

Perfusion culture and live cell imaging of Hepatitis C Virus Replicon cells in miniature bioreactor and analysis of shape of nuclei

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ABSTRACT

Biological systems, on the macro scale (organs, tissues) to the micro scale (cells, organelles) continuously respond and adapt to their environment. The study of mechanics of these systems is important in many perspectives. The ongoing research in the field of mechanobiology involves understanding of the fundamental nature of how cells sense and respond to mechanical stimuli.

When cells are subjected to mechanical stimuli, some forces are created which may alter the morphology of the cells and their organelles. For example, fluid flow induces shear stress on cells and this may affect the shape of their organelles. The aim of this project is to study the effect of flow on the shape of nuclei of liver carcinoma cells. A perfusion culture system was used to maintain the flow and live cell imaging was used to observe the shape of the nuclei. The size and shape of the nuclei were represented by the area and eccentricity of the nucleus which was computed from the live cell images using ImageJ software.

Keywords – Shear flow, nuclear mechanics , live cell imaging

1. INTRODUCTION

The study of mechanical properties of cells dates back to the 1940s where physical properties of cytoplasm of chick embryos were studied using magnetic particles [1, 2]. More recent studies show that forces applied on the cells are transmitted to the nucleus through the cytoskeleton [3]. It has been proposed that the nucleus may be involved in responding to these mechanical signals. The induced forces may cause a change in shape of the nucleus which further might alter the packaging or organization of DNA inside the nucleus. Any change in the structure or organization of chromatin is crucial for cell behavior as it can affect the biological functions of the cell [4]. Hence it is important to study nuclear mechanotransduction.

Here we intend to study the effect of mechanical forces applied to the cell membrane on the nuclei of liver cells. The shear stress induced by flowing media over the cells was used as the mechanical stimulus. The cross sectional area and eccentricity of the nucleus measured using live cell imaging was taken as the response to these forces.

There are three main aspects in this project – (i) Perfusion culture for inducing mechanical stimulus on the cells (ii) Live cell imaging for recording the size and shape of the nucleus (iii) Analysing the images for computing the changes induced.

1.1 Perfusion culture

Perfusion culture systems are used to mimic the *in vivo* conditions as closely as possible in tissue culture. Here we use such a system previously developed in the lab (M²D² laboratory, Department of mechanical engineering, Indian Institute of Science, Bangalore) for inducing mechanical stimuli on to cells. This set up has been designed and fabricated for live cell imaging assays. It consists of a miniature bioreactor with a coverslip where cells can be cultured and a miniature peristaltic pump which drives continuous flow of media into the bioreactor [5].

The bioreactor and pump parts have been designed in Solid Works and manufactured by 3D printing using VeroWhite [5]. The bioreactor consists of a base, body and a cap. The base has a provision for placing a coverslip where the cells are seeded. A polydimethyl siloxane ring (PDMS) is present below the coverslip to provide cushioning to it. A silicone-O ring is placed in between the base and the cap to ensure that the bioreactor is water tight. To allow passage of light during imaging and for exchange of gases, the cap is made with a PDMS layer in the middle. The bioreactor has been designed in a way that facilitates easy removal and fixing of the parts for cleaning, seeding cells and adding media. A BOROSIL glass container is used as media reservoir and biocompatible silicone tubings are used to circulate the media. These tubings are inserted into BOROSIL glass tubes which are immersed in the reservoir. The peristaltic pump consists of housing, rotor and motor. It also has an inlet and outlet through which the tubing passes. When an external voltage is applied, the pump drives flow of media inside the tubings. The media is sucked from the reservoir due to action of the pump and it passes through a syringe filter before reaching the bioreactor. The purpose of using a filter is to avoid any particulate matter from entering the bioreactor. The media from the bioreactor flows back into the reservoir. The inlet to the bioreactor is at a lower height than the outlet. The speed of the motor and voltage of power supply can be adjusted to get different flow rates of the media.

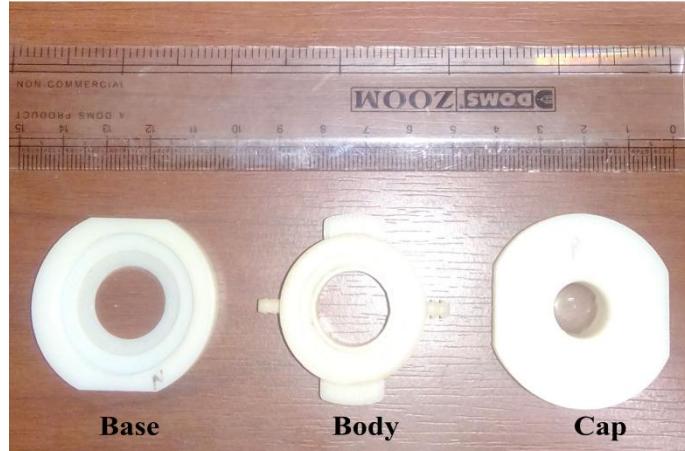


Figure 1. Parts of the bioreactor

1.2 Live cell microscopy

Fluorescent microscopy is used to observe specific cell organelles, proteins or other components of a cell. This enables us to take a quantitative approach to analyse the morphology of cell organelles. Hoechst stain is a fluorescent dye which can be used to stain nuclei of live cells. When the dye is excited with ultraviolet light, blue light is emitted which makes the stained nuclei observable under the microscope.

An inverted microscope has its objective below the stage of the microscope, pointing upwards and the light source and condenser on the top, pointing downwards. This is useful in observing cells that are located at the bottom of a petri dish or a culture flask. Since in this case the cells are seeded on the coverslip at the bottom of the bioreactor, an inverted microscope is suitable for imaging.

This imaging system is equipped with CO₂ and temperature control to provide the required environment to the cells in the bioreactor. 5% CO₂ and a temperature of 37 °C are generally maintained. The microscope also comes with a camera for capturing the images. A separate water bath is used to maintain the media reservoir at 37 °C.

A desktop is linked to the imaging system which will display whatever is seen through the eyepiece. This facility is used to capture and save the desired images.

1.3 Image analysis

It is required to identify quantifiable features of the nucleus to understand its morphology. This is done using the ImageJ software which enables the measurement of area and eccentricity of the nucleus.

2. MATERIALS AND METHODS

2.1 Perfusion cell culture

2.1.1 Cleaning and collagen coating

The bioreactor parts, media reservoir, glass tubes, pump parts and tubings were ultrasonicated once in 70% ethanol and twice in de-ionised water. This was followed by autoclaving and UV sterilization overnight. The coverslips were first cleaned using a detergent (3% extran) and then, using 70% ethanol at 80 °C for an hour. Next, they were dried at 80 °C and UV sterilized for 24 hours inside a laminar air flow hood. The assembly plate, circuit and pump parts which could not be autoclaved were washed with 70% ethanol and UV sterilized overnight. The bioreactor parts were assembled and collagen was coated onto the coverslip placed in the bioreactor for 4 hours at 37 °C. From a stock of 30 µg/mL of Type I collagen, about 700 µL was used for coating. Excess collagen was removed and the bioreactor was washed with autoclaved de-ionised water.

2.1.2 Cell seeding and staining

Around twenty to thirty thousand cells (Rep2a) grown in Dulbecco's Modified Eagle Medium (DMEM) were seeded on the coverslip. The cells were allowed to attach for 24 hours by keeping the bioreactor in an incubator maintained at 37 °C and 5%CO₂. Hoechst stain was used to stain the nuclei of these live cells. The stain and PBS were mixed in the ratio of 1:200 and 200 µL of this mixture was added to the bioreactor and incubated for 10 minutes. Excess stain was removed and 1 ml of DMEM was added. The components of the perfusion culture set up were assembled inside the laminar air flow chamber.

First, the tubing was inserted into the inlet port of the pump housing and removed from the outlet port. The rotor was fitted and pressed into the case in the housing such that the tube went around the roller and was pressed to the housing. The bottom was covered by a casing using bolts. The motor was fitted into the groove on top of the rotor. Once the pump parts were assembled, the pump was secured on the assembly plate and connected to the circuit. The motor was switched on to check the direction of rotation for deciding the inlet and outlet tubings. The inlet tube was immersed in the media reservoir through a glass tube. A tag was tied to the inlet tube close to the pump to avoid the tube from coming out of the housing during perfusion. The outlet tube from the pump was connected to a syringe filter. Another tubing leaving the syringe filter was made to go through a hole of a glass plate before connecting it to the inlet of the bioreactor. A third tubing was connected to the outlet of the bioreactor, the other end of which was made to go through another hole in the same glass plate before it was inserted into the media reservoir. The media reservoir was filled three-fourth with deionized water and perfused for some time under UV, as a precaution to avoid contamination. Next, the media reservoir was filled with about 10 ml of medium (DMEM) and perfusion was started to ensure that there was no leakage of media. The setup was then transferred to the microscope for imaging.

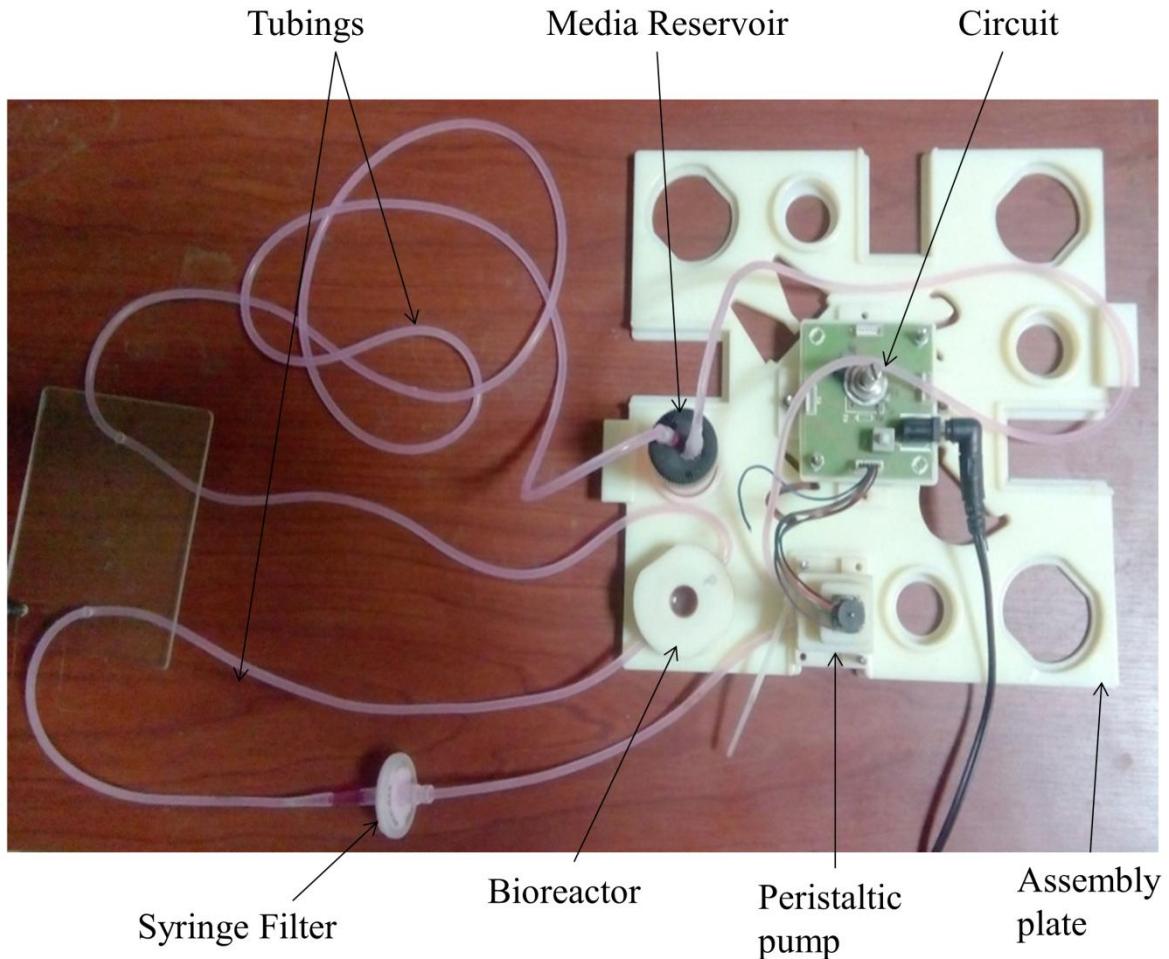


Figure 2. Assembly of perfusion culture set up

2.2 Live cell imaging

An inverted fluorescent microscope (Leica 16000B) was used for imaging. The microscope along with temperature and CO₂ control were started an hour before beginning imaging, to allow it to stabilize. The water bath was also switched on beforehand and was placed on a table close to the microscope. After these units were stabilized, the bioreactor was placed on the stage of the microscope. The media reservoir was made to sit inside the water bath. The assembly plate with the pump was placed next to the microscope on a table. The microscope consists of a motorized stage which can be moved to different positions to view the cells located in different regions of the coverslip (inside the bioreactor). Different positions near the inlet, outlet and center of the bioreactor were considered and marked for imaging. The desired position was focused and marked on the screen using the Leica software. This position was saved and could be revisited when the image had to be captured. Two positions each at the inlet, center and outlet regions of the bioreactor were marked. The first set of images was taken before starting perfusion at each of these positions with 20x magnification. Following this, perfusion was started at a flow rate of about 0.8ml/min. Images were taken after every hour at each of the set positions. Four time points were considered.

Care was taken to ensure that perfusion was taking place and that there was no leakage of media, by frequently checking the flow.

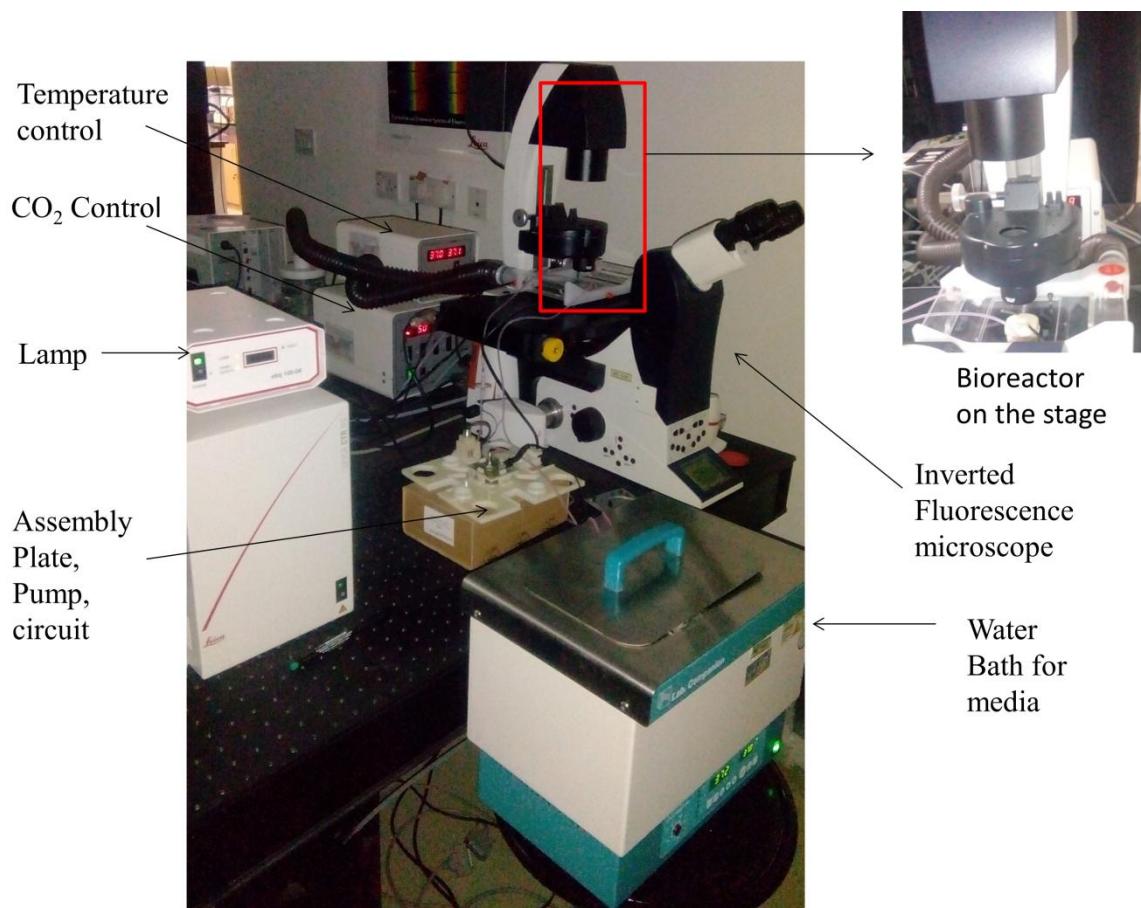
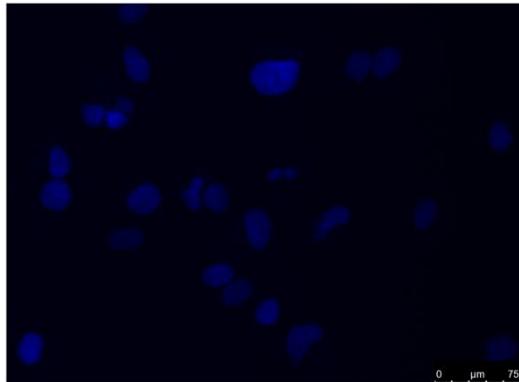
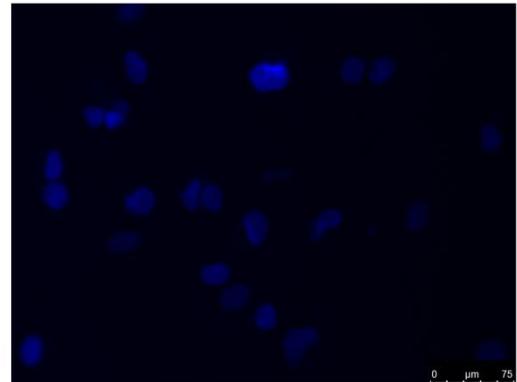


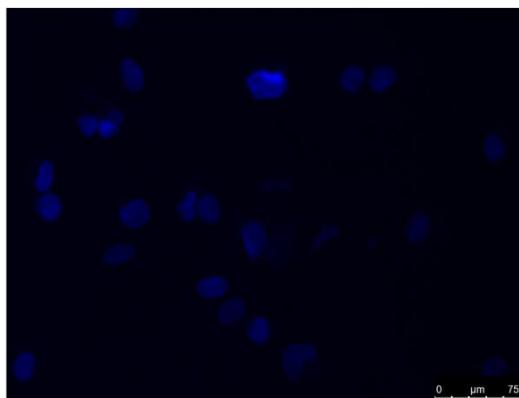
Figure 3. Set up for live cell imaging



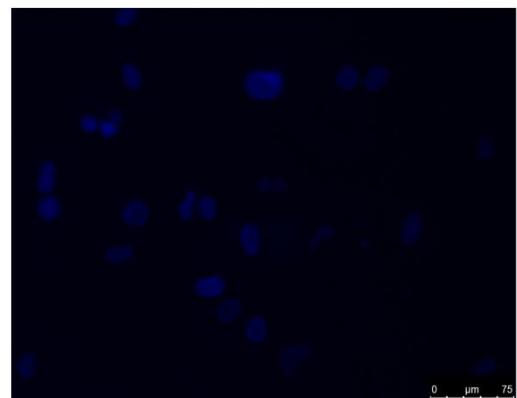
4 (a)



4 (b)



4 (c)



4 (d)

Figure 4. Live cell images captured at different time points.

4(a) Before perfusion, 4(b) 1 hour after perfusion, 4(c) 3 hours after perfusion, 4(d) 4 hours after perfusion.

2.3 Image processing

The images were analyzed using the ImageJ software. The idea was to fit an ellipse to each nucleus and find its area and eccentricity. The captured images were first converted to 8 bit and adjusted for brightness and contrast. Then the threshold was adjusted and they are converted to binary images. The next step was to set the scale. The scale bar on the images was used to convert the scale from pixels to micrometers. The area, major and minor axes measurements were selected. Particles larger than 30 micrometers were analyzed. The option for displaying the results was selected. From the data obtained, the eccentricity was calculated for each of the nuclei. A graph of area versus eccentricity was plotted to analyze the shape of nuclei. The graphs were plotted at different time points as well as for different regions of the bioreactor.

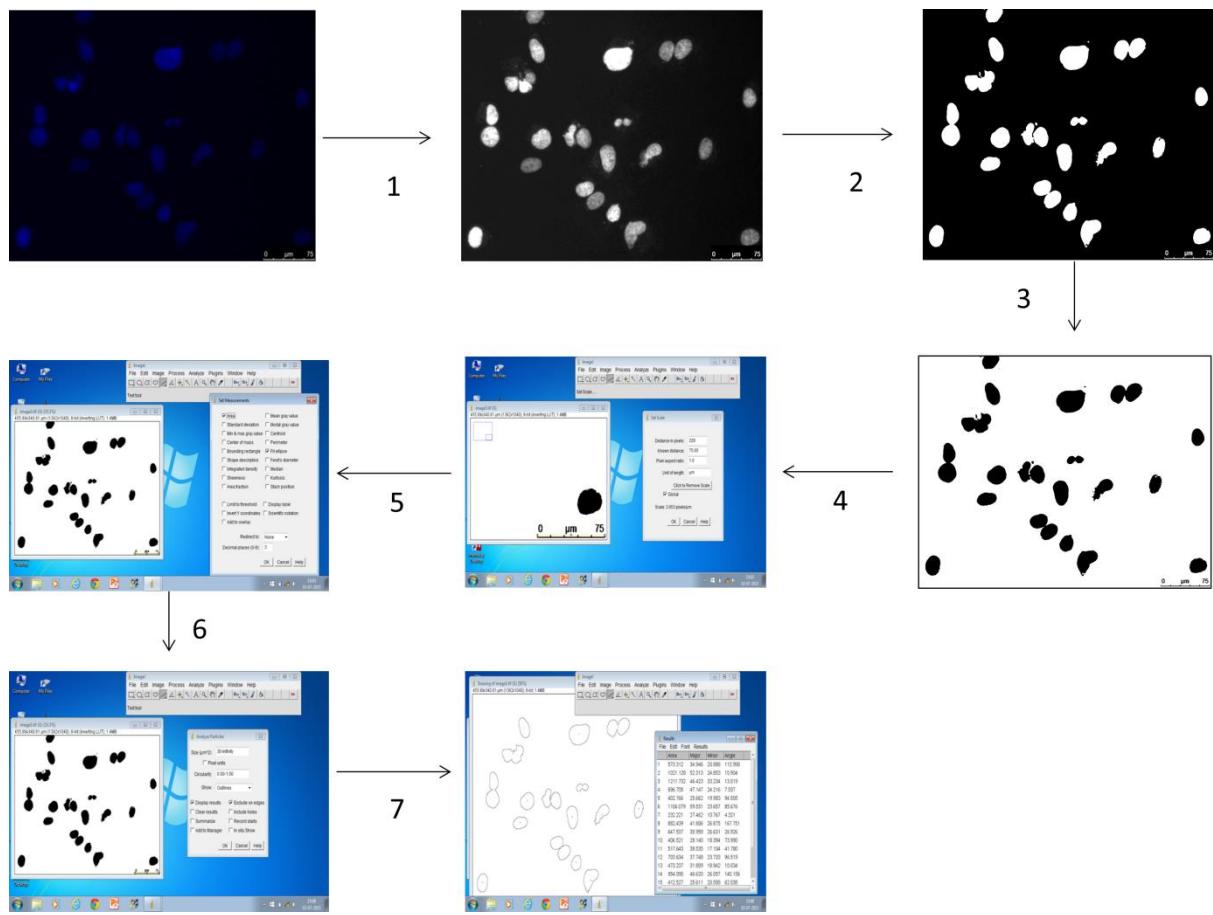


Figure 5. Steps involved in image processing. 1. Conversion to 8 bit and adjustment of brightness and contrast. 2. Adjustment of threshold. 3. Conversion to binary. 4. Setting the scale. 5. Selecting measurements. 6. Selecting size of particles. 7. Displaying the results.

2.4 Measurement of flow rate

The perfusion can be set at different flow rates. The flow rate was measured at three positions – low, medium and high and it was calculated at two different voltages – 3 V and 4.5 V. In the perfusion set up, two reservoirs were used instead of one. One of them was filled with de-ionised water. The tubing was taken from this reservoir to the pump and the other end of it was inserted into the second empty reservoir. The empty reservoir was completely dried and its weight was measured using an accurate weighing balance. Perfusion was started and the water was collected in the empty reservoir for a known period of time. The filled reservoir was weighed again and the change in weight was noted. From this, the volume of water was calculated. Flow rate was calculated as the ratio of volume to time. This procedure was repeated 3 times and the mean flow rate was computed. This way the flow rates were determined for each position at the two different voltages.

3. RESULTS AND DISCUSSIONS

3.1 Measurement of flow rate

The measured flow rate for different positions of the potentiometer at two different voltages is shown in Fig. 7. It was found that the flow rates were almost linearly increasing and there was significant difference between the flow rates at 3 V and 4.5 V. The very low standard deviations of the flow rates indicated that the measurement was quite accurate.

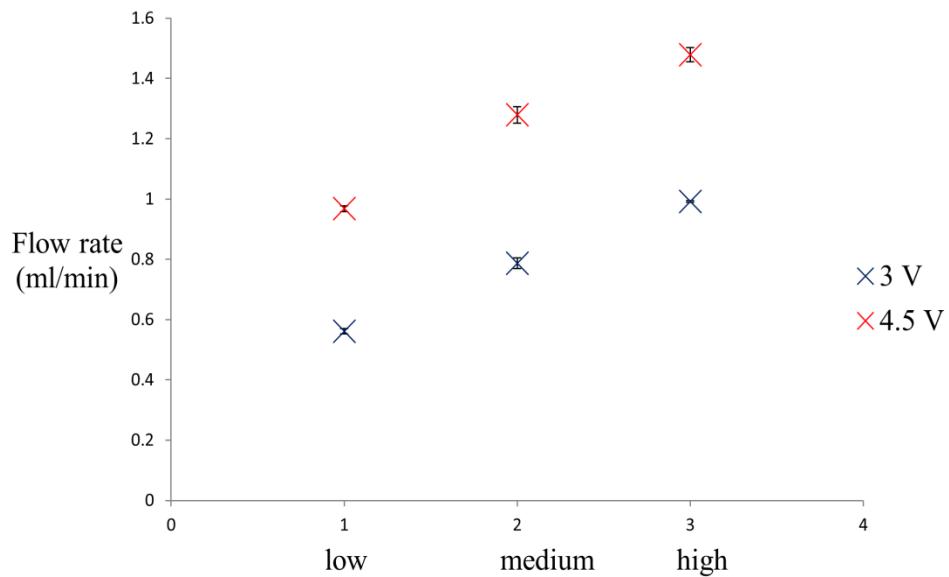


Figure 6. Plot of mean flow rates at different positions

3.2 Nuclear shape analysis under shear flow

The area of the nucleus is plotted against its eccentricity at various locations in the bioreactor for different time points (Fig. 7). From the graphs 6(a) and 6(b) it was observed that the area of nuclei had decreased with time. There was no significant change in eccentricity.

The experiment was carried out only for four hours. It is required to run the experiment for longer hours to check the time point at which significant change in shape of the nucleus takes place.

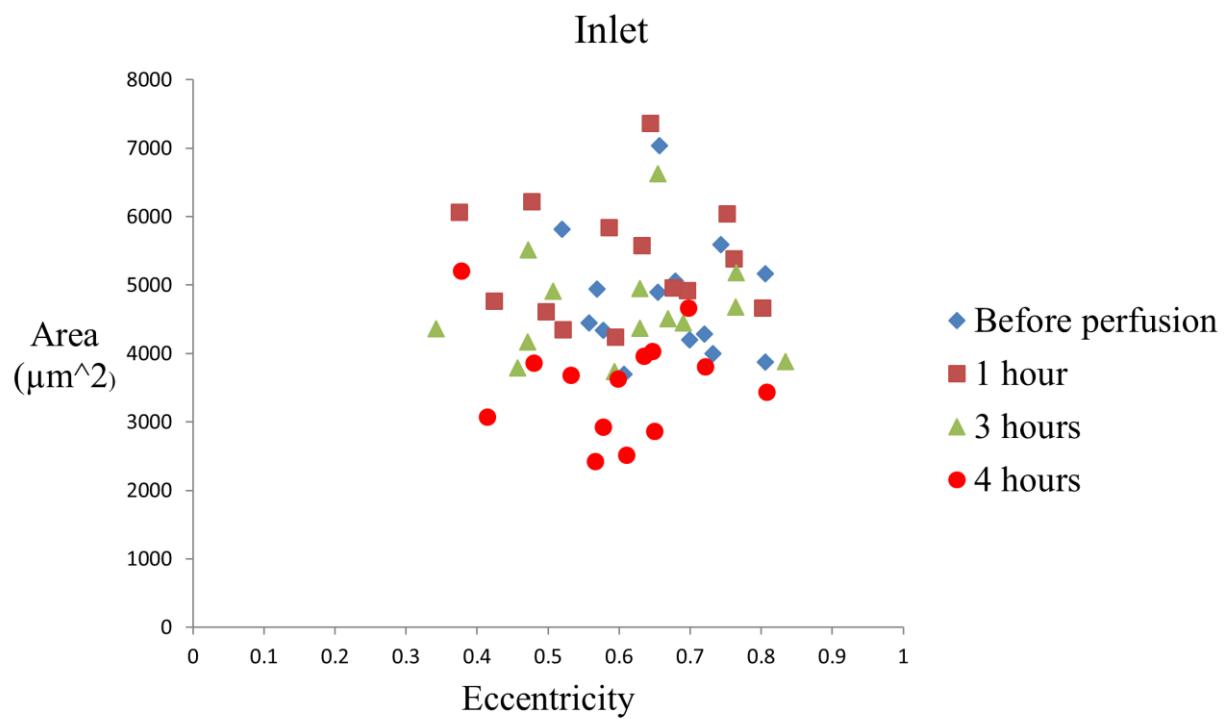


Figure 7(a). Plot of area versus eccentricity for nuclei at the inlet of the bioreactor

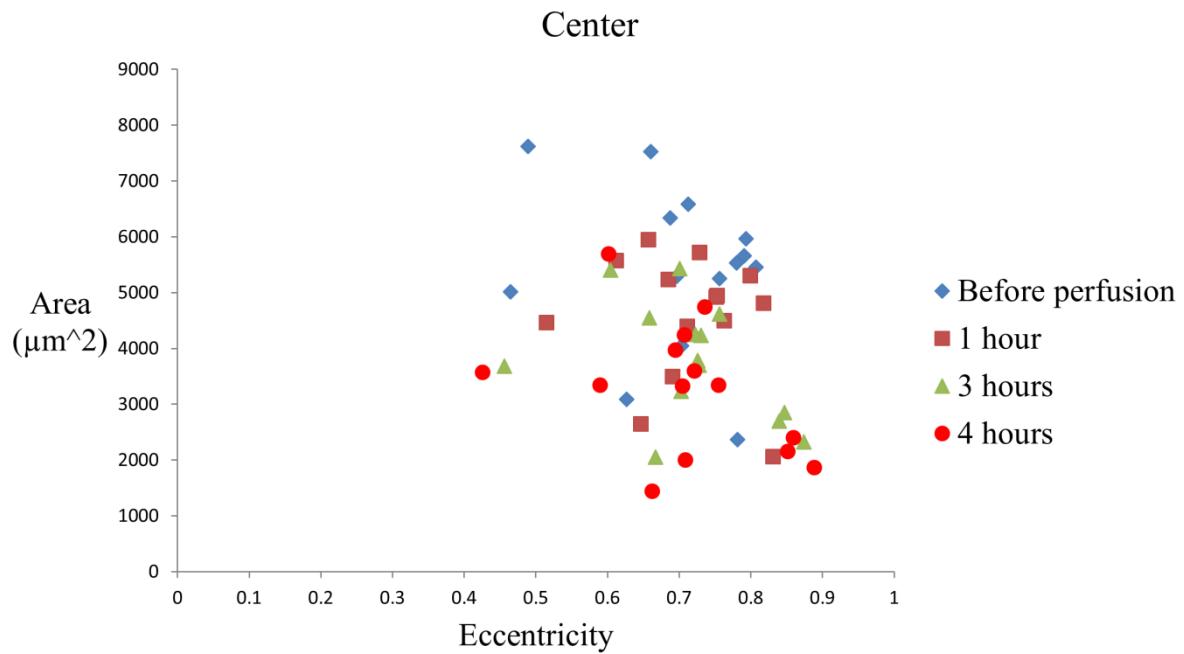


Figure 7(b). Plot of area versus eccentricity for nuclei at the center of the bioreactor

The experiment can be extended in different ways in the future -

1. It can be repeated at different flow rates of the media to see how they affect the shape of nuclei.
2. It can be used to compare different cell lines.
3. Other cell organelles such as mitochondria can be studied.

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