DEVELOPMENT OF FLOW-THROUGH PERFUSION CHAMBERS IN THE STUDY OF SPERM MITOCHONDRIAL METABOLISM AND FERTILIZATION COMPETENCE

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Abstract

Sperm are small, motile cells which are hard to capture and study effectively. The consequence of research into these elusive cells could be one of the keys to unlock the answer behind the driving forces of fertility, and to create better methods of sperm selection for In-Vito Fertilization (IVF) procedure. To effectively study the movement of both individuals and populations of sperm cells low cost and effective flow-through perfusion chambers must be created for the volume and specificity of experiments. Using multiple iterations with continuous quality improvements, perfusion chambers showed promising results to continue the work of studying mitochondrial metabolism and fertilization competence. The different iterations of the perfusion chamber are presented chronologically, and their drawbacks are discussed until a final form is displayed as a suitable method for experimentation. The chambers work by allowing sperm to explore the space free from external influence and collect in a collecting part of the chamber having experienced selective pressure based on distance and time. Microscopy was used to take pictures and videos of the cells which can be used to extrapolate data about their distribution of phenotypes.

Background

In recent years there has been an increased interest in the biochemical pathways that underlie fertilization competence in sperm. One way that scientists have begun to study metabolic changes has been through a flow-through perfusion chamber. This type of hardware applies controlled flow of various media that provides cells with different signaling molecules or metabolites. Throughout the experiment sperm explore the environment, and this journey gives insight into how cells interact alone, and in large populations, to gain information about their environment. This information ultimately transforms the cell into a fertility-competent gamete. Additional uses for this technology could be observation of physiological responses that cells undergo in response to the changing environment within the chamber by controlling what is perfused through it. This method of studying cells has provided valuable insights into the mechanisms of sperm motility, capacitation, acrosome reaction, and fertilization. Despite the challenges associated with this technique, namely, the effects of microflow, shear stress on cells, and variability in conditions, this method of experimentation has proven invaluable to study sperm mitochondrial metabolism, fertilization competence, and cell population dynamics.

There have been several recent studies that have demonstrated the effectiveness of flow through perfusion as an experimentation method in a variety of cells. Anderson and Tate made an innovation in perfusion chamber design with their specially created gaskets and dual flow chambers that reduced shear stress and could direct mouse embryonic cell development through controlled fluid flow[1]. Using perfusion chambers to study individual sperm cells, however, is a new and evolving topic in cellular biology. Suarez and Wu advocate for increased usage of microfluidics in their article that describes the recent advances in the study of the factors that most influence fertilization. They recount the struggles to create an effective flow-through perfusion chamber that accurately simulates the microenvironment of the Human reproductive tract while also being easily manipulated to test different variables[2]. Phiphattanaphiphop proposed a novel

sperm sorting device that is high efficacy and cost-effective. Using computer simulations and engineering processes, the team designed a 96% effective sorting method that would divide motile and non-motile cells for study[3].

Given recent advancements and interests with perfusion chambers, a process was undertaken in the Dr. Cameron Schmidt Cellular Energetics Lab to design cost-effective and customizable perfusion chambers which would provide real world data for computer models being simultaneously developed throughout this study. Currently there are only two types of chambers available to conduct sperm research. The cheaper and least customizable option would be the Grace Biolabs Coverwell Perfusion Chambers which offer different shapes and sizes and are adherent to microscope slides but offer little personalization to specific experimentation. On the other end of the spectrum are PDMS-based chambers which require expensive and specialized equipment to produce and often require 3rd party development to perform soft lithography. Both options were insufficient for the quick turnaround and relatively high degree of customizability that was needed for current projects. The chambers that are detailed in this report are much less precise at small scales but are much cheaper and better suited to individual experimental needs. The main goal of this study was to develop a consistent method of perfusion chamber creation that could be used to perform microscopy experiments and provide valuable insight into real-world sperm behavior in a physical model that could be simulated with virtual computer models.

To develop a flow-through perfusion chamber, a unique physics problem that develops at small scales must be overcome, that is, the obstacle of microflow physics. Microflow physics is the study of devices that have height and depth of less than 1 millimeter but more than 1 micron. Microflows can affect energy, momentum, and the distribution of mass in a small system, so it is important to find ways to counteract these effects to mitigate their impact. The microflows that are created from oversights in chamber design are unintended and, in fact, negatively impact experimental

results and validity. This relates to the original intention of beginning to design a new form of flow through perfusion chamber, namely to optimize the control of flow, rather than let experimentation be at the whim of accidental flows that arise at small scales due to capillary action. Interfacial slip is one effect considered in the development of the chambers. To accurately calculate the flow of Newtonian fluids, it is assumed that the velocity of the fluid with respect to the container wall is zero. Any change to the chamber walls whether that be the introduction of lubricant, surface features that are not smooth, or divots within the path of fluid flow enables interfacial slip that influences tangential velocity of the fluid with respect to the wall. Hydrophobic wall substances produce a greater interfacial slip than do other forms of containment. Vega-Sanchez, Neto (2022) describes the phenomenon of interfacial slip and presents solutions to this problem. They suggest varying the lubricant used on the sides of the chambers, along with continuously pumping new lubricant along with the working flow during experimentation[4].

Another unique aspect of microfluidics is that flow at the small-scale ceases to be turbulent and is dominated by laminar flow. This type of flow is described as fluid layers following smooth paths and not mixing between layers, rather, if molecules are to be transported, they are transported by diffusion. To determine if a flow is laminar or turbulent, the Reynolds Number is employed. The Number is calculated as density, velocity, and length multiplied together and divided by dynamic viscosity. The lower the Reynolds Number, the more laminar the flow. At low values, the fluid exhibits Stokes Flow behavior. This behavior describes a fluid where viscous forces dominate over inertia flows. Both sperm ejaculate and the environment of the Reproductive Tract are viscous, so measures must be taken to ensure low Reynolds Number in the perfusion chamber, mainly by low velocity of fluid flow after injecting the sperm. Thomas, Covington, and Wall reduced Reynolds number in their chamber designed to perfuse tissue samples with multiple different streams and varying times by making the entry chamber longer with a high surface area-volume ratio. The maximized ratios and lengths of chambers reduced turbulence and mixing of the

different perfusates[5]. Turbulence was also a factor in sperm perfusion chamber design for future experimental implementation whereby differing metabolites would perfuse through the chamber to be uptaken by cells and their effluent measured for metabolic changes. Less turbulence and mixing would show discrete cause and effect activity for the different signaling molecules and their effect on sperm respiration and motility.

Perfusion Chamber Development

At the start of the study CoverWell™ Perfusion Chamber-PC3L-1.0 were used and were suitable as a proof of concept to study sperm within a perfusion chamber. Using a bolus of concentrated sperm, cells were directly injected into one of the three chambers for observation. To have customizable experiments that resembled created mazes within computer simulations, a more personalized method had to be developed. There were many different iterations of the chambers that will be successively shown:

Iteration 1

The first iteration of perfusion chambers was developed out of a necessity for customization. Rather than purchasing thick rubber spacers, it was decided to design a method to create unique chambers. Cricut Cutter software allowed for digital design and engineering and offered a malleable way to cut into Cricut Permanent Vinyl sheet using the Joy cutter model. The mold was created by removing the middle part which had been cut and carefully extracted using Cricut transfer tape and placed on a Fisherbrand Plain Microscope Slide Precleaned with dimensions 25x75x1mm. Next, a Scotch Self-Seal Laminating Pouch was cut to dimensions to cover the gap to create the perfusion chamber. To allow inflow and outflow through the chamber, 1 mm holes were drilled using a Hardell Mini Cordless Rotary Drill on opposing sides of the chamber to cover the input and output portions. Grace Biolabs Press Fit Tubing Connectors with 5 mm diameter and 0.5 mm aperture were affixed to the holes that were drilled. Intramedic Clay Adams Brand

Non-Radiopaque Polyethylene Tubing with dimensions of 0.58mm internal tube diameter were threaded topside through the tubing connectors for both ends. Since the laminating pouches were inherently adhesive, a simple force pushing the pouch cutout and glass slide/vinyl combination together results in a perfusion chamber. The chamber withheld a flowthrough of dye with minimal leakage. The largest malfunction with this design is that the adhesive from the laminate is toxic to cells and affects the chamber microenvironment which is not conducive to simulating the Reproductive Tract. Another drawback was the design of the chamber itself. The chamber consisted of two identical squares with sides of length 7.62 mm attached together by a 25.4 mm long passage. When the dye was observed under the microscope, it was noted that the fluid would not reach all the way into the 90-degree corners of the square towards the other end of the chamber, rather, microflows created eddies that prevented uniform flow of fluid.

Iteration 2

The second iteration reworked the chamber design to mitigate cell exposure to toxic chemicals, a decision was to make the top portion of the chamber another glass slide drilled with the same holes as iteration 1 and attempt to stick the top and bottom portions of the chamber using Molykote Vacuum Grease which could be painted to the perimeter of the chamber to allow the pieces to stick together. Hypothetically, this would not impact the internal environment of the chamber. In some cases, this approach worked. The chamber could withstand one or two experiments using live cells, this gave a great first look into the viability of the customizable perfusion chamber idea. There were many drawbacks to this iteration as well, however. The vacuum grease caused increased bubble formation and much greater likelihood of the fluid channeling out through the grease causing leakage. Additionally, the grease application process required skill and a lengthy amount of time to ensure the process was successful. Even with special care and attention, the grease would often leak during experimentation and behave unpredictably.

Iteration 3

The next iteration of the perfusion chamber was created using thin (0.08 mm) adhesive sheets by Dynta cut by a programmed Cricut cutter to specifications particular to the experiment. The adhesive is then attached to a PET Sheet made by HTVRONT cut to 75x25mm. Another plastic slide cut to the same dimensions and is drilled with holes filled with tubes like the previous iterations and then is then firmly placed on the adhesive to create an ultra-thin perfusion chamber without the hassle of vacuum grease or the toxicity of the laminating pouches. This final iteration seen in Figure A allowed for the sperm to be inseminated into the chamber, the bolus stopped directly at the aperture of the tube adapter and allowed for the sperm cells to discover the space without any external aid from microflows. Using this setup, the inside of the chamber can be customized to fit experimental needs.

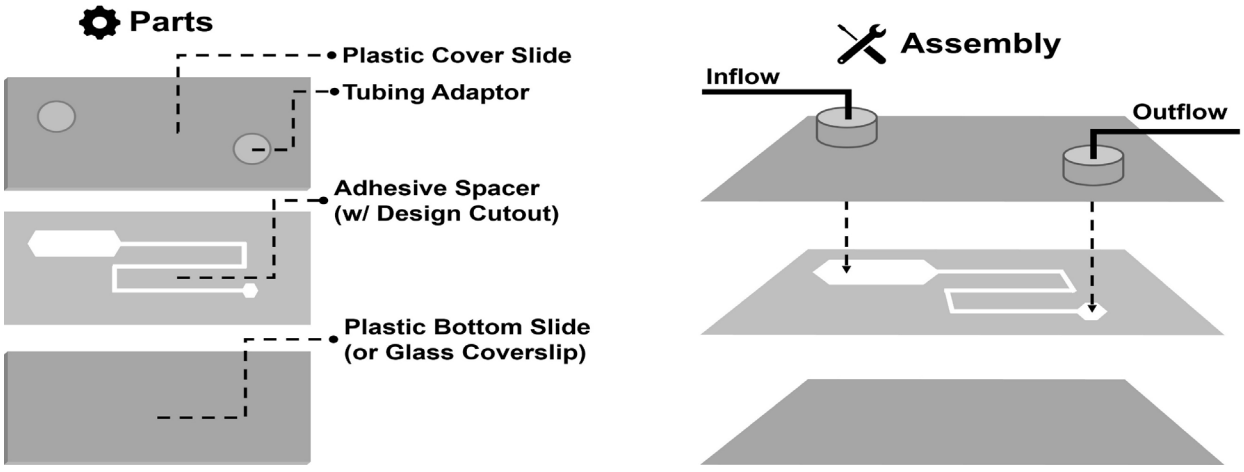

Figure A Pictorial Description of Perfusion System

Methods

For Cauda Epididymis Extraction

To extract sperm cells for experimentation, a uniform protocol was followed. After sacrificing the breeder CD1 male mice using ether as the anesthetic, the epididymides were isolated in 1X PBS. The Cauda was separated and opened in HTF media (+ D-glucose 2.78 mM + lactate 10 mM +

HEPES 10 mM + EGTA 1 mM) and centrifuged at 100 rcf for 1 minute. The supernatant was transferred and the pellet discarded. SNARF-1 is added to a final concentration of 20 uM and incubated at 37 degrees Celsius (non-CO2) for 30 minutes. The mixture is then centrifuged again at 800 rcf for 2 mins. The supernatant is discarded and the pellet is resuspended in 3 mL of Bovine Serum Albumin (BSA).

For Micro-Insemination

The perfusion chamber is placed on the microscopy viewing table which has been preheated to human body temperature, 37° C, using Linkam LTS420 heating platform. A syringe of BSA is perfused through the chamber to fill it using a slow flow using a KDS Series 100 syringe pump. The syringe is then swapped for a syringe with a bolus of sperm in a media of BSA. The sperm are also perfused slowly until the bolus reaches the aperture of the tubing connectors and visible under microscopy.

For Microscopy Experimentation

Using a Zeiss Axio microscope and Zen Blue software, images and videos were taken of cells as they swam through the chamber. Data is analyzed using ImageJ.

Results and Discussion

An important hurdle to overcome, as previously mentioned, is the effort to prevent bubble formation as seen in Figure B. Throughout early stages of development when the adhesive layer of the chamber was thicker, many times air would become easily dislodged into the interior of the chamber which would interrupt active experimentation.

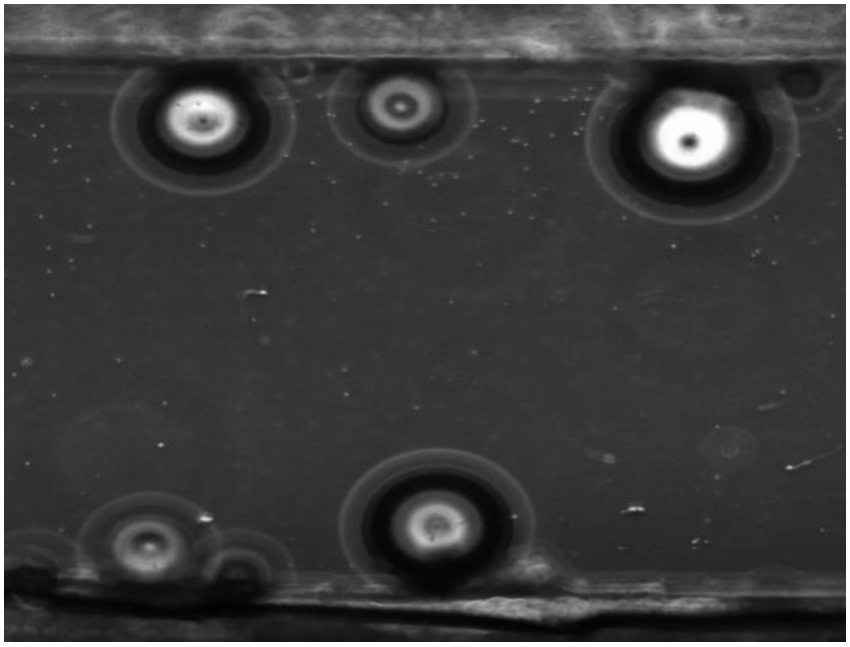

Figure B Bubble Formation in Perfusion Chamber

Figure C shows a population of sperm cells being injected, a process called micro-insemination. It was observed that sperm cells would enter the chamber and without external influence explore the space exhibiting a vast array of phenotypes. The perfusion liquid would stop abruptly and precisely where needed which would otherwise have influenced the movement of cells. In figure D, the different phenotypes are shown overlayed with colors showing how the cells moved through space and time, with the more blue part of the path being the furthest away in time while the more white color represents more recent movement. Even within a small population, different phenotypes can be seen, and phenotypes can rapidly change with a single cell.

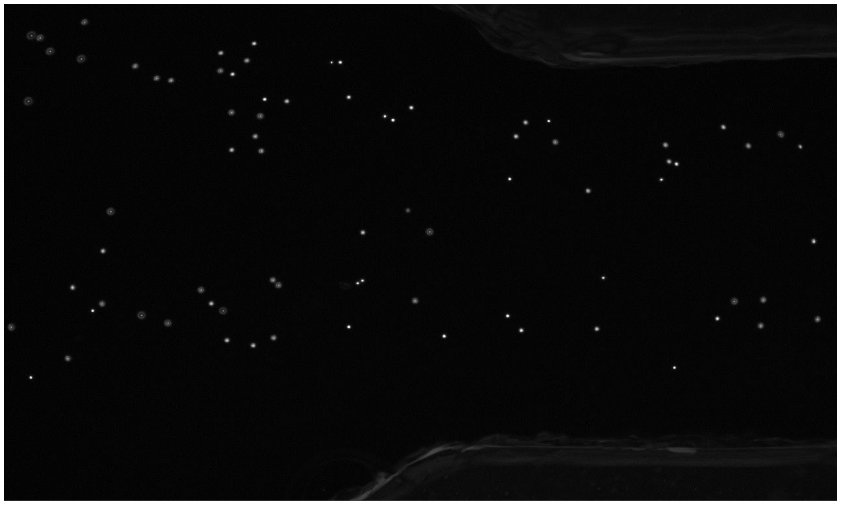

Figure C Fluorescent Beads Entering Perfusion Chamber

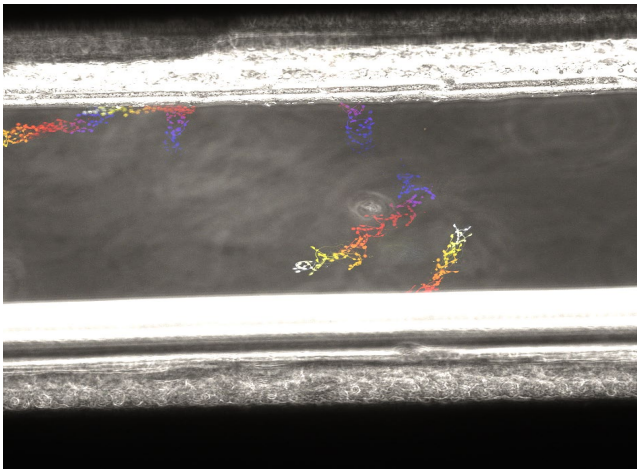

Figure D Cells Overlayed with Movement Over Time Data

Using this novel method, cells are easily seen and perfused through the chamber. The cells can be pushed through using the fluid media to any point in the chamber and stopped immediately to allow cells to move on their own. Careful attention must be made to ensure that the adhesive cutouts are firmly placed on the slides to avoid air bubbles that eventually move to the inside of the chamber and interfere with experimentation. Cells can freely explore the space and future experimentation may involve quantitative analysis of eluent.

Conclusion

The issues highlighted through a deep dive into microfluidics physics challenges were addressed through a systematic approach of trial and error. This approach created successive models that refined the design and operation of the chamber. One of the key modifications of the process was the reduction of sharp edges which are known to increase the likelihood of bubble formation, which is a common and inconvenient problem with microfluidics devices. The chamber's thickness was also successively reduced to more closely resemble the pseudo-two-dimensional representation of the computer simulation on which the chambers are based. Not only do smaller chambers make the system more compact and efficient, but it also enhances heat transfer which better facilitates thermal equilibrium and maintains a temperature consistent with the Female Reproductive Tract. Lastly, the pressure of the chamber during the micro-insemination process was reduced to maintain the device's integrity and functionality. Higher pressure syringe insemination would cause the chamber to burst and would also be harder to follow a single cell through its journey through the chamber if the fluid was moving too fast. Having higher pressure

in the chamber also would allow the cell to move further than it would on its own which invalidates results making them unable to be compared to the computer simulation.

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