Synchronized Cellular Mechanosensing due to External Periodic Driving

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Abstract

The response of cells to periodic driving is important for many biological processes, particularly blood flow and heart functioning. Our research analyzes the collective shear stress response of fibroblast cells due to a periodic driving frequency. When cells experience shear stress, they release calcium into the cytosol. To quantify the shear stress response of the cells, we use calcium imaging and single-cell level analysis. We had previously observed that the average collective calcium response of a monolayer of fibroblast cells had a different frequency than the applied periodic shear stress. Upon further testing, we ran into difficulties with air bubbles that caused the previous results to not be repeatable. We developed a new, more stable procedure including the use of a flow controller and a bubble trap. This work analyses the old procedure, the previous data, and the new procedure. With the changes made, we were able to successfully developed a new procedure to run the periodic shear stress tests.
Declaration

The work in this thesis is based on research carried out at the Cell Biophysics Lab, the Department of Physics, Corvallis Oregon. No part of this thesis has been submitted elsewhere for any other degree or qualification and it is all my own work unless referenced to the contrary in the text.

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Chapter 1

Introduction

1.1 Motivation and Objective

The response of cells to periodic driving is important for many biological processes, particularly for proper blood flow and heart functioning. My research analyzes the collective shear stress response of fibroblast cells due to an applied periodic driving force. When cells experience shear stress, they release calcium into the cytosol. While the cells experience an oscillatory shear stress over time, the calcium levels in the cytosol will fluctuate. Calcium dye is added to the cells to track the shear stress response of the cells. While the cells experience the applied shear stress, they are imaged using fluorescence microscopy. The intensity profile of the calcium within the cells is then derived from the images. Then the intensity profile of the calcium can be compared to the shear stress profile applied to the cells to understand the response of the cells to the shear stress. We are interested in how the collective cellular response compares to the singular cell response. Also, we want to understand the role cellular communication plays in the collective mechanosensing of the fibroblast cells.

1.2 Shear Stress

Shear stress is simply a force per area or a pressure that is felt when one medium is in contact with and moves parallel to another medium. A biological example
of shear stress would be endothelial cells that line blood vessels. They constantly experience shear stress from the flow of blood through the blood vessel that they comprise. As such, shear stress and the effect it has on cells is very important for proper function. For our experiments, we will be using Newtonian fluids and laminar flow. Newtonian fluids are fluids such that the viscosity is independent of the amount of shear applied to the fluid. Laminar flow occurs when the fluid does not interfere with itself and this results in a constant velocity throughout the fluid. For a Newtonian fluid, the shear stress equation is:

\[ \tau(\vec{u}) = \mu \nabla \vec{u} \]  \hspace{1cm} (1.2.1)

Where \( \tau \) is the shear stress and it is proportional to the shear rate, \( \nabla \vec{u} \), by a factor of the viscosity \( \mu \). The shear rate is the gradient of the velocity, \( \vec{u} \), of the fluid. Figure 1.1 shows a flow rate, \( Q \), being applied along the tops of cells in a single channel device similar to the experimental setup we will use. The flow rate supplies the shear stress to the cells.

Figure 1.1: The diagram shows a monolayer of fibroblast cells inside a channel or device. A laminar flow of fluid moves through the channel with flow rate \( Q \). The flow of the fluid along the tops of the cells causes the cells to experience shear stress \( (\tau) \) in the horizontal direction [1].

1.2.1 Cellular Response to Shear Stress

While the exact mechanism for cellular response to shear stress is unknown, one part of the response is the release of calcium into the cytosol of the cell. There are two pathways that this can occur by. The first is for calcium to be released from
the endoplasmic reticulum (ER) via the sarco/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) pump. The second is that the plasma membrane Ca$^{2+}$ ATPase (PMCA) pump on the cell membrane will take extracellular calcium and release it into the cytosol. The pathway used for shear stress response has yet to be discovered. It could be one of these pathways or both of them. A diagram showing this response can be seen in figure 1.2.

Figure 1.2: The diagram shows the calcium response of a cell in a monolayer. As the cells experience shear stress (τ), calcium is introduced into the cytosol. This calcium may come from outside the cell or from the endoplasmic reticulum (ER) within the cell [2].

### 1.3 Cellular Communication

Cells can communicate in a multitude of ways. Gap junction communication is a very common form of communication and is important for our research. Gap junctions are common in many animals and cells in tissues communicate using them [3]. Cells can directly transfer small molecules and ions from the cytoplasm of one cell to another using gap junctions (figure 1.3). The gap junctions are composed of two connexons that connect across the intercellular space and through both cell membranes [3]. The movement of small molecules and ions occurs via passive diffusion [3]. For normal function of organs, gap junction communication is important for regulating events...
1.4. Fluorescence Microscopy

between cells [3].

Figure 1.3: *Cells can communicate with each other through gap junctions. A gap junction consists of two connexons that allow small ions and molecules to transfer from the cytoplasm of one cell to the cytoplasm of another cell [4].*

1.4 Fluorescence Microscopy

A fluorescence microscope is similar to a conventional light microscope, but it uses a higher intensity light source that excites a fluorescent tag or dye in a sample which emits a lower energy light that produces a magnified image (5). A diagram of the basic setup of a fluorescence microscope can be seen in figure 1.4. To prepare a sample for fluorescence microscopy, a fluorescent tag or a fluorescent dye must be added to the sample. The fluorescent tag and dye contain fluorophores which absorb the high energy light they are illuminated with and emit lower energy light allowing for the substance to be imaged. A fluorescence microscope usually has an emission filter which allow a specific wavelength of light through. This wavelength corresponds to the fluorescing material being used in the experiment so that only the light emitted by the fluorophores will be detected (5).
Figure 1.4: The object being imaged is labeled with a fluorescent substance called a fluorophore. A higher energy source is produced using an excitation filter. The light source is then used to illuminate the object. This light is absorbed by the fluorophore and lower energy light is emitted. Emission filters are used to exclude the surrounding radiation and only image the light emitted by the fluorophore [5].
Chapter 2

Initial Methods

2.1 Experimental Tests

The experimental tests run for this research project consist of making a single channel device\(^1\), fibroblast cell preparation, applying an oscillatory shear stress to the cells, and using fluorescence microscopy to image the resulting calcium dynamics of the cells. A diagram of the single channel device can be seen in figure 2.1.

Figure 2.1: The single channel device is shown from a top view and side view in the diagram. The completed device consists of a glass slide on the bottom, an open channel with an inlet/outlet at both ends, and PDMS surrounding the channel.

\(^1\)A single channel device is similar to a microfluidic device which deals with the flow of fluids through micrometer sized channels. The device used here is on the millimeter scale and consists of a simple single channel that is encased in PDMS and bounded on the bottom by a glass coverslip.
2.2 Device and Cell Preparation

2.2.1 Making the Devices

The single channel devices are created by making the polydimethylsiloxane (PDMS) mixture, pouring it over the stainless steel device mold, curing the PDMS, cutting out the devices, and assembling the final product. The PDMS mixture is composed of 11 parts of a base to 1 part of a curing agent. All bubbles are removed from the mixture by using a small vacuum chamber. The PDMS is then poured over the device mold and this mold is cured in an oven at 65 °C for 3 hours. After the PDMS has been cured, the devices are cut out of the mold, holes are punched for the inlet/outlet, and then the PDMS device is bound to a glass slip via corona treatment. To ensure a strong bond, the devices are then placed on a hotplate with weights added on top for approximately 15 minutes.

2.2.2 Cell Culturing

The NIH Native 3T3 mouse fibroblast cells used for these experiments are kept in an incubator with growth medium (Dulbeccos modified Eagle medium (DMEM) supplemented with 10% bovine calf serum and 1% penicillin). The cells are subcultured using TrypLE Select, centrifuged, and then re-suspended in growth medium. This cell mixture is inserted into the devices and left to incubate over night.

2.2.3 Calcium Dye

In the morning, the cells are treated with a mixture of 1 mL of a 2x concentration of calcium dye and 20 μL of probenecid. The cells are put into the incubator for 20 minutes and then spend 10 minutes covered with tin foil on the counter. After this time, the excess calcium dye can be removed and replaced with growth medium.

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2.3 Pressure Pump-Device System

The shear stress tests are conducted using a pressure pump system to apply the oscillatory shear stress profile. A diagram of the experimental setup can be seen in figure 2.2. The pressure pump and vacuum are connected to the computer and the tubing system which is filled with growth medium. The pressure pump applies a negative pressure that pulls liquid from the growth medium reservoir on the right, through the single channel device, and into the reservoir on the left. The flow of liquid through the device applies a shear stress along the top of the cells in the device. The single channel device is connected to a microscope and imaged with fluorescence microscopy.

Figure 2.2: The pressure pump system consists of a pressure unit and vacuum. These are connected to a computer to run the pressure profile and to a tubing system which connects to the device and liquid reservoirs. The tubing and reservoirs are filled with growth medium. The pressure pump system applies a negative pressure which pulls liquid from the reservoir on the right, through the device, and into the reservoir on the left [6].
2.4 Fluorescence Imaging and Data Analysis

2.4.1 Fluorescence Imaging

To measure the calcium in the cytosol, we use calcium imaging. An important part of calcium imaging is fluorescence microscopy. To use this type of microscopy technique, a sample must be labeled with a fluorescent substance. In our experiments, we use a calcium dye (Fluo4, Life Technologies) which binds fluorophores to calcium molecules. The sample is then illuminated with a high energy light source, obtained by using an excitation filter, and the fluorophores absorb this light and emit a lower energy light. Using emission filters to ensure surrounding radiation is excluded, the light emitted by the fluorophores can be imaged.

After the device has been connected to the pressure pump system, the location to be imaged within the sample is found using the microscope. The lights are turned off and the imaging and pressure pump profile are turned on at the same time. An image is taken every 2000 ms. Due to the small time between images, auto focus is turned off. The tests generally run for 30 minutes with the sinusoidal pressure profile and then the pressure is set to 0 mbar for another period. The images are saved to the computer.

2.4.2 Data Analysis

For the intensity profile analysis, the first image in a set is used and markers are placed on each cell (sample image shown in figure 2.4). This image is used in a Matlab code which tracks the location of each marker throughout the data set to get intensity profiles from each cell. From this, plots are made of the individual cellular intensity profiles and the average intensity profile. The locations of each cell are specified by a blue dot (markers). There are approximately 2,000 cells per data set and it takes two to three hours to mark the locations of all cells per data set. The Matlab code only takes a couple of minutes to run.
2.4. Fluorescence Imaging and Data Analysis

Figure 2.3: This is the first image in one data set which has been edited to place blue dots on each cell. The image is approximately 1 mm by 1 mm and has around 2000 cells in a monolayer. A scale bar is added to show the size of 100 µm in reference to this image. The blue dots are used as location markers in a matlab code to track the intensity in those location over the full data set. From this, the intensity profiles of each cell and the average intensity profile of all of the cells can be found.
Chapter 3

Initial Results

To test the validity of these methods and calibrate the process, we collected preliminary results. The preliminary results consist of intensity profiles of the cells and are collected over a range of varied parameters.

We ran a set of 8 tests changing either the amplitude of the pressure profile (2 mbar or 4 mbar) or the period of the oscillation (2 minute or 4 minute). A table of the tests can be seen in table 3.1.

<table>
<thead>
<tr>
<th>Amplitude of Pressure</th>
<th>Period of Oscillation</th>
<th>Number of Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mbar</td>
<td>2 minutes</td>
<td>2</td>
</tr>
<tr>
<td>2 mbar</td>
<td>4 minutes</td>
<td>2</td>
</tr>
<tr>
<td>4 mbar</td>
<td>2 minutes</td>
<td>2</td>
</tr>
<tr>
<td>4 mbar</td>
<td>4 minutes</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.1: *Table of the eight experimental tests completed. The amplitude of the pressure profile, the period of oscillation of the pressure profile, and the number of tests completed at these values are specified for all eight tests.*

After processing the data, we plot the intensity profile of 30 randomly chosen cells from our data set (this ranged from 1,500-2,500 cells for the 8 tests run), the average intensity profile over all of the cells, and the applied oscillatory pressure profile. One of the 8 graphs generated can be seen in figure 3.1.
Figure 3.1: The plot shows the relative intensity of the calcium response over the duration of the experiment. One frame was taken every 2 seconds. The blue curves are 30 randomly selected individual cell responses, from the approximately 2000 individual cell responses in the data set. The black curve is the average intensity response of all of the cells (collective response). Both the black and blue curves correspond to the left y-axis. The yellow curve corresponds to the right y-axis and is the pressure profile of the applied shear stress. A key feature of this graph, if that the average response (black curve) appears to have twice the frequency of the applied pressure (yellow curve) after some time.

The graph in figure 3.1 shows the intensity responses of 30 randomly chosen cells (in blue), the average response of all of the cells (in red), and the pressure profile applied (in green). It is important to notice that the red curve does not show a simultaneous response to the applied pressure and that the frequency of the average response (red curve) after approximately frame 400 is twice the frequency of the applied pressure (green curve).

From the intensity data shown in chapter 3, it is important to notice that the
behavior of the average response is not uniform throughout the whole data set. The oscillatory response is not simultaneous in the beginning, but with time the response becomes well synced and has a frequency of oscillation that is twice the frequency of oscillation of the applied pressure profile. This suggests that the cells require a time period to sync to the applied shear stress. It also brings up the question of cellular communication in producing the synced and doubled frequency that is seen later. To fully understand this behavior, more tests will need to be run, testing more periods of oscillation and testing the role of cellular communication.

When we tried to repeat these tests we ran into complications with our experimental setup. The biggest complication was air bubbles entering the tubing system and altering the intensity images rendering the data useless. Another complication was that the pressure pump only supplies the pressure profile dictated but does not account for the height of the reservoir. Due to these complications we have worked to get new equipment and change our experimental setup to incorporate them. With the proposed changes we will have a more reliable and easier to repeat experimental setup.
Chapter 4

Improved Methods

4.1 Changes Made

Four major changes were made to our experimental setup to have a more stable and more easily repeatable experimental method. The changes are the addition of a flow rate controller, the addition of a bubble trap, the replacement of the devices, and the change in direction of the pressure. A diagram of the new experimental setup can be seen in figure 5.1.
4.1. Changes Made

Figure 4.1: *The pressure pump system consists of a pressure unit and vacuum that are connected to a computer and the tubing system which is filled with growth medium. The major changes in the experimental setup are the addition of the bubble trap and flow controller, the change in direction of the pressure, and the new devices being used. The flow controller monitors the flow rate in the tubing system and connects to the computer so that the pressure pump can be changed to produce the desired flow rate profile. The bubble trap releases bubbles from the tubing system. The new devices have luers to lock the tubing into the devices to avoid air bubbles and growth medium leakage [6].*

4.1.1 Flow Rate Controller

The flow rate controller (Bronkhorst Mini CORI-FLOW) measures the flow rate in the tubing system. The controller is connected to the computer which allows the controller and computer to change the pressure if needed to keep the desired flow rate profile. This addition allows us to have a more precise shear stress profile in our devices which is based on the actual flow rate and not the pressure we apply. We now do not need to worry about the change in height of the growth medium reservoirs effecting the flow rate during testing.

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4.1.2 Bubble Trap

Having bubbles in our single channel device can change the shear stress the cells experience and it changes the intensity of the light we image which makes our images useless for the region of time a bubble is in the device. To fix this complication, we purchased a bubble trap which connects to our tubing system and releases the air trapped in the tubing. The bubble trap feeds in two pieces of tubing which reach a semi-permeable membrane inside the trap. On the other side of the membrane is a tunnel for air bubbles to escape out of. The liquid in the tubing cannot pass through the membrane and so it is transferred to the other piece of tubing. Figure 5.2 shows a simple diagram of the bubble trap.

![Figure 4.2: The bubble trap connects to the tubing system and releases the air trapped in the tubing out the back of the bubble trap so that the tubing leaving the bubble trap has no air bubbles in it. It does this through the use of a semi-permeable membrane which allows the air bubbles to leave but not the fluid [6].](image)

4.1.3 New Devices

The new devices are ibidi $\mu$-slides. These have the same basic design as the previous devices but can vary in the height and volume. The key change is that they have easily connected Luer adapters which will give us a better seal going from the tubing to the device to prevent leakage or bubbles entering the tubing. An image of the device with the Luers is shown in figure 5.3.

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4.1. Changes Made

Figure 4.3: The ibidi $\mu$-slides are single channel devices that have an inlet and outlet and are very similar in structure to the single channel devices shown previously in figure 2.1. There are minor differences in the dimensions between, but the major difference between the two devices is that the ibidi slides have luer adapters. These adapters connect the tubing to the inlet and outlet and allow for a tighter seal which helps prevent air bubbles and leakage of growth medium [7].

4.1.4 Direction of Pressure

Finally, we changed the direction of the pressure to reduce the likelihood of bubbles entering the tubing system. With a negative pressure, any leak in the tubing would result in air being sucked into the tubing system. With a positive pressure, we reduce the likelihood of this happening.
Chapter 5

Discussion

Our preliminary results showed interesting behavior in the collective response of cells due to shear stress. These results motivated us to explore this response more. However, when we tried to repeat the results using the original method, we ran into multiple difficulties. The main complication was from air bubbles entering the tubing system and altering the images we took, changing the shear stress on the cells, and possibly damaging the cells. To fix this, we introduced a flow rate controller, a bubble trap, changed the devices, and changed the direction of the pressure applied. With these changes in place, we developed a more stable and easier to repeat experimental setup. The flow rate controller ensures that the shear stress within the device is exactly what we want it to be. The bubble trap helps prevent any bubble errors resulting in a loss of data. The new devices help prevent bubbles and leakage of fluid. Finally, the direction of pressure also helps prevent against bubbles entering the tubing system. With these new changes in place, we are now ready to run experiments and continue learning about the collective cellular response of cells due to oscillatory shear stress.
Chapter 6

Conclusion

We investigated the collective response of fibroblast cells due to an applied shear stress. The response of cells from shear stress is important in many biological systems, for example in blood vessels. Understanding the response of cells can help us to understand how these cells function. To investigate this situation, we ran experiments where fibroblast cells are arranged in a monolayer and are subjected to shear stress. Using calcium imaging techniques, we can analyze the calcium response from the shear stress. From the preliminary results, we saw interesting behaviors in the frequency of the response compared to the frequency of the applied shear stress. When trying to repeat these results and run more tests, we ran into experimental complications with bubbles entering the system. After fixing these complications and implementing a new experimental setup, we now have a better and more reliable procedure.

Now that we have a new experimental setup, we want to run tests varying the period of the shear stress, decreasing the density so that cells cannot communicate with each other, and using a chemical to block one of the calcium pathways. For all of these tests we will analyze the data the same as before to compare the individual cellular responses to the collective response. These tests should help us understand how cells respond to shear stress and the role of cellular communication in the response.
Bibliography


[7] Figure adapted from https://www.ibidi.com.