

University of Toronto

Faculty of Applied Science and Engineering

BME440 Biomedical Engineering Technology and Investigation

Lab 4: Fluorescence Microscopy & Image Analysis of Cells

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Name: Deniz Uzun

Student Number: 1006035005

Lab Partner: Darsh Jain

1.0 Introduction & Scientific Objective

Fluorescence microscopy is a commonly used technique in biomedical sciences that utilizes fluorescent dyes and an optical device (microscope) to visualize small cellular and molecular structures [1]. Through magnification and fluorescent elements used in this technique, one can identify the sub-cellular components with high specificity [1]. Cellular staining allows for visualization of cell components [1]. The cytoskeleton of a cell is mainly composed of protein filaments such as actin, tubulin and talin [2]. Therefore an actin stain such as TRITC-Phalloidin can be used to highlight the cell cytoskeleton. DAPI (4',6-diamidino-2-phenylindole) emits blue fluorescence upon binding to AT regions of DNA, therefore is commonly used as a nuclear counterstain for fluorescence microscopy [3]. Photobleaching is a process that occurs due to cells being excited for elongated periods, which may cause fluorophores to oxidize and become less emissive [1]. The specific problem that will be addressed in this lab is to visualize and analyze specific cellular components by mainly focusing on nuclei and cytoskeleton, of fixed U87 human primary glioblastoma cells. The scientific objective of this lab is to determine the intensity of the GFP signal in each cell, which are stained with TRITC-Phalloidin for cytoskeleton and DAPI for nucleus visualization, by counting the number of cells in each image frame under different factors of magnification, in addition to observing the effects of photobleaching. As a result, we expect to obtain clear and distinct images of the actin and nuclei that yields accurate quantitative data such as cell count and GFP intensity and to observe a linearly decreasing fluorescence intensity under red illumination as a result of the photobleaching.

2.0 Materials and Methods

In this lab, U87 Human Primary Glioblastoma cells were used as the primary sample, which were fixed 3 wells (A1-A3) in a 96-well plate. Using a p200 pipette with 200 μ L pipette tips, 100 μ L 0.2% Triton X-100 in PBS were added to each well for cell permeabilization and incubated for 10 minutes. Afterwards, Triton X solution was discarded and cells were washed 3 times with PBS. 50 μ L 100 μ g/mL TRITC-Phalloidin in 1% BSA in PBS and 50 μ L 0.1 μ g/mL DAPI in PBS were added to each well for staining actin filaments and nucleus of the cells, respectively. Then, the plate was covered with foil and incubated for 20 minutes at room temperature. Afterwards, staining solution was aspirated and cells were washed 3 times with PBS. For the second part of this lab, the stained 96-well plate was imaged using Leica DMI 4000 Microscope using 10x, 20x and 40x lenses. The images were captured using blue, red and green illumination for each well. During this process, since we have captured images of different locations in the well, the overlay of these images could not be done, which is needed for analysis. Therefore, in the rest of this report, the images from one well that was provided by the TA will be presented and discussed. Additionally, photobleaching was recorded over 20 minutes using red illumination and 20x lens, at 2 minute intervals. Lastly, image analysis such as overlaying the provided images and adjusting thresholds for the inspection of number of nuclei and investigation of the effect of photobleaching were done by using the FIJI software.

3.0 Results

1. Provide representative images of your cells with the DAPI and TRITC-Phalloidin channels merged at 10x, 20x, and 40x. Please include the scale bar for each image.

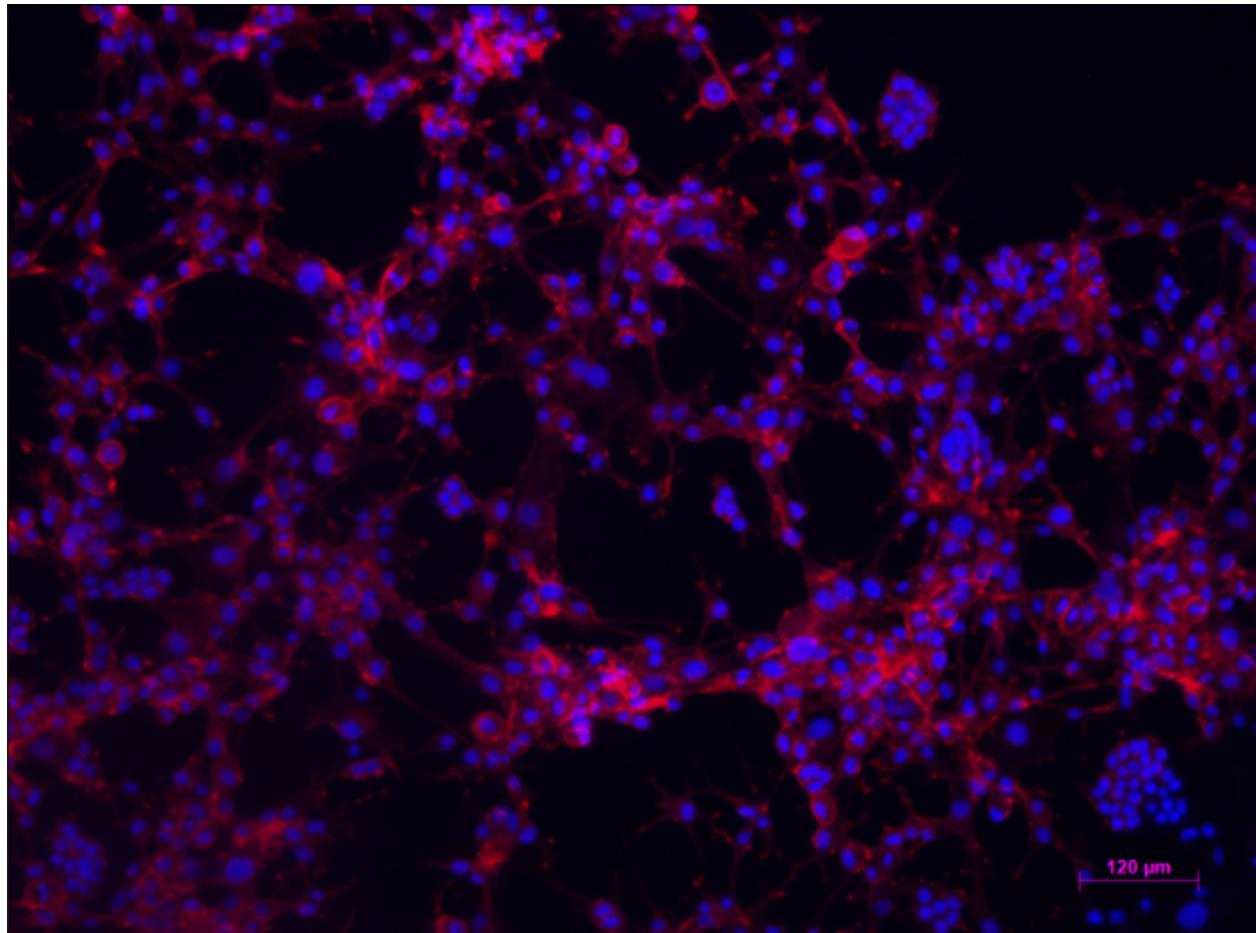


Figure 1: Overlaid image of DAPI and TRITC-Phalloidin channels of fixed U87 cells taken using Leica DMI 4000 microscope at 10x magnification, merged using ImageJ. DAPI stained nuclei appear blue and TRITC-Phalloidin stained cell actin cytoskeleton appears red. Scale bar is provided on the bottom right of the image.

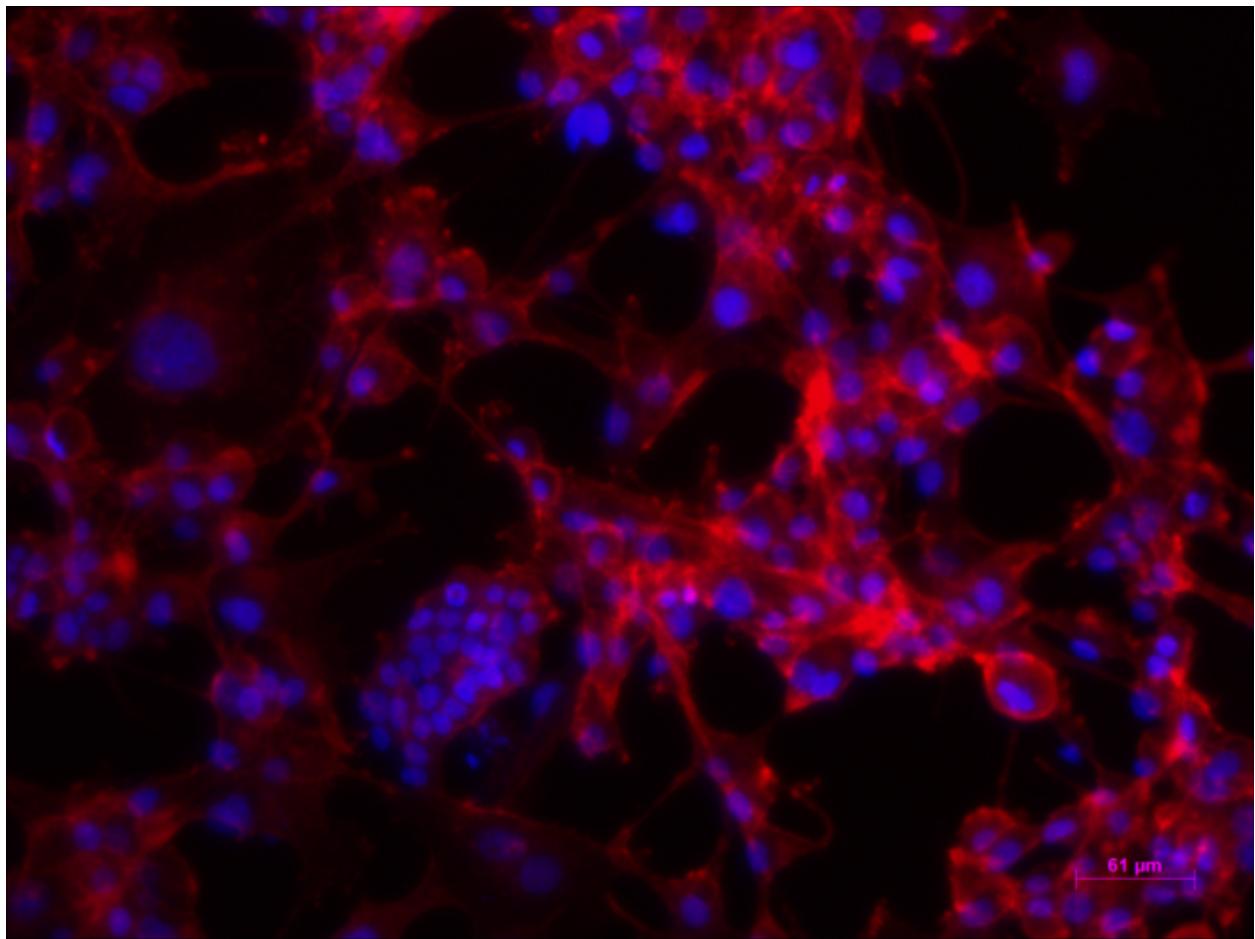


Figure 2: Overlaid image of DAPI and TRITC-Phalloidin channels of fixed U87 cells taken using Leica DMI 4000 microscope at 20x magnification, merged using ImageJ. DAPI stained nuclei appear blue and TRITC-Phalloidin stained cell actin cytoskeleton appears red. Scale bar is provided on the bottom right of the image.

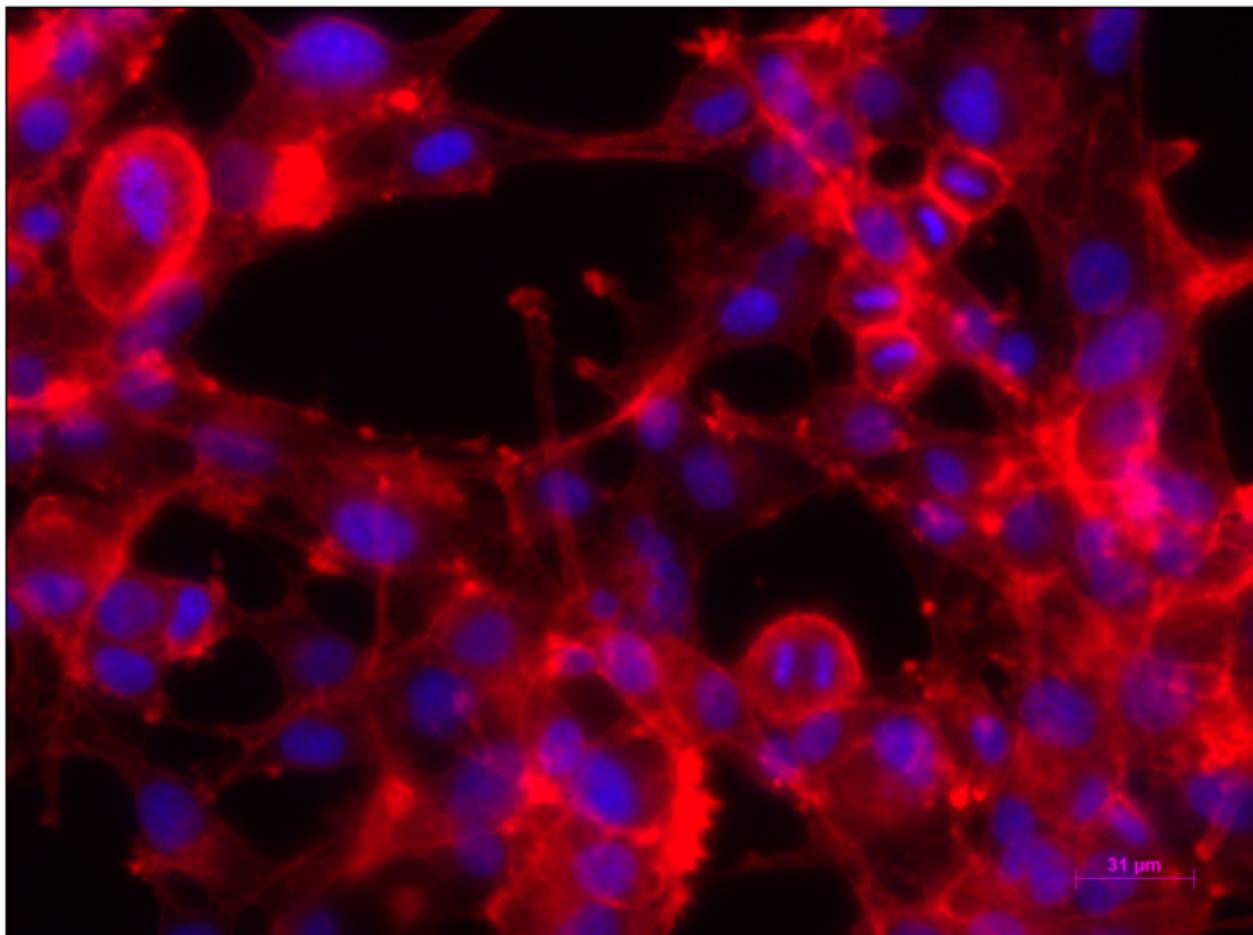


Figure 3: Overlaid image of DAPI and TRITC-Phalloidin channels of fixed U87 cells taken using Leica DMI 4000 microscope at 40x magnification, merged using ImageJ. DAPI stained nuclei appear blue and TRITC-Phalloidin stained cell actin cytoskeleton appears red. Scale bar is provided on the bottom right of the image.

2. Estimate the number of cells in a well using image analysis with ImageJ. Please include:

a. Representative output images (thresholded and counted) from your image analysis for 10x, 20x, and 40x.

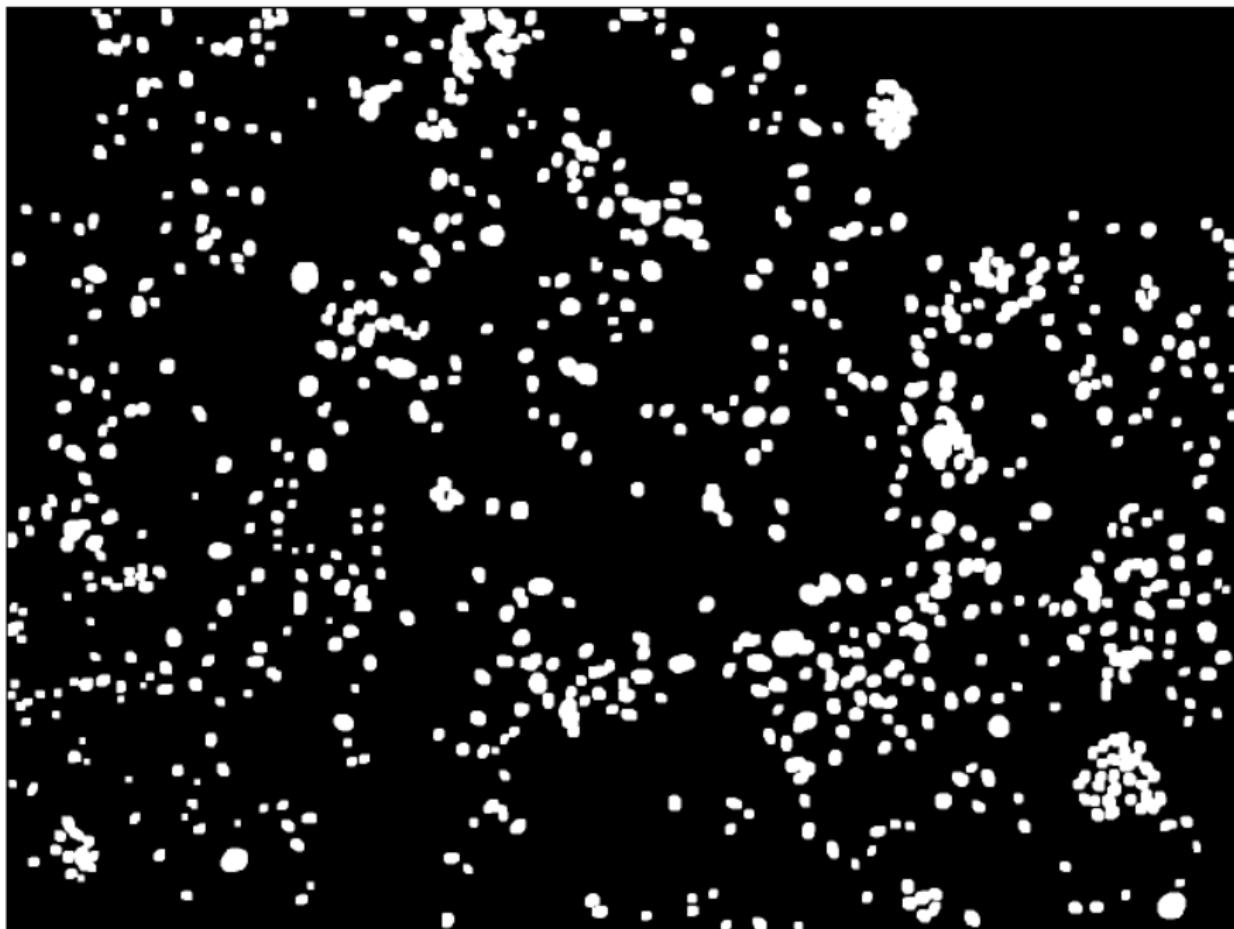


Figure 4: Thresholded output image of DAPI channels of fixed U87 cells taken using Leica DMI 4000 microscope at 10x magnification. The cell nuclei appear white with a dark background. 3 erosions and dilations were performed with 1 more additional dilation at the end, prior to analysis.

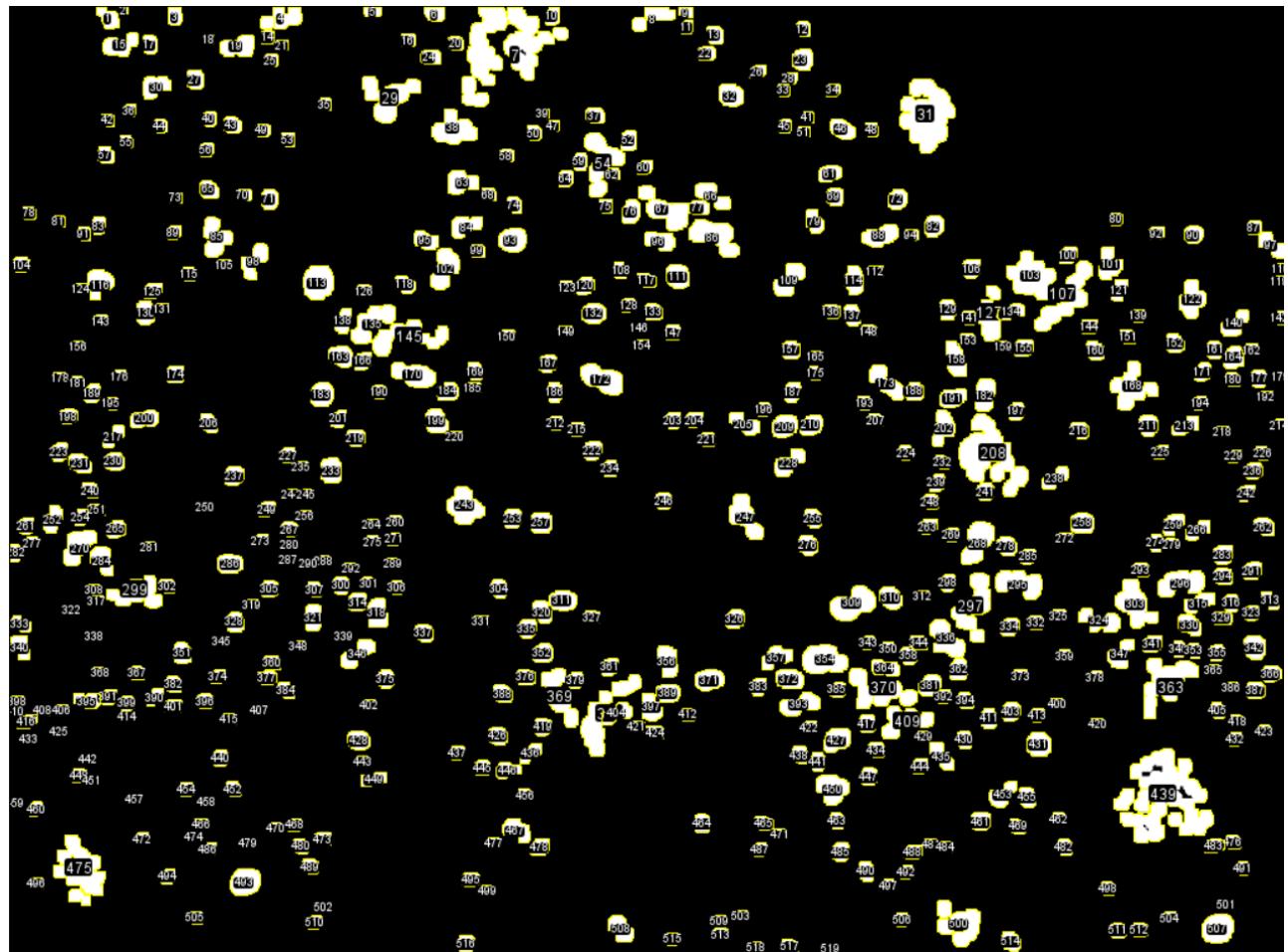


Figure 5: Thresholded and counted output image of DAPI channels of fixed U87 cells taken using Leica DMI 4000 microscope at 10x magnification. The cell nuclei appear white with a dark background. 3 erosions and dilations were performed with 1 more additional dilation at the end, prior to analysis. 519 nuclei were recorded using the ImageJ. Closely clustered cells were counted inaccurately as one by the software, ImageJ.

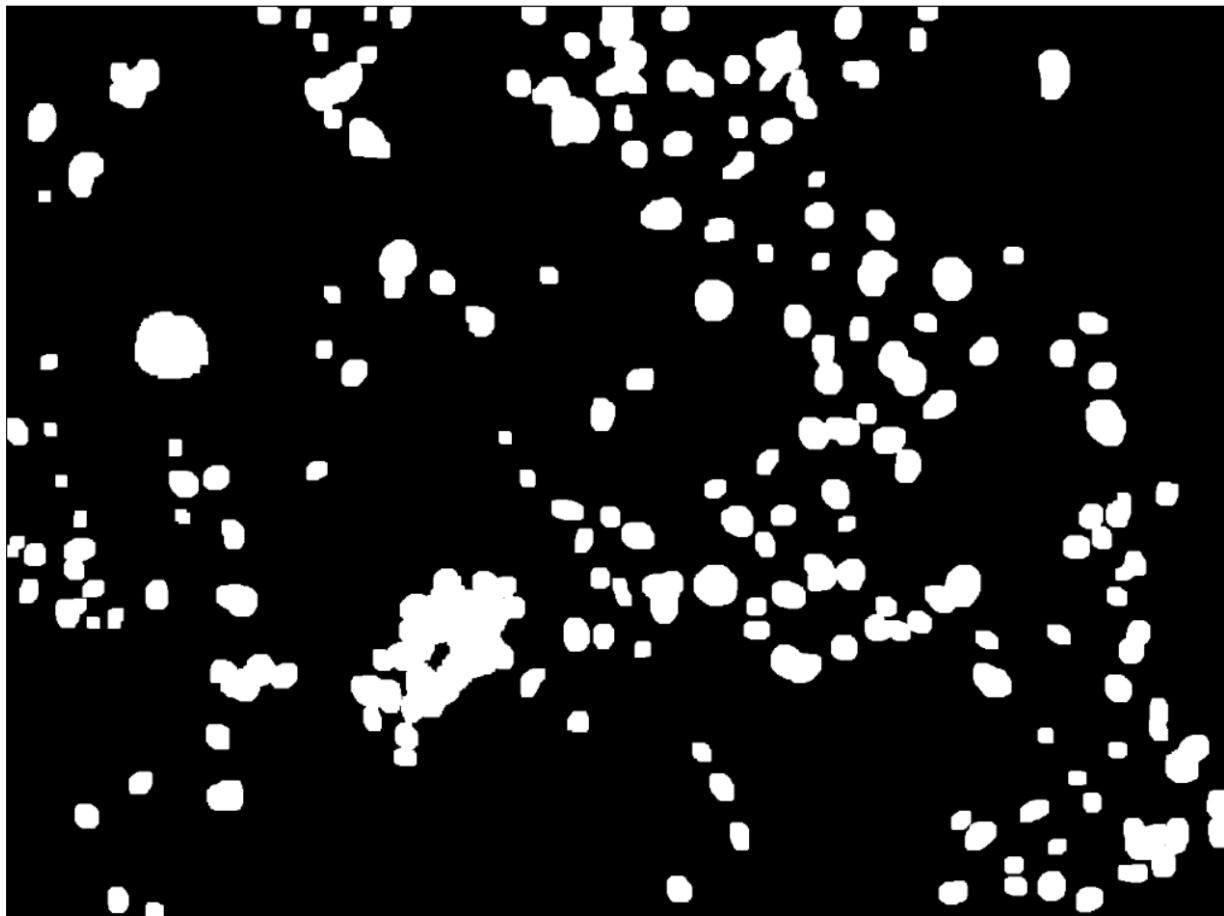


Figure 6: Thresholded output image of DAPI channels of fixed U87 cells taken using Leica DMI 4000 microscope at 20x magnification. The cell nuclei appear white with a dark background. 5 erosions and dilations were performed with 1 more additional dilation at the end, prior to analysis.

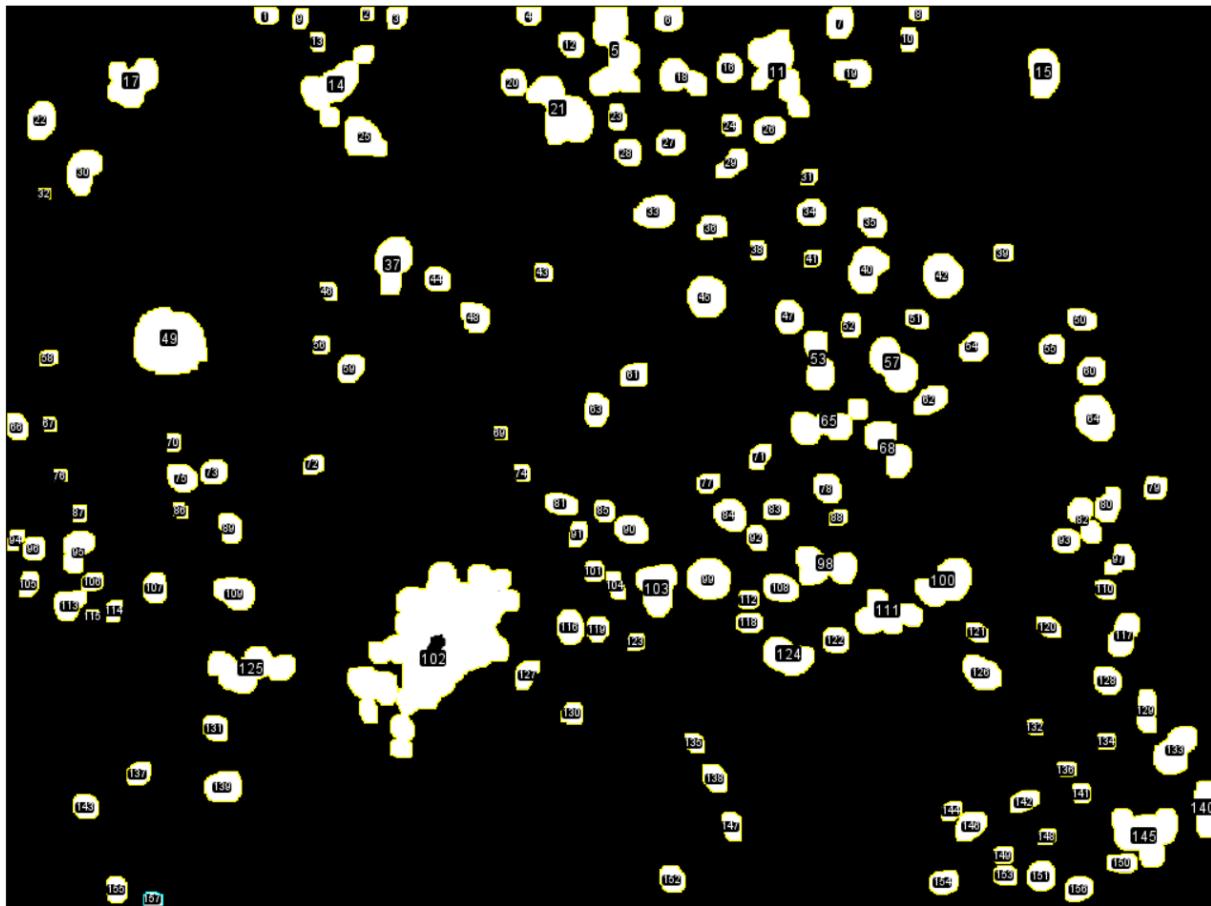


Figure 7: Thresholded and counted output image of DAPI channels of fixed U87 cells taken using Leica DMI 4000 microscope at 20x magnification. The cell nuclei appear white with a dark background. 5 erosions and dilations were performed with 1 more additional dilation at the end, prior to analysis. 157 nuclei were recorded using the ImageJ. Closely clustered cells were counted inaccurately as one by the software, ImageJ.

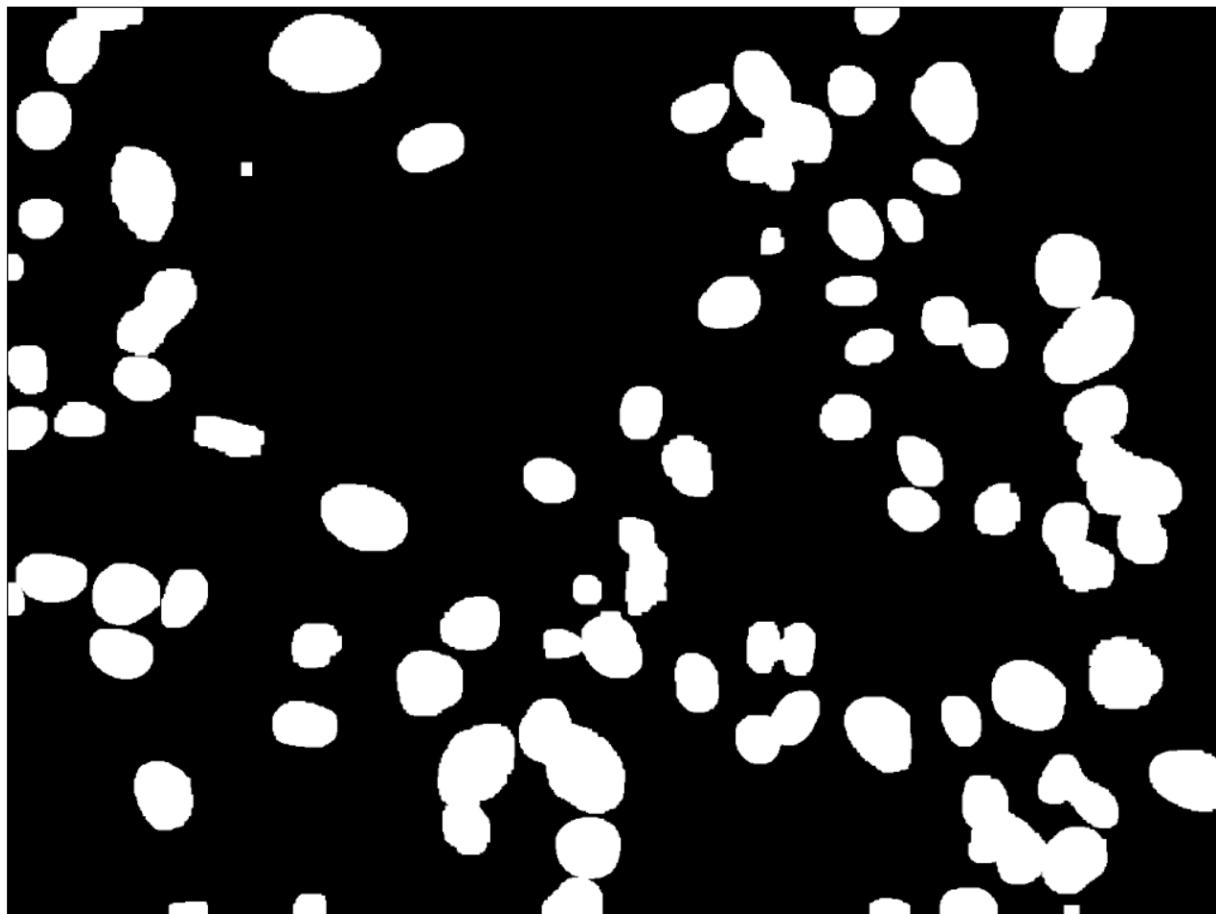


Figure 8: Thresholded output image of DAPI channels of fixed U87 cells taken using Leica DMI 4000 microscope at 40x magnification. The cell nuclei appear white with a dark background. 4 erosions and dilations were performed with 2 more additional dilations at the end, prior to analysis.

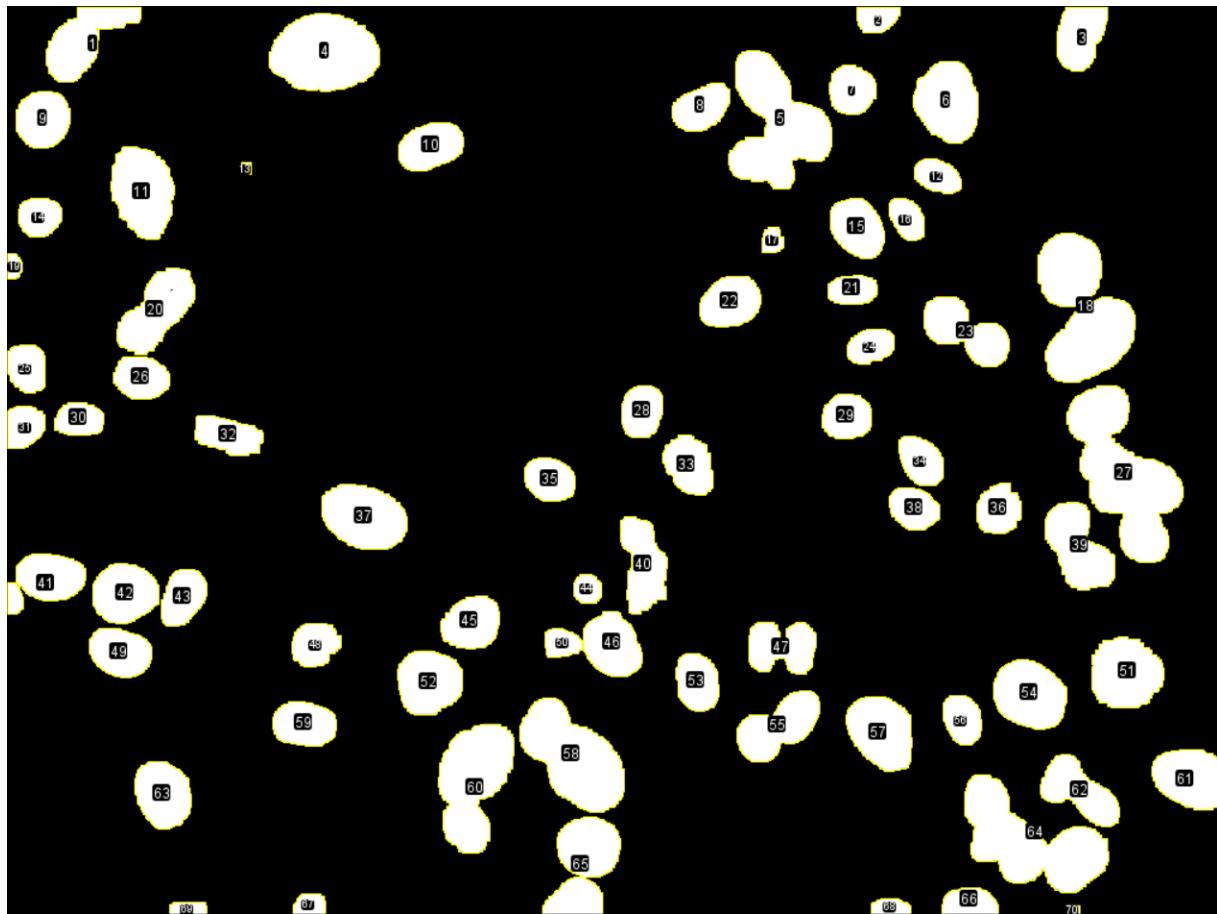


Figure 9: Thresholded and counted output image of DAPI channels of fixed U87 cells taken using Leica DMI 4000 microscope at 40x magnification. The cell nuclei appear white with a dark background. 4 erosions and dilations were performed with 2 more additional dilations at the end, prior to analysis. 70 nuclei were recorded using the ImageJ. Closely clustered cells were counted inaccurately as one by the software, ImageJ.

b. A sample calculation for how you obtained your estimated number of cells per well for the 10x objective. State any assumptions you make.

The well surface area of a 96-Well Eppendorf Cell Culture Plate [4] is calculated below:

$$A_{well} = \pi\left(\frac{d}{2}\right)^2 = \pi\left(\frac{7*10^{-3}}{2}\right)^2 = 38.48 \text{ mm}^2 \text{ where the diameter of the well is } d = 7 \text{ mm.}$$

From *Figure 5*, the number of cells in the well are measured to be $n = 519$ at 10x magnification.

Using the 120 μm scale bar (see *Figure 1*) and the ‘Measure’ tool in ImageJ, the total area of the image in *Figure 4* (which is the same area as *Figure 1*) is found to be $1264.25 \mu\text{m} \times 923.149 \mu\text{m} = 1167091 \mu\text{m}^2 = 1.1672 \text{ mm}^2$ and the area that is covered by cells is approximately 13.96% of the total image area.

Therefore, the area of an average individual cell can be calculated as below:

$$A_{avg\ cell} = A_{cells}/n = (1167092 * 13.96\%) / 519 = 313.9470 \mu\text{m}^2$$

For 100% confluency in the rest of the well, we can estimate the number of cells as:

$$n_{cell, 100\%} = A_{well}/A_{avg\ cell} = 38.48 * 10^6 \mu\text{m}^2 / 313.9470 \mu\text{m}^2 = 122250 \text{ cells per well}$$

By taking the average confluence under different magnifications (see Table 1) and rounding it to the nearest value, we assume 15% confluency for the rest of the well:

$$n_{cell, 15\%} = 122250 * 15\% = 18337 \text{ cells per well}$$

Table 1: Measured values needed to estimate number of cells in the well for magnification factors 10x, 20x and 40x. The estimated number of cells with the assumption of 15% confluence is provided in the last row, for each magnification factor.

	10X	20X	40X
Confluency (%)	13.96%	12.09%	17.84%
Cell count (n)	519	157	70
Area of the Image (mm^2)	1.1672	0.5933	0.3015
Estimated Average Cell Area ($A_{avg\ cell}$) (μm^2)	313.9470	456.9341	768.6371
Estimated Number of Cells with 15% Confluency ($n_{cell, 15\%}$)	18337	12599	7490

c. A bar plot for your estimated number of cells per well for 10x, 20x, and 40x. Remember to plot your data with error bars.

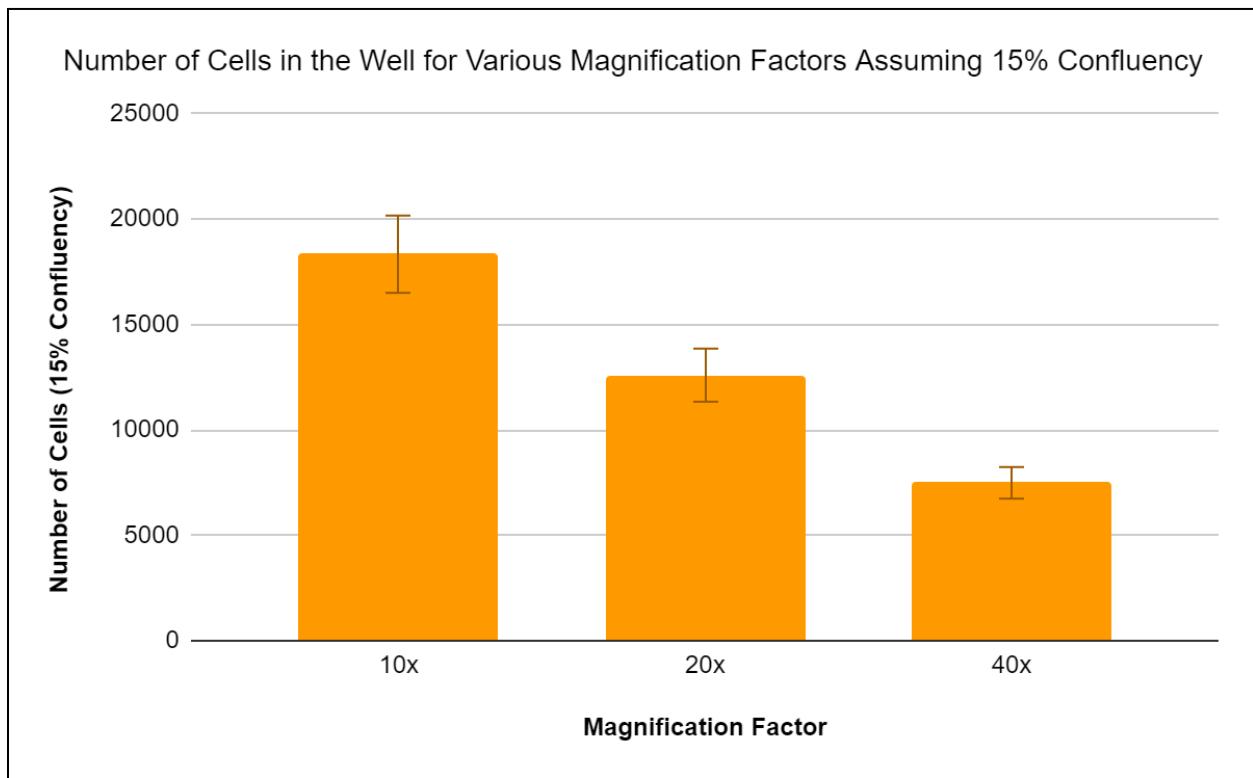


Figure 10: Bar plot of estimated number of cells in one well of the 96-well plate using TRITC-Phalloidin stained cytoskeleton and DAPI stained nuclei images of fixed U87 cells at 10x, 20x and 40x magnification. The number of cells and their area were calculated using ImageJ. The measured values that were used in the estimations are provided in Table 1.

Note:

As mentioned in the Materials and Methods section, since the images we obtained in the lab were from different locations in the wells, we weren't able to use and analyze our images. We were provided with the images from only one well to use in this report, therefore the bar plot provided in *Figure 10* only includes data obtained from one well instead of 3 separate wells.

3. Plot how photobleaching affects TRITC-Phalloidin fluorescence over 20 minutes. Please include:

a. Images of TRITC-Phalloidin fluorescence after 0 minutes, 4 minutes, 10 minutes, and 20 minutes of photobleaching.

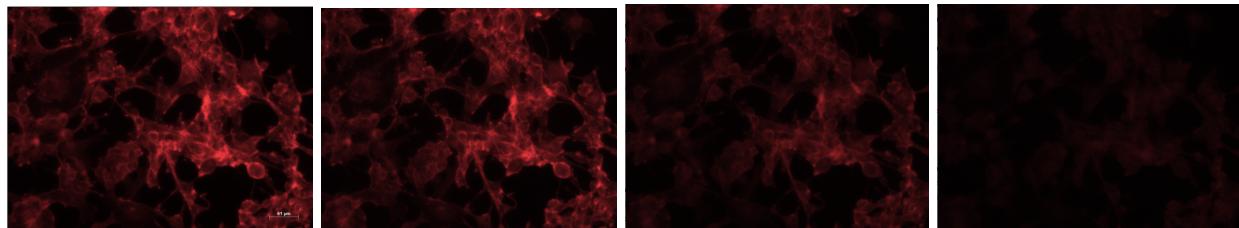


Figure 11: TRITC-Phalloidin fluorescence images of fixed U87 cells taken using Leica DMI 4000 microscope under red illumination after 0, 4, 10 and 20 minutes of photobleaching (from left to right, respectively). Decreasing fluorescence intensity is observed over time.

b. A line plot of how photobleaching affects the fluorescence intensity of TRITC-Phalloidin over time on a per cell basis. Your analysis should be done with ImageJ.

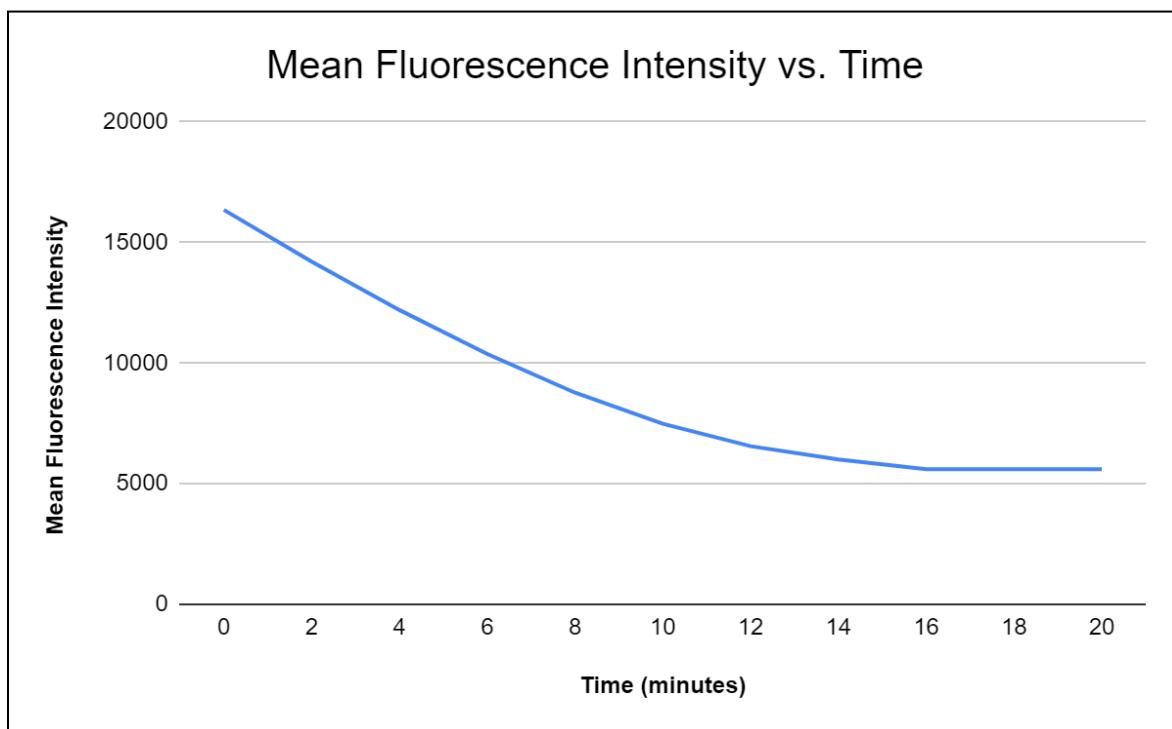


Figure 12: Line plot of fluorescence intensity of TRITC-Phalloidin over a 20-minute time period, with 2 minute intervals, during photobleaching. Fluorescence intensity is measured on a cell basis using ImageJ. Decreasing intensity is observed over time.

4.0 Discussion

1. The estimated number of cells per well is provided in Table 1, as 18337, 12599 and 7490 with the assumption of 15% confluence for 10x, 20x and 40x magnification factors. By inspecting *Figures 5, 7 and 9*, we observe that ImageJ provides more accurate results of the cell count at higher magnification (e.g. 40x), since it is easier to distinguish between closely clustered cells. On the other hand, lower magnification (e.g. 10x) covers a larger field of view (FOV) enabling it to capture more cells, although it lacks the resolution to count individual cells accurately.

Therefore, the number of cells at 20x is most likely to be accurate, since it has a balance of reasonable FOV and detail of resolution. The estimates are likely to be underestimated, since ImageJ cannot distinguish closely clustered cells and counts them as one, even at higher magnifications.

2. When estimating the size of an individual cell the field of view and magnification is a limitation, since the chosen field may not represent the rest of the well. At lower magnifications, lower resolution could lead to inaccurate measurements. Cell overlapping and clustering makes it difficult to distinguish between individual cells, which can lead to underestimation of the cell size. Difference in the shape of the cells also makes it challenging to estimate a single average size of the cell. Additionally, image processing artifacts may occur due to over or under thresholding, which may exaggerate or diminish the actual size of the cell. Analyzing the cell at multiple magnifications, using high-resolution imaging techniques, manually measuring a sample and using image segmentation techniques to distinguish overlapping cells can be used to improve the quality of approximation of the average cell size.

3. Uneven distribution of actin filaments in the cell and the varying degree of stain absorption of cells could result in homogeneous TRITC-Phalloidin distribution, which may result in inaccurate measurements of the photobleaching. The light intensity and uniformity also affects the rate of photobleaching and could introduce errors in measurement if brightness varies across the measured area. The size and shape of cells may affect the illumination of the cell as well. As discussed previously, resolution, image processing artifacts and the limitations of our software ImageJ could introduce errors when taking precise measurements. Lastly, the rate of photobleaching strongly depends on the specific environment [5], which may be challenging to quantify accurately on a per-cell basis. To avoid these errors, different imaging techniques can be used for better resolution and more advanced image analysis softwares can be utilized for precise quantification of fluorescence in individual cells.

4. The sharpness of our images can be evaluated based on how clear and well-defined the cell structures are, such that actin filaments and nuclei are presented with a high level of detail and are clearly distinguishable. Our images in *Figures 1, 2 and 3* are fairly sharp, since actin (in red) and nuclei (in blue) are clearly distinguishable with good contrast. At higher magnification (e.g. 40x, see *Figure 3*) the resolution is the highest, although the image appears to be more blurrier. Assuming the sample is stable and the plate is laying flat, this blurriness may be attributed to inadequate lighting. To further improve the resolution of the image, we can use higher numerical aperture (NA) to capture more light and increase resolution, to obtain a clearer image

5.0 References

(1) Chan, W.; Zhang, X.; Fiddes, L.; Syed, A. M.; Hoang, G.; Poon, W.; Fernandez-Gonzalez, R.; Kilkenny, D.; Shukalyuk, A.; Albanese, A.; Keith, B.; Mansouri, F.; Ming, A.; Chou, L., *2023-2024 BME440H Lab 4: Fluorescence Microscopy & Image Analysis of Cells, Lab IV*, University of Toronto, Department of Biomedical Engineering, 2023.

(2) *Cytoskeleton Structure*

https://www.thermofisher.com/ca/en/home/life-science/cell-analysis/cell-structure/cytoskeleton.html?gclid=CjwKCAiAsIGrBhAAEiwAEzMlCyJKibPyp9AYN6zrs8Ha24nb6cCPG-QNBRs3AnT-NeBae0f_KbkMThoC3HEQAvD_BwE&ef_id=CjwKCAiAsIGrBhAAEiwAEzMlCyJKibPyp9AYN6zrs8Ha24nb6cCPG-QNBRs3AnT-NeBae0f_KbkMThoC3HEQAvD_BwE%3AG%3As&s_kwcid=AL%213652%213%21657171723547%21p%21%21g%21%21phalloidin%212031782395%2170575884303&cid=bid_pca_iva_r01_co_cp1359_pjt0000_bid00000_0se_gaw_nt_pur_con&gad_source=1 (Accessed Nov 24, 2023).

(3) *DAPI protocol for fluorescence imaging*

https://www.thermofisher.com/ca/en/home/references/protocols/cell-and-tissue-analysis/protocols/dapi-imaging-protocol.html?gclid=CjwKCAiAsIGrBhAAEiwAEzMlC70o6XJdhJ57D6gCjgRZ1WexvO5V07DivsBPxac7YxOOzA8ZUuLwxoCtR0QAvD_BwE&ef_id=CjwKCAiAsIGrBhAAEiwAEzMlC70o6XJdhJ57D6gCjgRZ1WexvO5V07DivsBPxac7YxOOzA8ZUuLwxoCtR0QAvD_BwE%3AG%3As&s_kwcid=AL%213652%213%21447292198721%21%21g%21%2110506731179%21109642167331&cid=bid_pca_iva_r01_co_cp1359_pjt0000_bid00000_0se_gaw_dy_pur_con&gad_source=1 (Accessed Nov 24, 2023).

(4) *Eppendorf Consumables Technical Data Sheet Cell Culture Plate 96-Well*; Eppendorf, 2013; pp. 1-2.

https://www.eppendorf.com/product-media/doc/en/90696/Eppendorf_Consumables_Technical-data_Cell-Culture-Plate-96-Well.pdf

(Accessed Nov 24, 2023).

(5) Hummert, J.; Yserentant, K.; Fink, T.; Euchner, J.; Ho, Y. X.; Tashev, S. A.; Herten, D.-P. *Photobleaching step analysis for robust determination of protein complex stoichiometries* <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8693960/> (Accessed Nov 24, 2023).