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Impedance Spectroscopy of Zebrafish Sperm

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Impedance Spectroscopy of Zebrafish Sperm

by

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Undergraduate honors thesis under the direction of

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Department of Biological and Agricultural Engineering

Submitted to the LSU Honors College in partial fulfillment of

the Upper Division Honors Program.

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Louisiana State University

& Agricultural and Mechanical College

Baton Rouge, Louisiana

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Abstract

Sperm analysis is a necessary technique to predict male fertility as indicators of malnutrition and gene pool integrity. Sperm analyses are also used to determine the effectiveness of cryopreservation protocols. Developing cryopreservation methods demands reliable analytical methods for determining healthiness of sperm samples. For these reasons it is useful to understand which characteristics define healthy cells and which characterize diseased or compromised populations. Visualization of motility using imaging techniques has traditionally been used as the indicator of sperm viability. Path velocity is an extension of motility as a quantitative parameter of viability. Specifically the Computer Assisted Sperm Analsyis (CASA) programs perform motion analysis on sperm samples and can rapidly provide many parameters related to sperm health.

The majority of these new devices are calibrated for mammalian use. However, it is possible to adapt the technologies to fish sperm, which are important for aquacultural and biomedical research. The small size and short lifespan of fish sperm samples present hurdles to adapting the technology. Alternate technologies need to be developed which characterize sperm cell integrity in addition to motility. We designed electrical analysis chambers by adapting CASA slides with platinum electrodes. Using electrochemical methods such as impedance spectroscopy, we aim to monitor the ionic changes in motile zebrafish samples. Zebrafish sperm rely upon osmotic changes in their environment to become active and motile. Using this electrochemical method, we monitored impedance differences in the cells which can and cannot be rendered motile through osmotic changes compared to isotonic storage buffer and pure water. Analyzing the impedance measurements and phase changes at varying frequencies of the

samples showed no differences between buffer and live cells. However, dead cells had lower impedance and phase values than the buffer and live cells. This difference is greatest between 100-1000 Hz. Comparing sperm from various collections, we see changes in impedance suggesting cell concentration may be a critical factor. Other controls such as nonconductive buffers and fixed concentrations need to be implemented to ascertain the causes of these changes in impedance and phase. These differences provide merit into further impedance studies of zebrafish sperm physiology to eventually be used in the aforementioned applications.

Chapter 1

Background and Introduction

Sperm Analysis Motivations

The characterization and understanding of whole sperm cell viability is vital to the fields of reproductive biology, cryopreservation, and fertility. Although the quantitative parameters amongst species vary, similar qualitative markers exist for normal, healthy sperm. Two of the most common measurements of sperm viability are motility and morphology (Hirano 2003). Flagellation and cell size are important for morphology studies. Velocity and displacement are important for motility studies. Although these estimates were once performed manually, technologies allowing for automation are becoming common. These provide new protocols for sperm analysis while reducing human error (Wilson-Leedy 2007).

For fields such as fertility and reproductive biology, sperm quality is important to quantitatively understand the male reproductive output for comparison between individuals and populations. Reproductive output is not determined solely by genetics. Nutrition and environment also affect sperm quantity and quality. Therefore, information on sperm motility and morphology can indicate individual or population health. It is necessary to monitor both human and animal sperm parameters. Proper livestock management involves rationing food to prevent overfeeding while simultaneously ensuring the animals receive the proper nutrition. It has been shown that the dietary intake of farm animals directly affects their reproductive capabilities. Improper diet can lead to barren animals unable to contribute to future generations. However, nutrition dependent fertility also affects humans. Like livestock, improving the diet of

men can aid in reproductive output. Comhaire, et. al., noted increasing fatty acids in the human male diet can increase fertility (Comhaire 2003).

The individual's surroundings can affect male fertility. Excess pollutants and toxins have been documented as estrogenic compounds which act as reproductive inhibitors. The once common pesticide dichlorodiphenyltrichloroethane (DDT) was made famous for its effects on the development of avian eggs. Likewise, DDT has been shown to decrease male output. Kilian et.al. studied DDT's effects on rats and their progeny (2007). It was shown the toxin would not fatally harm the individual animals. However, each successive generation of animal exhibited lower sperm counts. Sperm monitoring of populations in areas where DDT is still in use (i.e. Africa) is necessary to understand how the supposedly helpful pesticide harms the population. Industrial contaminants are another class of estrogenic compounds reducing sperm viability. These consist of occupational hazards and man-made environmental hazards. Chlorinated by-products from drinking water have been the focus of studies, but did not show correlations between their presence and abnormal sperm count (Luben 2007). Toxic heavy metals are often blamed for sperm impairment. Mercury has been shown to decrease bovine bull sperm by impairing proper acrosome formation (Arabi 2005). Similar harmful effects have been noted in sea bass (Abascal 2008). Mercury buildup in the environment is the result of several industrial processes. Welding materials and lead have been implicated as causes of male infertility (Kenkel 2001, Hsu 1998).

Aside from population health, fertility tests are required to maintain livestock. It is common to monitor sperm samples from bovines prior to reproductive activity. This is done in conjunction with artificial insemination to increase agriculture stocks. Methods of sperm

viability methods which differentiate between good and poor producing bulls more accurately would allow for increased livestock control by furthering selective breeding (Chapwanya 2008).

The field of sperm cryopreservation also requires methods of sperm analysis to monitor the effects of the freezing process on the reproductive cell's post-thaw viability. Cryopreservation processes are currently used to store the genetic material of laboratory animals in place of keeping stocks of live, reproducing populations (Asturiano 2007, Hagedorn 2008). These methods conserve both money and space. The field of applied zebrafish biology is currently developing new methods for cryopreservation (Hagedorn 2008). Zebrafish are used to model genetic disease variants. Due to the increase in the library of animals, it is almost mandatory to preserve their sperm, rather risk losing a single breeding population (Danilova 2008). Frozen genetic material would also allow the specimens to be transferred between labs. Freezing sperm would also aid in livestock management. These methods would allow for the healthiest specimens to mate with several generations (Rubio-Guillen 2007).

For these reasons automated processes of sperm analysis have been developed. Studying individual and population sperm quality can reveal information about the environment, nutrition, and overall health. Likewise, sperm analysis methods are useful for the laboratory setting as researchers investigate toxic, hormonal, and estrogenic effects on male fertility (Christensen 2004). The furthered development of this technology will also help other burgeoning technologies such as cryopreservation.

Current Sperm Analytical Techniques

Until the past decade sperm analytical techniques focusing on morphology and viability were largely manual using standard optical microscopy. These manual, subjective

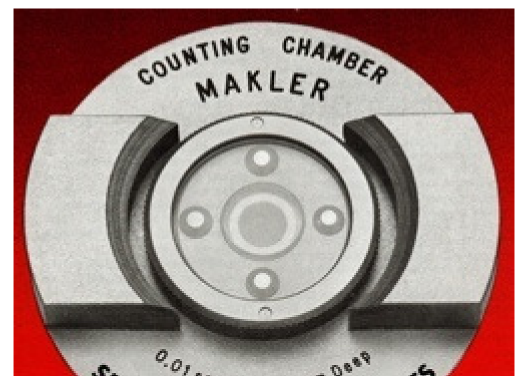

Figure 1.1: The Makler Counting Chamber is a common subjective analytical technique (ZDL, Inc.).

methods lead to disagreements over fertility rates. Several studies have pointed out the large variations between analysts and laboratories even if they inspected the same animal (Dunphy 1989). One common subjective method is the Makler counting chamber (Figure 1). The sperm sample is placed in the chip and a technician can then count the non-motile and motile specimens (ZDL, Inc). However it still relies upon the user's determination of motility. These inaccuracies lead to the development of many objective, automatic methods.

Coulter Counter

The analytical technique known as the Coulter Counter relies upon an electrical principle known as the Coulter Principle. The Coulter Principle is also known as the "electrical zone sensing technology." As particles displace electrolytic fluid between two electrodes, the volume of the displaced fluid is measured. The displaced volume creates a voltage potential peak recorded by the Coulter Counter. The particles are drawn through an aperture opening in the system. The diameter of this aperture can be used to limit the measured particle sizes (Beckman Coulter 2002). Figure 2 shows a typical output of a Coulter Counter analysis.

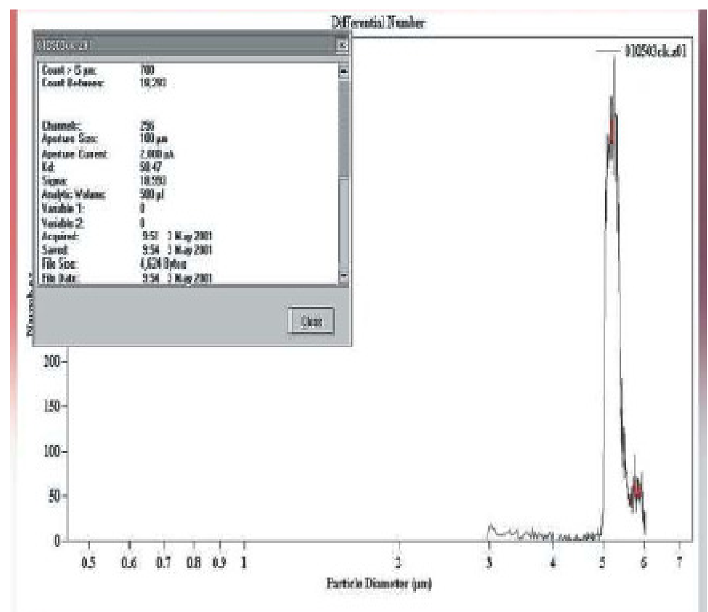

Figure 1.2: Coulter Counter output. The system can accurately measure particle diameter while simultaneously counting the total number of particles (Beckman Coulter).

This technique is not restricted to sperm analysis. Rather it is commonly used to study erythrocytes and lymphocytes and has found utility in the hospital community (Peng 2007).

However it does not discriminate between cell (or particle) type and therefore can be used to precisely measure the size and volume distribution of sperm samples. Using the Coulter Counter requires any extracellular particulate debris to be limited due to possible false positives.

The Coulter Counter technique can not be used to probe sperm motility. It is only used to monitor sperm volume morphology. Sperm are often considered “perfect osmometers,” as their volume reflects the ion concentration of the surrounding fluid (Yeung 2002). The Coulter Counter technology can be used to exploit this theory. Sperm samples can be prepared in a range of physiological (and extreme) osmotic buffered solutions. The Coulter Counter can then be used to measure the differences between the individual cell volume after exposure to these changes (Hagedorn 2008, Yeung 2002).

Although the Coulter Counter cannot provide direct information on sperm motility, it is useful in acquiring data on sperm volume and morphology. As a non-discriminatory method, it does not need to be calibrated to different species of sperm. Rather, it is possible to use the system on various cell types. The objective operation is an improvement over subjective cell staining methods common for membrane integrity studies (Rijsselaere 2002).

Sperm Quality Analyzer (SQA)

Another method of sperm analysis is the Sperm Quality Analyzer (SQA). The device calculates concentration, progressive motility, and normal morphology. The computer compares the measured parameters to human standards set by the World Health Organization (Medical Electronic Systems 2008). A 20 uL sperm sample are placed into a capillary tube. The sample is then inserted into the analytical device. As the sperm move single file through the capillary tube, they pass a light source.

The interruption of the light source caused by the sperm is measured by the on-board instrumentation. The computer programming converts the light interference caused by the passage of cells to calculate percent motility. The programming determines motility based upon rapid fluctuations in light intensity measured by the diode system. Rapid fluctuations in the light intensity are caused by the flagellated tails moving in and out of plane with the light path. An analog processing algorithm translates this motion to motility. Therefore a normal motile (i.e. fertile) specimen would be characterized by a series of alternating 'peaks' on the chart. A non-fertile specimen would exhibit a near stable minimum on the light intensity scale (Figure 3). This is because the non-fertile sperm are exhibit less flagella beatings as the move through the Sperm Quality Analyzer's optical measuring system (Medical Electronic Systems 2008).

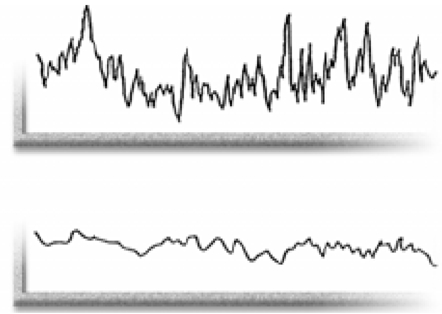

Figure 1.3: On board measurement of light beam interference on SQA platforms of a fertile (top) and non-fertile (specimen). Axes are intensity vs. time (Medical Electronic Systems).

Due to the dependence on the proper algorithmic conversion optical measurements, several commercial Sperm Quality Analyzers are not sold for use with non-human samples (Medical Electronic Systems 2008). However the concept has been proven to work with other animal species such as dogs (Rijssleare 2002) and turkeys (Neuman 2002). This rapidly progressing analytical technique is becoming a popular method to individually measure sperm motility and morphology.

Computer Assisted Sperm Analysis (CASA) Systems

A third, common technique for sperm viability studies utilizes computers to process sperm velocity and motion. These computer assisted sperm analysis (CASA) systems are hybrid microscope/video imaging systems which combine high resolution capabilities with imaging software. Like SQA technology, CASA systems rely upon computer software to calculate sperm parameters allowing for objective measurements. To operate the system, the user places the sperm sample on a glass slide on the microscope stage. The CASA image processing software measures sperm velocities and sizes while calculating the percentage motile, average speeds, directionalities, and axial diameters. The CASA output data include the traced path travelled by the motile sperm cells. This is useful in tracing the overall distance travelled by the individuals, something SQA devices lack. The CASA systems are capable of calculating several different velocities based upon the sperm path. It can be used to calculate curved velocity and straight velocity which are integral in understanding motility (Hamilton Thorne, Inc. 2008). CASA systems are sensitive enough to allow for studies of “motion characteristics in a sperm subpopulation to an unprecedented degree of sophistication” (Quintero-Moreno 2007).

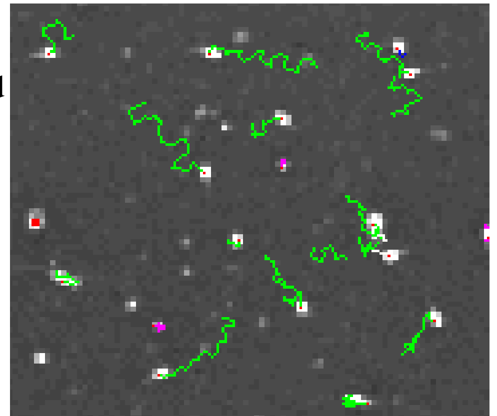

Figure 1.4: CASA output with sperm paths (green). The data allows the user to observe the geometry and distance travelled by motile individuals (Hamilton Thorne, Inc.)

Fuse, et. al., claim the CASA system is too expensive and complicated to gain usage in many laboratories and research hubs (Fuse 2005). However, the added benefit of sperm tracking is worth the “extra technical skill” necessary to operate these devices. To combat these claims, companies such as Hamilton Thorne Inc. are producing easier to operate instruments which are more customizable for use (2008). Because CASA is a thorough technique, updated SQA systems are continuously compared to the benchmark (Suzuki 2002, Fuse 2005). CASA allows

for the imaging measuring parameters to be modified for specific use. This allows for one system to be easily compatible with many different species unlike the SQA (Hoflack 2007, Rijssleare 2007, Hagedorn 2008).

Electrochemical Biological Interrogation Methods

The applied principles of electrochemistry have seen extensive use in characterizing and quantifying biomaterials. Many herald the advantages of electrochemical interrogation's lack of labeling and inexpensive materials, particularly when compared to optical detection methods (Laczka 2007). The ease of miniaturization of necessary components facilitates the use of application of electrochemical principles to the fields of microfluidics and lab-on-a-chip devices. Electrochemical sensors rely upon an electrode's ability to detect minute changes in potential or current flow. These changes are initiated by alterations of the medium due to biological events. Immobilization of the biological analytes on the electrode surface can be used to improve detection. Biosensor development utilizes electrochemical methods as a transducer. The immobilized bio-active elements 'recognize' specific analytes. The recognition of analytes often produces byproducts or current flow measurable with electrodes. The three dominating electrochemical methods are Potentiometry, Amperometry, and Impedimetry.

Potentiometry

Potentiometric measurement methods directly measure ions produced by reactions using ion selective electrodes. These electrodes measure the development of an electrochemical potential across the selective membrane due to biological reactions. Interrogation methods relying on potentiomentry often use enzymes to breakdown species of interest into ionic constituents measurable by these probes. Potentiometry has been applied to monitoring

pathogenic organisms and specific oligonucleotide binding (Laczka 2007). Quantifying potentiometric responses is done best using the Nernst equation:

$$E = E_0 + \frac{RT}{zF}\ln[i]$$

Where E and E_0 are the measured potential and the baseline potential respectively. RT/zF represents constants of the measurement system. R is the gas constant (8.314 J/mol K) and T is the absolute temperature in Kelvins. F is the Faraday constant 96485 (Coulombs/mole) and z refers to the ionic charge of the species of interest. The statement $ln\ [i]$ is dependent on the ionic concentrations of the disassociated species in solution (Chapman 2004).

Amperometry

Amperometry monitors the current flow as a potential is applied across an electrode setup. This setup consists of either two or three electrodes. Noble metals such as gold, silver, and platinum are often used to create these electrodes due to their long term stability. The process of amperometric testing involves the excitation of the sample using a controlled potential. This excitation potential then interacts with the analytes of interest, often reducing them at the anode. This process creates a measurable current which is often linear to the concentration of the analyte. Due to this excitation process, amperometry requires specific knowledge of the electrochemical reactivity of the samples. Nonspecific reduction may occur leading to incorrect calculations of analyte concentration or sample characterization. Because amperometry requires electron transfer across the electrodes to monitor the current, electron mediators are often used. This complicates the manufacturing of these measuring methods. Despite this amperometry is the basis of the majority of electrochemical sensing and measuring

devices of biological reactions and systems. The most famous (and commercial) amperometric sensor is the glucose biosensor used to monitor blood glucose levels.

Impedimetry (Impedance Spectroscopy)

Impedimetry interrogation measures the offset between incoming potential waveforms compared to measured current waveforms. In DC systems, this relationship is resistance, where ideal resistors follow Ohm's Law:

$$V = IR$$

where *V* represents the measured voltage, which is the produce of the current (*I*) and the resistor (*R*). Ideal resistors follow Ohm's Law for all voltages, currents, and frequencies applied to it. In the AC domain, this equation becomes:

$$V = IZ$$

where *V* and *I* are the applied sinusoidal voltage and and measured sinusoidal potential respectively. These sinusoidal forms of voltage and current are dependent on phase and frequency (Figure 1.5). The calculated impedance *Z* is therefore dependent on the frequency and phase shift between these two waveforms, or phasors.

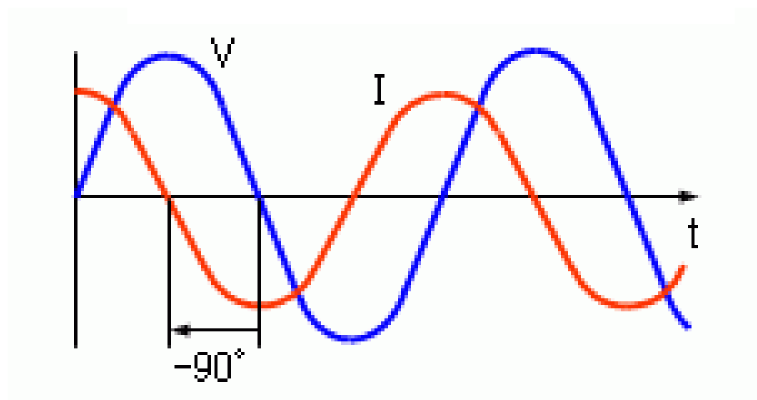

Figure 1.5: Phase lag in impedance systems. An offset of 90° indicates a current lag in the system. The incoming voltage leads the current. (NEC Electronics Corporation 2008)

Impedance spectroscopy is presented in several forms. First, Nyquist plots are used to demonstrate how the sample responds to varying frequencies. Nyquist plots utilize frequency as a parameter and graph the results relative to phase and amplitude in polar coordinates. This plot is often used in controls design. Another example of impedance data presentation is the Bode Plots. Bode Plots are a set of diagrams illustrating the changes in impedance and phase (separately) with respect to the frequency. These presentations simplify the understanding of the frequency dependency of the sample materials.

The non-ideal resistive nature of many systems (non-biological and biological) has made impedance spectroscopy an advantageous analysis method. Impedance spectroscopy found use in analyzing metallic coatings, thin film deposition, and battery characterization (K'Owino, 2005). Like other electrochemical methods, it has been applied to study biological materials and biological events. The technique offers advantages over other electrochemical methods by employing a small excitation voltage which does not interfere with normal sample processes. This nondestructive method allows for further characterization of the material using additional techniques or to use the material for its intended use. This is ideal for biological systems because it allows for the interrogation of the bio-system (tissue, cells) without destroying necessary components. Impedance spectroscopy has been used to monitor the viable state of organs (Gersing, 1998), blood glucose (Tura, 2007), cell cytotoxicity (Ceriotti, 2007), freshness of commercial seafood fishes (Niu, 2000), and detection of pathogenic microorganisms such as *Salmonella typhimurium* (Yang, 2004). Since impedance measurements do not interfere with the biological systems, researchers can augment the environments and monitor changes in the biological materials.

Zebrafish Sperm Morphology and Mechanisms

Zebrafish (*Danio rerio)* are an important species used for genetic testing as a model organism. The species offers insight into developmental biology due to a translucent, easily observable embryo stage. Research laboratories have created a multitude of transgenic specimens to study effects of genetic modifications on development and disease (Danilova, 2008). To mass produce transgenic individuals, researchers can implement genetic changes in the sperm. The male reproductive cells are also cryopreserved for storage.

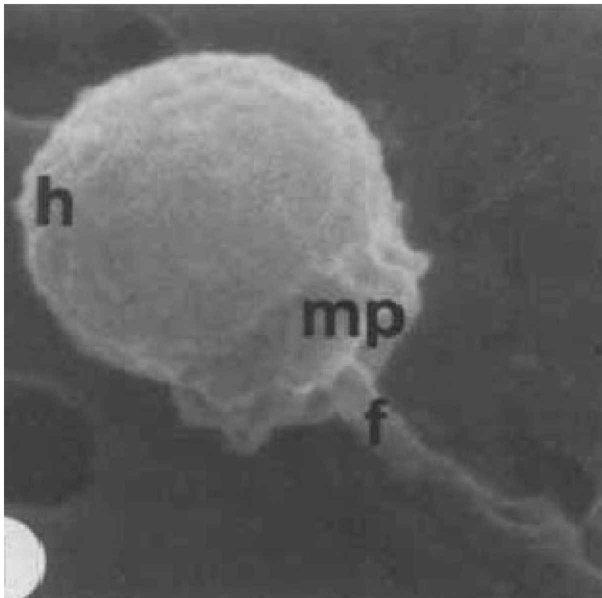

Figure 1.6: SEM image of Zebrafish sperm head and tail portion. H *designates the head region of about 2 um diameter,* f *is the flagella (Wolenski 1987).*

Zebrafish sperm consist of two distinct parts: the head and tail (Figure 5). On average the sperm head is about 2 um in diameter and the tail is roughly 30 um long. (Hagedorn 2008, Wolenski 1987). To analyze viability, the sperm are often 'activated' and motility measured using a number of sperm analysis techniques. Activation renders the sperm motile. When sperm are collected, they are placed in a buffer solution isotonic to the sperm cells. In this environment (300 mOsm/kg), the sperm is immotile (Hagedorn 2008). Dilution of this medium (beneath 200 mOsm/kg), resulting in a hypoosmotic environment, renders the sperm motile (Takai, 1995). This motility is likely the result of intracellular uptake of water, reducing the concentration of potassium ions. In examining the effects of ions and osmolality on activation, it was demonstrated solution osmolality is the dominant factor in determining motility (Wilson-Leedy 2009).

Research Significance and Project Objectives

Current trends in sperm analysis are dominated by Coulter Counters, Sperm Quality Analyzers, and Computer Assisted Sperm Analysis software. Each of these three technologies aims to reduce subjective error in analyzing sperm viability, morphology, and motility (ZDL, Inc. 2008, Hamilton Thorne Inc., 2008, Beckman Coulter 2008). Further advancements in sperm analysis focus on further integrating the measurement platforms and making them more user friendly while simultaneously reducing associated human error. These additions to the sperm analysis toolkit benefit those who monitor reproductive cells to study toxicology and reproductive biology (Quintero-Moreno 2007). One major improvement would be the application of what other cells have had the benefit of: development of integrated objective, electrochemical analyses of membrane integrity in a single system (Dittami 2008). This work aims to lay the foundation to non-optical sperm analysis using impedance spectroscopy. Applying this electrochemical technique, characteristics about cell viability, defined as motility, will be studied. Impedance spectroscopy measurements allow for further studies of the same sample, such as motility, using previously described, accepted methods.

Chapter 2

Impedance Spectroscopy of Zebrafish Sperm Cells

Introduction

Zebrafish are often used to model genetic engineering due to development and genetic expression similar to mammals and humans. As such, they are frequently used as disease models (Danilova, 2008). Instead of keeping a reproducing population of fish in the laboratory, it is becoming important to store the genetic material of zebrafish. Exploiting cryopreservation, researchers have created libraries of genetic variants by freezing sperm. It is less costly to store sperm than to keep large numbers of fish alive in aquaria. Databases also facilitate the transfer of these unique genetic variants useful for labs around the country (Hagedorn 2008). Successful cryopreservation protocols are marked by recoverable, viable sperm stocks. Several parameters have been used to determine viability. Research has linked the percent motility of the sperm within a sample to sperm viability (Lahsteiner, 1998).

Currently, Computer Assisted Sperm Analysis (CASA) "is one the most objective and comprehensive quantification currently available (Wilson-Leedy, 2007)." These systems apply motion analysis to determine percent motility, velocity, and trajectory of sperm samples. It is an objective method which aims to reduce human error in analysis by employing computer programs to screen for and track pixilated images of specific sizes. Although CASA is a widely acceptable method, its dependence on sperm motility is a limitation. Membrane integrity studies require separate methods. These methods involve dyes to characterize membrane integrity. Cellular uptake of dyes indicates membrane compromise. These two characterization methods

are dependent on optics. Alternate analysis methods utilizing the principles of electrochemistry, specifically impedance, could be used to reveal similar information about membrane integrity and motility. Hypoosmotic changes in the environment induce motility in the zebrafish sperm. As the ion concentration decreases, motility increases (Wilson-Leedy 2009). This electrolytic dependency confers electrical properties upon the sperm samples. We can harness this property for biological interrogation to determine live and dead cells. In this study, we define live cells as those which have the ability to become motile. Dead cells are those which lack the ability for motility. This difference should convey a difference in polarity of the cells measurable using the principles of impedimetry and impedance spectroscopy.

Impedance is the electrical property of a material to inhibit current flow from an applied potential in AC systems. In DC systems, this property is known as resistance. AC circuits employ sinusoidal voltages to induce sinusoidal currents in circuit. These sinusoidal waveforms follow the general formula:

$$x(t) = X\sin(\omega t + \theta)$$

where X is the current or potential magnitude in amperes and volts, respectively. The angular frequency ω is dependent on the product of $2\pi f$ (f is the applied frequency to the system). θ is the lag between waveforms. Impedance measurements allow for better understanding of complex electrical systems consisting of non-ideal, frequency dependent elements. Frequency dependency is of interest for many systems. Changes in impedance measurements at specific frequencies can indicate slight changes in organized systems. This is beneficial for biological systems since the biomaterials are sensitive to changes in environment and health potentially causing measurable changes in impedance. This method has been demonstrated as sensitive

enough to measure changes in living populations in pathogenic bacteria in commercial fruit drinks, measure toxin effects on cell growth, and organ degradation with time (Gersing, 1998, Ceriotti, 2007).

Applying impedance spectroscopy, differences in sperm solutions will be detected as changes in phase and impedance of the electrical waveforms. These changes may be induced by several factors. As already mentioned, live cells contain an intracellular electrolytic gradient which is the trigger for motility. Dead cells lacking this electrolytic gradient should provide different impedance measurements. Fragmented cells should occlude the electron path. This increase in sample debris should increase impedance by reducing the number of direct current paths between the electrodes. Successful characterization lays the foundation for alternative technologies for sperm analysis to be used in addition optical analysis.

Methods and Materials

Materials

All reagents necessary for buffers and platinum wire (76 um diameter) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All zebrafish sperm were collected from live fish stocks at the Louisiana State University Agricultural Center Aquaculture Research Station as described below. 2X-CEL slides were purchased from Hamilton-Thorne (Beverly, MA, USA). Impedance measurements and signal processing were performed using the Chemical Instruments Electrochemical Workstation Model 660B (Austin, TX, USA). Amicon centrifugation filters were purchased from Millipore (Bedford, MA, USA).

Methods

Zebrafish Sperm Collection

Zebrafish sperm was collected using two methods: non-survival surgical excision of the testes and squeezing. Using surgical excision, a euthanized male is quickly decapitated and its gonads are removed. The testes are placed within a centrifuge tube and 20 uL of Hank's balanced salt solution (HBSS) are added to the sample. The testes are crushed with tweezers in the solution to release the sperm cells. Sperm extraction through squeezing does not involve donor death. In squeezing, the male is anesthetized using a diluted MS-222 solution. Then the sperm sample is extracted by adding pressure to the male using tweezers. Sample was diluted to 20 uL using HBSS. In the non-survival collection method, normally only one or two testes are used. The squeezing method involves 3-5 males to procure enough sperm for testing.

Electrode Manufacturing and Impedance Measurement Control Setup

Electrodes were manufactured using 76 um platinum wire and Hamilton-Thorne 2X-CEL slides. The 2X-CEL slides have a 80 um deep viewing chamber. Pt wire was immobilized onto the glass slide using a conductive epoxy. Larger copper leads were epoxied to the Pt wire for connections to the impedance measurement controller.

The CHi Electrochemical Workstation controlled and processed input and output signals from the electrical system. All measurements were performed using an amplitude of 0.005 volts and an excitation signal of 0.02 volts. Frequency ranges from 0- 100000 Hz were scanned to observe phase and impedance changes due to the sample composition.

Sample Preparation and Interrogation

To determine immotile, dead sperm, 1 uL of sample was placed on a glass slide. 10 uL of water was added and motility was observed using a standard inverted microscope. To test the effect of buffer effects on 'dead' measurements, the sperm cells were filtered out of solution using Amicon filters. Samples were placed in 1 uL volumes directly onto the electrodes. Impedance of the 1 uL sample was measured. All samples were then diluted to 11 uL using Millipore H_2O. Impedance was measured again. Different volumes were used to measure the effects of diluting the sample on impedance measurements.

Results

Frequency sweeping revealed the non-ideal nature of the tested systems. However impedance spectroscopy could not discern between several sample compositions. Measurements taken for samples diluted to 11 uL showed differences between the deionized, Millipore H_2O and all other samples containing buffer (Figure 2.1). This is likely due to the large dilution of the sample of interest. The dead and live sperm samples appear to take on the properties of the HBSS buffer. To overcome this limitation, phase changes for the samples were also monitored at 1 uL volumes.

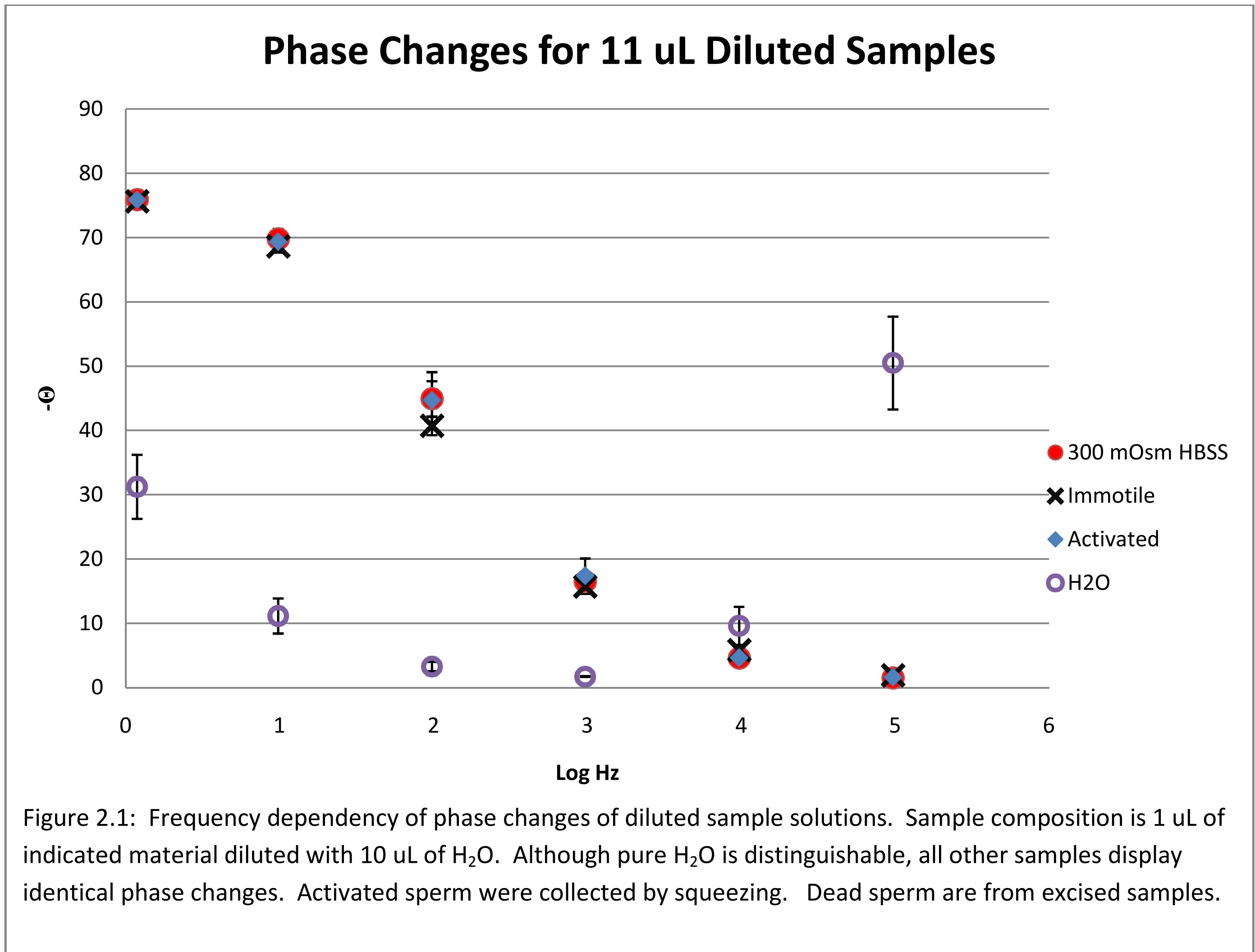

Figure 2.1: Frequency dependency of phase changes of diluted sample solutions. Sample composition is 1 uL of indicated material diluted with 10 uL of H_2O. Although pure H_2O is distinguishable, all other samples display identical phase changes. Activated sperm were collected by squeezing. Dead sperm are from excised samples.

Phase changes of undiluted 1 uL samples indicate differences between dead cells and live cells/HBSS buffer (Figure 2.2). Despite the presence of cellular membranes, HBSS buffer and the immotile live cells display identical frequency dependencies for the phase change. However, the phase difference of the dead cells suggests a compositional difference affecting the biological capacitance of the cells. The 100-1000 Hz for both volumes show the largest degree of separation.

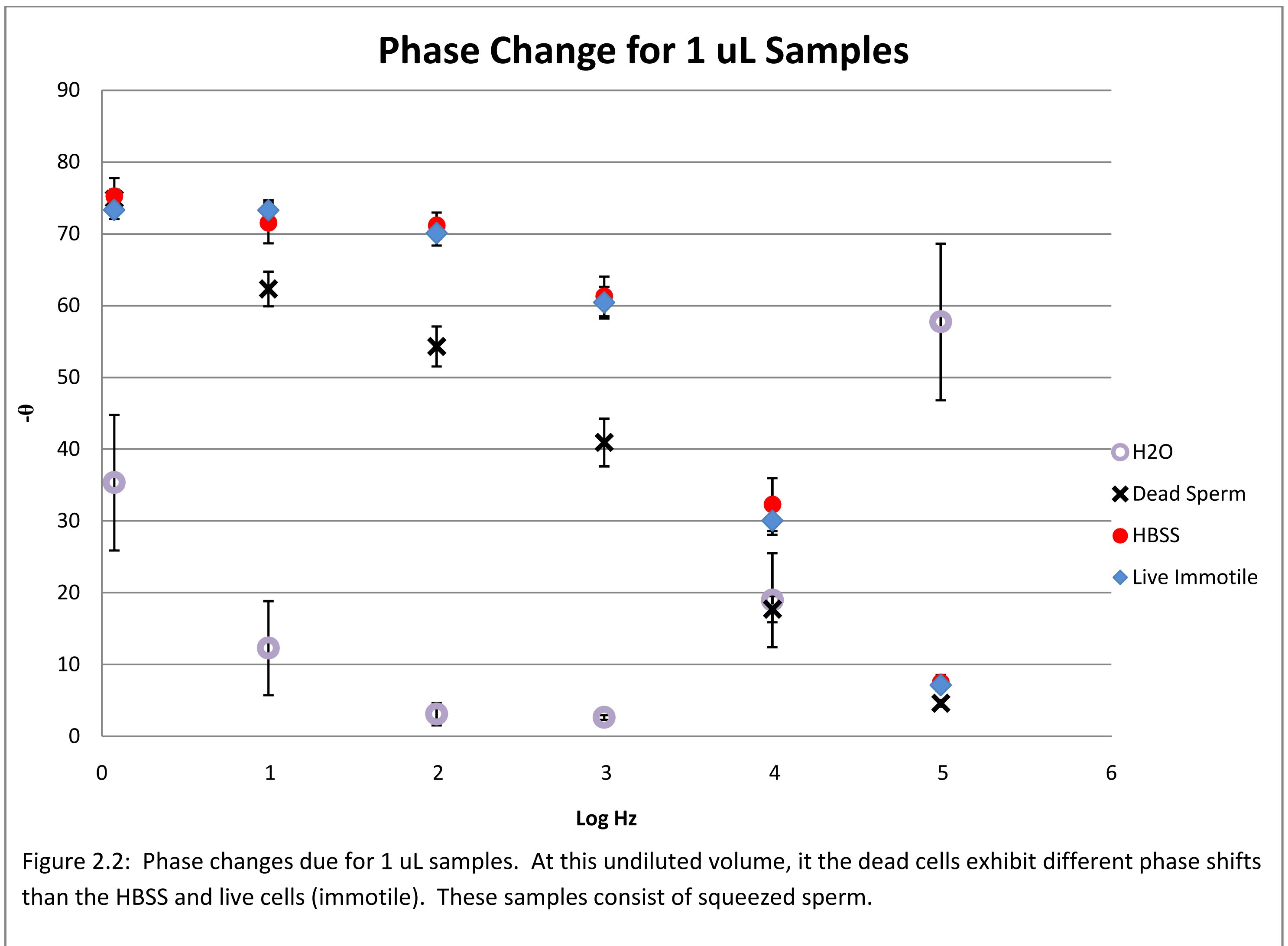

Figure 2.2: Phase changes due for 1 uL samples. At this undiluted volume, it the dead cells exhibit different phase shifts than the HBSS and live cells (immotile). These samples consist of squeezed sperm.

Impedance magnitudes were also measured for 11 uL and 1 uL samples (Figures 2.3, 2.4). Millipore H_2O showed the highest impedance for both samples. The smaller volume of water exhibited different magnitude/frequency differences. For both samples, live cells and buffer result in identical impedance changes while dead cells show the lowest impedance change.

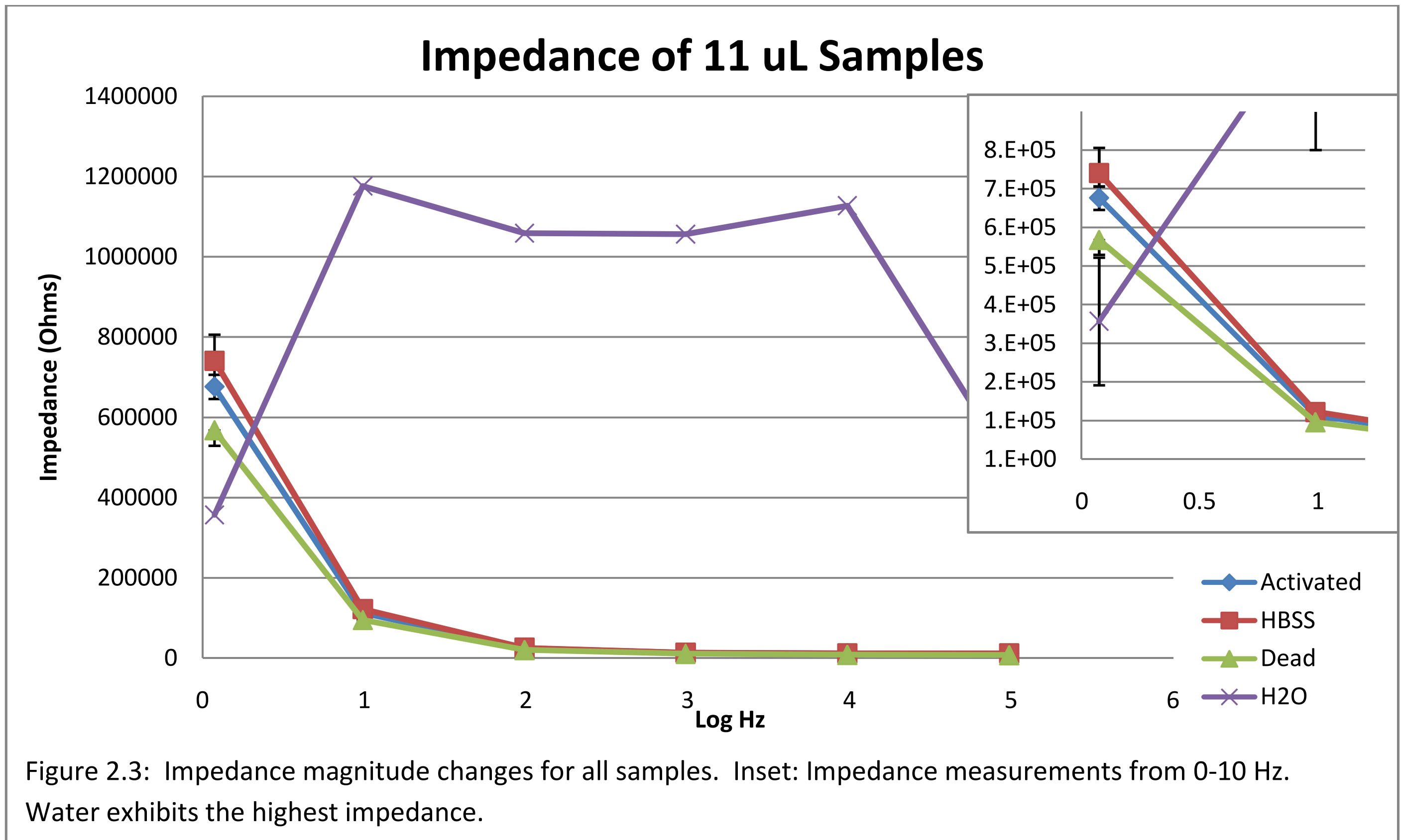

Figure 2.3: Impedance magnitude changes for all samples. Inset: Impedance measurements from 0-10 Hz. Water exhibits the highest impedance.

Impedance for 1 uL Samples

Impedance (Ohms)

100000000
90000000
80000000
70000000
60000000
50000000
40000000
30000000
20000000
10000000
0

0 1 2 3 4 5 6

Log Hz

2000000
1500000
1000000
500000
0

0 0.5 1 1.5 2

Live Immotile
Dead
HBSS
H2O

Figure 2.4: Impedance magnitude dependency on frequency. At lower frequencies, the impedance increases. Like 11 uL samples, water displays largest impedance magnitudes. There are distinct different impedances for dead and live samples at low frequencies.

In order to ascertain the repeatability of measurements between stocks, immotile sperm measurements were taken for other stocks. Figure 2.3 shows the phase changes of a separate, squeezed immotile sperm stock. The two immotile stocks share similar frequency dependencies with different changes in phase magnitude. However it is still greater than the dead cell samples. This decrease in phase change approaching a medium between the original and new data reveals complexities in the impedance measurement system.

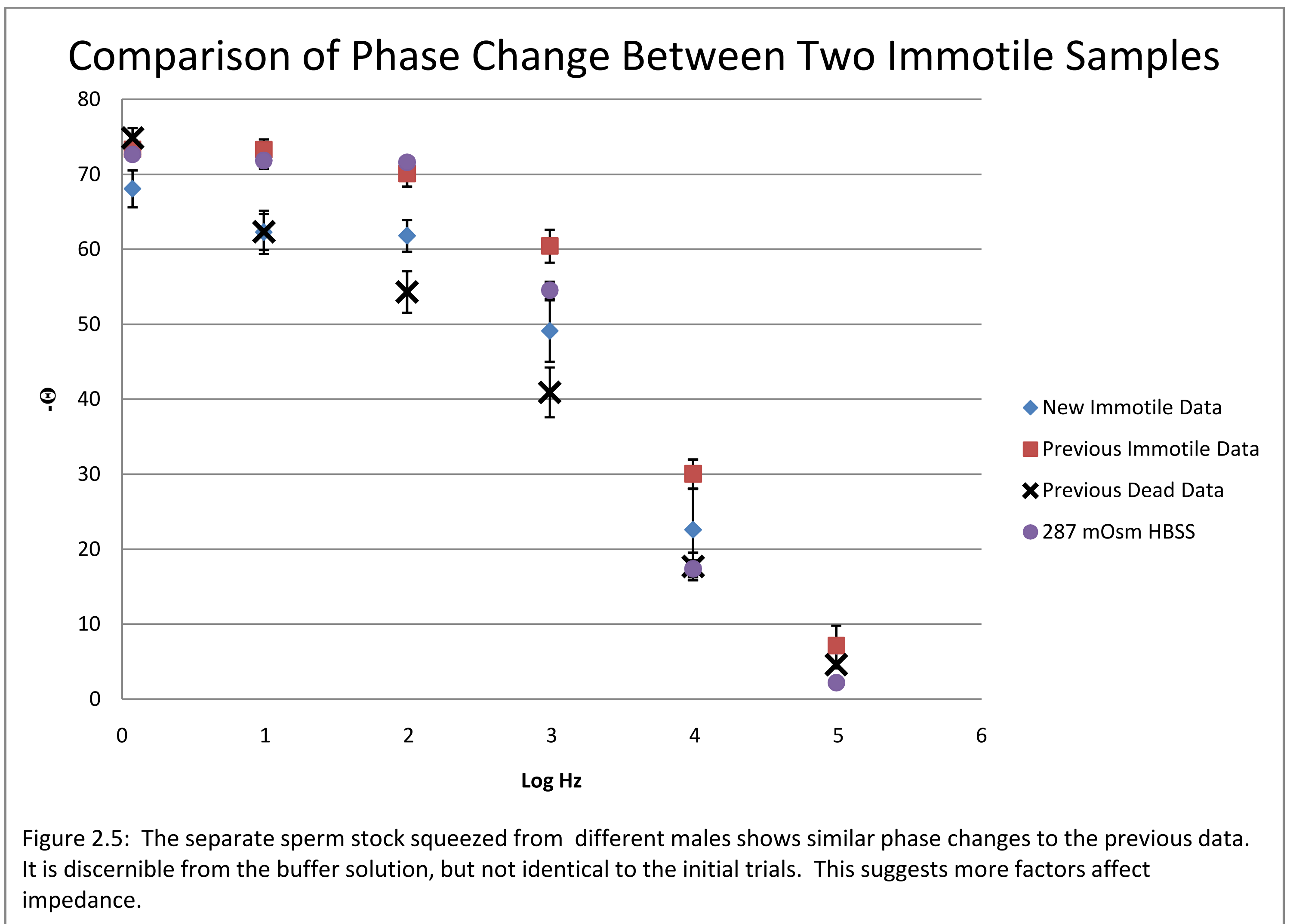

Figure 2.5: The separate sperm stock squeezed from different males shows similar phase changes to the previous data. It is discernible from the buffer solution, but not identical to the initial trials. This suggests more factors affect impedance.

Discussion

Electrochemical measurement of biological materials and systems are inherently complex due to the composition of the test subjects. This complexity prompts the use of impedance spectroscopy because it is sensitive to the composition of the measured systems. Impedance is a ratio of the inhibition of the flow of electrons across a medium due to an applied potential. In cell measurement, the concentration of cells should affect the impedance by prevent less direct paths of electron flow from one electrode to the other. Other factors which can potentially affect impedance measurements are membrane integrity, electrolytic concentration, and extracellular debris. Intact membranes create isolated, polarized regions in the medium. These should obstruct current. Electrolytes can assist the flow of electrons and lower impedance.

The impedance measurements and phase changes for these samples do not correlate well with these statements. While water shows high impedances from low ionic activity, the other samples measurements were less predictable. Low impedances are expected for the highly electrolytic buffer HBSS. However, they are statistically higher than the buffered dead sperm samples. The samples containing dead sperm should impede current flow due to the presence of cellular membranes and debris. The presence of cells in the buffer, in theory, would create electrochemically distinguishable measurements in sample containing the buffer, live, and dead samples. These cells would increase impedance due to concentration. Live cells should affect phase changes due to capacitive properties of the intact membranes. The variability between the two stocks (Figure 2.5) suggests cell concentration instead of membrane characteristics is a controlling factor in these measurements.

Phase changes and impedance changes are detected between the samples. At this point it is impossible to discern the composition of samples from these measurements alone. However we can pinpoint frequency ranges of interest. Frequencies between 100 and 1000 Hz result in the largest phase separations. This separation could be useful in future studies. In addition to this separation, a potential isosbestic point exists at the 10000 Hz range. This isosbestic point could see use in concentration studies.

Detecting changes in phase and magnitude justifies the use of impedance spectroscopy. The constructed system however may not be ideal for these measurements. The measured impedance and phase changes during this study are due to changes in the sample solution. Although no definitive conclusions can be made about impedance or phase relationships with live or dead cells, the system has proved sensitive enough for further study.

Future Studies and Conclusions

Although the results of this study do not support the intended purpose, further research into this area is promising. Errors associated with this experiment provide opportunity for future work and methods to optimize the system and understanding of the data. This preliminary work identified frequency ranges of interest (100-1000 Hz) which could be used for further work at these lower impedances. The experimental setup must be optimized to fully exploit the range of magnitudes for future studies. The optimization process involves examining buffer effects on impedance measurements. According to the data, the HBSS buffer has the same frequency dependent electrical properties as the buffered live cells. This compromises the ability to obtain significant data about the biological systems separately from the buffer. In anticipation of future studies, other buffers and media must be characterized to fully understand their impedance properties. First, we will begin by measuring simpler electrolytic buffers (NaCl) of similar osmolarities. Examining impedance changes of other buffers with and without cells will allow for selection of the optimal medium to perform these measurements. It will be difficult due to the high ionic concentrations to preserve live cells in their immotile state.

In examining data collected from separate fish stocks, it becomes evident cell concentration may be a driving factor for these bulk measurements. It will be worth measuring concentration of cells in the samples to support this theory. Parallel research is developing optical density protocols for zebrafish sperm measurement. These relations will expedite and aid in the understanding of impedance spectroscopy of the sperm cells. In addition to cell counts, it is necessary to further characterize discrete regions in solution. To do this, non-biotic, non functionalized polystyrene beads will be introduced to the electrode system. These polystyrene beads would mimic the presence of the cells in solution by simulating volume and particle changes. Similar studies can be performed employing vesicles. These polystyrene beads and

vesicles would not have the same membrane potential as the sperm cells; nevertheless, they will be useful to study concentration effects on impedance measurements. Increasing impedance and concentration measurements will be confirmed.

The Chemical Instruments Electrochemical Workstation used in this study has limitations. It is only capable of performing measurements up to 10^5 Hz. For most biological impedance studies, it is high and low impedance ranges that are of interest. As mentioned, the data reveals measurement gaps at lower frequencies. Yet, to study 1 MHz ranges and above, other instruments must be used. The Agilent E4980A precision LCR meter can be used to apply higher frequencies to the system. These higher frequencies should produce different impedance measurements and phase changes in the samples. Preliminary experimentation has already been performed using this instrument (Figure 3.1, 3.2). These are dead cell samples and buffer magnitude and phase at 2 MHz and 1 MHz. Multiples trials must be performed to further assess measurements at higher frequencies.

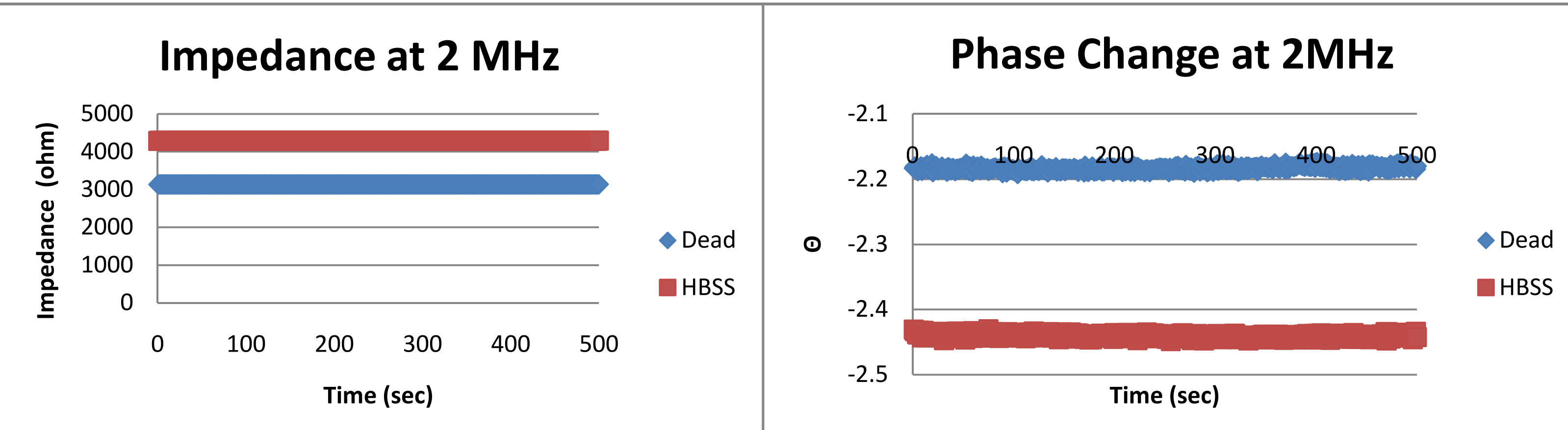

Figure for 3.1: Impedance and phase measurements for dead cells and buffer. The measurement was taken with respect to time. They are time independent. Although the two samples are discernible due to the differences, further testing must be done.

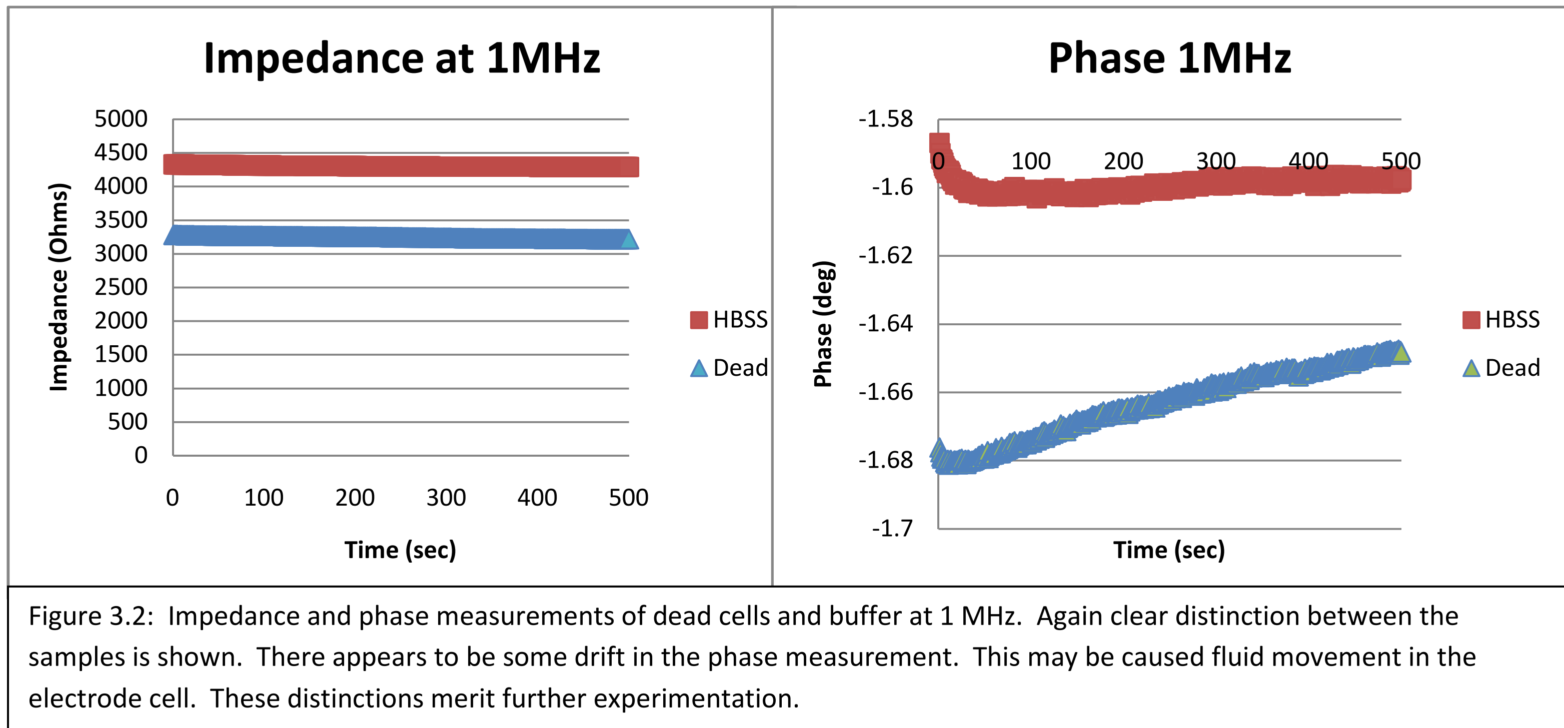

Figure 3.2: Impedance and phase measurements of dead cells and buffer at 1 MHz. Again clear distinction between the samples is shown. There appears to be some drift in the phase measurement. This may be caused fluid movement in the electrode cell. These distinctions merit further experimentation.

Finally, another factor in these measurements has been the sample size. The current measurement system allows for samples as small as 1 uL due to the distance between the electrodes. Microfluidic systems would be ideal to measure this volume and smaller ones. In these systems, the electrode gap can be made smaller. This effectively reduces the working volume of the system. Sample would be introduced to the electrodes using syringe pumps. Measurements would be performed in the same described methods. These systems would allow for more repeatable sample introduction and measurements by controlling sample volume and electrode configuration.

This study shows the sensitivity of impedance spectroscopy to differences in sample media containing sperm cell, buffer, and water. This sensitivity indicates the potential utility of the technique for characterizing these media. Due to this sensitivity, several parameters need

further research and optimization. Beads and vesicles will be employed to study discrete particles effects on impedance. Concentration will be further correlated to the measurements. Microfluidics and higher frequencies will be used to reduce error in sample geometry, volume, and noise.

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