AN IMAGE PROCESSING APPROACH TO DETERMINE THE MORPHOLOGICAL CHANGES IN CELL NUCLEUS

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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By

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CERTIFICATE

This is to certify that the research project report entitled "**An image processing approach to determine the morphological changes in cell nucleus**" submitted by **Soham Mukherjee**, in partial fulfillment of the requirements for the award of the Degree of Master of Technology in Biotechnology and Medical Engineering with specialization in Biomedical Engineering at National Institute of Technology Rourkela is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

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CONTENTS

Abstract

Confocal imaging has been a powerful tool for scientists over the decades for visualization of cellular architecture and behavior. However, the quantitative inference drawn from the confocal images typically relies upon image processing. So far many image processing tools are available that can quantify various image parameters and image characteristics i.e., intensity, area, shape, volume, perimeter, etc.. However the success of the existing techniques depends on high picture clarity and efficient noise removal. Now in this regard, lots of scope remains over the increase of the quality of the native image.

Moreover, the existing procedure of image processing fails to quantify morphology distortion properly. Though pattern recognition algorithms can often be used to measure the changes, but is seldom able to provide reliable data when the input elements are taken from a pool of varying diversity. Keeping this perspective in mind, we have developed a MATLAB image analysis program for processing of universal confocal images and quantification of cell shape factors. The confocal images were properly processed for efficient noise removal and then subjected to skeletonization algorithm for quantification of cell nucleus shape change.

The program was tested to quantify the nuclear mechanotransduction. The nucleus is a key component of the cell and its shape changes with a change in environment. Scientists have now discovered that such variation leads to altered cellular and nuclear function.

CHAPTER 1

INTRODUCTION

1.1 Cell and its Components.

The term cell is derived from Latin word *cella*, meaning “small room”. It is the fundamental, structural, functional, and biological component of life. Cells are the smallest unit of life that can reproduce independently, and are also called the "building blocks of life." So, the basic ailments in a living organism can be traced down to its cellular level. A cell can be divided into two 2 tyes the prokaryotic cell (DNA devoid of Membrane or nucleus) and the Eukaryotic cell (DNA embedded in a membrane or nucleus). Human cells are eukaryotic in nature and contains various components like Plasma Membrane, Centriole, Endoplasmic Reticulum, peroxysome, lysosome, mitochondria, Golgi apparatus, Ribosome, Nucleus, Etc.

1.2 Brain of the Cell: Nucleus

Although each of the cellular components have their specific tasks related to the proper functioning of the cell and each one is as important as the other, the Nucleus is known as the control center or the brain of the nucleus. The cell nucleus is a double membrane bound cell organelle which contains the hereditary information of the cell in the form of chromatin. The nucleus performs the function of genetic information storage, retrieval and duplication of genetic information. The main component of the nucleus is deoxyribonucleic acid or DNA. The nucleus has various subcompartments having separate functions in the course of DNA replication and controlling of gene expression during the cell cycle. The nucleus also performs other duties such as transcription and post-transcriptional processing of pre-messenger ribonucleic acids(mRNA).

Primarily, the major content in a cell nucleus is chromosome, interestingly for a particular species though chromosome number in the cells is fixed but the shape and size of nucleus varies from cell to cell depending up on its origin and function. This leads to provoke the people to think what are the factors leading to differences in gene expression, nuclear transport, and other nuclear activities in both pathological and physiological events. This

curiosity was quenched when cell behavior was investigated under different forms of mechanical stress.

1.3 Cellular and Nuclear Mechanotransduction: Overall Effects

The role of cellular deformation in transduction of mechanical forces into biochemical responses has been the subject of numerous investigations in recent years [1-11]. When the cells are subjected to a mechanical stimulus, they show a concomitant volume reduction in extracellular matrix, nucleus, mitochondria, etc. only golgi bodies were able to survive intraorganelle water loss which suggests that changes in the volume of an organelle is driven primaryly by osmotic interactions and the changes in shape are brought about by other structural factors, i.e, cytoskeletal interactions that can occur due to compression in the extracellular matrix [12]. In nuclear mechanotransduced isolated nuclei, the emerin, and lamin A-C underwent post translational modifications, which directly had an impact on the nucleoskeletal structure and its functions [13-15]. When the cells are subjected to mechanical stimulus, the nuclear and cytoskeleton alignments can rearrange centromeres through deformation of the nucleus and in turn effect both morphological and biochemical cellular behavior in cells [16-18]. Various researchers have found that the nucleus is more rigid in structure than its surrounding cytoplasm. Research has established that the modulus of the nucleus is 10 times more rigid than its surrounding chondrocytes [19-20]. The nuclear alignment is also a critical part of plant genomics. Various studies also found that the nucleus is mechanically integrated with the physical entity of the cell via intermediate filaments; thus active or passive cell extension may lead to nucleus deformation [21]. It has already been hypothesized that the nucleus reacts to mechanical stimuli after it passes through the cytoskeleton and this reaction to the stimuli may contain the key to control gene transcription [22-23]. It is also found that the nucleoskeletal response to mechanical stimuli may directly have an impact on chromatin study [24-26]. The direct effect of nuclear mechanotransduction

is found in nuclear morphology which in turn effects the gene expression and and nuclear transport. Researches have proved that the nucleoskeletal coupling is an essential part of 2D and 3D cell migration hence mutation in LINC complex associated protein can bring about various human diseases. The majority is caused by LMNA gene, which encodes lamins A and C. Mutation in these genes causes diseases in striated muscles, adipose tissue, or peripheral nervous system. Some of the common diseases include Emery-Dreifuss muscular dystrophy, limb-girdle muscular dystrophy, dilated cardiomyopathy, familial partial lipodystrophy, Charcot-Marie-Tooth and the accelerated aging disorder Hutchinson-Gilford progeria syndrome [27]. Although the role of cellular and nuclear morphology changes due to transduction of mechanical forces such as fluid flow and others forms of shear stress into biochemical responses has been thoroughly investigated in recent years [28-30]. Various sensors should be developed and perfected to calculate and predetermine the amplitude and frequency of stress experienced by the nucleus in pathological and physiological contexts [31-32]. There even have been numerous studies to examine the insitu cellular and nuclear morphological changes in reaction to mechanical stimuli of articular cartilage. But in the recent years there are no proper researches focusing on the pattern of changes in cells (in in-vitro) condition when the cells are exposed to any kind of mechanical stress [11, 33].

1.4 Confocal Imaging and its bottlenecks

For the better part of the last decade, the confocal imaging system has been of immense use to the scientific and research community for visualization of biological, chemical and other samples. Its high resolution, easily controllable and highly maneuverable depth of field, reduces the unnecessary background information situated away from the focal plane which in turn leads to image enhancement, and the capability to record 3D images from specimens,

etc.. For this project work to determine the nuclear morphological changes we are using confocal microscope by Leica imaging systems. Although confocal imaging is the best way of determing the effects and morphological changes occurring during nuclear mechanotransduction, this process has its limitations. The process of cell fixation and staining should be done with great care and patience else there comes a series of noises in the confocal images which makes it intricate to differentiate morphological changes in mechanotransduced nucleus and makes it even harder to see for any pattern formation in the changes in nuclear morphology. Other problems faced while working with the confocal microscope generally deal with the manual error during the handling of the confocal microscope. Each time the parameters of imaging may differ and hence determination of morphological changes may again be hindered. So, to avoid this problem and make the process better and to quantify the amount of changes and detect patterns in changes of the nucleus we take an image processing approach.

1.5 Image Processing Approach

Image processing is a field of study which takes an image as an input, and its output may be a ranging from an improved image to any parameter analyzed from the image. It can be said that the image processing is an advanced part of signal processing where the input image acts as an input signal. Exactly like digital or analog signal, the processing of image can also be done in a spatial or frequency domain. In many studies, the image processing approach gives clear and conclusive proves to the theories, and it also reduces human error in analysis. Also, the image processing approach reduces human effort in the area of computation and quantification. In this study, we are concentrating mainly on the morphological changes in the nucleoskeleton of the cell.

1.6 Recent years in field of Cellular imaging and Image Processing

Over the past few years there have been many image processing approaches on confocal microscope images such as segmentation of fibrin, detection and removal of noise and other distortion from confocal images of neurodegenerative diseases, quantitative analysis of cardiac tissue through image processing of confocal images. Cell components segmentation using fluroscent imaging [34]. automated detection of cell nuclei from pap smear cell images, etc. [35-38]. Relavent work is also going on the field morphological reconstruction, segmentation, advanced noise removal, and picture clarity of 2D, 3D and 4D imaging from Confocal Images [39-40]. There also have been some commendable work done on changes in morphology, cytoskeleton, and protein expression changes in various cells under external stimuli [41].

1.7 Why MATLAB Platform?

For this study, the process of image processing is done on MATLAB platform due to its various features which are exclusively found in MATLAB. As, MATLAb is a general purpose programming language, all of its code is in the form of function, scripts, etc. So, there is an advantage that there final results can be re-evaluated and replicated by others. It also gives everyone the opportunity to scrutinize and implement small changes if needed without any hassle. If any person wants to understand the algorithm and process, he/she can just directly see the script/function in a plain text format. Another huge advantage of using MATLAB as an image processing platform is due to its high numerical precision. Other image processing tools generally cannot handle pixel intensities in double ar floating point integers, hence for an 8-bit image the intensity values are only from 0-255. But MATLAB can handle floating point integers and hence where there is a high difference of intensities in

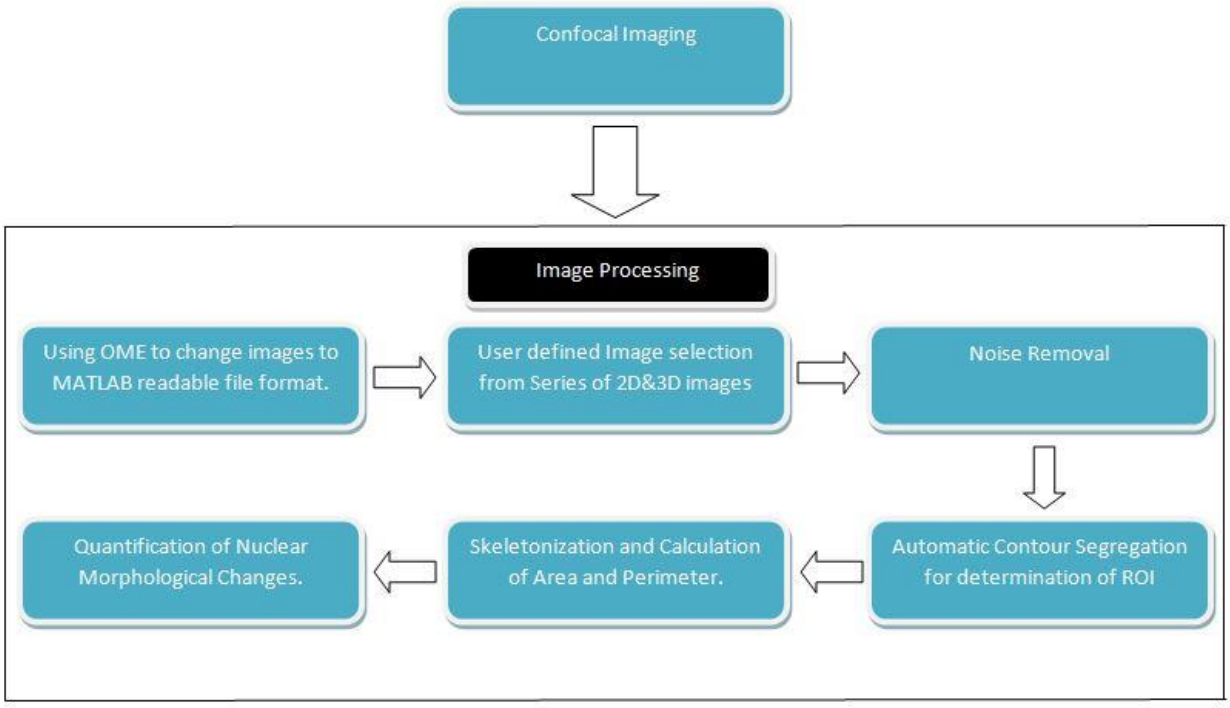
edge, MATLAB doesn't need to truncate the values to 0-255 and hence no information is lost. The inbuilt image processing toolbox in MATLAB is also very accurate and easy to use.

1.8 Rationale and Objective:

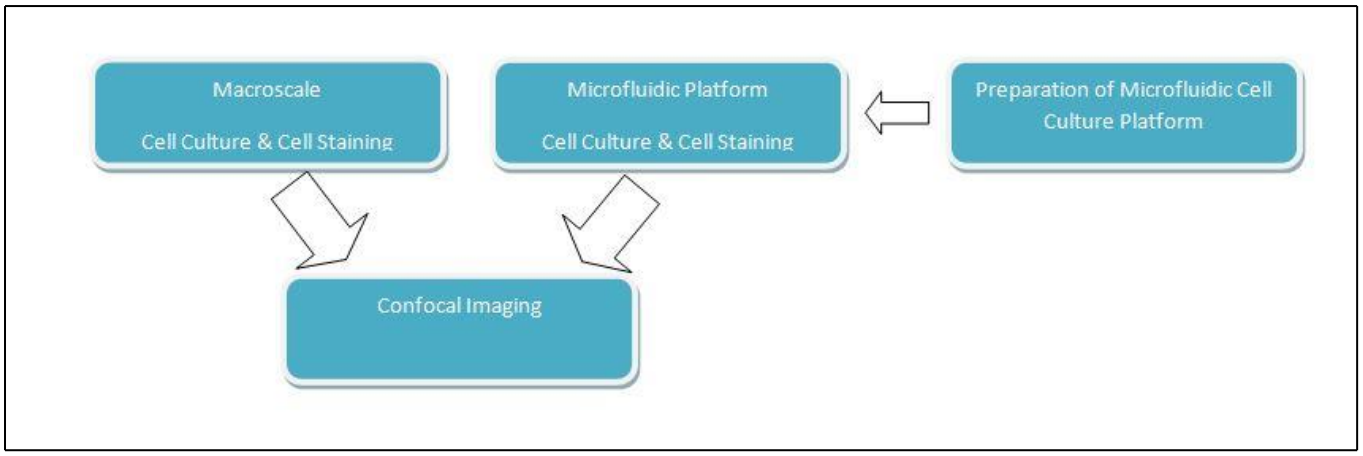
In this study, our main objective was:

- Development of an image analysis program for processing of universal confocal images and quantification of cell shape factors.
- Application of the program for morphology based quantitative analysis of nuclearmechano-transduction.

1.9 Work Plan (Phase-I)



(Phase-II)



CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

HaCaT and MG63 cell lines were procured from NCCS, Pune. PDMS was bought from Dow Corning.Dulbecco's Modified Eagle's Media (DMEM), Dulbecco's Phosphate Buffer Saline (DPBS), Trypsin-EDTA solution, Fetal Bovine Serum, Antibiotic-Antimycotic solution were purchased from Himedia, Mumbai, India. TRITC Phalloidin and DAPI were bought from Sigma-Aldrich, India. Syringe pump (model: SP-1000) was purchased from Ningbo Annol Medical Device Technology Co. Ltd. The Images were processed using a program coded using MATLAB 2012b(Licenced under NITRKL). Confocal Microscope was From Leica Microsystems ( ).

2.2 Methodology

2.2.1 Preparation of Microfluidic Channel.

For the preparation of microchannels, we are using Polydimethylsiloxane (PDMS) in microchannel moulds and baking it to solidify. Firstly, PDMS is taken in a 50ml Falcon and crosslinker (Sylgard 184,Dow Corning) is added to it with the ratio 10% weight of PDMS. Then the whole content of the falcon is stirred vigorously until it turns into a milky white fluid. The mixture is then centrifuged at 4 degree Celsius at the rate of 5000 RPM for 5 mins. The microchannel mould is cleaned properly and sterelized with the help of 70% Ethanol solution, and then the mixture was poured very carefully in the mould. The fluid with the mould was then kept in a dessicator for further degassing of the PDMS crosslinker mixture. After all the gas bubbles from the fluid from the mould are properly removed, the whole system is then kept at a Hot air Oven(Labotech, India) at 80 degree celcius for an hour. When the PDMS has solidified, the mould with PDMS is taken out of the oven, and the microchannel is cut out with the help of a heated scalpel.

2.2.2 Preparation of Oxidized Cover slip Base

Firstly a Piranha solution is prepared by thoroughly mixing Concentrated Sulphuric acid and Concentrated hydrogen per-oxide(1:1 (v/v) ratio). and pouring it on a petri plate. Rectangular cover slips are then taken and dipped in the petri plate containing the piranha solution and kept there for an hour. The cover slips are then taken out and properly washed in distill water before being stored.

2.2.3 Finishing the Micro-channels

A scalpel blade is firstly heated and then used to cut the thick ends of two 100 microlitre pipette tips. Make two holes at both the ends of the microchannels which will serve as inlet and outlets. With the help of PDMS paste, the microtip ends over the holes in the microchannel. The whole system is then again baked in a hot air oven for an hour at 80 degree celcius. After the microtip ends are properly fixed, the microchannels are flushed with 70% alcohol solution with the help of a syringe. To make the channels ready for cell adhesion, the microchannels are flushed with 3 different concentrations of gelatin and stored at 37 degree celcius, 5% carbn dioxide, and 95% humidity.

2.2.4 Cell culture in Microfluidic Channel

HaCaT and MG63 cell line was procured from NCCS (Pune, India). The cells were maintained in Dulbecco's modified Eagle's medium(DMEM, HiMedia), supplemented with 10% FBS (GIBCO)and 1% antibiotic & antimycotic solution (HiMedia) in a humidified (95%), CO_2 (5%) incubator at 37^{o}C.The cells were harvested using 0.25% Trypsin EDTA (Himedia) [42].Thereafter, 10µl of the cell suspension ($1x10^6$cells/ml) was seeded into the microchannel, and the inlet & outlet reservoirs were filled with media. The seeded microchannel was placed in CO_2 (5%) incubator at 37^{o}C and 95% humidity for 24 hours for proper cell adhesion.

2.2.5 Cell Preparation and Staining

The cells seeded in microchannels were stained with TRITC Phalloidin and DAPI for fluorescent tagging of cytoskeletal and nuclear organization, the cells. In brief, the cells in the microchannel were washed with PBS (pH 7.4), fixed with Paraformaldehyde (4%) for 15 minutes and again washed with PBS. Thereafter, the cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min and stained with TRITC Phalloidin (1:300) and DAPI (1:1000). After the cells had been stained, they were exposed to shear stress by a continuous fluid flow of .6ml\hr for 6 hrs using a syringe pump(model:SP-1000, Ningbo Annol Medical Device Technology Co. Ltd.). Static channel (without flow) was taken as a control for examining the variations in the cells [43].

2.2.6 Image Processing using MATLAB 2012b

The confocal images were processed and analyzed using functions from the image processing toolbox and Bioformats package from Open Microscopy Environment. Open Microscopy Environment or OME develops open-source software and data format standards for the storage and manipulation of biological microscopy data. It is a joint project between universities, with LOCI at the University of Wisconsin-Madison, University of Dundee and Glencoe Software, research establishments, industry and the software development community. For automatic contour segmentation code from MATLAB Central File Exchange by Shawn Lankton.

2.2.7 Conversion of images to MATLAB recognizable formats using OME.

Open Microscopy Environment or OME develops open-source software and data format standards for the storage and manipulation of biological microscopy data. It is a joint project

between universities, with LOCI at the University of Wisconsin-Madison, University of Dundee and Glencoe Software, research establishments, industry and the software development community.

Bio-Formats is a standalone Java library for reading and writing life sciences image file formats. It is capable of processing both pixels and metadata for a large number of formats, as well as writing to several formats. The primary goal of Bio-Formats is to facilitate the exchange of microscopy data between different software packages and organizations. It achieves this by converting proprietary microscopy data into an open standard called the OME data model, particularly into the OME-TIFF file format.

It is software codes collection for accessing, processing and analyzing various formats of image data using standardized, open formats. Bio-Formats is a community driven project with a standardized application interface that supports open source analysis programs like ImageJ, CellProfiler and Icy, informatics solutions like OMERO and commercial programs like Matlab.

Bio-Formats package helps in handling of files related to High Content Screening, time lapse imaging, digital pathology and other complex multidimensional image formats.

CHAPTER 3

RESULTS

3.1 Collection of Confocal 2D, 3D images and other Bioformats.

The program was created in such a way that it can read, process and analyze both bio-formats file format i.e., .lif(Leica Imaging File) and also any matlab readable file formats for ease of access. The program has many user input variables such as Threshold limit, confocal channel, Z-Plane no.(useful in case of 3D images), etc. As a single program cannot be guarrenteed to work equally good for all types of images as the environment, focus, manual error and other parameter can differ every time, some of its features are also kept as user's need choice.

3.2 Enhancement of image features and removal of noises

The confocal microscope uses a system of Excitation laser on the fluorescent dyes on the cell components (DAPI for nucleus) to get electrons emitted from the fluorescent surface. A photomultiplier tube is used to collect the electrons and find the region of fluorescence emission. This procedure can cause many human errors and form a noisy image. Hence, for proper analysis of the image, we should remove all the false positives and noises from the image keeping the sharpness and texture of the ROI intact. Sometimes, the parameters of the images are often needed to modify to get a better image. The whole process is generally done keeping the morphological information intact. This is usually done by image intensity thresholding, and histogram equalization. Both of these procedures are typically important as the intensity of confocal images may vary from one image to another. By thresholding, we remove the high or low intensity noises of an image. The threshold value is set by the user as the threshold value differs from image to image and also accordin to users choice. Histogram equalization is done when the intensity of the confocal images are very low. In histogram equalization the intensity of the image is multiplied with the cumulative distribution function to enhance the intensiy of the image, to make the ROI visible properly and help in better noise removal and processing of the image. As, the process of histogram equalization is not

needed in case of each and every image hence the feature is used only if the user thinks there is a need for it.

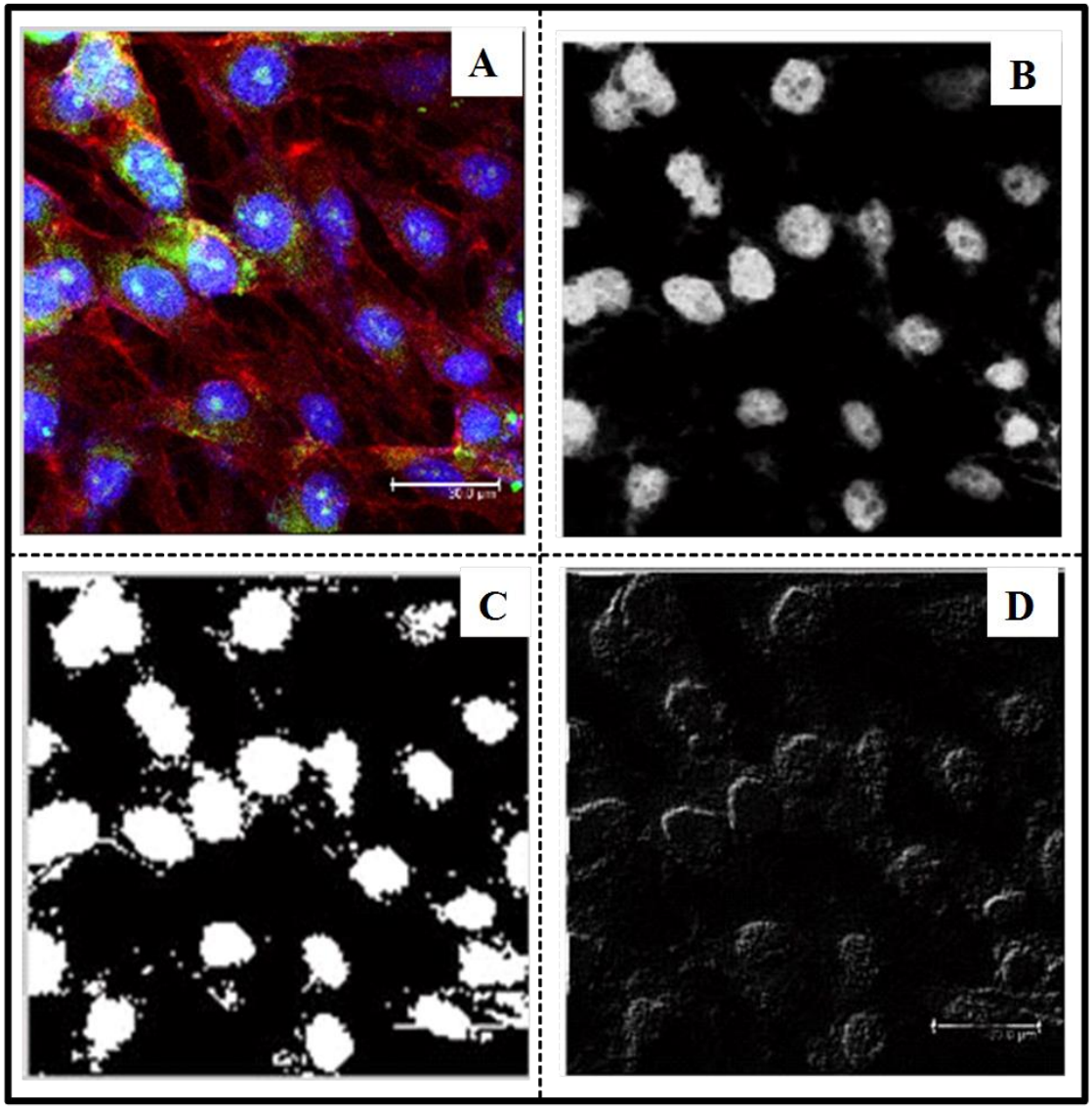


Fig. 1: A) Original Image B) Effect of Smoothning Filter C) Improper Thresholding D) High Pass filtering effect

The images are firstly segregated into the respective planes and then all the noise from that particular plane is removed using an algorithm which uses a square mask of various lengths and detects the noise from the original texture by comparing its size to the ROI.This step is repeated several times for more accurate results and greater noise removal.

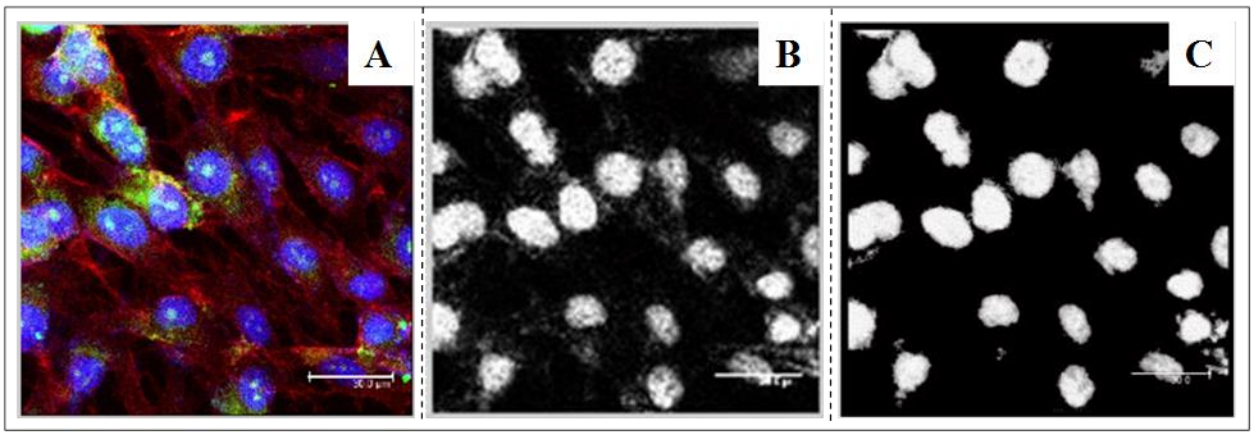


Fig. 2: A) Original Image B) Blue Plane C) Noise Free Image

When the cells are subjected to various flow rates occasionally there are cases when the cell nucleus gets ruptured and the fragments of the nucleus is detached from the surface andfloat around in the media which causes unwanted noises in large part of the image which in turn makes proper imaging and analysis of the nucleus shape and size very difficult.

The images also contains many Salt and pepper noises which are primarily found on the borders of the ROI and improper removal of the noise via Gaussian,mean or any other orthodox filters can degrade the contour of the ROI and give many false positive results. It will also hamper the quantification of the morphological changes in the ROI.

So, the process of noise removal is done in 2 steps

- Algorithm to Compare noise/false positive size and thus removal of noise
- Automatic contour segmentation using Chan Vese model for greater accuracy in contour detection and removal of salt and pepper noise along the boundary.

The normal significant noises are automatically removed when the program is executed. For removal of Salt and pepper noise from the ROI, greater care should be taken.

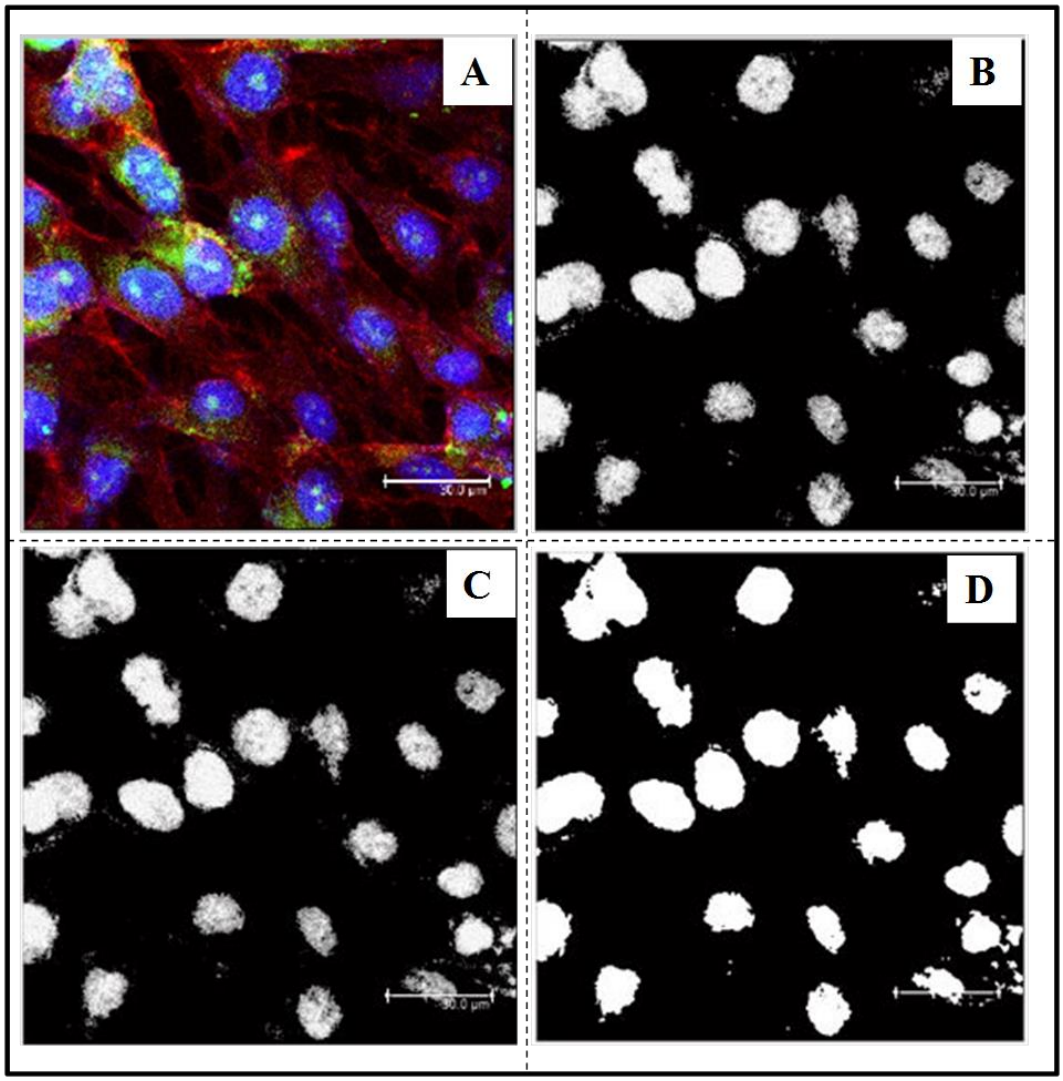


Fig. 3: A) Original Image B) Salt noise free Image C) Pepper noise free Image D) Noise free Image

- If there is salt noise near the contour of the ROI then draw the mask on the ROI in such a way that the mask in enclosed in the ROI and no salt noise is present in the initially drawn mask.
- If there is pepper noise inside the contour of the ROI, then draw the ROI mask in such a way that all the pepper noise in enclosed within the initial mask. By this way, the pepper noises are eliminated in the final output image.
- In any case, the variable mask noise removal Algorithm is executed again to remove any accidental salt noises created.

3.3 Removal of streaking noise

Due to improper incubation of cells and application of dye, there are times when the excessive dye is left behind in the microchannels which causes the presence of various streaking noises in the Images. Although the presence of streaking noises in the ROI compleatly damages the image and it doesn't give accurate or appropriate results, still various processing can be done to reduce the damage and increase the accuracy of the morphological change quantification. For this purpose, an algorithm consisting of proper opening and matrix multiplication of the image is done by use of various structural elements to reduce the damage and replicate the original contour. The structural element used is generally dependent on the shape and size of the streaking noise.

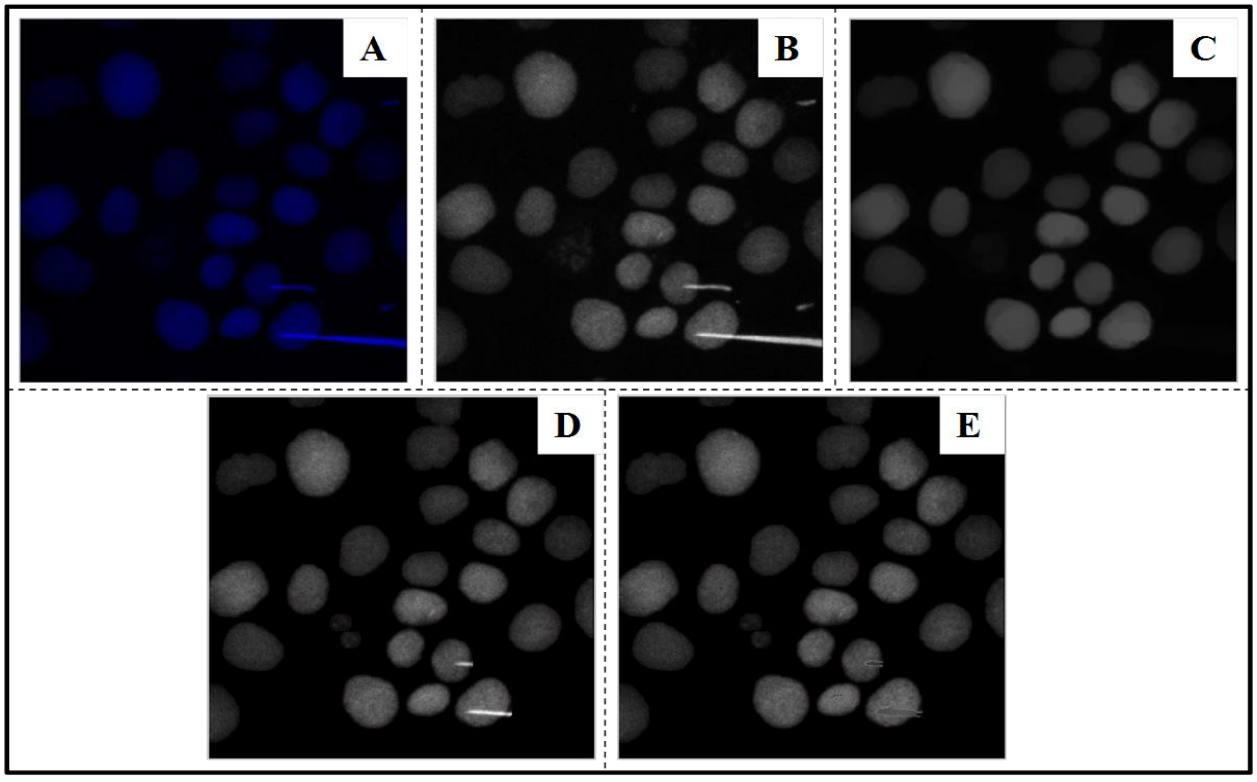


Fig. 4: A) Original Image with streaking noise B) Blue Plane C) Closing With structural objects D) Recovery of the original texture E) Reducing intensity of noisy pixels

3.4 Programming for Automatic Segmentation of ROI

As for the study we are using registered MATLAB version MATLAB 2012b we are facing problems regarding the active contour segmentation function which is present in version MATLAB2013b onwards. So, for active contour segemtation using the Chan Vese method we are implementing the paper "Localizing Region-Based Active Contours" by Lankton et al. (2008) [44]. By use of this program package we are able to successfully and perfectly segment the nucleus from its surrounding and hence better analyze the changes in the cell nucleus. It also helps upto some extent in the removal of salt & pepper noises and also in some cases spur pixels.

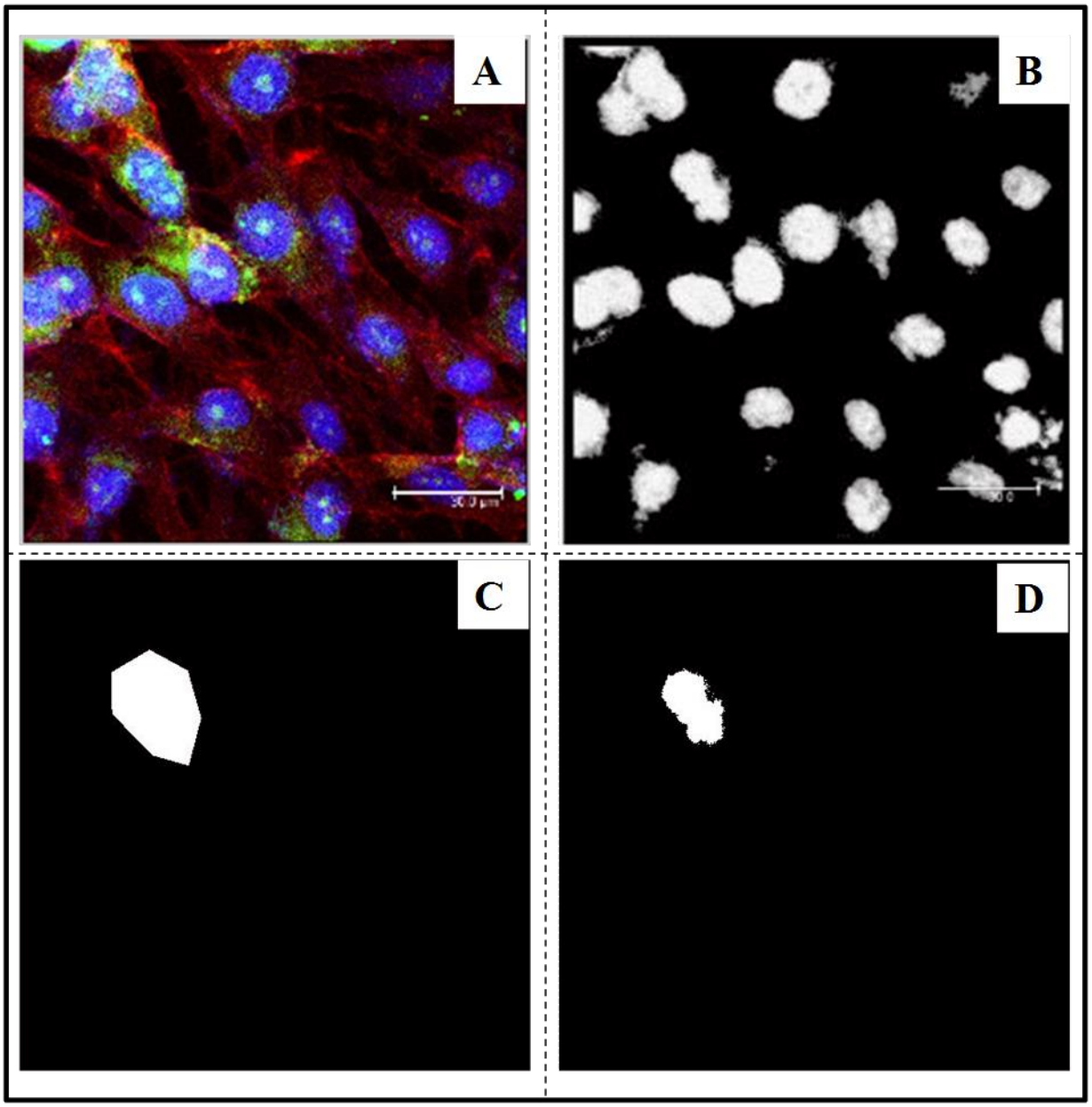


Fig. 5: A) Original Image B) Noise free image C) Initial Mask D) Segmented image

3.5 Programming for spur pixel removal

Sometimes during confocal imaging if the images are taken without proper line averaging or in a high laser power the nucleus images are surrounded by unwanted spur pixels which can cause the automated segmentation to form a faulty mask and create extra branching while skeletonizing of the nucleus hence while processing the images, these spurs should be removed.

The process of removing the spur pixel is quite easy. It is done by the use of black & white morphological function inbuilt in MATLAB

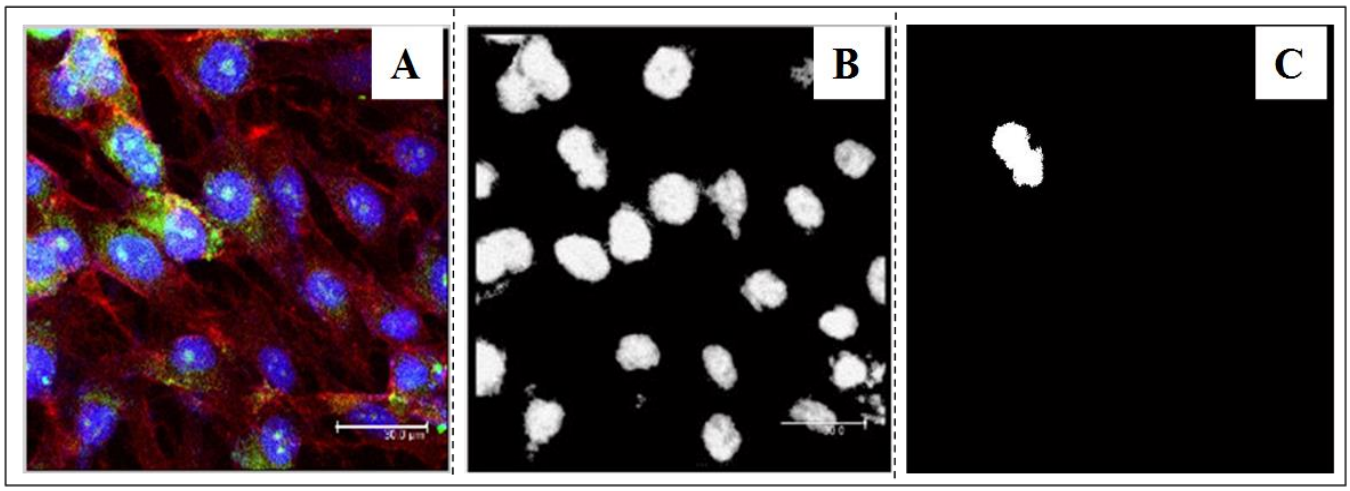


Fig. 6: A) Original Image B) Noise free image C) Spur pixel free Segmented image.

3.6 Programming for skeletonaization

For proper quantitative analysis of the shape, size and morphology of cell nucleus I am using the MATLAB inbuilt black & white morphological function. Skeletonization is a repetitive thinning process which decreases the foreground portions of a binary image to a single pixel width line or skeleton which maps the total contour of the original image. It works by discarding most of the original image pixel. Another way to think of the skeleton is as the loci of the centers of bi-tangent circles that fit entirely within the foreground region being considered.

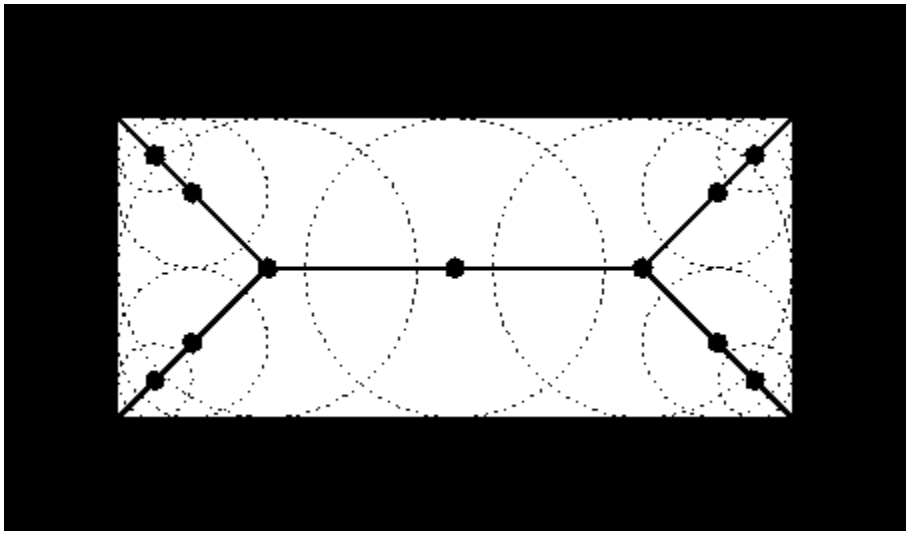
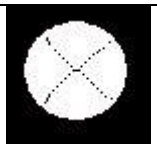
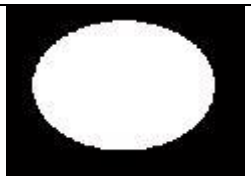
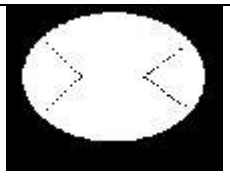
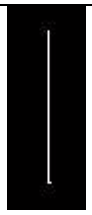
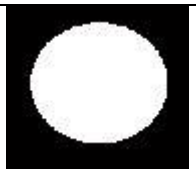
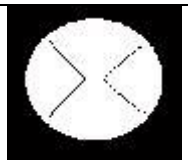
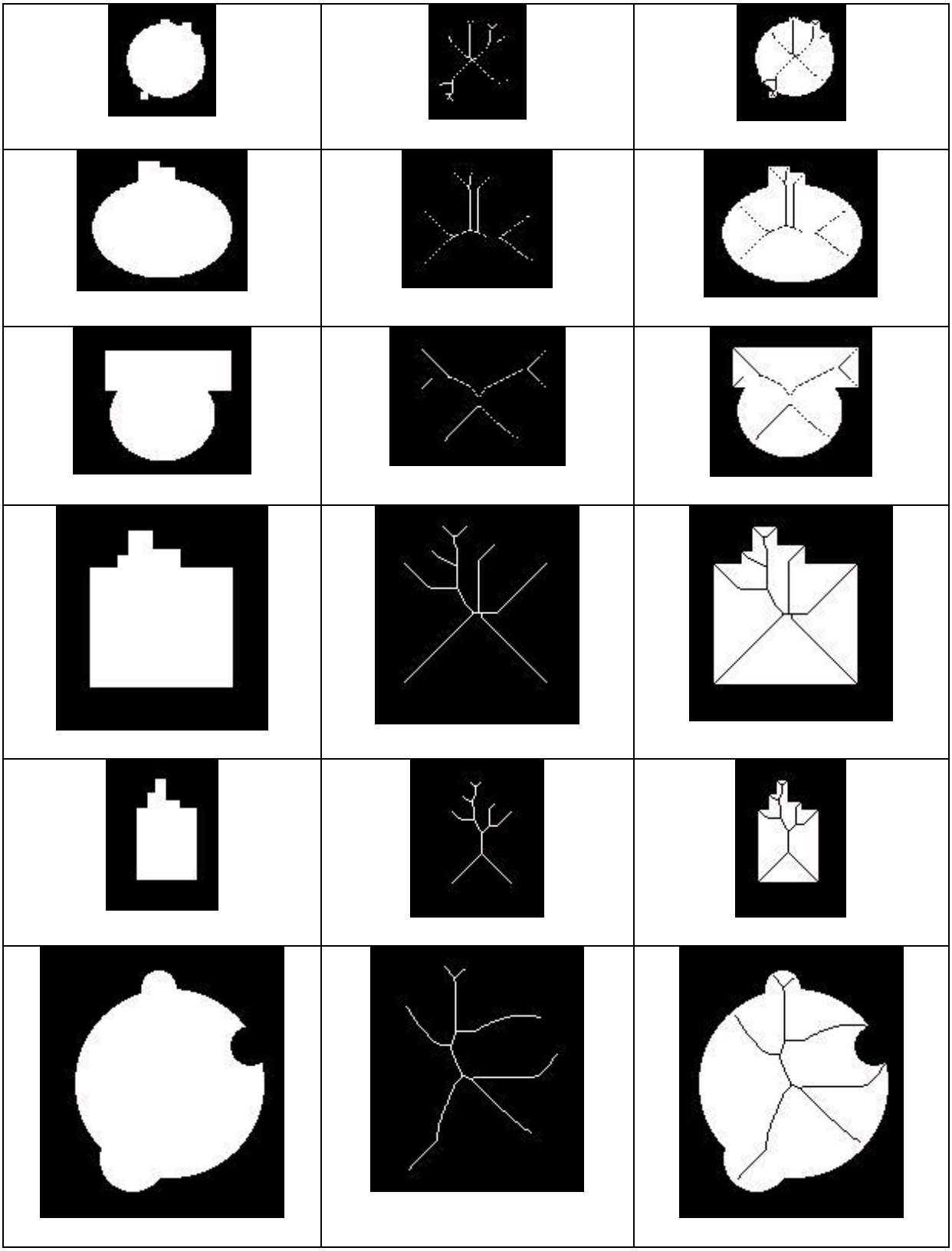


Fig. 7: Skeletonization of a rectangle

Below given are some examples how the skeleton changes with the change in shape of the ROI.

Table 1: Effects of Skeletonization on various shapes

Original Image	Skeleton	Image+skeleton
		
		
		
		



Skeletonization of Cell nucleus after noise removal

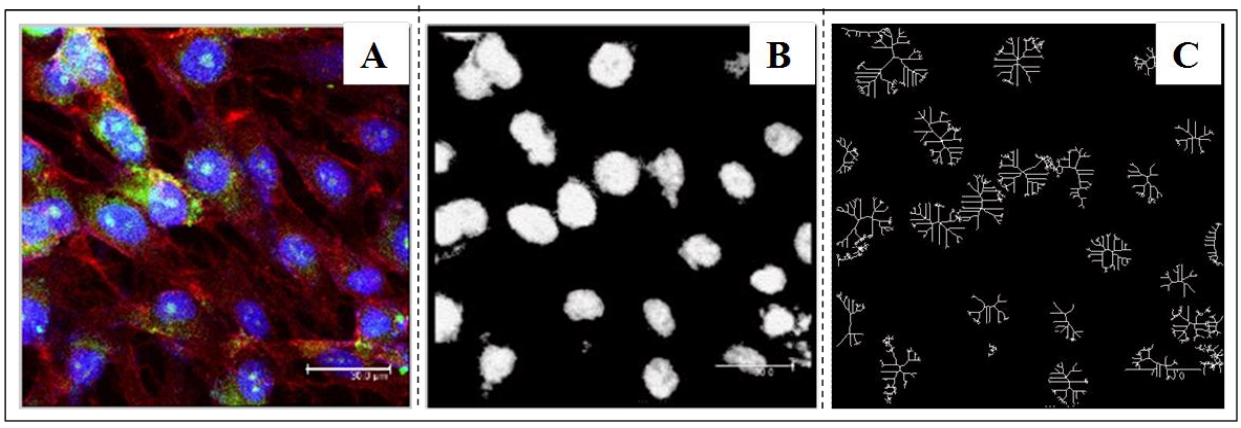


Fig. 8: A) Original Image B) Noise Free Image C) Skeletonized Image

3.7 Incorporating the scale

To efficiently quantify the morphological parameters, one important aspect of image analysis is scale. Without incorporating the automatic implementation of scale it is vry difficult to measure the area, perimeter, etc. so, to implement the scale in the image we first interactively ask the user to tag or crop the scale portion of the image by use of the crop function. When we get the image of the scale we use various algorithm to eliminate its various features such as the end points, units, values, etc. then we use another algorithm to count the number of pixels in a 2 pixel width line left behind after processing. The count is divided by 2, and the value is stored and the user is asked to enter the value of scale without the unit. A simple unitary method is then used to connect pixel count to length. When we segment a nucleus, we can easily cunt its pixels and hence find the area, perimeter, etc.

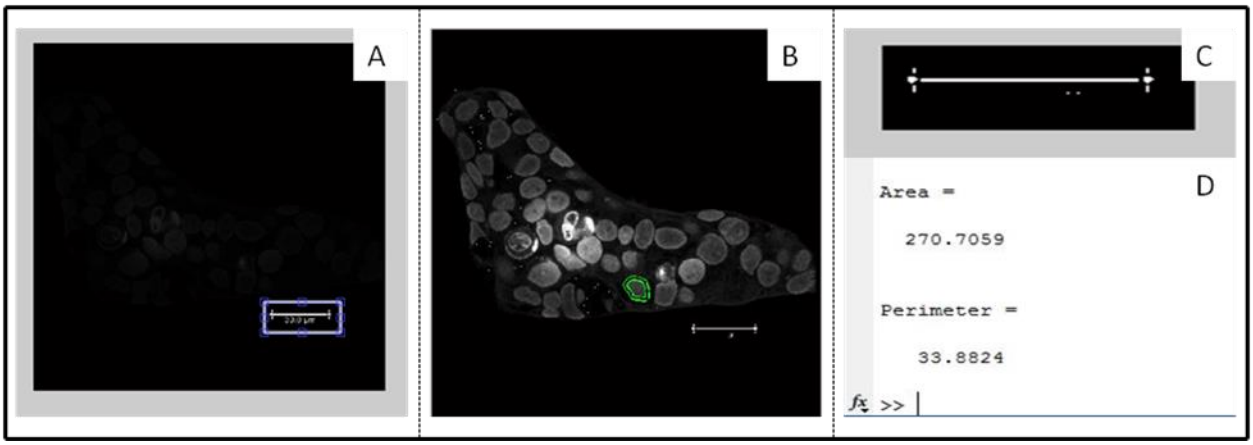


Fig. 9: A) Tagging the scalee **B)** Selecting ROI **C)** Scale processing D) Area and Perimeter

3.8 Programming for Quantification of morphological information

The process of skeletonization only detects the changes in the morphology of the cell nucleus. But, for effective quantitative analysis of the cell nucleus, we must see the nature of the branching in a cell nucleus image, number, position, clustering of branches in a cell nucleus image. We will also have to see the position, number, and clustering of endpoints of the branches in a cell nucleus image. By calculation of all those factors only proper quantitative morphological analysis of the cell nucleus can be done.

3.9 Preparation of microchannels.

The microfluidic channels were prepared as described in the materials and methods section.

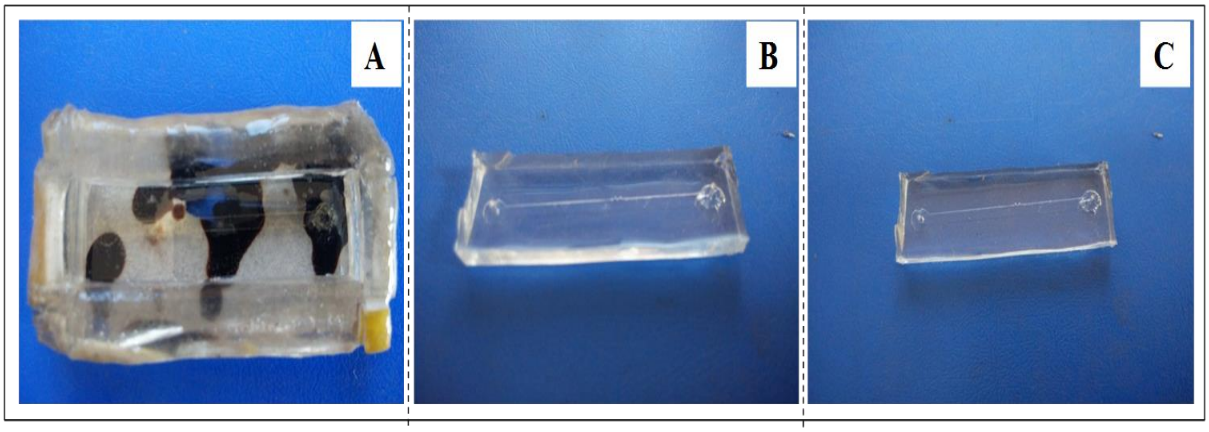


Fig. 10: A) Micro channel Mould B) & C) Microchannels

3.10 Finishing the Micro-channels

The microfluidics channels were prepared as described as in the materials and methodology.

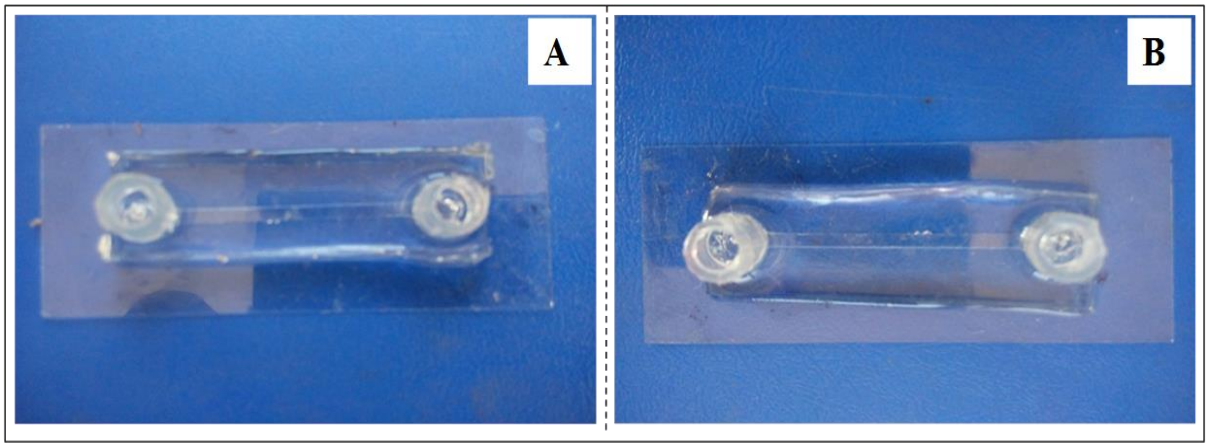


Fig. 11: A) & B) Finished Microchannels

3.11 Cell Cultures

HaCaT Cells and MG-63 Cells were cultured in macro and microenvironment as described in the methodology section and the growth, adhesion, and other factors were seen using a fluorescent optical microscope.

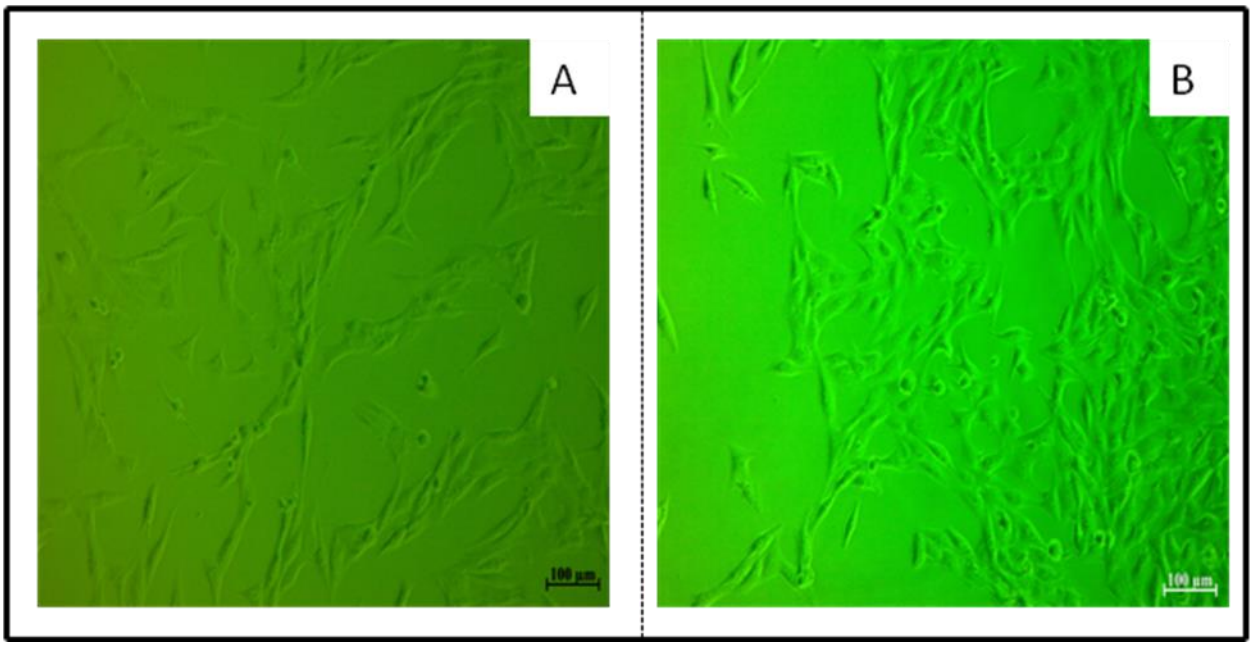


Fig. 12: A) & B) HaCaT cells after subculture.

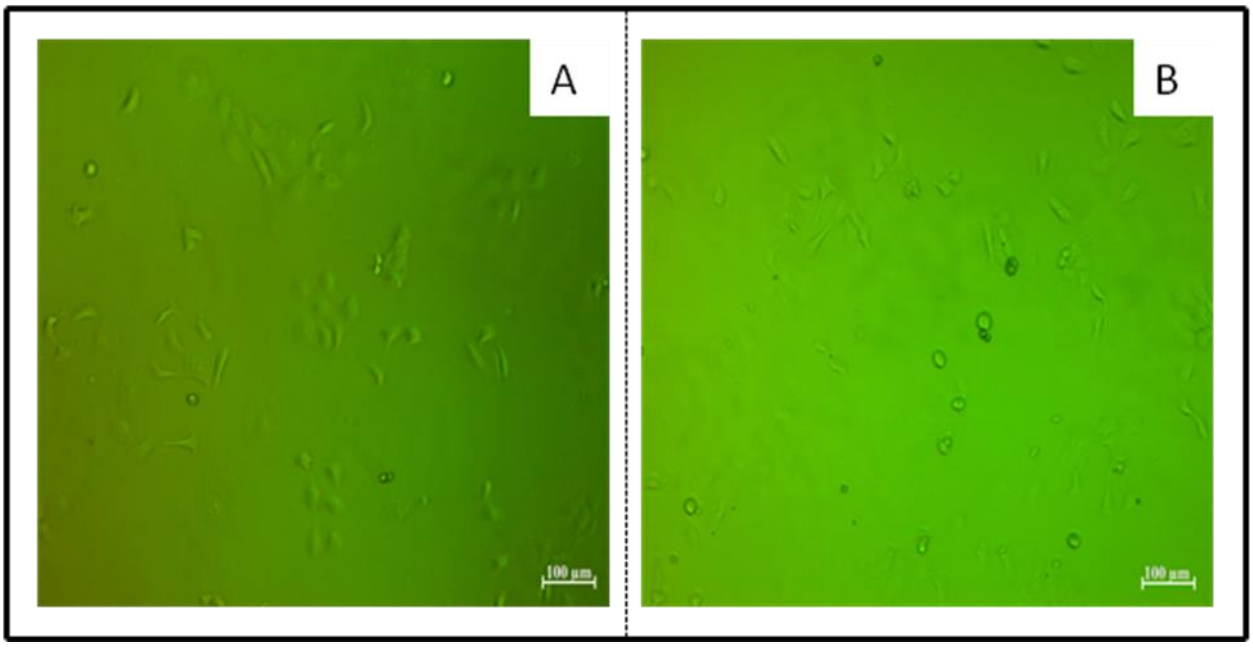


Fig. 13: A) & B) MG-63 cells after subculture

3.12 HaCaT Cells exposed to Flow at the rate of 0.6ml/hr

The HaCaT cells were cultured in a flask and then seeded into the microchannels. After successful adhesion, a syringe pump was used to flow complete media through the microchannels at the rate of .6ml/hour. After 6 hours, the microchannels were placed under the confocal microscope and images were taken and processed by the MATLAB program.

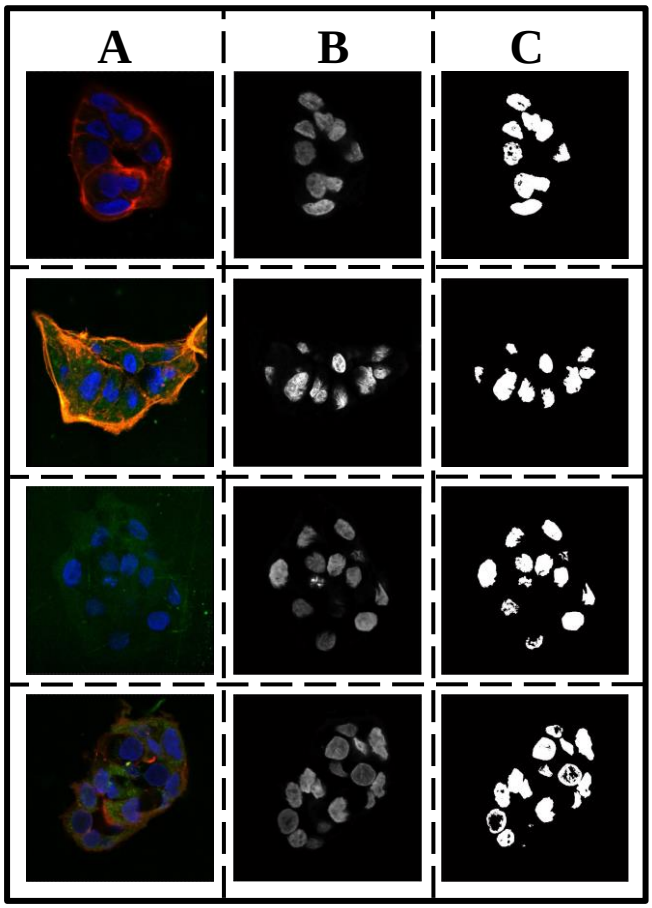
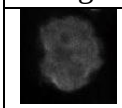
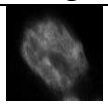
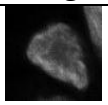
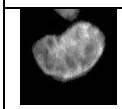
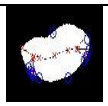
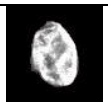
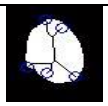
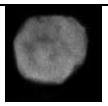
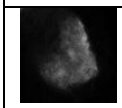
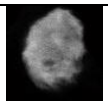
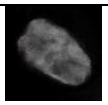
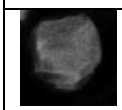
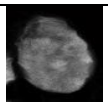
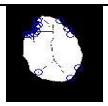
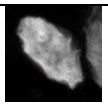
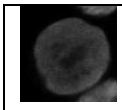
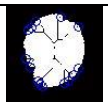
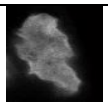
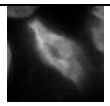
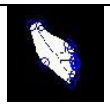
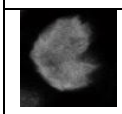
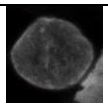
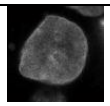


Fig. 14: A) Original image B) Blue plane image C) Noise free image

Table 2: Effects of Skeletonization on HaCaT flow nucleus

Image	Skeleton	Quantity	Image	Skeleton	Quantity	Image	Skeleton	Quantity
		27			21			12
		11			5			7
		18			16			11
		10			13			10

		14			14			11
		18			13			6

3.13 HaCaT Cells in Static Micro-environment

The HaCaT cells were cultured in a flask and then seeded into the microchannels. After successful adhesion, the microchannels were placed in a confocal microscope, and the images taken were processed in the MATLAB program.

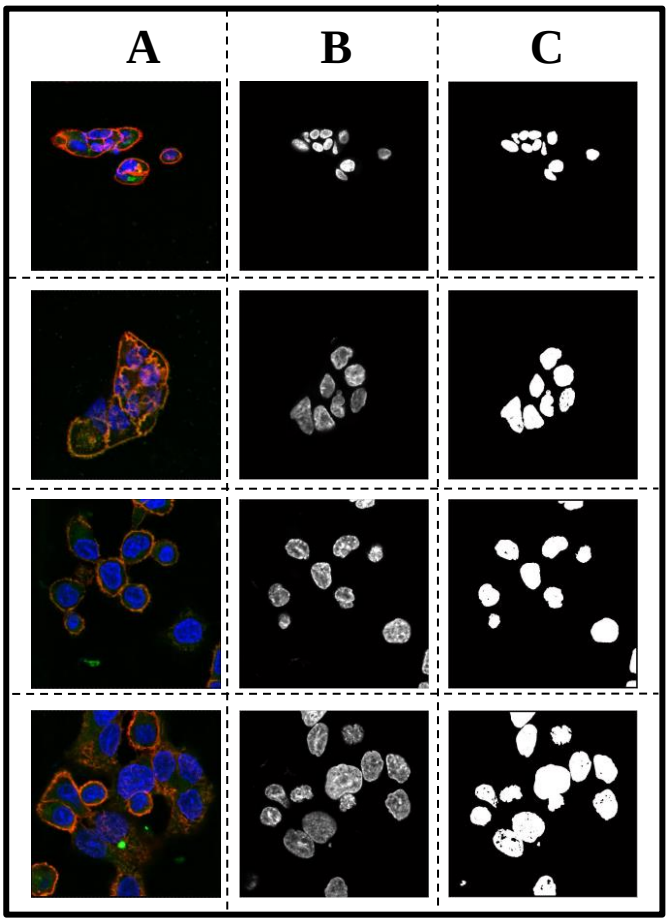
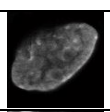
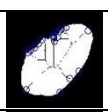
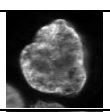
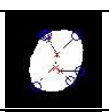
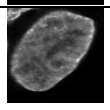
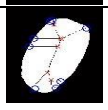
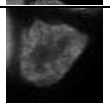
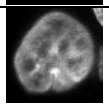
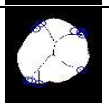
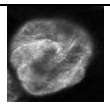
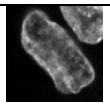
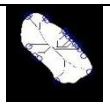
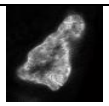
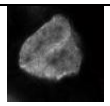
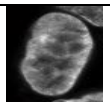
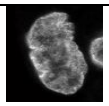
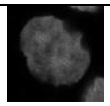
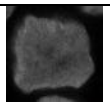
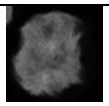
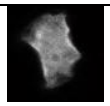
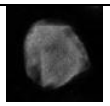
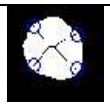
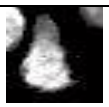
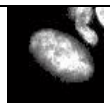
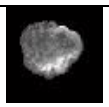
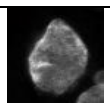
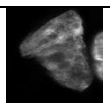


Fig. 15: A) Original image B) Blue plane image C) Noise free image

Table 3: Effects of Skeletonization on HaCaT static nucleus

Image	Skeleton	Quantity	Image	Skeleton	Quantity	Image	Skeleton	Quantity
		17			9			6
		8			8			9

		10			17			15
		8			6			9
		19			6			11
		10			13			4
		7			10			7
		11			6			6
		9			23			10
		14			8			6
		8			6			6
		8			6			6
		8			7			9
		4			5			7

3.14 MG-63 Cells in Macro-environment

The cell culture flask were taken from the incubator and kept under Confocal Microscope, and the images were processed and analysed using the MATLAB code.

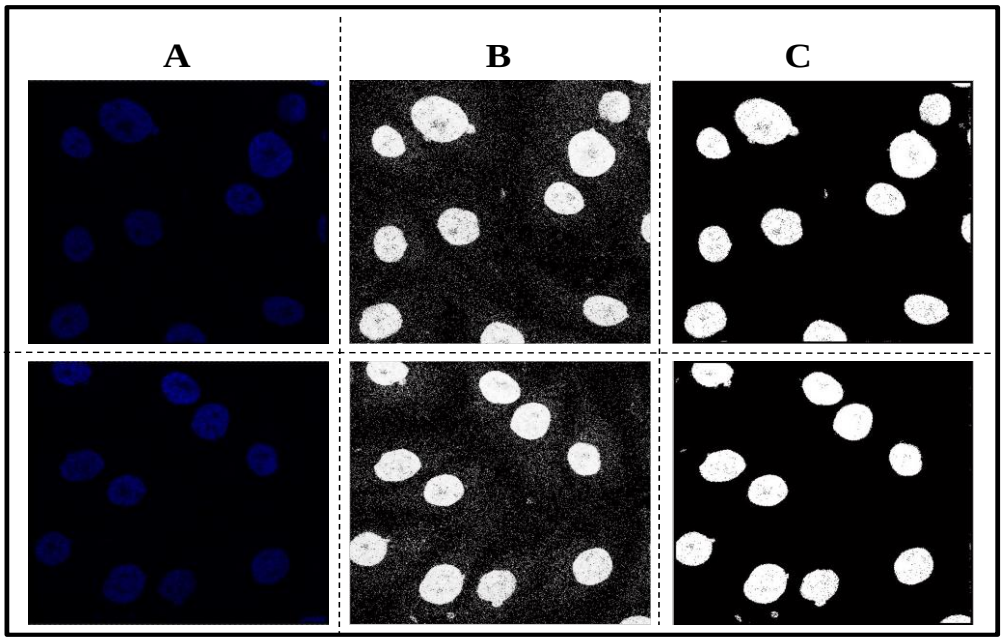


Fig. 16: A) Original image B) Blue plane image C) Noise free image

Table 4: Effects of Skeletonization on MG-63 nucleus in macro-environment

Image	Skeleton	Quantity	Image	Skeleton	Quantity	Image	Skeleton	Quantity
		36			49			24
		28			31			33
		31			45			46
		27			33			37
		42			38			

3.15 HaCaT Cells in Macro-environment

The cell culture flask were taken from the incubator and kept under Confocal Microscope, and the images were processed and analysed using the MATLAB code.

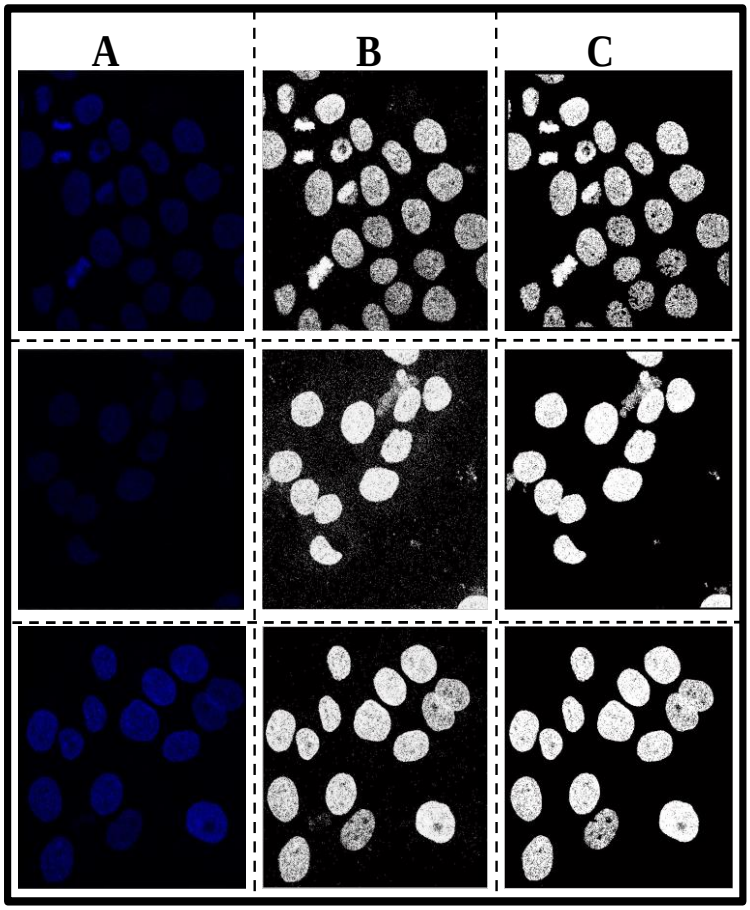
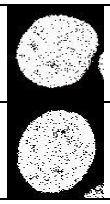
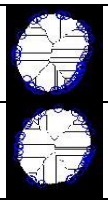
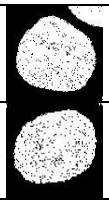
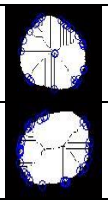
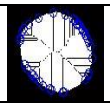


Fig. 17: A) Original image B) Blue plane image C) Noise free image

Table 5: Effects of Skeletonization on HaCaT nucleus in macro-environment

Image	Skeleton	Quantity	Image	Skeleton	Quantity	Image	Skeleton	Quantity
		36			30			38
		20			22			29
		15			7			14
		18			19			19
		16			20			41
		21			24			25
		33			42			21

		37			28			35
		29			26			

3.16 HaCaT Vs MG-63

The quantity of changes found from the table above was plotted in a graph to search for any distinctive pattern of changes between MG-63 and HaCaT cells which were fixed in macro-environment.

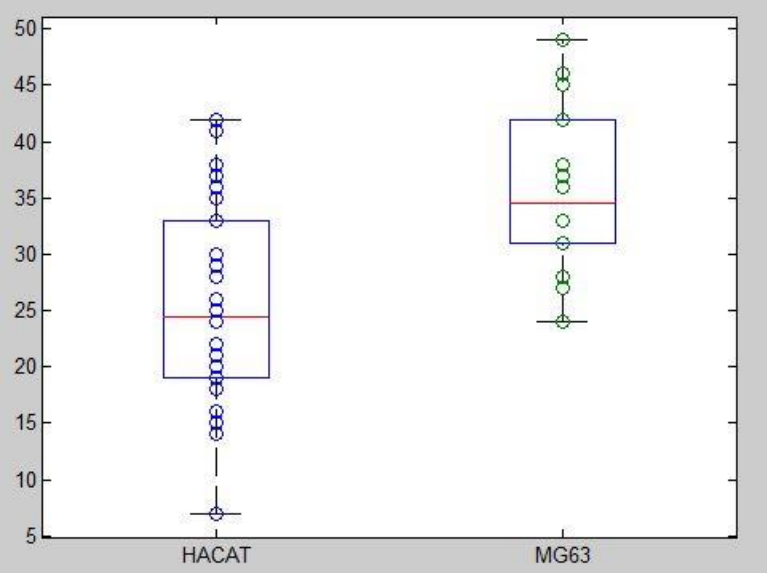


Fig. 18: Morphological change in HaCaT and MG-63

The above boxplot clearly indicates that the overall shape of the nucleus of MG-63 and HaCaT differs by a large margin. As, endpoints in skeletonization show change in the shape profile of a nucleus hence we can say that on an average MG-63 has more crease and protrusion (changes) in nucleus periphery than HaCaT cells.

3.17 Comparisons of cell nucleus under various environment(Flow:Static:Macro)

The quantity of changes found from tables were plotted in a graph to find out any distinctive pattern of changes in HaCaT cell's nucleus when they are seeded in a micro-environment, macro-environment, and subjected to mechanical stress via fluid flow of .6ml/hr.

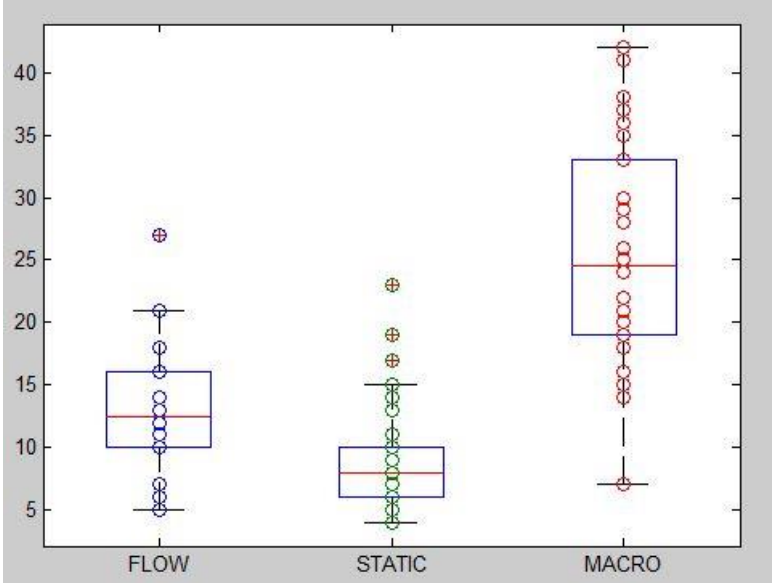


Fig. 19: Morphological change in HaCaT under macroenvironment, microenvironment, and flow

From the graph, we can see that there was a drastic reduction in the amount of changes in the shape of cell nucleus from macro-environment to micro-environment. But, there is very little difference in the quantity of changes in the nucleoskeleton when the cells are subjected to flow in micro-environment or just seeded in microchannels.

CHAPTER 4

DISCUSSION

4.1 Discussion

The MATLAB code created was used to process a variety of confocal image of cells and cell nucleus, and the code was also used to analyze over 100 nucleus images for morphological changes. The program was found to have some limitations. They were mainly in forms of overlapping nucleus. The overlapping nucleus made the automatic contour segmentation in adequate as there was no distinct change in background and foreground pixel intensities, the program grouped both the nucleus as one and hence giving error results. The procedure for imaging by confocal microscope must also be modulated so that most of the images are good in nature without high distortion and noise. The process of cell staining should also be done properly to avoid any additional noises. For the program to run properly, the computer should have more than 6GB of RAM. From the data we got, we can say that all the cells will show different number of shape changes in their macro-environment. Hence, if we analyze a large number of the nucleus and make a data bank, then shape change can be treated as a parameter to differentiate between normal, mutated and diseased nucleus. In case of cells in micro-environment, this shape change quantification can be used as a parameter of differentiating between normal cell nucleus, and mechanotransduced nucleus. If the LINC complex associated protein is mutated in cell nucleus and it hampers the nucleocoupling of the nucleoskeleton with the actin then it will give a difference in shape change of the nucleus in various environment and can hence be detected by the use of the program.

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