Abstract

Understanding a cell’s contents and their location within the cell is a key step to understanding a cell’s function. We are trying to develop time of flight second ion mass spectroscopy, or TOF-SIMS, for this very purpose. TOF-SIMS has long been used as a tool to investigate the quality of semiconductors. We are interested in using TOF-SIMS to determine what protein and lipids are present in a cell, and where these components are localized. We also hope to create an image of the location of these molecules in a cell. To do this, we have examined *Drosophila* embryos as well as adult gonads via TOF-SIMS. For these analyses, two separate TOF-SIMS processing techniques, static and dynamic were employed. Static, or ion milling TOF-SIMS, is a technique where a focused ion beam is targeted on a specific location of the biological sample. Ion milling can be used to penetrate through a sample at a particular point or to remove a monolayer. Ion milling is conducted on cryostat sectioned samples. The dynamic technique, or sputtering TOF-SIMS, is a technique that uses a stream of primary ions to shave off a layer of tissue at a time. Sputtering was conducted on unfixed tissue.

To test the capacity of TOF-SIMS for analysis of biological samples *in vivo*, we have analyzed both adult gonads and *Drosophila* embryos via TOF-SIMS. These systems were chosen for analyses due to their relative simplicity and ease of large sample collection. Through these analyses, it was determined that the dynamic technique of sputtering was not an effective way to get high resolution spectra and ion images. Ion milling did, however, produce high resolution spectra and ion images. Furthermore, both techniques were able to detect lipid signals in *Drosophila* embryos. Thus, our analyses provide
proof of principle that TOF-SIMS can be used to detect lipid fragments in biological tissue. As TOF-SIMS technology is adapted for analysis of proteins, we hope it will become a viable technique for whole-lipid and proteomic analysis on a sub-cellular level.

**Introduction**

Scientists from developmental biologists, to cell biologists, and cancer researchers are interested in the biochemical and molecular pathways involved in determining a cell’s function. In particular, they are interested in understanding what proteins and lipids are involved in performing specific cellular functions. To gain insight into this question, scientists need to develop technology that allows them to examine protein/lipid content and the location of these molecules within a sample all at once. Why? The data from this technology would allow scientists to have good indicators as to what receptor proteins are located at a specific region in a cell. This is a good indication that a particular protein is acting in the cell in a given way. TOF-SIMS, or time of flight second ion mass spectroscopy, is a technique that could possibly give this type of information. The purpose of my project has been to determine whether time of flight second ion mass spectroscopy or TOF-SIMS can give biologically relevant data using a simple biological specimen. Specifically, we have attempted to produce a three dimensional image of a developing *Drosophila* embryo or an adult gonad using TOF-SIMS. The overall goal for imaging is to have the protein and lipid information superimposed over this image, so as to understand where these molecules are localized within the cell or tissue. In the following sections, a discussion of the broad range of techniques used to collect biological information is provided. This is followed by an overview of the biological
systems, *Drosophila* embryos and gonads, that we have attempted to analyze via TOF-SIMS.

**Techniques for the investigation of protein and lipid content in biological tissues:**

There are many proteomic techniques used to investigate protein and lipid content in cells or whole tissues. However, they all vary in the type and amount of information they are able to provide. Depending on the technique scientists can gain information about the types of proteins and lipids present in a tissue, where these molecules are located, and in what concentration they can be found. Additionally, the morphology of a tissue can be investigated. The following sections discuss the information each different technique collects and its relative advantages and disadvantages. A detailed description of TOF-SIMS and its benefits and disadvantages is also discussed.

*Protein analysis using gel electrophoresis*

Gel electrophoresis is a technique that allows scientists to separate proteins in solution by their molecular weight and/or charge. Coupling gel electrophoresis to western blotting allows for the detection of a specific protein that reacts to an enzymatically labeled antibody. Gel electrophoresis can be used for a total analysis of the proteins that are present in solution. Furthermore, coupled with cell fractionation; it can give some information about where proteins are located in a tissue sample or a cell. Standard gel electrophoresis techniques do not, however, provide high resolution pictures of where proteins exist in a cell, or with what components they co-localize. Furthermore, detection
of specific proteins separated via electrophoresis, then revealed through western blotting, is limited to available antibodies. Thus, while gel electrophoretic techniques can sometimes combined with mass-spectrometry techniques for identifying unknown proteins, typically this technique is limited to analysis of already known proteins.

**Immunohistochemistry and confocal microscopy techniques for investigation of proteins**

Immunohistochemistry is a technique to help identify localization of proteins in a cell or tissue. Through this technique, tissues are mixed with antibodies that bind specifically to a given protein. By using antibodies that have been conjugated to fluorescent molecules or enzymes that produce a colored precipitate, the site of antibody binding to its target protein within a cell or tissue is revealed. When using fluorescently conjugated antibodies in conjunction with high-resolution microscopy techniques, this technique can be particularly powerful for determining a protein’s location in a cell.

One such technique for assessing sub-cellular localization of proteins is fluorescence confocal microscopy \(^1\). Unlike, conventional fluorescence microscopy in which emissions from a fluorescently conjugated antibody are collected from all areas of a sample at once, confocal microscopy optically sections samples (in 3-dimensions) so that proteins localized above each other (along the Z-axis) in a given cell or tissue can be optically resolved \(^2\) \(^1\) \(^3\). By using multiple fluorescently conjugated antibodies and/or fluorescent dyes that bind directly to a specific lipid or protein, each of which fluoresces at a different wavelength, both conventional and confocal fluorescence microscopy can be used to examine co-localization of multiple molecules within a cell. These techniques are
therefore very powerful. However, like western blotting analyses described above, they are limited by the existence of antibodies and dyes used for detection of a specific molecule. Furthermore, because there are a limited number of fluorescent molecules that can be conjugated to each specific antibody, scientists are only able to examine co-localization of only a few proteins at a time.

**MALDI-MS**

Matrix assisted laser desorption ionization mass spectroscopy, or MALDI-MS, is a mass spectroscopy technique that has been used to investigate both proteins and lipids. MALDI-MS is currently used to perform proteomic analyses on biological tissue. This is because MALDI-MS has the ability to detect high mass proteins that other mass spectroscopy techniques cannot detect. MALDI-MS is a technique that uses a laser that emits high energy photons which hit the tissue sample. These high energy photons hit a tissue sample that is embedded in a matrix consisting of salts and proteins. When high energy photons impact with the sample, this results in proteins fragmentation into small peptides which are then ejected from the sample. All molecules from the sample and the matrix have a charge associated with them due to the high energy impact of the photons. Because of differences in mass and charge, each ejected fragment emitted from the sample flies toward a detector at a different speed. The detector records when the fragment hits the detector and, based on the time it took for the fragment to be ejected from the tissue, the mass to charge ratio is determined. Based on this ratio, the protein sequence can be identified. Furthermore, by
identifying multiple fragments with overlapping sequence, the full length protein sequence can be assembled.

While MALDI-MS is a powerful technique for examining protein content in a tissue, it also has many disadvantages. MALDI-MS can only be used to examine large regions of a tissue which are hundreds of micrometers in area. By MALDI-MS, using a relatively large scanning area of laser beam, it reduces the amount of fragmentation of proteins and allows for better detection. Also, depending on the type of matrix used, low sensitivity is observed. While this problem can be alleviated, by increasing the ratio of sample to matrix this results in increased fragmentation. Additionally, MALDI-MS is an extremely inefficient system for detecting smaller mass molecules, like lipids.

X-ray photoelectric spectroscopy

X-ray photoelectric spectroscopy (XPS) is a spectroscopy technique that employs x-rays to inspect the surface composition of a material. This technique has been used to examine metals along with biological tissue. It has the great benefit of being able to give detailed data about the chemical composition of a material such as its molecular weights. This is a great benefit because it allows scientists to determine what types of molecules are located in a tissue.

XPS, however, also has many disadvantages. Furthermore, resolution for small molecules is relatively poor, and it is limited by the size of the molecules that it can detect along with the limited size of the tissue sample that it can investigate. XPS can
only look at tissue that is one centimeter by one centimeter. XPS also does not provide any three dimensional image of the tissue.

**Protein BioChips**

Protein BioChips would be able to tell scientists the protein make up of a sample very quickly without destroying the sample. Protein BioChips have the added benefit over mass spectroscopy in that they would be capable of being adapted into a high throughput assay. By using multiple protein BioChips in tandem, researchers are able to investigate many samples at once and then be able to easily compare the proteomic map of the samples against each other. This is not the case with any of the techniques mentioned above, except for gel electrophoresis.

Protein biochips, like DNA biochips need to have a defined substrate that is attached to the chip in a certain known pattern. DNA chips use cDNA to bind anti-sense DNA sequences placed on a gene chip. Attached to the cDNA is a fluorophore that when the DNA binds to its corresponding DNA strand will fluoresce. Protein biochips must function in a similar way. Protein biochips like ELISA must have a substrate that the proteins can bind to. In the case of protein biochips antibodies specific to an array of proteins are arrayed at different locations on a biochip. A fluorophore is bound to the antibody just like in immunostaining and when the protein that specifically binds to the antibody binds the molecule and fluoresces.
The disadvantages of protein biochips are currently that only proteins that have antibodies made for them can be detected. Secondly not all antibodies bind covalently with their corresponding protein at the same conditions. Antibodies differ in the pH and the temperature at which they bind most efficiently with their specific protein.

**Time of Flight Second Ion Mass Spectroscopy**

TOF-SIMS is a technique that was first developed for use in the semiconductor industry to examine the surface of semiconductors for imperfections. Recent work, however, has focused on adapting this technique for the investigation of biological tissues. TOF-SIMS could be used by scientists to identify proteins and lipids, where they are located in a cell or tissue, and at what concentration they are found. TOF-SIMS displays this information in the form of a spectra with mass to charge ratios of fragments, and an ion image with positional information as to where a fragment came from. The information given by the TOF-SIMS would enable biochemists, molecular biologists, and developmental biologist to gain a better understanding of what function certain proteins and lipids have within a cell. TOF-SIMS is currently very useful at investigating lipid content, but not very good at investigating what proteins are present. However, scientists are working to develop this technique for investigation of proteins and other high-mass molecules.

TOF-SIMS collects data by detecting the secondary ions that are ejected from a sample due to a primary ion from either a gold or argon ion gun impacting the tissue resulting in
the ejection of secondary ions. Secondary ions are fragments of proteins and lipid from the sample. Each fragment has both a mass and a charge associated with it. The ejected secondary ions fly through the instrument towards a detector (Figure 1). Each secondary ion flies through the instrument at a different rate due to the fact that each one has a different mass to charge ratio. As such, the secondary ions hit the detector at different times. Based on the time of impact, a spectra is created and the origin of the fragments can be ascertained (Figure 2)

When performing TOF-SIMS, there are two different types of methods of collecting data. There is the static technique of ion milling and the dynamic technique of sputtering. Both techniques result in a spectra and ion image being generated.

The first way of milling through a sample is to bombard the tissue sample with a few ions from an ion gun or photons from a laser. Many analyses, including ours, utilize a gold ion gun to bombard tissue samples. The primary ions or gold ions are targeted on a small portion of the sample between the lengths of 25 x 25 micrometers to a depth of 5 nm (Figure 3). This high resolution allows for easy investigation of specific locations on a tissue sample. With this technique, a monolayer at a time can be removed. When this happens, a crater of destruction where the primary ion hit the tissue sample. The damage is due to the high energy impact of the primary ion which leads to fragmentation of the bottom layer and the intense energy can lead to those fragments and tissue burning. The secondary ion fragments from these tissue layers that are not recovered by the detector are embedded in the bottom layer causing contamination or matrix effects in lower layers of tissue.
The second method, called dynamic TOF-SIMS, is a technique whereby layer after layer of tissue is sputtered through by a constant bombardment of primary ions. The primary ions from the sputtering gun mill through multiple layers. The benefit of TOF-SIMS compared to a lot of other forms of mass spectroscopy techniques is that it has an increased sensitivity due to only secondary ions from the sample being generated because the sample is not imbedded in a matrix. It also provides more sensitive results, allowing scientists to investigate tissues for protein and lipid content that are very small.

The spectra that is generated by each technique contains all of the information about what molecules are present in the tissue sample while the ion image contains all the positional information about where those molecules originated from in the sample. The ion image collects data on where the secondary ion was ejected from and how many secondary ions of a certain type originated from that location (Figure 3).

Two different ion images can be displayed. The total ion image which is a display of all the secondary ions that were ejected from the sample over the total time the sample was run in the TOF-SIMS. The second image is of only a particular secondary ion and where it was ejected from. This can help scientists to see if there is a particular place in the tissue where that fragment is localized (Figure 4). This can help scientists to see where molecules are localized and possible lead to an understanding of what the molecules do.
What TOF-SIMS does that other mass spectroscopy techniques cannot, is to give positional information about fragments from proteins or lipids ejected from the sample (Table 1). These fragments are displayed in the form of an ion image (Figure 4) that shows the localization of the fragments. TOF-SIMS has the potential to give information about hundreds of molecules present in a sample at once, provided they can be identified. However besides being a qualitative technique TOF-SIMS can also be semi quantitative by measuring the quantity of secondary ions. This can only be done when matrix affects are taken into account using calibration curves. Currently, TOF-SIMS is very good at identifying lipid content in a tissue sample, while further work must be done to adapt it for proteomic analyses. Currently scientists in Korea are working on improving TOF-SIMS as a technique to investigate proteins and other higher mass molecules\(^{13}\). They have been putting thin layers of pure streptadivin on silicon wafers and collecting images and spectra data. The purpose of their experiments are to see if the TOF-SIMS can detect the protein and if so can they visualize the positional information in the form of an ion image. While this is a good step in the right direction there is still more work needed to do before TOF-SIMS will be an affective technique to investigate protein content in a biological tissue sample.

**The biological system**

The first step in trying to develop TOF-SIMS as a technique to investigate biological tissue was to pick a model system that could help us determine if TOF-SIMS provided any relevant biological data. In Dr. Wawersik’s lab, a major area of interest is the regulation of germline stem cell (GSC) behavior in adult gonads. Specifically we are
interested in the signals that control the balance between maintenance of a continuously dividing population of GSCs, and the capacity of these cells to produce progeny that differentiate into either sperm or eggs. We therefore, began our TOF-SIMS analyses on adult gonads, while also examining both fertilized and unfertilized Drosophila embryos as an even simpler system for testing the efficacy of TOF-SIMS as a technique for analyzing lipid content in biological systems.

**Adult Drosophila Gonads**

The primary function of testes and ovaries is to produce the sperm and eggs required for sexual reproduction (Figure 5-6) \(^{14}^{15}^{16}\). The continuous production of sperm or egg requires the asymmetric division of a GSC to produce one cell that is maintained as a GSC, and one daughter cell that differentiates step-wise into gametes. In Drosophila gonads, this balance between GSC maintenance and differentiation is controlled by signals for the surrounding micro-environment, termed the GSC niche. In testis, the GSC niche, or hub, is comprised of a group of somatic cells at the apical tip of the testis, to which 5-9 GSC are docked. Both adhesion to, and signaling from, the GSC niche/hub are responsible for regulating asymmetric GSC division. While a number of the signals that regulate this process have been identified, other signals are yet to be determined. We are, therefore, interested in developing TOF-SIMS as a technique to identify these signals. Specifically,
we are interested in using TOF-SIMS to locate proteins and lipids found at different concentrations in the polarized GSC which might help us to determine the molecular mechanisms that regulate GSC maintenance vs. differentiation.

In the *Drosophila* ovaries, the stem cell niche is very similar to the hub in the testis (Figure 5-6)\(^{17,16}\). It is comprised of a small group of somatic cells, termed cap cells, localized to the apical tip of each ovariole. Each ovary consists of 15-16 individual ovarioles oriented in parallel such that all GSCs are localized at the apical end of the ovaries, while differentiated eggs are found at the opposite end, where they are pushed into the reproductive tract and fertilized. As in testes, adhesion to and signals from the GSC niche regulated the balance between GSC maintenance and differentiation in each ovariole. Similarly, the signals that regulate this balance are still being described. Once again, we hope that TOF-SIMS can help us to gain insight into the proteins and lipids that are located at each end of the polarized GSC in order to obtain a better understanding of the molecular mechanisms that regulate asymmetric GSC division.

*Drosophila Embryo*

*Drosophila* embryos were picked as our model system because of their relative simplicity. They are among the simplest *in vivo* biological systems that we could investigate. The drosophila embryo is approximately 350 micrometers in length by 150 micrometers in diameter and width. Because of their large size, the relative simplicity of their outer membranes, and the fact that the developmental stages of the their biologically system have been extensively investigated, *Drosophila* embryos are well suited for our
purpose of performing a “proof of principle” experiments to determine if TOF-SIMS can be used as a tool to gain information about the protein and lipid content of biological tissues.

The developing *Drosophila* embryo is comprised of two outer tissue layers that protect it from the external environment. Each of these layers has a different composition and thickness. The inner layer, or vitelline membrane, is a layer of wax approximately 300 nm thickness, that has been secreted by support cells of the developing egg. The outermost layer, termed the chorion, is a water impermeable layer consisting primarily of cross-linked proteins along with wax. The chorionic membrane consists of three sub-layers including the externally localized exochorion (300-500 nm thick), the middle endochorion (500-700 nm thick) and the chorionic layer (40-50 nm thick). Each of these layers is characterized by different concentrations of cross-linked proteins (double check this, this is just a guess on my part). Altogether, the chorion consists of 6 major proteins, A1, A2, B1, B2, C1 and C2, as well as 13 other proteins found at lower concentrations. Because we know the concentration at which these proteins are found in different external tissues layers, this tissue is an ideal system for initial *in vivo* analyses conducted with TOF-SIMS. As current TOF-SIMS technology is not effective for protein identification we first decided to focus on lipid content in the plasma membrane. However, in the future we hope to investigate the protein content of the outer membranes of Drosophila embryos.

The plasma membrane is the last barrier between the embryo and its environment. The plasma membrane is located below the vitelline membrane. The plasma membrane
is actually comprised of many membranes from several support cells that surround the inside of the embryo. The lipid composition of each of the support cells is very similar. The plasma membrane of *Drosophila* malanogaster support cells has been found to be comprised of very similar lipids to those found in humans. The major difference between the lipids found in the *Drosophila* membrane and those in humans is that flies have shorter fatty acid chains. The *Drosophila* plasma membrane is comprised of Ergosterol making up about 69% of the total membrane, phosphocholine, phosphoethanolamine, phosphoserine, phosphoinostol, and sphingomyelin.

To determine the best way to show that we can get relevant biological data from TOF-SIMS, we decided to focus on investigating the lipid content of the Drosophila plasma membrane in fertilized and unfertilized embryos. This was decided for two reasons. First, that TOF-SIMS is very good at collecting data on lipids. Secondly, the plasma membrane is easily accessible by TOF-SIMS, and its contents are known. Thus, it would provide a proof of principle if we got spectra that contained data about the lipid composition that was the same or similar to that in the literature. Specifically, we wanted to compare the spectra from fertilized and unfertilized embryos to see if there were any differences in the lipid content. We hypothesized that there would be a difference between the two spectra in that the lipid content of the unfertilized embryo would be much higher than that of the fertilized embryo. While the lipid content overall between the fertilized and unfertilized embryos would be the same, there would be differences in the distribution of lipids throughout the fertilized embryo as a result of developmental changes that are occurring. Furthermore, any morphological changes that occur during
development, should be detectable in the ion image. A detailed description of the developmental stages of fertilized embryos is given in Table 2.

We decided to test to see if both the dynamic and static techniques of TOF-SIMS could generate high resolution spectra and images. We were particularly interested in observing if both techniques could detect secondary ions from lipids present in the tissue. We discovered that both techniques can detect fragments from lipids. However, the ion milling technique is able to generate high resolution spectra and ion images due to its ability to detect secondary ion fragments over a wider mass range than the dynamic technique of sputtering. The sputtering technique proved to lose too many of the secondary ions as the sputtering time increased. As a result of the loss of secondary ions the sputtering technique could not generate high resolution spectra and ion images.

**Methods**

*Embedding of Drosophila embryo’s*

Drosophila embryo’s fertilized and unfertilized were first laid by YW1118 wildtype drosophila adults and virgin adults. The virgin flies were identified and collected. Two cages were set up. The first was a cage of adult YW1118 flies. The second was of the virgin YW1118 which once they reached adulthood would lay unfertilized embryos. The cages consist of plastic tubing with a wire mesh on one end. An apple juice plate with yeast was placed at the open end of the tube. The flies being attracted to the yeast food lay their eggs on the apple juice plate. The eggs were removed using a paint brush
that had its bristles cut so that they were about 3mm in length. Once the eggs were collected off of the apple juice plate the eggs were placed in a strainer. This is so that eggs were not lost during the washing period. The excess yeast and apple juice agar were removed from the eggs using PBT 1X. This is a dilute detergent that is good at removing the yeast and apple juice agar from the eggs. The unfertilized and fertilized eggs were then embedded in four different ways in two types of gelatin. The two types of gelatin were Gelatin porcine skin from Sigma Aldrich and in TBS tissue freezing medium from triangle biomedical sciences. Fertilized and unfertilized eggs after being washed with PBT 1X were then washed with water and embedded in one of the mediums. Fertilized and unfertilized eggs were washed in a solution of 50% bleach to 50% water to remove the chorion and then washed with water and embedded in one of the gelatins. These samples were marked and contained in a plastic Petri dish and were placed at -80°C.

_Cryostat sectioning_

Drosophila tissue was cryostat sectioned after freezing at -80°C. The cryostat internal temperature was kept at -20°C while the cold plate was kept at -40°C. The drosophila samples were removed from the -80°C freezer and were cut to fit the mounting plates in the cryostat sectioning machine. Excess gelatin was poured over the sample. The mounted sample was then put on the -40°C cold plate in the cryostat sectioning machine in order for the new gelatin to freeze insuring the sample stayed on the mounting plate. A new Fisher cryostat sectioning blade was put into the cryostat machine. The sectioning device was set to cut thick sections. The sections were placed onto silicon wafers and coated glass slides that were at room temperature approximately 25°C. These sections
were then transferred to the Applied Research Center surrounded by liquid nitrogen to keep them cool.

_Sputtering experiments_

In these experiments the drosophila embryo was bombarded by 5K eV argon ions from the argon gun. A secondary ion spectrum was determined along with an ion image.

_Comparison between sections of Fertilized and Unfertilized embryo’s_

From cryostat sectioning of both fertilized and unfertilized embryo’s four mid sections were taken and placed on silicon wafers to be analyzed in the TOF-SIMS. The sections were placed under vacuum in the TOF-SIMS and were millied through by the gold ion gun at 22 K eV. A secondary ion spectrum was taken to be analyzed.

_Results_

In order for TOF-SIMS to be developed into a technique to investigate biological systems, an investigation had to be conducted as to which techniques, dynamic and/or the static TOF-SIMS could collect data that could generate high resolution spectra and ion images. Within the spectra, we wanted to identify specific lipid peaks previously shown to be present in the _Drosophila_ plasma membrane, in order to prove that we could collect
data about the lipid composition of a biological sample. An overview of our experimental process is contained in Figure 7.

_Sputtering Experiments on Drosophila Testis and Ovaries_

The dynamic technique of sputtering through a tissue sample was first investigated due to the benefit of being able to run an unfixed sample in the TOF-SIMS. The drosophila testis and ovaries were first chosen as the biological system to be investigated because we wanted to be able to investigate the lipid content of _Drosophila_ germline stem cells. We wanted to be able to investigate the lipid composition of GSC cells because some lipids such as cholesterol play important roles in the modification of proteins that are important in the differentiation and maintenance of GSC cells. GSC cells however were too difficult to investigate due to their small size and their location within the testis and ovaries. As a result, it was decided that the lipid content of drosophila testis and ovaries would be investigated.

_Drosophila _testis were investigated using TOF-SIMS sputtering with an argon gun. Data was collected over a range from 0 m/z to 400 m/z (Figure 8). Polymer contaminates dominated the low mass region of the spectra between 0 and 100 m/z. A low intensity phosphocholine peak was detected in testis. A few fatty acid peaks were detected between 200-300 m/z (Figure 6). A high resolution total ion image was created from the secondary ions ejected from the _Drosophila_ testis (Figure 9). This ion image tells us that we are having a loss of secondary ions which results in poor quality of the ion image.
However at the same time we were able to see some morphological information from the testis ion image.

Drosophila ovaries were investigated and found to have a similar lipid content as testis. It was found from the spectra that secondary ions were recovered from 0 m/z to about 400 m/z. It was found that the polymer contaminates present in the TOF-SIMS were the main secondary ions species that were at the low mass range between 0 m/z and 100 m/z (Figure 8). A high intensity phosphocholine peak was detected at 180 m/z as well as fatty acid signals between 200-300 m/z and large R group fatty acid tails between 500-600 m/z (Figure 10). A total ion image of the Drosophila ovary was created (Figure 11). No morphological information could be ascertain from the ion image.

From the comparison of the testis and ovaries spectra, it was determined that there were differences in the amount of phosphocholine present with the testis showing a higher level of phosphocholine in ovaries than the testis showed. There were also large R groups that comprise the side chain of a fatty acids present as well. The difference in the amount of phosphocholine was not expected. There were still many difficulties with the Drosophila testis and ovaries. Specifically, only low mass secondary ions were recovered. We therefore decided that we would investigate Drosophila embryos because they were a much simpler biological system where we believed we could detect many different lipid signals that are known to be present in the drosophila embryos plasma membrane.

*Sputtering experiments on Unfertilized Embryos*
From our sputtering experiments conducted on Drosophila testis and ovaries we determined that the system was too complex. We decided to move on to a simpler biological system: the Drosophila embryo. The dynamic technique was performed on unfertilized Drosophila embryos. An argon ion gun at 5 K eV was used to sputter through the unfertilized embryos. The sample was bombarded with gold ions for a second to generate an initial spectra. Afterwards sputtering was conducted at 5 second intervals from 10-30 seconds with secondary ion images and spectra taken at each interval (Figure 12). With argon sputtering of the sample there were many secondary ions recorded from 0 m/z to 300 m/z.

For the low mass range, there was an overall reduction in the intensity of the peaks present with the disappearance of few secondary ion species. In the low mass range between 0 m/z and 100 m/z there were low mass fragments of lipids and polymer contaminates present (Figure 13). The polymer contaminates were the dominant secondary ion species in this mass range. The polymer contaminates had masses ranging from 0 m/z to 60 m/z. The low mass fragments of lipid were above 60 m/z to approximately 100 m/z. With the initial sputtering of 10 seconds, it was observed that all low mass fragments of lipids disappeared from the spectrum and were not observed again. Also the polymer contaminant at 60 m/z was lost as well. However, over the course of the sputtering time from 10 seconds to 30 seconds there was only a gradual reduction in the intensity of the other polymer contaminates. There was not disappearance of their signal from the spectra. This means that the sputtering technique is
destroying the tissue sample, resulting in fewer secondary ions being detected especially of the small fragments from lipids. However, the polymer contaminates continue to persist which means that there is a large quantity of the material present in the TOF-SIMS from some location.

For the high mass range, there was an overall reduction of peak intensity with the eventually disappearance of the fatty acid signals (Figure 14). Fatty acid signals are present between the mass range of 200-300 m/z. Masses between 200-300 m/z were determined to be fatty acids by comparison to literature data. With initial bombardment with the gold ion gun there were many fatty acid species present in the spectra. After 10 seconds of sputtering there was a drastic reduction in the high mass fatty acid secondary ions peak intensities between 200-300 m/z. With every additional 5 seconds of sputtering there is a gradual reduction in their intensity until there were no secondary ions recovered between 200-300 m/z at 30 seconds of sputtering. Thus the dynamic technique of sputtering leads to the loss of secondary ion signal at the high mass range where most lipids of interest lay.

We were interested if a lipid signal could be recovered from a sample after the initial analysis of unfertilized embryos by the sputtering technique. We wanted to see if we were able to recover a lipid signal after the sample had been placed into the TOF-SIMS a drosophila unfertilized embryo that had been sputtered through with argon a month previously and found that a weak lipid signal between the mass range of 200-300 m/z appeared. What we found was that, indeed, there was a return of a low fatty acid lipid signal between 200-300 m/z (Figure 15). This return of ion signal was at very low
intensity only approximately 3 to 4 counts. The fatty acid peaks were of very low intensity.

Ion images were also collected for each of the sputtering times during the experiment. Between the initial pulsed bombardment with the gold ion gun and 10 second sputter ion image there is a drastic reduction in the amount and intensity of secondary ions that are detected by the TOF-SIMS. With the increase of each 5 second interval there is a gradual reduction of the total ions recovered. This is indicated by the reduction in the number of yellow and red spots on the ion image and the increase of black spots indicating that no secondary ions were ejected from that location.

From the data collected about the dynamic technique of sputtering, we determined that it was not an efficient technique for the investigation of lipids in biological tissue samples because of the loss of secondary ion signal. Even though there was a retention of secondary ion signal at low mass, there are few lipid secondary ions that are located in that low mass range. The combination of the secondary ion images along with the drastic reduction of fatty acid secondary ions detected led us to determine that the dynamic technique was too destructive to be used to collect data that could be used to create high resolution spectra and ion images.

*Ion Milling Experiment of Cryostat Sectioned of Fertilized and Unfertilized Drosophila Embryos*
As a result of our conclusion about the dynamic technique, we decided to move on and test the static technique of ion milling. We were interested in two things with the ion milling technique. First, we were interested to see if the ion milling technique could generate high resolution spectra and ion images (Figure 16). We were also interested to see if we could see a discernable difference between the fertilized and unfertilized embryo spectra (Figure 16). Fertilized and unfertilized Drosophila embryos were embedded and cryostat sectioned as described (see methods). Two sections of both the fertilized and unfertilized embryos were taken to be analyzed using the ion milling technique.

A high resolution spectrum and total ion image was generated for the fertilized Drosophila embryo (Figures 17-18). The polymer contaminates still dominated the low mass range of the spectrum between 0 m/z and 100 m/z. These peaks are omitted from all spectra pertaining to the cryostat sectioned fertilized and unfertilized embryos. There were many different kinds of lipid secondary ions recovered from ion milling of the fertilized embryo (Figure 15). An intense 6000 count phosphocholine peak was observed at 180 m/z along with fatty acid peaks between 200-300 m/z. A low intensity cholesterol peak at around 369 m/z was also observed. Large R groups (which are the carbon chains) from fatty acid were observed between 500-600 m/z. A sphingomyelin fragment at 614 m/z was also observed along with Phosphatidic acid peaks between 700-800 m/z (Figure 17). All mass to charge ratio data was compared to literature values to ascertain what lipid species were present.
A high resolution spectrum and total ion image was also generated for the unfertilized Drosophila embryo (Figure 19-20). Once again the polymer contaminates still dominated the low mass range. A phosphocholine peak at 180 m/z was observed at an intensity of approximately 4300 counts (Figure 14). A slightly more intense cholesterol peak was also observed. The same large R groups (which are carbon chains) from fatty acid were observed between 500-600 m/z as the fertilized embryo. A small sphingomyelin fragment was observed at 614 m/z along with Phosphatidic acid peaks between 700-800 m/z (Figure 19).

There was little observable difference between the fertilized and unfertilized spectra. The same lipid signals were observed in both tissue samples. The only major difference between the two spectra was that the lipid signals in the fertilized spectra were more intense than those found in the unfertilized spectra. The phosphocholine peak at 180 m/z was significantly higher in the fertilized embryo spectra in comparison to the unfertilized embryo spectra. By looking at the ion images no morphological differences could be ascertained from the data because the ion images were of a particular section of the embryo and it was very difficult to compare the two tissue samples.

From our investigation into the static and dynamic TOF-SIMS technique, we determined that both were able to detect lipid secondary ion signals in their spectra. The static technique was better at generating high resolution spectra because it recovered more secondary ions over a larger mass range. The static technique was also better than the dynamic technique at generating high resolution ion images because the dynamic technique resulted in loss of secondary ion signal as the time of sputtering increased.
Discussion

Through our experimentation we evaluated the ability of the dynamic and static TOF-SIMS techniques. It was determined that both techniques resulted in recovery of lipid secondary ions. It was determined that while both techniques could recover secondary ions from lipids, that the ion milling technique was the better technique for generating high resolution spectra and ion images. This can be seen by the increased mass range of detection from 0 m/z to 800 m/z as opposed to the 0 m/z to 300 m/z range for sputtering. Also more types of lipids were detected by the ion milling technique than by the sputtering technique. The ion milling technique was also found to be less destructive than the sputtering technique. By sputtering of unfertilized embryos it was found that there was a loss of secondary ions as the time of sputtering increased. This resulted in the dynamic technique of sputtering being unable to generate high resolution spectra and ion images. At the present time the static ion milling technique is the better technique for the investigation of biological tissue samples.

Sputtering of Drosophila Testis and Ovaries

From the Sputtering experiments on testis and ovaries we were able to prove that the dynamic TOF-SIMS technique of sputtering can be used to investigate the lipid content of a tissue sample. Fatty acid peaks were found in both spectra. However, the fatty acid signal in the testis was very weak. This might be due to the destruction of the morphology of the testis during the flash freezing process. This destruction could have
led to lower secondary ion yields. The lower amount of fatty acids present might also be
due to the fact that they are not as important in the testis as in the ovaries. There needs to
be further investigation into this.

An interesting difference between the testis and ovary spectra was the high intensity of
phosphocholine in the ovary spectra. This high intensity might be an indicator of the
importance of phosphocholine in the ovary but not in the testis. Phosphocholine has been
found to be important in the cellular processes of oogenesis and morphogenesis in
*Drosophila* ovary development. Phosphocholine is used by follicle cells which help
support development of the *Drosophila* GSC cells into an egg. Phosphocholine (CPT) is
modified by cytidylyltranferase (CCT) to become phosphatydchocholine the second most
abundant lipid in the plasma membrane of cells in the *Drosophila* ovary. This might be
worth further investigation in the future.

One considerable problem that occurred when investigating the testis and ovaries was
that secondary ions were only collected between 200-300 m/z. We know from the
literature, that there are many more types of lipids that are present in the Testis and
ovaries. Our inability to detect the higher mass secondary ions might have been a result
of the complex morphology of the testis and ovaries. Due to the complex three
dimensional structures there could have been electromagnetic affects that resulted in
some secondary ions not following a trajectory that would allow them to be detected by
the TOF-SIMS. Another possibility for the low secondary ion yield is that the sputtering
technique resulted in the secondary ions being fragmented to just atoms which are not
detected by the TOF-SIMS. This destruction of tissue the tissue resulting in the inability
to collect secondary ions. To test the sputtering technique further we decided to sputter unfertilized embryos a relatively simple biological system.

**Sputtering of Unfertilized Drosophila Embryos**

From our testing of the dynamic technique of sputtering we determined that it was possible for secondary ions from lipids to be detected. However, we only detected secondary ions between the mass range of 200-300 m/z where fatty acid peaks are present. We also observed a phosphocholine peak at 180 m/z. There, however, was a loss of signal of these ions as the sputtering time increased from 10 seconds to 30 seconds at 5 seconds intervals. The reduction and eventual loss of lipid secondary ion signals between 200-300 m/z along with the disappearance of the low mass lipid fragments between 60 m/z and 100 m/z could have been due to factors either fragmentation to the atomic level of molecules or matrix affects. Possible the high energy argon ions impacting the tissue sample resulted in such high fragmentation of the secondary ions that only single atoms were ejected which leads to identification of what molecule those fragments came from very difficult. Another possible reason for the loss of secondary ions is due to matrix affects which is the imbedding of secondary ions from the previously sputtered monolayer of tissue into the next layer of tissue. Matrix affects lead to the perturbation of other secondary ions ejected from the sample because they can either block the ejected ions path or they can create electromagnetic fields that perturb the trajectory of the secondary ion. Which ever the reason may be the dynamic technique of sputtering does not produce at this time high resolution spectra and ion images due to loss of secondary ions over the duration of sputtering.
From our ion milling experiments high resolution spectra and ion images were generated due to the ability of the technique to be able to detect over a mass range from 0 m/z to 800 m/z. This allowed for many more lipid molecules to be observed in the spectra. The reason why the static technique of ion milling produces better spectra and ion images than the dynamic technique of sputtering might be because of the tissue being pre-sectioned. A good test to see if this is the case would be to ion mill through un-sectioned tissue samples. Also pre-sectioning simplifies the morphology of the system. The tissue sample is flat after sectioning instead of being three dimensional. However another reason why the ion milling technique might be better than the sputtering technique is because the primary ion beam in ion milling is focused on a particular part of the tissue at a time so data is recorded from each part that is bombarded by primary ions. This increases the recovery of secondary ions because there are fewer secondary ions to detect form a smaller area while the sputtering technique must collect the data from the entire tissue at a time.

The results of comparing the fertilized and unfertilized spectra were a little bit surprising to us. We had hypothesized that there would be minor differences since the plasma membranes of cells in either are comprised of the same lipids. The differences we expected to see would have been due to a difference in the distribution of lipids throughout the plasma membrane. Rather, what was observed was that the fertilized
embryo spectra and the unfertilized embryo spectra were almost exactly the same. The only difference was that the fertilized embryo spectra had a higher intensity for its lipid content than did the unfertilized spectra. The higher intensity of secondary ions from lipids in the fertilized embryo spectra, than in the unfertilized embryo, spectra might be due to the fact that during development lipids are taken from the gut region and used in the plasma membranes of developing cells. By there being more cells present in the fertilized embryo there must be more lipid used to create those plasma membranes to protect the cells. There are just not as many cells in the unfertilized embryo so not as much lipid is necessary. The best way to tell if there is a difference in lipid composition between fertilized embryos and unfertilized would be to investigate the yolk lipid composition in the gut. The lipid composition of the yolk in the fertilized embryo should be very different from that in the unfertilized embryo because lipids from the yolk in the fertilized embryo are used for the plasma membranes of developing cells in the embryo.

**Future Directions**

Based on the conclusion that ion milling is a good technique for generating high resolution spectra and ion images, we plan to conduct ion milling of testis and ovaries and see if better spectra and ion images are generate than when the sputtering technique was used. Ion milling should also be conducted on un sectioned tissue samples to see if it also has a reduction in the intensity of secondary ions due to the morphological complexity of the tissue. We will also do a more in depth investigation of the role of phosphocholine in ovaries based off of the ovary and testis spectra that shows phosphocholine at higher levels in ovaries than in the testis.
Another possible experiment is to try a hybrid technique between TOF-SIMS and MALDI-MS where we would imbed a tissue sample in a matrix and then use the TOF-SIMS to produce secondary ions. We could use this technique in combination with the sputtering or ion milling to see if we could get larger of molecules which would help us to observe the presence of large lipids and possible even small proteins. Sputtering experiments need to be carried out again.

Fertilized and unfertilized embryos will continue to be sectioned and then run in the TOF-SIMS using the static technique of ion milling. We hope to be able to generate high resolution spectra and ion images for all sections. Then we hope to use computer software to stack all of the ion images together to have a three dimensional map of the lipid content in the Drosophila embryo. We hope to use the ion image with the correlated spectra to do an in depth analysis of the difference between the fertilized and unfertilized embryo. We want to specifically focus on the gut region of the embryo. This is because both fertilized and unfertilized embryos have a lipid rich yolk in the gut. In the fertilized embryo the lipids from the yolk are used in developmental processes while in the unfertilized embryo they are not. This would lead to the composition of the lipids in the yolk to be very different in the fertilized embryo compared with the unfertilized embryo. We hope to generate high resolution spectra and ion images from the data. We hope to use this as a proof of principle that TOF-SIMS can be used to observe the differences in composition and morphology between two biological samples.
Our research when combined with the research done in Korea on developing TOF-SIMS as a technique to investigate protein content could result in scientists being able to generate a three dimensional image that with its spectral information can tell a scientist what proteins and lipids are present and where they are located in the sample. This could lead to great breakthroughs in developmental and cancer biology.

References


17 Thomas B Brody, (Society of Developmental Biology 2006).
