Effects of Cholesterol Depletion on Membrane Capacitance & Cytoplasmic Conductivity

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Applied Science
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Abstract

Cholesterol is a key molecule in a wide variety of cellular functions and serves as an important indicator for cellular properties. Cholesterol can be measured directly using fluorescent techniques or indirectly through biophysical properties, such as capacitance. To explore the relationship between membrane cholesterol depletion and electrical properties, single cell impedance spectroscopy was used. HEp2 cells were exposed to methyl-β-cyclodextrin, a cholesterol-depleting molecule, for varying treatment times. The level of depletion was determined using a Filipin-III fluorescent assay. A frequency sweep was performed in a microfluidic constriction channel, allowing the electrical properties to be extracted by fitting the data to a circuit model. Depletion of cholesterol was shown to affect both the membrane capacitance and the cytoplasmic conductivity. The capacitance decrease is thought to be caused by a decrease in surface area and the conductivity by changes in the ion concentration of the cytoplasm.
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1 Introduction

1.1 Background Information

1.1.1 Cholesterol

Cholesterol was first discovered in gallstones in 1769 by Poulletier de la Salle [1]. As shown in Figure 1, this unique molecule consists of four rings with side chains and methyl groups located above the plane, giving this molecule a rigid core with a flexible tail [2].

![Figure 1 - The molecular structure of cholesterol.](image)

Cholesterol is vital in maintaining proper cellular function. This molecule can be used to synthesize hormones [3], is part of signaling pathways [4], and is present in high quantities as part of the cellular plasma membrane [5]. Cholesterol interacts with phospholipids present in the membrane, altering both the physical and biological properties [6]. These changes include regulating the permeability to ions [7] and water [8], controlling the membrane fluidity (i.e. viscosity) [6], and maintaining proper orientation and location of membrane proteins [9, 10]. Cholesterol is also necessary for different structures found on the cellular membrane, such as lipid rafts and caveolae. A lipid raft is a dense domain of sphingolipids, cholesterol, and certain
proteins depending on the lipid raft’s function [11]. These rafts are able to freely move around the cellular membrane to perform signaling functions [11]. For example, lipid rafts are involved in signal transduction of the tumor necrosis factor receptor family [12], to T cell antigen receptor involved in the immune response [13]. Caveolae are small invaginations on the membrane created through the interaction of cholesterol and caveolin proteins [11, 14]. Caveolae provide important functions for the cell including increasing cell membrane surface area [15], interacting with ion channels [16], responding to mechanical stimulation [14, 17] and are involved in signal transduction [14, 18]. Examples of these functions include insulin receptors being localized in caveolae during signaling [19] to cellular migration where they are polarized on migrating endothelial cells [20].

Given that cholesterol is important to many cellular functions there is no surprise that it is involved in a number of diseases. For instance, in cancer, cholesterol contributes to the adhesion/migration of cancerous cells by providing crucial lipid rafts on which adhesion proteins like CD44 rely on [21]. An increased level of lipid rafts in certain cancer cell lines has also been shown to have a correlation with cellular death when treated with methyl-β-cyclodextrin [22]. Mouse models fed with a high cholesterol diet had the cell cycle protein cyclin D1 present in high quantities indicating a more aggressive tumor formation [23]. In Alzheimer’s disease, it has been reported that the level of the caveolin protein is raised in the hippocampal region of the brain [24]. Lastly, human respiratory syncytial virus requires the use of lipid rafts in lung epithelial cells, which are dependent on cholesterol, to complete the viral cycle [25].

Adjusting cholesterol levels via therapeutic drugs present a viable treatment option for certain diseases. Statins, an inhibitor of HMG-CoA reductase and commonly prescribed, have been shown in randomized trials to reduce the 5-year risk of coronary disease and stroke by about one
fifth per mmol/L reduction in LDL cholesterol [26]. Novel cholesterol lowering drugs are continually being discovered such as berberine, a Chinese herb extract. Berberine has been shown to lower serum cholesterol by 29% in three months by increasing the low density lipoprotein receptor [27].

1.1.2 Cholesterol Depletion

To better understand how cholesterol affects different cellular functions, it is necessary to be able to raise or lower the cholesterol content in a cell. Experimentally, one method of depletion is to incubate cells in a solution of methyl-β-cyclodextrin (MβCD). MβCD, as seen in Figure 2, is a sugar ringed-shaped molecule that has a hydrophobic interior that has been methylated to further improve its water solubility [28]. While the exaction mechanism of cholesterol is not fully known, proposed models include cholesterol extraction via collision, diffusion into the hydrophobic interior or an activation-collision mechanism [28].
Figure 2 - The molecular structure of methyl-β-cyclodextrin.

The amount of cholesterol removed from the cell is dependent on both the concentration of MβCD and the incubation time. Previous research suggests that high concentrations (>10mM) and long incubation times (>30 minutes) favour cholesterol extraction from the entire membrane, whereas low concentrations (<1mM) and short incubation times (2-10 minutes) favour cholesterol extraction from lipid rafts [28].

If the MβCD is first loaded with cholesterol before exposing it to the cell, then the membrane cholesterol can be raised. The amount of cholesterol raised is dependent on the MβCD to cholesterol molar ratio, the MβCD-cholesterol concentration and exposure time [28]. This method can be used not only on cells where cholesterol has first been depleted, but also on cells with unaffected levels of cholesterol [28].
1.1.3 Cholesterol Measurement Techniques

Cholesterol is commonly measured using fluorescent biochemical assays. Quantitative fluorescent kits detect as low as 80 ng/mL of cholesterol using enzymatic methods [29]. Fluorescent kits also exist which detect the relative change of cholesterol using the compound Filipin-III. The Filipin-III assay can be considered a gold standard measurement because of its application in aiding diagnosis of Niemann-Pick disease type C [30]. Niemann-Pick disease type C is a lipid storage disease with abnormal levels of unestrified cholesterol located in the cell, hence necessitating the use of a Filipin-III stain [31].

In addition to the fluorescent methods it is also possible to correlate the change in cholesterol with mechanical and electrical biophysical properties. With respect to mechanical properties, cholesterol depletion has been shown in different cell models to affect membrane stiffness [32, 33]. The electrical property of capacitance has also been demonstrated to change depending on the amount of cholesterol present in the membrane, typically measured via the patch clamp method. Modulation of cholesterol content in artificial membranes has been demonstrated to cause changes in capacitance [34, 35, 36, 37]. In cells, cholesterol depletion has been shown to decrease [38, 39, 40] or have no effect [41] on capacitance.

1.1.4 Cellular Electrical Properties

Before any change in membrane capacitance can be measured, the cell needs to be described in terms of electrical circuit components. To model a cell electrically, several important assumptions are made to aid in analysis. A cell can be considered a conductive body (cytoplasm) surrounded by a capacitive boundary (plasma membrane) [42]. Therefore an appropriate circuit model can be seen in Figure 3, where $C_{\text{Mem}}$ represents the membrane capacitance and $R_{\text{Cyto}}$ represents the cytoplasm resistivity. Dividing the membrane capacitance by the cellular surface
area yields the specific membrane capacitance. Likewise if the cell’s volume is known, the cytoplasmic conductivity can be calculated from the resistance. Calculating the specific membrane capacitance and cytoplasmic conductivity removes any geometrical coupling and allows cells to be compared on an individual basis. Accordingly, the capacitance can be influenced by a cell’s membrane composition and the resistance by a cell’s cytoplasmic composition [42]. Various methods exist to measure the electrical properties of a single cell. For example there is the path clamp method, electro-rotation, and impedance spectroscopy.

![Diagram](image)

**Figure 3** - A simple electrical model of a cell.

1.1.5 Electrophysiological Measurement Techniques

1.1.5.1 The Patch Clamp Method

The patch clamp method involves gently applying suction on a piece of the membrane using a pipette [43]. This applied suction forms a high-resistance seal and electrically isolates the patch from the rest of the membrane [43]. Ionic currents can then be measured from this patch with the help of amplifiers and the use of a measurement configuration such as cell-attached recording, inside-out recording, whole-cell recording, and outside-out recording [43]. By changing the membrane voltage potential via amplifier feedback, electrical properties such as capacitance can be measured giving insight into the behavior of ion channels and therefore the membrane itself [43]. While a powerful technique, the patch clamp method does present several drawbacks. This method requires a long training time for the user to become skilled (e.g. three-dimensional
maneuvering of the pipette) and has a low throughput due to several time consuming steps (e.g. having to measure cells in series rather than parallel) [44].

1.1.5.2 Electro-rotation

Due to the inherent polarizability of a cell, it can be manipulated in a non-uniform electric field. Dielectrophoresis can be used to move and sort cells, whereas electro-rotation can characterize electrical properties [45]. Cells are modeled as idealized shelled-spheres that contain equivalent circuit components which describe their behavior in a changing field [45]. Much like the patch clamp technique, electro-rotation has its limitations. Electro-rotation relies on an idealized geometry which cells may not conform to [45], has a low throughput [42], and uses a non-physiological low-conductivity buffer that could affect cellular properties [42].

1.1.5.3 Impedance Spectroscopy

In impedance spectroscopy the goal is to analyze how the impedance of a sample varies over a frequency range. Sine waves of various frequencies are inputted to the sample and the output signal is measured. The output typically produces two plots, one representing change in impedance and the other representing change in phase angle as functions of frequency. Electrical parameters of interest can then be extracted by fitting the data to an appropriate circuit model. With the advent of microfluidics, single-cell impedance spectroscopy is possible and has been able to produce large sample sizes due to a high cellular throughput [42, 46]. Some disadvantages of single-cell impedance spectroscopy include microfluidic devices being prone to clogging necessitating filter structures in some cases [47] and potentially complex device fabrication (i.e. microfabrication) compared to previous mentioned methods.
1.2 Motivation & Objectives

The motivation for this thesis is to leverage the advantages of microfluidic impedance spectroscopy in measuring changes in electrical properties due to the depletion of membrane cholesterol. As previously mentioned measuring electrical properties is typically accomplished via the patch clamp method which drawbacks include a low throughput and requires a high level of operator skill. A microfluidic impedance spectroscopy method is used so that larger sample sizes are able to be obtained and reduce the skill demand on the operator. Larger sample sizes will help in illustrating important trends in analysis and to demonstrate the feasibility of this measurement method. Larger sample sizes and live cell monitoring are beneficial to research involving drug or molecular screening to evaluate efficacy.

Therefore, this work reports use of microfluidic impedance spectroscopy to investigate how cellular electrical properties change when membrane cholesterol is depleted. HEP2 cells, an epithelial carcinoma HeLa contaminant cell line [48], were depleted of cholesterol using MβCD for defined time points. A Filipin-III assay was performed to detect the relative change in cholesterol for each defined time point. A single cell was positioned inside a constriction microchannel in order to collect impedance data. This data was then fit to the appropriate equivalent circuit model in order for the relevant cellular electrical parameters to be extracted. Statistical testing and data analysis is then performed to identify trends and significant findings. Finally, potential mechanisms are proposed to elucidate why any changes were observed.

Specific objectives are as follows:

1. To determine the feasibility of using microfluidic single-cell impedance spectroscopy to measure electrical effects due to cholesterol depletion;

2. To characterize the device sensor properties;
3. To determine how the membrane capacitance is affected when cholesterol is depleted;
4. To determine how the cytoplasm conductivity is affected when cholesterol is depleted;
5. To explore mechanisms as to why any changes in electrical properties are observed.
1.3 Thesis Outline

The outline of this document is as follows:

- Chapter 2 covers the experimental outline including, but not limited to, device fabrication, culturing conditions and experimental protocols;

- Chapter 3 discusses the experimental results and subsequent analysis specifically focusing on mechanisms for capacitance decrease and potential limitations of the instrument; and

- Chapter 4 formally concludes the document, identifies the contributions of this research and provides direction for future work and improvement.
2 Material & Methods

2.1 Experimental Design Overview

To measure the capacitance of the cells a simple set up is used. An impedance spectroscope performs frequency sweeps on the microfluidic device to gather impedance and phase data. A simple constriction channel, such as in Figure 4, creates an area of high impedance thereby allowing impedances changes to be easily measured. The device is situated on an inverted microscope in order to view the channel and cells as they pass through the constriction. Once the cells have been prepared for the experiment, the channel is first filled with a physiological buffer solution followed by loading the cells. The electrodes are then placed in the inlet and outlet to complete the electrical circuit. Cells are then drawn into the constriction channel via negative pressure in order for a frequency sweep to be performed. When a cell is placed in the channel, it conforms to a well-known geometry defined by the channel dimensions and causes a large change in impedance.

![Diagram of a constriction channel](image)

**Figure 4** – A constriction channel features a narrow region of high impedance.

The output signal from the device is then passed through a trans-impedance amplifier to convert the output current to a voltage for the spectroscope to record. This simplified layout is illustrated in Figure 5. A picture of the cell is also taken at this point to measure the cell’s geometry. The resulting data can then be fit using appropriate circuit models to extract the resistance and
capacitance of the cell. By combining these values with the geometrical measurements, the specific membrane capacitance and cytoplasmic conductivity can be calculated.

**Figure 5** – A simplified layout of the electrical measurement setup.
2.2 Fabrication Methods

A previous SU-8 master created via standard soft lithography techniques was used to create the constriction microchannel devices [42]. Briefly, the device consists of one SU8-5 and one SU8-25 negative photoresist layer. Each layer was soft-baked, exposed to ultraviolet light with the appropriate mask, developed and hard-baked. Devices were then created by mixing polydimethylsiloxane (PDMS) in a 10:1 ratio (Sylgard 184 Silicone Elastomer Kit, Dow Corning) and baked at approximately 210°F for 25 to 30 minutes. A 2 mm punch was used to create the inlets and outlets. Devices were cleaned using tape, and rinsed with acetone, methanol then deionized water. The device was bonded to a glass slide via plasma treatment (100-E Oxygen Plasma Asher, TePla Technis).
2.3 Cellular Culturing

HEp2 cells, chosen due to availability, were cultured using Eagle’s Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS). Cells were seeded on a six-well plate at an approximate density of $2 \times 10^5$ cells/mL prior to experiments. To deplete cholesterol, cells were incubated at 37°C with 1mL of 10mM MβCD for varying durations (0-, 1-, 5-, 15-, 30-minutes). The 1-minute and 5-minute groups were excluded for reasons discussed in Chapter 3. Once the MβCD was removed, cells were trypsinized (500μL) and quenched (2mL) using the same EMEM with FBS solution. Depending on the length of the MβCD incubation time, a shorter trypsinization time was used. Cells were then spun down and suspended in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). The PBS solution was also used to fill the channels prior to loading the cells. A single biological replicate Filipin-III assay (Cholesterol Cell-Based Detection Assay Kit, Cayman Chemicals) was used to determine the percentage of cholesterol depletion by following the manufacturer’s directions. This assay was also used as a comparison for sensitivity of methods.
2.4 Experimental Protocol

To measure the cells’ electrical properties, an impedance spectroscope (HF2IS Impedance Spectroscope, Zurich Instruments) configured in a 4-terminal setup was used. The 4-terminal setup has the benefit of reducing the serial resistance found in the wired connections. The device was placed on an inverted microscope (IX81 Motorized Inverted System Microscope, Olympus) in order to view the channel. Cells were loaded into the channel and then the baseline frequency sweep was performed. This technique was used to minimize the risk of clogging due to repeatedly inserting and removing the electrodes, which generates PDMS debris. In order to adjust for channel to channel variation a baseline sweep was performed on each new channel with typical results shown in Figure 6.

![Figure 6](image)

**Figure 6** – A baseline frequency sweep was performed on each new channel to account for variations.
Since the impedance of the device is primarily determined by the constriction channel, it is assumed that the addition of cells has a negligible effect on the baseline impedance. A frequency sweep (1 kHz to 1 MHz) at 200mV was performed on single cells in the microchannel. Results of a typical sweep with a cell located in the channel can be seen in Figure 7.

**Figure 7** – Compared to Figure 6, a cell located in the channel drastically changes the impedance profile.

If needed, additional sweeps were performed on the same cell in an attempt to improve the quality of the recorded data. This decision was made qualitatively based on visual observations of the recorded curve. Pressure was controlled via a syringe to position the cell in the
microchannel as seen in Figure 8. A picture was taken of each cell in order for image processing measurements to be completed at the end of the experiment (Retiga EXi Fast 1394, Q Imaging).

**Figure 8** - By controlling the pressure, a single cell is positioned in the channel and a frequency sweep is performed.

Each treatment time group is the summation of three independent samples, with over 50 data points in each group. A new PDMS device was used for each treatment time or when a device became clogged due to PDMS debris.
2.5 Fitting & Electrical Parameter Extraction

The impedance data was fit via a nonlinear least squares algorithm (MATLAB, Mathworks) using both real and imaginary components from the frequency sweep simultaneously between 1 kHz and 500 kHz. The equivalent circuit models used to extract the cellular resistance and capacitance values are shown in Figure 9(a) (b), as previously described [42]. Briefly, when a cell enters the channel it displaces a certain amount of liquid, changing the impedance of the channel. As seen in Equation 1, the new channel resistance ($R_{Ch}'$) can be calculated by subtracting the missing amount of liquid (proportional to the cellular size) from the baseline channel resistance ($R_{Ch}$). The leakage resistance ($R_{Leak}$) is due to the cell being surrounded by a thin sheath of conductive fluid. At low frequencies, the capacitors in the circuit model will appear as open circuits. The measured low frequency impedance is the channel resistance and the leakage resistance in series. Since the channel resistance is known, the leakage resistance can be calculated as seen in Equation 2. For the purpose of this experiment the low frequency resistance was taken as the average of the first 10 recorded points. The channel capacitance ($C_{Ch}$) is assumed to stay constant regardless of whether a cell is present or not.
Equation 1 – Relationship between the new and old channel resistance when a cell is present.

\[ R'_{Ch} = R_{Ch} - \frac{l_{cell}}{\sigma_{Buffer} * A_{Ch}} \]

Equation 2 – Relationship between the low frequency resistances.

\[ R_{Leak} = R_{Low} - R'_{Ch} \]

Figure 9 - (a) Equivalent circuit model for the empty constriction channel. (b) Equivalent circuit model when a cell is located in the channel.
Due to the microchannel confinement, the cell’s geometrical model is considered to be an elongated box with two hemispherical caps with radius $r$ as seen in Figure 10. The length of the cell, $L$, and the path length of the end caps were determined by manually measuring the desired portions with image processing software (ImageJ, National Institutes of Health). These measurements can also be used to calculate the specific membrane capacitance, Equation 3, and cytoplasmic conductivity, Equation 4, as previously mentioned [42]. Briefly the specific membrane capacitance is determined by dividing the capacitance by the cellular surface area of one hemispherical end cap. The cytoplasmic conductivity is determined by assuming the end caps are part of the box-like main body therefore greatly simplifying the conductivity calculation.

**Figure 10** - The ideal geometrical model of a cell confined by the constriction channel. The cytoplasm is represented by the blue section with an exaggerated plasma membrane represented by the grey section.
Equation 3 – The specific membrane capacitance value removes any size influences allowing capacitances to be compared.

\[ C_{SM} = \frac{C_{Mem}}{2\pi r^2} \]

Equation 4 – The cytoplasmic conductivity value removes any size influences allowing capacitances to be compared.

\[ \sigma_{Cyto} = \frac{I_{Cell}}{A_{Ch} \cdot R_{Cyto}} \]

It should be noted that a constant-phase element was used in place of the channel capacitor as previously discussed [42]. Constant phase elements can be thought of as a hybrid resistor-capacitor element where at one extreme the element is purely resistive and at the other end purely capacitive. In between these two extremes, the element takes on both capacitive and resistive properties. This element replacement was found to reduce the error of the initial channel fit. For the curve fitting, the cellular capacitance and resistance values were scaled close to each other in magnitude. A scaling factor of \(10^{8.95}\) was used for a majority of the fittings, with \(10^{9.5}\) being used to fit one 30-minute biological replicate and one 15-minute biological replicate. Results were pooled and data points were excluded if the norm of their residual error during fitting was greater than \(10^{13}\). An example of a fit close to the cutoff is shown in Figure 11(a) (b). Statistical testing was performed using one-way ANOVA and Tukey’s HSD post-hoc test in MATLAB R2014a. A breakdown of the experimental results, with errors over \(10^{13}\) removed, can be found in the Supplementary Materials.
Figure 11 - Typical fit results by fitting the real (a) and imaginary (b) components of the frequency sweep simultaneously with an error of $9.15 \times 10^{12}$. 
3 Results & Discussion

3.1 Depletion of Cholesterol Leads to a Lower Membrane Capacitance

When the cells were exposed to MβCD, a decrease in capacitance was observed as seen in Figure 12. The change in capacitance depended on how long the cells were incubated. A one-way ANOVA test found a statistically significant difference between the treatment times [F (2, 231) = 53.49, \( p = 8.11 \times 10^{-20} \)]. Boxplots of the capacitance distributions can be seen in the Supplementary Materials. Tukey’s HSD post-hoc test was used to determine which groups had a statistically significant difference (\( p<0.05 \)). The 0-minute treatment time group (\( n = 84, M = 4.23\text{pF}, SD = 0.89\text{pF} \)), 15-minute (\( n = 79, M = 3.54\text{pF}, SD = 0.76\text{pF} \)) and 30-minute treatment time (\( n = 71, M = 2.58\text{pF}, SD = 1.29\text{pF} \)) all showed a statistically significant difference between each other. The results of this post-hoc test can be seen in Table 1. HEp2 cells were also exposed to 1-minute and 5-minute exposures of MβCD in an effort to find the lowest observable change in capacitance due to cholesterol depletion. While MβCD is primarily used for long time scales (> 30 minutes), short time scales have been used [28]. However, different cell lines will respond to treatment times differently. For example, low concentrations of MβCD raised cholesterol content in T lymphocytes acquired from young subjects, but not acquired from elderly subjects [28]. The chemical kinetics of MβCD on HEp2 cells on a relatively short-time scale is unknown. Therefore, the validity of this data is questionable and was excluded from the present analysis.
Figure 12 – A decrease in capacitance is observed when treated with MβCD.

Table 1 – Results of Tukey’s HSD post-hoc test using the membrane capacitance values.

<table>
<thead>
<tr>
<th>Comparison Groups</th>
<th>95% Confidence Interval – Lower Bound (F)</th>
<th>Difference Between Estimate Group Means (F)</th>
<th>95% Confidence Interval – Upper Bound (F)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-Minutes &amp; 15-Minutes</td>
<td>3.30*10^{-13}</td>
<td>6.94*10^{-13}</td>
<td>1.06*10^{-12}</td>
<td>2.37*10^{-5}</td>
</tr>
<tr>
<td>0-Minutes &amp; 30-Minutes</td>
<td>1.28*10^{-12}</td>
<td>1.65*10^{-12}</td>
<td>2.03*10^{-12}</td>
<td>9.56*10^{-10}</td>
</tr>
<tr>
<td>15-Minutes &amp; 30-Minutes</td>
<td>5.77*10^{-13}</td>
<td>9.57*10^{-13}</td>
<td>1.34*10^{-12}</td>
<td>1.14*10^{-8}</td>
</tr>
</tbody>
</table>


Cell size can affect the cell membrane capacitance value because capacitance is proportional to the surface area as demonstrated by Equation 5. To compensate for the size variation across cells, capacitance measurements are commonly normalized by their surface area and reported in the units of μF/cm², a quantity referred to as the specific membrane capacitance (C_{SM}).

**Equation 5** - Relationship between surface area and specific membrane capacitance

\[
C = SA \times C_{SM}
\]

Capacitance manifests itself when the electric field lines intersect the membrane (i.e. the electrical field lines are not parallel with the cell membrane), therefore it is assumed only the end caps contribute to the measured capacitance value. Ideally, all cells would have the same effective surface area of \(4\pi r^2\), allowing the capacitance to be decoupled from the surface area. However, many cells did not exactly conform to the ideal geometrical model and therefore possessed a different surface area as illustrated in Figure 13. Since this configuration strays from the ideal geometry, errors would arise in calculating the specific membrane capacitance because the membrane surface area varies between each cell.

![Figure 13](image-url) – An example of a cell that did not conform well to the ideal geometrical model.

This deformation also had the additional effect of making it difficult to determine the cellular length used for calculating such values as the change in channel resistance and cytoplasmic conductivity. To remove the uncertainty as to where to measure the cell length, as seen in Figure
10, the maximum length of the cell was used. The maximum cell length was therefore used to calculate the change in channel resistance and the cytoplasmic conductivity. The cross-section area is still assumed to be determined by the channel dimensions. An example of the measured cellular length can be seen in Figure 14.

![Cell Length Image](image)

**Figure 14** - The maximum length of the cell was used to calculate the change in channel resistance as well as the cytoplasmic conductivity.

A Filipin-III assay was performed in order to compare the sensitivity of the two methods. A one-way ANOVA test found a statistically significant difference between the treatment times \([F (2, 253) = 25.61, p = 7.43 \times 10^{-11}]\). Boxplots of the intensity distributions can be seen in the Supplementary Materials. Tukey’s HSD post-hoc test was used to determine which groups had a statistical significant difference \((p<0.05)\). A statistical difference was seen when the 0-minute \((n = 128, M = 2.6 \times 10^5 \text{ A. U.}, \text{SD} = 8.34 \times 10^4 \text{ A. U.})\) group was compared to the 15-minute \((n = 64, M = 1.94 \times 10^5 \text{ A. U.}, \text{SD} = 6.31 \times 10^4 \text{ A. U.})\) and 30-minute groups \((n = 64, M = 1.94 \times 10^5 \text{ A. U.}, \text{SD} = 6.13 \times 10^4 \text{ A. U.})\). No difference was noted between the 15-minute and 30-minute groups. The results of this post-hoc test can be seen in Table 2. It is interesting to note that while there is little change in the cholesterol content between these two groups, a large change in capacitance is measured. By normalizing the y-axis, the Filipin-III assay and microfluidic spectroscopy method can be compared by the slope of their linear fit. The sensitivity of the Filipin-III assay is -0.0085
A.U./minute and the sensitivity of the microfluidic assay is -0.013 A.U./minute. However, care should be taken when comparing these numbers due to their being little difference between the 15-minute and 30-minute groups for the Filipin-III assay affecting the linear fit.

Table 2 - Results of Tukey’s HSD post-hoc test using the fluorescent intensities.

<table>
<thead>
<tr>
<th>Comparison Groups</th>
<th>95% Confidence Interval – Lower Bound (A.U.)</th>
<th>Difference Between Estimate Group Means (A.U.)</th>
<th>95% Confidence Interval – Upper Bound (A.U.)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-Minutes &amp; 15-Minutes</td>
<td>39207.36</td>
<td>65618.44</td>
<td>92029.52</td>
<td>1.82*10^-8</td>
</tr>
<tr>
<td>0-Minutes &amp; 30-Minutes</td>
<td>39674.45</td>
<td>66085.54</td>
<td>92496.62</td>
<td>1.44*10^-9</td>
</tr>
<tr>
<td>15-Minutes &amp; 30-Minutes</td>
<td>-30029.8</td>
<td>467.0953</td>
<td>30963.98</td>
<td>1.00</td>
</tr>
</tbody>
</table>
3.2 Cholesterol Depletion Changes Cytoplasmic Conductivity

Unlike membrane capacitance, cytoplasmic conductivity is a size independent parameter. As mentioned before the maximum cellular length is used to simplify the measurement process and better compensate for the deformation of the hemispherical end caps. In order to verify the independence, the conductivity was plotted against the measured cellular length. As seen in Figure 15, these results are poorly correlated validating the conductivity measurement.

![Graph showing the relationship between cytoplasmic conductivity and cellular length.](image)

\[ y = 0.002x + 0.52 \]

**Figure 15** – As expected the cytoplasmic conductivity is not influenced by cellular length.

A change in cytoplasmic conductivity was noted when the cells were exposed to MβCD as seen in Figure 16. A one-way ANOVA test found a statistically significant difference between the treatment times \[ F(2, 231) = 37.83, p = 6.15 \times 10^{-15} \]. Boxplots of the conductivity distributions can be seen in the Supplementary Materials. Tukey’s HSD post-hoc test was used to determine
which groups had a statistical significant difference ($p<0.05$). No statistical significant difference was found between the 0-minute ($n = 84$, $M = 0.40$ S/m, SD = 0.06 S/m) and the 30-minute group ($n = 71$, $M = 0.36$ S/m, SD = 0.18 S/m). Both these groups showed a statistically significant difference from the 15-minute group ($n = 79$, $M = 0.57$ S/m, SD = 0.19 S/m). The results of this post-hoc test can be seen in Table 3. Unlike the capacitive measurement, which continues to decrease with an increasing treatment time, the cytoplasmic conductivity does not follow such a trend. At the 15-minute mark a notable increase in conductivity was observed, followed by a decrease to its original value.

![Figure 16](image-url)  

**Figure 16** – When treated with MβCD the cytoplasmic conductivity rises and then returns to its original value.
Table 3 - Results of Tukey’s HSD post-hoc test using the cytoplasmic conductivities.

<table>
<thead>
<tr>
<th>Comparison Groups</th>
<th>95% Confidence Interval – Lower Bound (S/m)</th>
<th>Difference Between Estimate Group Means (S/m)</th>
<th>95% Confidence Interval – Upper Bound (S/m)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-Minutes &amp; 15-Minutes</td>
<td>-0.22</td>
<td>-0.16</td>
<td>-0.11</td>
<td>9.85*10^-10</td>
</tr>
<tr>
<td>0-Minutes &amp; 30-Minutes</td>
<td>-0.02</td>
<td>0.04</td>
<td>0.10</td>
<td>0.258</td>
</tr>
<tr>
<td>15-Minutes &amp; 30-Minutes</td>
<td>0.14</td>
<td>0.20</td>
<td>0.26</td>
<td>9.56*10^-10</td>
</tr>
</tbody>
</table>

One of the benefits of using microfluidic impedance spectroscopy is that it is able to measure on a single cell basis. By combining the conductivity and capacitance, a distribution of the individual cells can be created as seen in Figure 17. This plot reinforces the effect that cholesterol depletion has on the membrane capacitance and the cytoplasmic conductivity.
Figure 17 – By leveraging single cell analysis the distribution of the membrane capacitance and cytoplasmic conductivity can be plotted.
3.3 Mechanisms Explaining the Changes in Electrical Properties

3.3.1 Membrane Capacitance

A simple electrical model of a cell membrane is that of a parallel plate capacitor described in Equation 6, where $C$ is the membrane capacitance, $d$ the distance between the plates, $\varepsilon_r$ the relative dielectric, $\varepsilon_0$ the vacuum permittivity, and $A$ the area of the parallel plates.

**Equation 6 - Capacitance of a parallel plate capacitor**

$$C = \frac{\varepsilon_r \varepsilon_0 A}{d}$$

Therefore changes in membrane thickness, membrane dielectric constant and membrane surface area can all lead to a change in membrane capacitance. Cholesterol is known to change membrane thickness, especially when interacting with proteins [49, 50, 51]. However, this research has reported the change in thickness due to the removal of cholesterol to be minimal. For instance, there was a 0.1 nm change in membrane thickness with cholesterol depletion and a 0.5 nm thickness change with protein depletion [50]. If the membrane is assumed to be 10 nm thick, this represents less than 5% change in the membrane capacitance. It is also important to note that cholesterol thickens the membrane resulting in a decrease in capacitance.

Previously reported values of the dielectric constant for cholesterol are typically around 2.3 [52, 53]. More recent work using electrostatic force microscopy has also reported a dielectric constant of cholesterol crystals to be approximately 2.3 [54]. This dielectric constant is slightly larger than the dielectric constant of phospholipids, which is around 2 [52, 53]. Due to the fact that the dielectric constant is proportional to capacitance, the ratio between the dielectric constants represents the change in capacitance. Assuming that the molar concentration of cholesterol is equal to the molar concentration of lipids [55], this yields an average dielectric constant of 2.15.
Therefore, the maximum change transitioning from a purely lipid membrane to one containing equal molar amounts of cholesterol is 7.5%. This change is insufficient to account for the drastic changes in capacitance revealed by the experimental data presented in Figure 17. However, the change of the dielectric constant could still represent a minor role in the overall capacitance change.

In previous studies of cholesterol depletion a decrease in capacitance, as measured by the patch clamp method, was attributed to a decrease in surface area by membrane internalization or exocytosis [38, 39]. In addition to the visual membrane, nanometer-sized features such as caveolae, microvilli and microridges also contribute to the total surface area of the cell membrane and thereby capacitance. For example, in adipocyte cells, caveolae contribute up to 50% of the surface area [15]. This mechanism has previously been discussed [39, 40], reporting that cholesterol depletion caused a decrease in membrane capacitance. Removal of cholesterol from the membrane is detrimental to this surface topography, translating into a reduction in surface area. The reduction in surface topography has previously been verified by fluorescence and electron microscopy imaging [56, 57, 58]. Furthermore, different cell types have different concentrations of these surface features [59] possibly explaining why, in some cases, no change in capacitance was found when cholesterol was depleted [41].

Electrostatic simulations also support the argument that changes in surface area can affect capacitance. By comparing different two-dimensional membrane configurations in COMSOL it is possible to calculate the amount of charge stored per meter. This value is proportional to the overall membrane capacitance. Thus an increase in coulomb storage per meter will indicate an increase in capacitance. To investigate how this property changes solely due to surface area, the membrane thickness (t=10nm) and dielectric constant (ε_r=2.7) were fixed. The first membrane
configuration, Membrane A, to be examined was one that did not feature any surface topography. Membrane A was compared against Membrane B, which featured an indentation in order to increase the effective length. Both these membrane configurations can be seen in Figure 18.

![Figure 18](image)

**Figure 18** – While the membranes have the same overall length, by adding a small depression the effective length of the membrane is increased. This change is analogous to an increase membrane surface area.

To calculate the charge storage of each configuration, one volt was applied across the membrane. The corresponding line integration across the top surface can be found in Table 4 along with the effective membrane length. While each membrane has an overall length of 200nm, by simply adding the depression the effective length is increased to 260nm. This increase corresponds to a 30% change in effective length. Likewise the linear charge density increases by about 28%.
Therefore, this supports the proposed mechanism that capacitance is proportional to cellular surface area.

**Table 4** – An increase in the effective length of the model membrane increases the linear charge density.

<table>
<thead>
<tr>
<th>Configuration</th>
<th>Linear Charge Density (C/m)</th>
<th>Effective Length (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$4.78 \times 10^{-10}$</td>
<td>200</td>
</tr>
<tr>
<td>B</td>
<td>$6.1 \times 10^{-10}$</td>
<td>260</td>
</tr>
</tbody>
</table>

Combining these three factors together can help illustrate the relative contributions to the capacitance change as seen in Equation 7.

**Equation 7** - Change in capacitance can be described by changes in the surface area, dielectric constant, and membrane thickness

$$\Delta C \propto \Delta S_A, \Delta \varepsilon_r, \Delta t^{-1}$$

As discussed above, inclusion of cholesterol thickens the membrane thereby decreasing the capacitance, by an estimated 5%. The increase in dielectric constant is approximately 7.5% which for the most part counteracts the decrease in capacitance from the thickness increase. Therefore, the change in surface area is responsible for 97.5% of the capacitance change. This change in surface area is could be from a reduction of the membrane via internalization or exocytosis, and also from the decrease in nanometer-sized surface topography.

While the above mechanism is feasible, previous research has shown that the removal of membrane cholesterol affects additional properties, including membrane proteins. For example,
the release of glycerophospholipids and glycoproteins from the membrane [60], disturbance of lipid rafts which can affect signaling pathways [61], changes in ion currents [62], and increased levels of water in the membrane [63]. Further research needs to be conducted to develop a more comprehensive dielectric model, such as through dielectric computer simulations [64, 65], and to address how cholesterol depletion changes the membrane in different types of cells.

3.3.2 Cytoplasm Conductivity

The effects of membrane cholesterol depletion impact the cytoplasm as well as the membrane. Previous research has shown the response to membrane cholesterol depletion is cell line specific. For instance, fibroblasts treated with 2% hydroxypropyl-β-cyclodextrin recorded an approximate 25% reduction in membrane cholesterol, with an 80% reduction in endoplasmic reticulum cholesterol; the opposite trend was observed with BHK cells treated with 10mM of MβCD [28]. Unlike membrane capacitance, which can be simplified to a parallel plate capacitor, the cytoplasm is more complex. This complexity is due to the presence of various organelles and their individual electrical properties. For the purpose of this analysis, the primary factor that is thought to affect the cytoplasmic conductivity is the ion concentration of the cytoplasm.

Changes in ion concentration have been shown to change the cytoplasmic conductivity. Previous research has shown that by treating cells with ion channel blockers, it is possible to identify the contribution of different ions measured via dielectrophoresis [66]. Additionally, the conductivity of Chinese hamster ovary cells dropped when treated with oligomycin, a compound that blocks mitochondria adenosine triphosphate production [67]. One of the roles of cholesterol is to modify the membrane permeability. If more ions are able to enter the cell from the higher conductivity buffer, the cytoplasmic conductivity should increase. Therefore a large change should be possible due to the conductivity of the surrounding PBS medium being much greater than that of
the cytoplasm. However, using pig erythrocytes it was shown that upon 35% removal of membrane cholesterol there was little change in membrane permeability [68]. More extensive depletion in mammalian erythrocytes resulted in an increased transfer rate of nonelectrolytes and organic acids [69]. As illustrated by the Filipin-III assay, after 30 minutes only 25% of cholesterol was depleted. Therefore, it is unlikely that any change in cytoplasm conductivity was due to an increase in membrane permeability. This mechanism could play a role in longer exposures to MβCD.

Ion channel activity can change based on the level of membrane cholesterol present. This change in activity could explain why cytoplasmic conductivity changed. Modulating cholesterol content has different effects on different ion channels. Ion channels such as inwardly-rectifying K⁺ channels, voltage-gated K⁺ channel, Ca²⁺ sensitive K⁺ channels, voltage-gated Na⁺ channels, N-type voltage-gated Ca²⁺ channels and volume-regulated anion channels have been shown to decrease in activity when membrane cholesterol was raised [70]. However, some ion channel’s activity decreased when membrane cholesterol was depleted such as Ca²⁺ channels and Na⁺/Ca²⁺ exchange channels both located in the scrolemema of striated muscle cells [62]. If the interior cholesterol level is affected during the MβCD exposure time, organelles might also contribute to the change in ion concentration. In isolated rat liver lysosomes, exposure to MβCD caused protons to leak and caused to potassium to enter the K⁺/H⁺ exchange resulting in an osmotic imbalance [71]. Therefore depending on the behaviour of ion channels present in the HEp2 cells, in addition to the response of organelles, it is conceivable that an increase and decrease of cytoplasmic conductivity can be observed. More research is needed to create a comprehensive model to identify why these changes are observed, such as including the effects of ion mobility and the response organelles due to cholesterol depletion.
3.4 Sources of Error

One reason for the large standard deviations seen in the capacitance arises due to the fact that these measurements are single-cell based rather than population based. Population measurements risk ignoring important sub-populations whose information would be lost in the averaging process, however, single-cell analysis gives rise to biochemical noise represented by outliers [72]. To account for the outliers, interquartile filtering was applied. However, the standard deviation in some cases is still greater than 20% of the mean’s value. Therefore, to determine whether the outliers are in fact outliers or another important sub-population, a larger sample size should be taken where feasible. Genetic drift of cells might also be more pronounced between samples at the single cell level [73]. The cells themselves contributed to the error as well. For example, cells that were close to the inlet and outlet of the constriction channel might have influenced the electrical measurements performed. Obvious cases can be excluded because the fitting will yield extremely high resistive values due to the cells being in series. However, subtle effects might not be noticed if the cell is just in the general vicinity of the constriction channel. Finally, some cells changed their morphology over the course of the measurement, thereby potentially inducing error. If this was observed, these cells were eliminated from further analysis.

Electrical noise was also present even though a 4-terminal setup was used to reduce serial resistance. Noise was also introduced to the measurement due to a relatively low gain of 1000 Ohms being used. This gain was chosen to prevent bandwidth limitations at higher frequencies. While noise did induce error, attempts were made to calibrate for it via the initial fit. A constant phase element was used instead of a capacitor for the initial baseline channel fit. This circuit element was found to improve the initial fitting parameters. For example, the residual norm error decreased from $1.48 \times 10^{11}$ to $1.08 \times 10^{11}$, a percentage change of almost 27%. Error arose due to
The scaling factor selected. The scaling factor was used to bring the resistive and capacitive values closer together for fitting. This value was not fine-tuned for each cell; instead a single scaling factor was used for each sample group. Therefore, it is not known what the optimal scaling factor for each cell is or what the sensitivity to this parameter is. In one case, a cell using a scaling factor of $10^{8.95}$ produced a residual norm error of $6.7 \times 10^{-12}$ with a capacitance of 0.33pF, whereas a scaling factor of $10^{9.5}$ produced an error of $2.7 \times 10^{-12}$ with a capacitance of 1.59pF. This change in scaling factor causes a large change in capacitive value. Error also exists within the electrical model used, suggesting room for improvement. Empty channel curve fitting, which is fairly well characterized, has errors typically on the order of $10^{10}$. Whereas curve fitting channels with a cell present has errors typically on the order of $10^{12}$. This error is also evident by the deviation between the recorded data and the fitted data at higher frequencies. Finally, error is introduced by choosing to use the maximum cell length. Using this length would decrease the channel resistance when a cell is present and increase the leakage resistance, changing the parameters for the curve fitting. For the purpose of calculating the cellular conductivity, using this cell length to approximate the cell as a box, neglects the complex morphology of the end caps.
4 Conclusion

4.1 Concluding Remarks and Contributions

Cholesterol is a diverse molecule whose role ranges from a building block in hormone synthesis to signaling pathways. Its presence in the plasma membrane reduces ion permeability, allows proteins to maintain their correct orientation, and is a key component in different membrane structures (e.g. lipid rafts and caveolae). In disease, cholesterol content can be an indicator of more aggressive tumor formation to providing lipid rafts needed for completion of the respiratory virus lifecycle.

This work reports the feasibility of using single-cell impedance spectroscopy to measure changes in electrical properties caused by cholesterol depletion. A single-cell treated with MβCD was placed inside a constriction channel where a frequency sweep was performed. The electrical properties were then extracted by fitting both the real and imaginary components of the data, simultaneously, to an appropriate circuit model. It was determined that cholesterol depletion results in changed membrane capacitance and changed cytoplasm conductivity values for HEp2 cells.

The reason for the decrease in capacitance was speculated to be primarily caused by a change in the membrane surface area. This change in surface area not only includes membrane loss visualized under optical microscopy, but also the loss of nanometer-sized surface features. Changes in cytoplasm conductivity were also observed with an increase at the 15-minute treatment time followed by a decrease at the 30-minute treatment time back to its original value. These changes were speculated to be caused by varying ion concentrations in the cytoplasm. This work opens the door for further research in monitoring cholesterol changes using single-cell impedance spectroscopy.
The primary contribution of this work is to demonstrate the feasibility of utilizing single-cell microfluidic impedance spectroscopy in detecting changes in electrical properties due to altered cholesterol levels. These changes have previously been measured via the patch clamp method which has a low throughput and a long training time for the user. By switching to a microfluidic platform, it is possible to measure a comparatively large sample size while reducing the skill needed. This method does not require the cell to be fixed or destroyed, as is typical in fluorescent measurements, thereby enabling long-term monitoring.
4.2 Future Research Directions

Future work can be broken down into two groups: technical improvements and scientific applications.

4.2.1 Technical Improvements

One improvement that would help to increase the measurement capabilities of the device would be to optimize the channel cross section depending on the type of cell used. To accomplish this change, a new SU-8 master will have to be created depending on the cellular dimensions. This optimization would allow cells to conform better to the geometrical model, induce less cellular deformation of the end caps, and minimize stress on the membrane which can cause cells to rupture at longer treatment times. Finding the optimal scaling factor for each cell’s frequency sweep will also reduce fitting error. By tailoring the scaling factor to each cell, the best electrical parameters will be extracted. To accomplish this task, the fitting results could be run a number of times while iterating the scaling factor in the background. Accounting for the deviation at high frequencies will also increase the accuracy of the fit. This deviation can potentially be corrected by adjusting the electrical model or modifying the experimental setup. Finally by increasing the sample size and number of biological replicates performed, the power of the study can be increased.

4.2.2 Scientific Applications

With respect to scientific applications, one major application of this device is to facilitate drug and molecular screening of novel compounds. Any drug that modulates membrane cholesterol has the potential to affect the capacitance or cytoplasmic conductivity. Therefore, by measuring these parameters the efficacy of the drug can be monitored. The ability to gather large cellular sample sizes will also present a better overall picture and can be used in conjunction with other
screening methods to fully evaluate novel compounds. Effects of cholesterol on cytoplasmic conductivity can be further explored as well. By using ion channel blockers or monitoring ion efflux it might be possible to determine which ions are responsible for the increase or decrease in measured cytoplasmic conductivity.
5 References


receptor (TCR) signalling," *Seminars in Immunology*, vol. 12, no. 1, pp. 23-24, 2000.


[31] M. Patterson, "Niemann-Pick Disease Type C," in GeneReviews, Seattle (WA), University of Washington, Seattle, 2013.


6 Supplementary Materials

**Table S 1** – Experimental results of the 0-minute treatment time group.

<table>
<thead>
<tr>
<th></th>
<th>March 1, 2016</th>
<th>March 10, 2016</th>
<th>March 15, 2016</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>37</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>Capacitance (pF)</td>
<td>4.28±1.04</td>
<td>4.41±0.80</td>
<td>4.05±0.69</td>
</tr>
<tr>
<td>Conductivity (S/m)</td>
<td>0.43±0.08</td>
<td>0.40±0.03</td>
<td>0.37±0.04</td>
</tr>
</tbody>
</table>

**Table S 2** – Experimental results of the 15-minute treatment time group.

<table>
<thead>
<tr>
<th></th>
<th>March 1, 2016</th>
<th>March 10, 2016</th>
<th>March 15, 2016</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>33</td>
<td>29</td>
<td>17</td>
</tr>
<tr>
<td>Capacitance (pF)</td>
<td>3.66±0.73</td>
<td>3.39±0.66</td>
<td>3.54±0.96</td>
</tr>
<tr>
<td>Conductivity (S/m)</td>
<td>0.49±0.04</td>
<td>0.75±0.19</td>
<td>0.39±0.04</td>
</tr>
</tbody>
</table>

**Table S 3** – Experimental results of the 30-minute treatment time group.

<table>
<thead>
<tr>
<th></th>
<th>March 1, 2016</th>
<th>March 10, 2016</th>
<th>March 15, 2016</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>41</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Capacitance (pF)</td>
<td>2.92±1.55</td>
<td>1.77±0.36</td>
<td>2.10±0.51</td>
</tr>
<tr>
<td>Conductivity (S/m)</td>
<td>0.35±0.21</td>
<td>0.35±0.10</td>
<td>0.61±0.15</td>
</tr>
</tbody>
</table>
Figure S 1 – Distributions of the measured membrane capacitances.

Figure S 2 - Distributions of the measured fluorescent intensities.
Figure S 3 - Distributions of the measured cytoplasmic conductivities.