Lab Report

Advanced Bio-Electronics

Quantifying Cell Morphology

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The shape of the cell is influenced by the external and internal forces acting on the cell cytoskeleton at any given moment. During the cell circle of life, the forces dynamic changes and therefore may represent the cell state. In this experiment, the influence of trypsin activity on two different fibroblastic cells concentration was examined. In the analysis of the results, we used two algorithms in the MATLAB software based on image processing methods. The results indicate that Trypsin activity causing morphology changes and all the cells that were observed become rounder. According to our results, we couldn't find clear differences between the trends in high and low confluence when examining the trypsin influence on cells shape; at the same time, when comparing the results to the initial state respectively, mainly for eccentricity parameter, it can be seen that in low confluence the cells become rounder.

1. Introduction

Prokaryotic and eukaryotic cells

A cell is the smallest working unit of all living organisms on our planet earth, which is capable of performing life functioning. Cells are of different shape, size and structure according to the function they needs to perform. Each cell type has its own role to play in helping our bodies to work properly, and their shapes help them carry out these roles effectively. There are two primary types of cells – Prokaryotic and eukaryotic. Prokaryotic cells are the simple cells of organisms like bacteria, whereas eukaryotic cells are the more complex cells that make up plants, fungi, and animals. Eukaryotic cells are cells that contain a membrane-bound nucleus. In addition to the nucleus, eukaryotic cells are characterized by numerous membrane-bound organelles such as the endoplasmic reticulum, Golgi apparatus, chloroplasts, mitochondria, lysosomes and others. Each of these organelles performs a specific function critical to the cell's survival. The physical structure of the cell is maintained by the cytoskeleton, an interconnected network of filamentous polymers and regulatory proteins. The cytoskeleton carries out three broad functions: it spatially organizes the contents of the cell; it connects the cell physically and biochemically to the external environment; and it generates coordinated forces that enable the cell to move and change shape.

The physical structure causing by cell's organelles

The shape of the cell is defined by the geometrical information of the space occupied by the cell and is determined by its external boundaries; the physical structure of the cell is based on a few important components: mainly by the cytoskeleton, also the membrane, Cytoplasm and Its Organelles, Endoplasmic Reticulum, Filament and Tubular Structures of the Cell.

Additionally, there are more organelles that maintain a major role in the cell functions as: Ribosomes, Golgi apparatus, Lysosomes, Peroxisomes, Secretory Vesicles, Mitochondria and the nucleus.

The importance of cell shape

The basic structure and function of all cells is similar, but there is a difference between them in relation to the unique functioning of each cell. In other words, there is a correlation between the structure of the cell and its function. The difference in the shape of the cells stems from the need to adapt them to function optimally. A muscle cell and a neuron, for example, differ in their functions and roles, which must be filled with different structures that allow for the idealization of the required functions. At the cellular level, the various organelles are designed to fill different roles in the cell cycle.

Cell's forces and conversion of cell's shape

Although the cell shape is usually fixed, there are changes as a result from the dynamic mechanical balance of the forces exerted on the cell membrane by intracellular components and the outside environment. Intrinsic forces exerted on the membrane are mostly the direct result of the reorganization of the cytoskeleton. Intracellular pressure of osmotic origin or generated by contraction of cytoskeletal networks can lead to cell deformations. Extrinsic forces exerted on the cell are mainly due to its adhesion to environmental components, such as

neighboring cells or the extra-cellular matrix (ECM) (Paluch, 15 September 2009). Shape change of individual cells, independent of their neighbors, contributes to different morphogenetic processes in development, such as the migration of single primordial germ cells towards the gonad. However, in most morphogenetic events, cell shape change is coordinated amongst hundreds of neighboring cells and drives shrinkage, extension, folding and movement of tissues. Cell shape undergoes conversion naturally, for example:

1. **Cellular differentiation** occurs numerous times during the development of a multicellular organism as it changes from a simple zygote to a complex system of tissues and cell types. Differentiation continues in adulthood as adult stem cells divide and create fully differentiated daughter cells during tissue repair and during normal cell turnover. Differentiation dramatically changes a cell's size, shape, membrane potential, metabolic activity, and responsiveness to signals.
2. **Immune cells** are cell that respond when the body is infected. To do their job, they need to be able to change shape. For instance, lymphocytes may need to move through body tissue to get the site of infection, so they change their shape to squeeze past tightly packed tissue cells. Some immune cells (such as neutrophils) engulf bacteria and viruses, so they need to change their shape to "swallow" them.

In addition, besides the effecting of mechanical properties on cell's shape, it also effects on cell phenotype. Different cells might change their functionality depending on their environment. For example, vascular smooth muscle cells display remarkable plasticity and can undergo phenotypic changes depending on their environment. (F.Liu, 2012)

Cells interaction

The nature of the interactions of a cell with other cells and a substrate is an important issue in biology. Cell adhesion is the ability of a single cell to stick to another cell or an ECM. Adhesion plays an integral role in cell communication and regulation. Cell adhesion is the process by which cells form contacts with each other or with their substratum through specialized protein complexes. Intercellular adhesion can be mediated by adherens junctions, tight junctions and desmosomes, whereas cells can interact with extracellular matrix molecules through focal adhesions. Commonly substrates are collagen, fibronectin, and laminin. Cells interact with these matrix components via cell surface receptors, such as integrins. Receptors bind to domains of collagen, fibronectin and laminin and trigger intracellular signaling pathways that facilitate adhesion complex formation, and can also trigger cell proliferation or differentiation. When the cells assemble and connect to each other by formatting cell-cell adhesion, the cell's shape changes.

Trypsin

Trypsin is enzyme that cleaves peptide bonds in proteins, in which serine serves as the nucleo philic amino acid at the active site. Trypsin catalyzes the hydrolysis of peptide bonds, breaking down proteins into smaller peptides [study.com]. The peptide products are then further hydrolyzed into amino acids via other proteases, rendering them available for absorption into the blood stream. Trypsin is the most commonly used enzyme in tissue culture to release the adherent cells from culture vessel surface and/or to disaggregate tissue into single cell suspension. Cell-cell and cell-matrix interaction depend on the adhesion molecules, which in turn, relies on the presence of calcium. Most trypsin solutions for cell culture also contain EDTA which acts as a chelator for calcium. By removing calcium from a solution with cells, cadherins (calcium dependent adhesion) which hold cells to each other, are broken and cells separate from each other as well as from the surface of the tissue culture plastic. Optimum activity is achieved at 37C, so pre-warmed trypsin speed up the detachment. Long term incubation with high trypsin concentration damage cells by striping cell surface proteins and kill the cells. It can be expected that if the cell-cell adhesion will be damaged the cells will separate from each other and the organized tissue will break up. The cells are expected to float in the medium and to receive a more rounded shape. This process may end in apoptosis.

2. Methods

Principles of quantitative characterization of shapes

According to the literature, conventional quantitative methods are mostly based on long/short axis of cell to derive the total area of cell spreading. The region of interest for quantifying the area is the inner part of the cell, while the boundaries of the cell are brighter in comparison to the outer part of the cell, which allows the understanding what is the wanted area for quantification.

In the current experiment we will examine fibroblast cells, which are elongated. In order to quantify the elongation of the cells there need to present basics parameters[11]. The following quantitative parameters of the cell culture that we based on in our analysis are:

- **Area:** After processing, the image is binary where 0= non cell pixel and 1= cell suspected pixel. The area is the sum of pixels in a region suspected to be cell pixels.
- **Major Axis**: Length of the major axis of the ellipse that has the same normalized second central moments as the region.
- **Minor Axis:** Length (in pixels) of the minor axis of the ellipse that has the same normalized second central moments as the region.
- **Eccentricity**: The eccentricity is the ratio of the distance between the foci of the ellipse and its major axis length. The value is between 0 and 1. (0 and 1 are degenerate cases. An ellipse whose eccentricity is 0 is actually a circle, while an ellipse whose eccentricity is 1 is a line segment.)
- **Perimeter**: Distance around the boundary of the region

Experiment design

This experiment focused on quantitative morphological characterization of cells which are cultured in 2D monolayers and exposed to a substance (Trypsin) which cause alteration in cell shape and cell dissociation from the substrate. Images of the cells were acquired at different time points for post analysis. Our hypothesis was that after exposure to trypsin, the cells change shape to a rounder one as seen in figure 2, due to the change in the

forces acting on the cells. In addition, it is observed that for lower cell density, we detected a faster change in the form because there are fewer intracellular connections when the distance between the cells is greater.

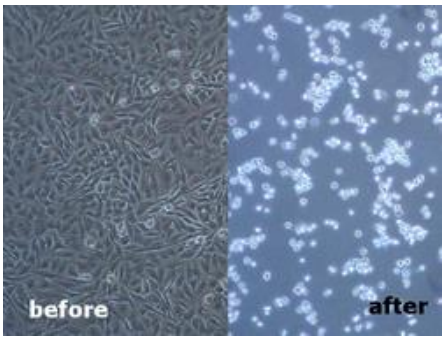


Figure 1: *Cells before and after trypsin treatment (biochrom)*

Equipment

- 2 wells of cell cultures (fibroblastic cells)
- Lab equipment (pipettes, test tubes, gloves, lab coats, laminar-flow hood, incubator)
- Growth medium (GM)
- Trypsin
- PBS (Phosphate buffered saline)
- Phase contrast microscope (Eclipse TS100; Nikon)
- Camera (DS-Fi1 camera; Nikon)

Our aim was to examine the influence of trypsin activity on different cells confluence. The experiment setup included PBS, trypsin and 2 wells of fibroblastic cell culture which are maintained in a growth medium. the wells differ from one another in the cell confluence: high and low. The experiment is divided into two parts:

1. The well of low cell confluence was taken out from the incubator, then GM was pumped out of the well. The dish was set on the microscope stage (Eclipse TS100; Nikon) and an image of the culture was captured at an original magnification of 20 (DS-Fi1 camera; Nikon). Immediately after the first image was acquired, five additional images were taken too at the same field of view in an interval of 1 minute.
2. The same process was done for the second dish which included high cell confluence.

The region of interest was contained a large number of cells so that the results were statistically validated and at this side the cell boundaries were as clear as possible in order to facilitate the processing of information in the MATLAB code. During the experiment, the cells were washed with PBS before trypsin treatment because most of the growth media (GM) for cell culture contains Fetal Bovine Serum (FBS). FBS contains protease inhibitors, such as α1-antitrypsin and α2-macroglobulin. These inhibitors stop the trypsin protease and act by inhibiting lysosomal peptidases that may be released. [symposcium]

1. •Taking the wells out of the incubator(37°c)
2. •Pumping out the GM of well
3. •Taking one image of the cell culture
5. •Adding Trypsin
6. •Taking five additional images of the cell culture in the same FOV and in interval of 1 minute.
7. •Continuing the same process for the second well (high confluence)

Diagram 1: *Schematic drawing of the experiment setup.*

Image processing

In this lab, we were asked to write an automatic algorithm for quantitative characterization of cell morphology. Advanced image processing tools such as dilation, erosion, and edge detection were used for image segmentation, allowing detection and separation of the cells from their environment. Finally, an automatic algorithm was written by using MATLAB program (**שגיאה! מקור ההפניה לא נמצא.**). It should be noted that an additional algorithm was written and it describes accurate segmentation than the first algorithm. However, all the results in this report have based on the first algorithm due to writing the additional algorithm after the results processing (more details of the second algorithm will be presented below). Regarding the first algorithm, the function output is segmented cells and morphological characterizations. In order to extract cell's properties a Regionprops MATLAB function was used. This function measures a set of properties for each labeled region in a binary image, such as area centroid, perimeter etc.

1. •Histogram Equalization- stretching the image to 256 gray levels.
2. •Edge detection - Canny function (output = binary image).
3. •Dilation: SE = Line in different angles.
4. •Useing Imfill function.
5. •Opening: SE = Disk with radius 6.
6. •Regionprops function.
7. •Filter out non-cell sturcture by defining area threshold.

Diagram 2: *Flow chart of the automated algorithm*

3. Results

The algorithm was applied to images of cells in different confluence (low and high) as well as on the ideal images received from the lab guide. For examination of morphological changes in the cell, 3 cells were selected. This amount was chosen because the cell culture contains the same kind of cells, therefore this amount is enough for analysis.

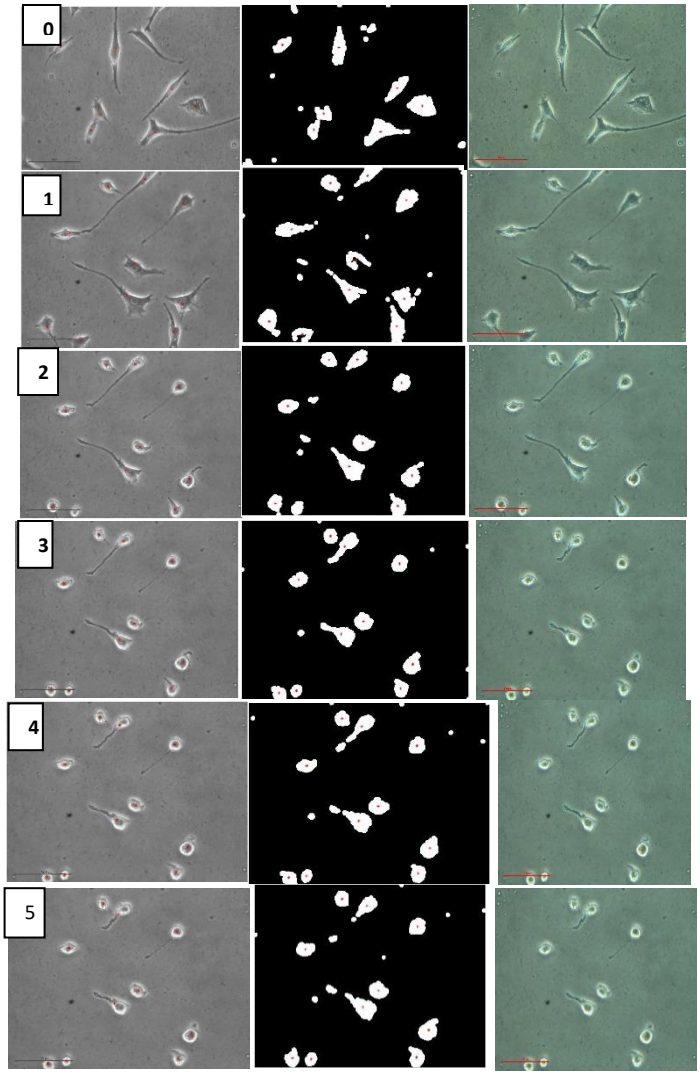


Figure 2: *Low confluence cell culture - segmented cells appearance according to time (0 – the first captured picture of cell culture and 5 – the last captured picture)*

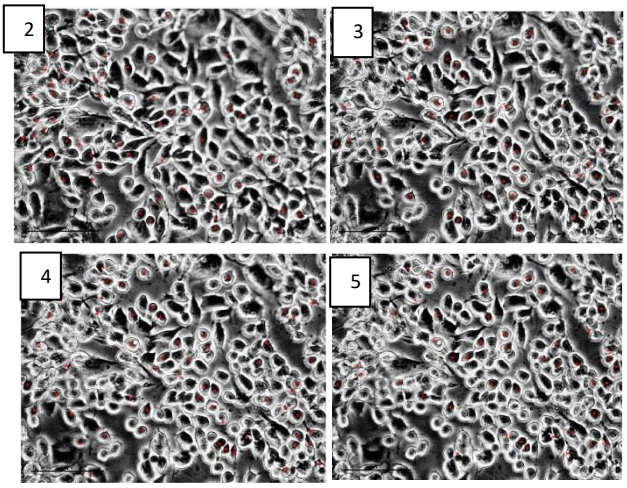
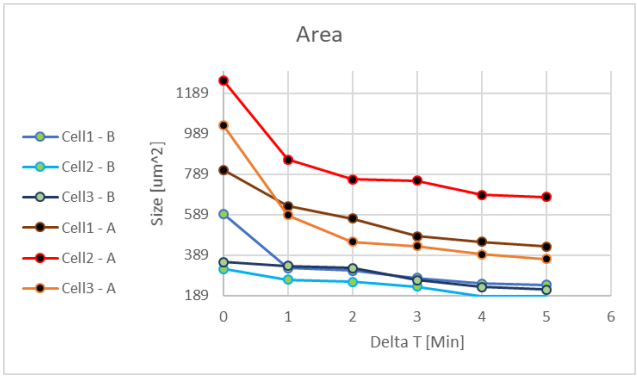
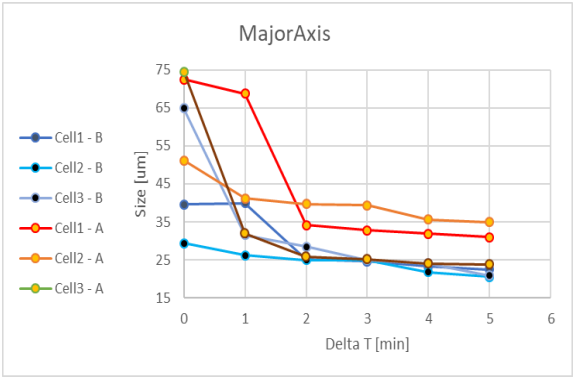


Figure 3: *High confluence cell culture - segmented cells appearance according to time (2 – image was captured after 2 minutes and 5 – after five minutes)*

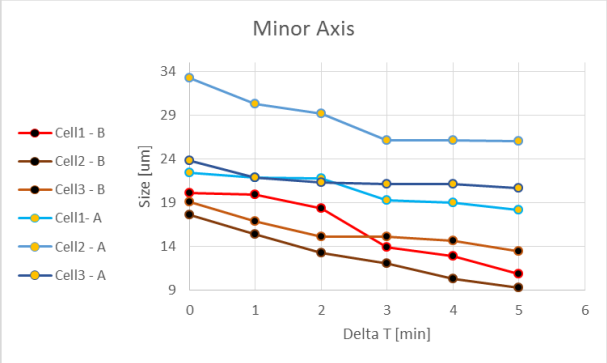
As can be seen in figures 3 and 4, regarding our expectations, the cell's shape changes and the cell become more round over time. It should be noted the cells from the low population were recognized clearly than in the high population. The reason for that is the high separation of the cells. The graphs below describe the trend of morphological parameters. These graphs based on data that are shown in the appendix.



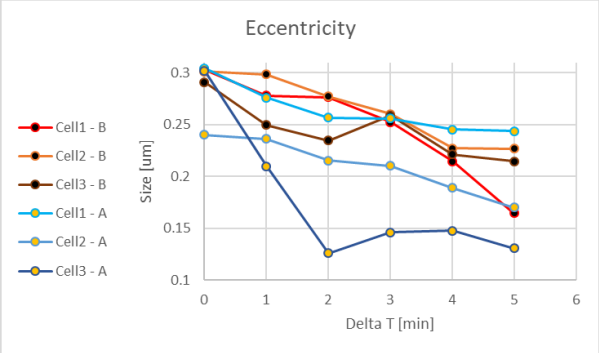
Grap1: *Area summation of the 3 cells in high and low confluence (B, A respectively)*



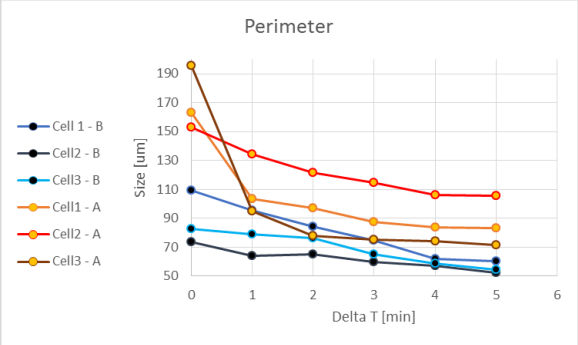
Graph 2: *Major axis of the 3 cells in high and low confluence (B, A respectively)*



Graph 3: *Minor axis of the 3 cells in high and low confluence (B, A respectively)*



Graph 4: *Eccentricity summation of the 3 cells in high and low confluence (B, A respectively)*



Graph 5: *Perimeter summation of the 3 cells in high and low confluence (B, A respectively)*

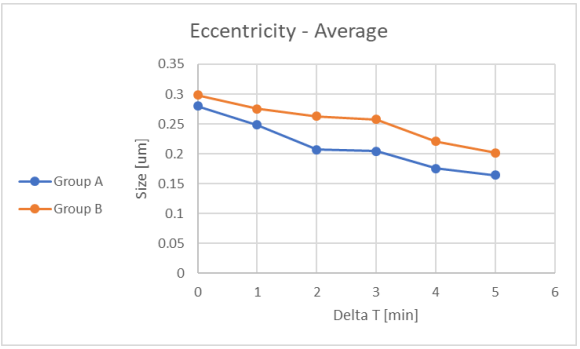
As can be seen, the results for low cells confluence as well as high confluence describe downward trend over time. In both cases, it was observed that all morphological characteristics would be changed and reduced because of the effects of trypsin, PBS and the degree of cell confluence. For high confluence cells, it was observed that more small changes would occur due to the high number of neighbors around each cell in comparison to the low cell confluence. As mentioned earlier, shape's changing of individual cells depends on their neighbors. Neighbor's cells exert extrinsic forces on the cell. Therefore, it was expected that in an area filled with cells, the cell would be tenser, thus, the shape would be changed less. The degree of changing in morphological characteristics both low and high confluence is shown in table 1. As can be seen, the results are settled with our expectations. Indeed, changes in low confluence relatively high than in high confluence.

The calculations in table 1 based on: $\frac{initial\ size - final\ size}{initial\ size} * 100$

Properties	Low cell confluence [%]	High cell confluence [%]
Area	55.6	49
Major axis	55.542	52.357
Minor axis	20.181	40.791
Eccentricity	41.22	32.324
Perimeter	49.159	37.146

Table 1*: Degree changes in morphological characteristic – low and high confluence*

In order to summarize the comparison between the populations, we chose to look at the average level of the eccentricity. This measure reflects the degree of roundness of the cell.



Graph 6: *Average Eccentricity compression. Red-high confluence, blue-low confluence.*

Ideal images

The automated algorithm was applied to the ideal images as shown below

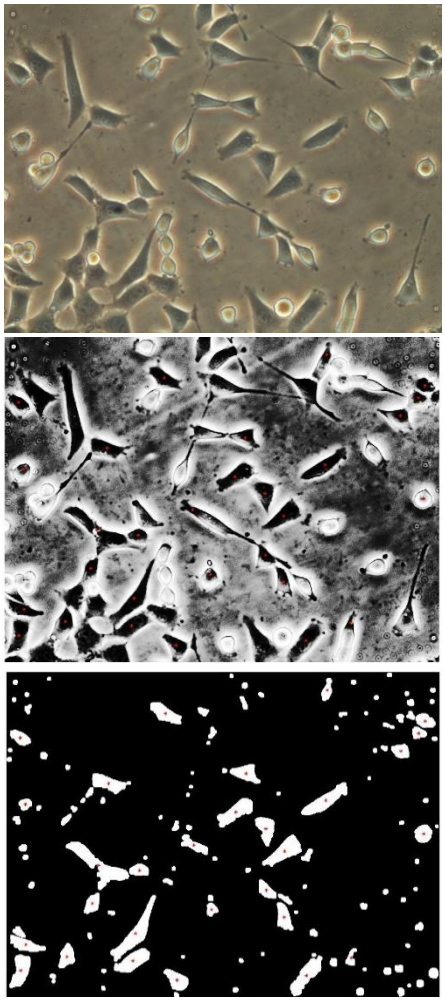


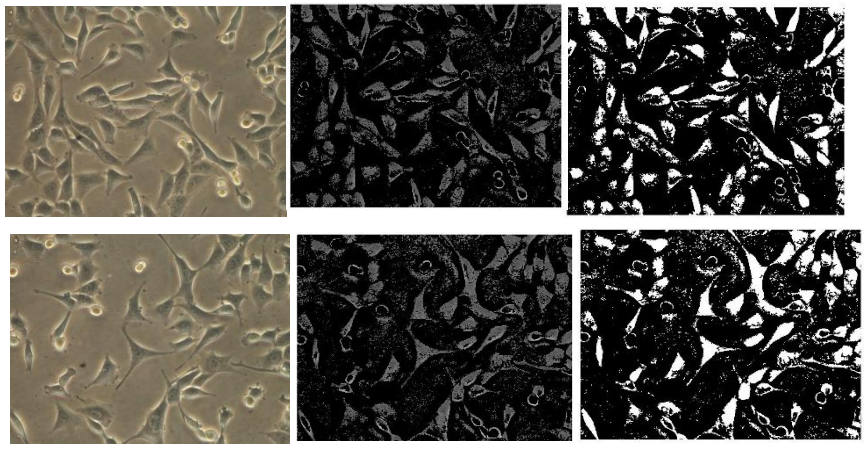
Figure 4: *example of the algorithm stages in ideal image (original image, detected cells, binary image respectively)*

In addition to the algorithm described above, another algorithm will be described which improves the quality of results. This algorithm based on segmentation using k means clustering. K-means clustering is a type of unsupervised learning, which is used when the data is unlabeled (i.e., data without defined categories or groups). The goal of this algorithm is to find groups in the data, with the number of groups represented by the variable K. The algorithm works iteratively to assign each data point to one of K groups based on the features that are provided. In addition, today there is a trend to segmented images using color images, mainly because color images can provide more information than gray level images. The accuracy in segmentation of a color image depends not only on the algorithm used but also on the color space selected. Effective segmentation is achievable through clustering. In this algorithm the LAB space was chosen due to the accuracy clustering.

1. • Reading image
2. • Converting Image from RGB Color Space to Lab Color Space
3. • Classifying the Colors in 'ab' Space Using K-Means Clustering
4. • repeating the clustering 3 times to avoid local minima
5. • Labeling Every Pixel in the Image Using the Results from K means
6. • Creating Images that Segment the cells Image by Color.
7. • Segmenting the cells into a Separate Image
8. • Converting to grayscale image
9. • Converting to BInary image
10. • Opening using small SE for removing noises
11. • Filling holes caused from opening

Diagram 3: *Flow chart of the automated algorithm*

Figure 6: *Algorithm performance on 2 ideal images. The original image(left) compared to the segmentation image(right).*



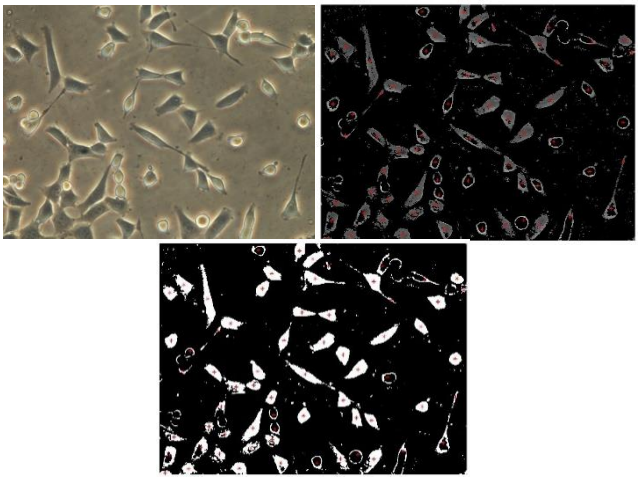


Figure 7: *example of the algorithm stages in ideal image (original image, detected cells, binary image respectively)*

4. Discussion

In this experiment, we have focused on quantitative morphological characterization of cells which were cultured in 2D monolayers and exposed to Trypsin. As mentioned before, Trypsin is an enzyme commonly found in the digestive tract, can be used to "digest" the proteins that facilitate adhesion to the container and between cells. When trypsinization process is complete the cells become in suspension and appear rounded.

Morphological changes in cells are related to modifications in the cytoskeleton and ECM components. Alterations in cytoskeletal and ECM components are related to cell function, stiffness, and morphology through physical interactions of cells with their environment and/or surrounding cells. Here, we used a method for quantifying these morphological changes throughout the trypsinization process. This process damages the cell-cell adhesion and cell -ECM. Regarding research [Sung Sik Hur, 2012] about cell confluency and tension, the tension is significantly higher for confluent monolayers than for subconfluent cells, as well as the intracellular tension decays rapidly from the cell junction to the cell edges in subconfluent cells. Therefore, our expectations are to minor changes in morphologic parameters in high confluence comparing to low confluence.

In the current experiment, the effect of trypsin appears to be similar in both cell populations which were checked. While examining the minor axis and the roundness we can identify relatively moderate change while the major axis changes relatively immediately and also indicate a change in the cell area (a separate graph). Although the trends are similar, while examining the eccentricity it can be seen that in low confluence the cells became rounder than the other group compared to the initial state. It may be influenced from two reasons; first, the amount of the trypsin was equal both low and high cells confluence, but in relation to the degree of density, less trypsin impact on each cell in the high concentration (surface-to-volume ratio). Therefore, a little difference appeared between the plats. Another reason is that less cells effect on other cells (fewer intracellular connections) while the density is lower, thus the shape is rounder when there is no specific force in some directions. In high concentration it might take more time to identify the trypsin activity by changing in the shape.

As written in the introduction, changes in cell size and shape are strongly influenced by the life cycle of the cell. Therefore, the algorithm we developed based on image processing techniques, and allows the analysis of quantitative parameters for monitoring the cell is an important method and means to continue many studies. The algorithm we developed serves as an indication of the general trend but contains errors that result from the use of morphological operators. Since the trend we have received among the different cell populations is similar, we will recommend reducing the time intervals. It may be that, in shorter periods of time, the effect of cell density on the ability of trypsin to reach and sever all connections. In addition, we recommend increasing the duration of time for taking photos, perhaps, in this way we might observe high differences between the two populations. In our opinion, different algorithm based on clustering might yield clearly differences between the two population. However, the automated algorithm we developed gives the correct trend and expose us to the changes of cells.

5. Reference

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6. Appendix

Delta T	Cell1	Cell2	Cell3	Cell4
0	811.2301	1252.294	1031.113	1180.198
1	632.8873	862.3564	588.052	837.8917
2	569.5786	762.7001	455.5431	603.5297
3	483.5028	756.6089	433.0755	552.5032
4	454.6444	688.0078	393.1331	423.5892
5	431.2781	675.6257	370.965	418.9958

Table 2: *Area changes of cells high confluence (um^2)*

Delta T	Cell1	Cell2	Cell3
0	594.213004	322.1462	355.8435
1	325.464076	267.401	337.2841
2	312.296208	257.3437	326.0862
3	276.939964	233.3927	266.7789
4	249.048968	188.0828	231.9411
5	241.894772	187.7717	219.0843

Table 3: *Area changes of cells low confluence (um^2)*

Delta T	Cell1	Cell2	Cell3
0	39.59613083	29.37311	64.90812
1	39.88666442	26.17754	31.48707
2	25.19776253	24.92661	28.42018
3	24.61034521	24.91759	24.91759
4	23.22214341	21.71403	24.00843
5	22.46963257	20.46821	20.84537

Table 4: *Major axis length changes of cells high confluence (um)*

Delta T	Cell1	Cell2	Cell3	Cell4
0	72.53075	51.18416	74.59148	57.9508
1	68.72515	41.20859	31.89558	48.75886
2	34.14013	39.71268	25.70702	32.60267
3	32.73034	39.39716	25.07109	32.58746
4	31.87724	35.57668	23.92666	29.45788
5	31.01534	34.92645	23.73855	24.24573

Table 5: *Major axis length changes of cells low confluence (um)*

Delta T	Cell1	Cell2	Cell3
0	20.08265531	17.65068	19.12505
1	19.95263856	15.41482	16.88677
2	18.34044836	13.28488	15.14562
3	13.92939575	12.0511	15.14004
4	12.91428909	10.36409	14.62661
5	10.88529781	9.324514	13.45546

Table 6: *Minor axis length changes of cells high confluence (um)*

DeltaT	Cell1	Cell2	Cell3	Cell4
0	22.42402	33.214	23.84122	29.50859
1	21.8972	30.25888	21.90419	24.90307
2	21.73886	29.18916	21.30223	24.7521
3	19.26938	26.14491	21.1485	22.71319
4	19.04624	26.07961	21.1485	22.44043
5	18.18337	26.02339	20.63827	22.14796

Table 7: *Minor axis length changes of cells low confluence (um)*

Delta T	Cell1	Cell2	Cell3
0	0.303416294	0.30129	0.291118
1	0.278207781	0.298604	0.249842
2	0.276493963	0.277453	0.234934
3	0.252661897	0.260272	0.258994
4	0.214710835	0.227366	0.221335
5	0.164744012	0.226785	0.214728

Table 8: *Eccentricity changes of cells high confluence (um)*

Delta t	Cell1	Cell2	Cell3	Cell32
0	0.304437	0.240433	0.302068	0.271965
1	0.276155	0.236389	0.209915	0.271676
2	0.256987	0.215471	0.126222	0.229536
3	0.255997	0.210191	0.146049	0.205669
4	0.245546	0.18916	0.147792	0.119652
5	0.243657	0.170042	0.130652	0.113332

Table 9: *Eccentricity changes of cells low confluence (um)*

Delta T	Cell1	Cell2	Cell3
0	109.247516	73.78694	82.60137
1	95.456256	64.24802	78.72642
2	84.303464	65.28872	76.20194
3	74.439316	59.90166	64.90812
4	61.867148	57.00173	58.73538
5	60.346664	52.29087	54.32526

Table 10: *Perimeter changes of cells high confluence (um)*

Delta T	Cell12	Cell22	Cell32
0	163.3518	153.2335	195.6343
1	103.2088	134.2159	95.11726
2	96.79459	121.4344	77.6254
3	87.24823	114.6973	75.14543
4	83.98142	106.3233	74.02426
5	83.27548	105.5712	71.57021

Table 11: *Perimeter changes of cells low confluence (um)*