

Plot the MTT results. Plot error bars.

Averaging the data between replicates, and plotting these averages as a function of concentration yields the following plot. The error bars for relative error (in %) are shown as well to observe the fit in data. As discussed in Question 1, the 500,000 cells/mL data point for non-docetaxel NPs was discarded since it was an outlier.



A clear linear trend between concentration and absorbance can be seen. This is expected since the larger the concentration, the more number of viable cells exists in solution. Thus, their absorbance is high since more MTT can be converted to formazan.

Also, the slope of non-docetaxel encapsulated NPs is much higher than that of docetaxel-encapsulated NPs. This is exactly what we expect since the docetaxel is an anti-cancer therapeutic drug that kills off a large number of cancer cells. These cells therefore have a smaller viability and thus a smaller slope.

Question 3

If a 384-well microplate was used instead of a 96-well microplate, what other treatments could be tested to obtain a comprehensive picture of the effects of polymeric drug delivery vehicles on human cancer cells?

In a 96-well microplate (12x8), we are unable to use the outer 36 wells on the edges due to reasons explained in the pre-lab. However, we still had 60 wells to conduct our experiment on. This allowed us to try out 6 different concentrations with 3 replicates for each concentration and treatment.

In a 384-well plate (24x16), we will be unable to use the outer 76 wells for the same reason. But we will still have 308 wells left for our experiment. This allows us to use up to 14 different concentrations instead of 6. And if we maintained 3 replicas for each treatment, we will be able to try out up to 7 different treatments instead of just 3. This gives us an extra 4 treatments we can try out.

With these extra 4 treatments, we could try out different sizes of PLGA-PEG nanoparticles. In this lab, we used nanoparticles that ranged in size from 50-70 nm. But the biodistribution of NPs is largely determined by their physical and biochemical properties such as particle size, and surface properties.

We could also try out other polymeric vehicles and different polymer-drug conjugation models. This will allow us to investigate different pharmokokinetic pathways, and drug stability and degradation models. In particular, we could try out anti-cancer drugs encapsulated in non-polymeric and inorganic vehicles, such as platinum-type and organometallic drug systems, and compare how much safer the transport is compared to polymeric PLGA-PEG vehicles.

Question 4

Large scale mammalian cell cultures are established for the purposes of process development and the manufacturing clinical materials such as vaccines, recombinant DNA-derived proteins and monoclonal antibodies. Give an overview of how large scale mammalian culture is carried out. Support with literature.

Large-scale mammalian culture requires very precise and highly optimized aseptic control techniques. No amount of contamination is acceptable. To this end, manufacturing industries

use advanced aseptic control techniques that are steam-based. In a steam-based aseptic technique, the aseptic state is maintained by a positive in-system pressure. The oxygen supply is usually lowered for better aseptic control. The cultures are periodically treated with enzymes to ensure rapid bacteria degradation. Large-scale mammalian culture factories also control the temperature very accurately to maximize cell growth and reproduction [1,2].

Summary and Conclusions

In this laboratory experiment, a clear linear trend between concentration and absorbance was seen. This is expected since the larger the concentration, the more number of viable cells exists in solution. Thus, their absorbance is high since more MTT can be converted to formazan. Also, the slope of non-docetaxel encapsulated NPs is much higher than that of docetaxel-encapsulated NPs. This is exactly what we expect since the docetaxel is an anti-cancer therapeutic drug that kills off a large number of cancer cells. These cells therefore have a smaller viability and thus a smaller slope.

In all, this laboratory was a success since it establishes our hypothesis from Lab 5 as true. This means that the docetaxel anti-cancer drug was not prematurely killed by macrophages and was indeed uptaken by the cells. It also means that this slow uptake did indeed result in inhibited cancer cell growth and possible cell death.

Appendix A – Data Collected

Lab 4

Table 2 Lab 1 Live Cell Counts Lab 4

1 – 29	**2** – 19	**3** – 24
4 – 26	**5** – 19	**6** – 27
7 – 16	**8** – 24	**9** – 23

Total live cells = 29+19+24+26+19+27+16+24+23 = **207**.

Table 3 Lab 1 Dead Cell Counts Lab 4

1 – 0	**2** – 0	**3** – 0
4 – 0	**5** – 1	**6** – 0
7 – 0	**8** – 0	**9** – 0

Total dead cells = **1**

Lab 5

Table 4 Lab 1 Live Cell Counts Lab 5

1 – 66	**2** – 64	**3** – 161
4 – 82	**5** – 93	**6** – 159
7 – 83	**8** – 129	**9** – 138

Total live cells = **975**.

Table 5 Lab 1 Dead Cell Counts Lab 5

1 – 0	**2** – 1	**3** – 1
4 – 2	**5** – 2	**6** – 0
7 – 2	**8** – 1	**9** – 1

Total dead cells = **10**

References

[1] "Large-scale mammalian cell culture: methods, applications and products", Raymond E. Spier, University of Surrey, Guildford, Surrey, UK. *Current Opinion in Biotechnology* Volume 2, Issue 3, June 1991, pp 375-379.

[2] W. R. Tolbert and J. Feder. U.S. Patent 4,059,485 (1977).