Sea Grant Undergraduate Research Opportunity Program Final Report

Megan Chesnut

Faculty Advisor: Terrence Tiersch, PhD

January 31, 2016

This final report has been formatted as a manuscript in preparation for future submission to the peer-reviewed journal, *Theriogenology*

Development of a Disposable Microfabricated Counting Chamber for Standardization of Sperm Concentration Estimates in Aquatic Species

Megan Chesnut,[ab*] Amy Guitreau,[b] Jacob Beckham,[a] Adam Melvin,[c] William Todd Monroe[a] and Terrence R. Tiersch[b]

[a]Department of Biological and Agricultural Engineering, Louisiana State University, Baton Rouge, LA, USA
[b]Aquatic Germplasm and Genetic Resources Center, School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, LA, USA
[c]Cain Department of Chemical Engineering, Louisiana State University, Baton Rouge, LA, USA

[*]Corresponding author.
E-mail address: mchesn1@lsu.edu
Postal Address: Aquatic Germplasm and Genetic Resources Center
2288 Gourrier Ave, 70820
Baton Rouge, LA, USA

Abstract

Quantification of sperm concentration is widely utilized in reproduction research of aquatic organisms to determine quality for cryopreservation and *in vitro* fertilization protocols. Currently, there is a need to extend these protocols to eliminate variation caused by technical factors and to standardize methods among laboratories and hatcheries. Methods of acquiring accurate sperm concentration for aquatic species typically involve the use of fragile and expensive devices, which are often not appropriate for use in the field or in hatcheries. To address this, an economical counting chamber for reliable estimate of the concentration of fish sperm was developed. The Microfabricated Enumeration Grid Chamber (MEGC) is a microdevice composed of a platform and a grid-patterned coverslip, and supports are used to set the height of the coverslip at approximately 10 μm above the platform, restricting sample volume to a monolayer of sperm. To fabricate the coverslip, a master mold was made using standard single-layer photolithography with SU8 negative photoresist, which was cast with polydimethylsiloxane (PDMS). High resolution 3-D printing and two-step photolithography were methods investigated to fabricate a mold for the base component of the microdevice, and two-step photolithography was determined the most reproducible method to achieve a precise 10-μm height difference between the posts and the platform. The accuracy and precision of concentration estimates from the MEGC were determined by comparison with manual counts of zebrafish (*Danio rerio*) sperm. The disposable microdevice can significantly advance the capabilities of aquatic cryopreservation and help provide much-needed standardization to this field. Also, this device can assist management efforts to restore genetic diversity and contribute to the development of germplasm repositories to protect the genetic resources of fish and shellfish.

Keywords: counting chamber, aquatic species, sperm concentration, 3-D printing, microfabrication

1. Introduction

Sperm cryopreservation technology facilitates preservation of aquatic genetic diversity, which allows for a type of genetic time travel. The cryopreservation process involves low temperature cell preservation, cryogenic storage, and thawing of living material [1]. Since 1953, research has aimed to improve these protocols [2], and recently, the need to extend techniques past the laboratory setting and into the field for cryopreservation on-site has become a necessity. On-site cryopreservation functions to eliminate the variation caused by environmental factors, and possibly lessen the difference in motility between fresh and thawed samples, which will play an essential role in preservation of genetic diversity, especially after environmental catastrophes.

By allowing much of the cryopreservation process to take place in close proximity to the natural habitat of the fish, endangered and declining aquatic species would not need to be transported prior to sperm collection and freezing. This will allow for gene pools to be preserved in a repository and later used to repopulate affected areas. However, cryopreservation studies remain inconsistent due to lack of standardized reproducible methodology [1][2][3]. This inhibits work even at well-established laboratories.

Sperm quality is a limiting factor of reproductive success after cryopreservation [4] as a result of the biological, chemical, and physical stresses experienced throughout the procedure. These stress factors can reduce the numbers and quality of motile sperm in a thawed sample [3]. For quality assessment of cryopreservation, this reduction is characterized through comparison of motile sperm concentration between fresh and thawed samples [3][4]. This protocol serves to standardize cryopreservation, as it allows for evaluation of experimental conditions such as collection methodology, composition of dilution medium, and sperm storage conditions [4].

In past studies, evaluation of sperm motility and concentration has lacked accuracy and precision, and is characterized by inconsistent and conflicting results, which can be attributed to a number of complex factors, such as the lack of standardization, and genetic, physiological, and environmental effects on sperm samples [5]. There have been multiple studies describing disagreement between sperm concentration measurements using available methods [6][7]. Although the Word Health Organization puts forth the hemacytometer as the “gold standard” for determining sperm concentration in humans [8], the accuracy of the hemacytometer has been disputed because the typical depth, 100 μm, permits multiple layers of sperm to overlap, and significant variance between the different models available has also been demonstrated [6][7][8].

To standardize an accurate method of determining sperm concentration, the Makler® counting chamber was introduced in 1978 as a replacement for the hemacytometer, and this device has been used in laboratories worldwide [7]. This device permits only a specific volume of sperm within the chamber, which eliminates variation by creating a cell monolayer [10]. It consists of a thick glass coverslip, etched with a grid of 100 squares of 0.1 x 0.1 mm each, held up by posts on a glass base for uniform distribution of sperm [10]. Sperm are counted in 10 of these squares to evaluate the concentration of the sample [10]. Although the Makler® counting chamber has been proven an accurate device for determining sperm concentration [11][12] and is used in many aquatic reproductive biology laboratories, because of its fragile nature and cost of about US $700, it is expensive and, consequently, not conducive for on-site cryopreservation. Damage to a

device would mean significant loss; therefore, the ability to objectively standardize the protocol for obtaining quality and quantity of sperm on-site requires a practical and economical alternative that allows for accurate estimation of concentration and motility.

The goal of this project was to develop a disposable microdevice for reliable estimation of sperm concentration to standardize determination of sperm concentration and motility. The objectives of this project were to: 1) fabricate a reproducible and inexpensive counting chamber, 2) evaluate fabrication precision and device reproducibility, and 3) determine the accuracy and precision of the device in estimating the concentration of sperm cells. The counting chamber comprises the components essential to concentration measurement: a grid pattern to count cells, and a space of known volume to restrict samples to a single layer of cells. For the counting chamber to be practical for use on-site, polydimethylsiloxane (PDMS) was chosen as an alternative material to etched glass, as it is less expensive and more flexible, and can be easily replicated from a master mold, allowing for rapid prototyping of many designs and inexpensive fabrication [13]. The material is also optically transparent, waterproof, and nontoxic to cells, which makes it ideal for biological applications. The PDMS counting chamber reduces the cost of a counting chamber drastically, roughly 1000-fold, from US $700 to US $0.07, which will have an immense impact on the availability of a standardized device to estimate concentration and motility commercially and on-site.

2. Materials and Methods

2.1 Counting Chamber Coverslip Fabrication

To fabricate the coverslip for the counting chamber, a master mold was made using standard single-layer photolithography (Figure 1) using permanent epoxy negative photoresist (SU8 2025, MicroChem Corp., Newton, MA, USA). Optimization of each step in this manufacturing technique was an iterative process. About 20 different prototypes were developed over a period of months, culminating in the development of a single process detailed below.

To prepare the master mold, a 100 mm N-type silicon wafer (UniversityWafer, South Boston, MA, USA) was rinsed with acetone and isopropyl alcohol (IPA) for 10 s, dried with nitrogen gas, and centered on a spin coater (WS-650-23B, Laurell). Approximately 4 mL of SU-8 2025 were dispensed onto the center of the wafer. The wafer was spin coated with photoresist at 500 rpm for 10 s at 100 rpm/s and followed by 3000 rpm for 30 s at 300 rpm/s to achieve a thickness of 25 μm.

After spin coating, the build-up of photoresist on the edges of the wafer (approximately 1 mm of SU8) was removed with acetone to prevent contamination of the hot plate and allow

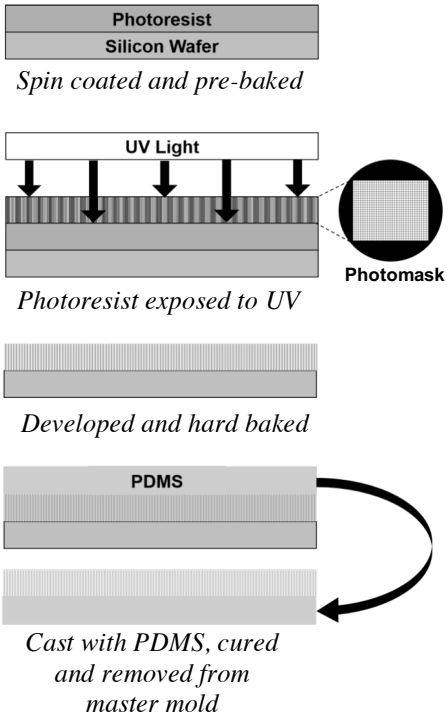

Figure 1. Microfabrication procedure for the grid-patterned coverslip.

the photomask to be in uniform contact with the photoresist. The wafer was baked on a hot plate at 65 C for 10 min and at 95 C for 1 hr. Once cooled to 25 C, the wafer was positioned in a custom exposure system that utilizes a Blak-Ray B-100 series UV lamp (UVP, LLC; Upland, CA, USA), and exposed through a chrome photomask (designed with AutoCAD software and printed by Front Range Photomask, LLC, Palmer Lake, CO, USA) to a 365-nm Ultraviolet light with an intensity of 2 mW/cm^2. The coverslip master mold was exposed to an effective dose of 175 mJ/cm^2. The mold was baked on a hot plate at 65 C for 10 min and at 95 C for 1 hr, and cooled to 25 C.

To selectively dissolve the photoresist not exposed to UV light, the wafer was immersed in SU8 developer solution (Microchem Corp., Newton, MA, USA), and developed for 4 min. The wafer was rinsed for 1 min with fresh developer, for 10 s with isopropyl alcohol, and dried with nitrogen gas. The master mold was hard baked at 150 C for 90 min. Once cooled to 25 C, the master mold was placed into a vacuum chamber with a glass petri dish for the purpose of silanization via vapor deposition. A Pasteur pipette was used to dispense 5 drops of trichlorosilane into the glass petri dish, and the chamber was placed under vacuum for 10 min before the master mold was removed, hard baked at 150 C for 90 min, and cooled to to 25 C. This process altered the surface chemistry of the wafer to allow for PDMS replicas to be removed evenly, with no residue.

To create replicas of the master mold, polydimethylsiloxane (PDMS, 184 Sylgard Silicone, Dow Corning, Midland, MI, USA) was mixed at a ratio of 10:1 (base: curing agent), placed in a vacuum chamber at -600 mm Hg for 15 min, cast on the master mold, and cured in an oven at 75 C for 90 min. Once cooled to 25 C, the PDMS replica was carefully peeled from the master mold. A 20 mm x 20 mm section containing the coverslip grid features was cut from the replica. The PDMS and a clean glass slide (76.2 x 25.4 x 1mm, Fisher Scientific) were each treated with a plasma cleaner (PDC-32G, Harric Plasma, Ithica, NY, USA) for 30 s at 1.8 W, and the PDMS was irreversibly bonded to the coverslip.

2.2 Counting Chamber Base Fabrication

The base of the counting chamber is composed of a platform and and supports, which are used to set the height of the coverslip at 10 μm above the platform. The controlled volume restricts sample volume to a monolayer of sperm. The design required a uniform 10-μm height difference between the platform and posts, and two methods of achieving such a design were investigated: high resolution 3-D printing and two-step photolithography.

2.2.1 High Resolution 3-D Printing

A mold of the counting chamber base was designed with Autodesk Inventor Professional software and printed by EntreScan 3D Printing, New Orleans, LA, USA (Figure 2). To create replicas of the 3-D printed mold, polydimethylsiloxane (PDMS, 184 Sylgard Silicone, Dow Corning, Midland, MI, USA) was mixed at a ratio of 10:1 (base: curing agent), placed in a the replica. The PDMS and a clean glass slide

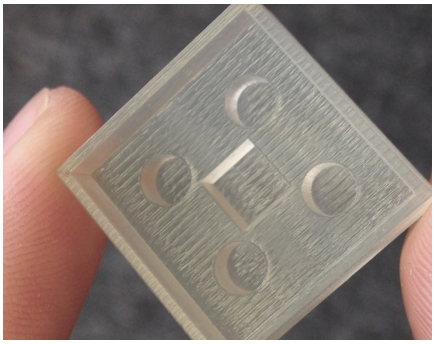

Figure 2. 3-D printed mold for counting chamber base

(76.2 x 25.4 x 1mm, Fisher Scientific) were each treated with a plasma cleaner (PDC-32G, Harric Plasma, Ithica, NY, USA) for 30 s at 1.8 W, and the PDMS was irreversibly bonded to the coverslip.

To test reproducibility of fabrication, a non-contact optical profiler (Wyko NT 1000 3-D optical profiling system) was used to characterize surface topography of the platform and confirm the 10-μm height difference between the platform and posts of multiple PDMS base components fabricated using this method.

2.2.2 Two-step Photolithography

To fabricate the base component of the counting chamber using two-step photolithography (Figure 3), a master mold was made using permanent epoxy negative photoresist (SU8 2010, MicroChem Corp., Newton, MA, USA). To prepare the mold, a 100 mm N-type silicon wafer (UniversityWafer, South Boston, MA, USA) was rinsed with acetone and isopropyl alcohol (IPA) for 10 s, dried with nitrogen gas, and centered on a spin coater (WS-650-23B, Laurell). Approximately 4 mL of SU-8 2010 were dispensed onto the center of the wafer. The wafer was spin coated with photoresist at 500 rpm for 10 s at 100 rpm/s and followed by 3500 rpm for 30 s at 300 rpm/s to achieve a thickness of 10 μm.

After spin coating, the build-up of photoresist on the edges of the wafer (approximately 1 mm of SU8) was removed with acetone to prevent contamination of the hot plate and allow the photomask to be in uniform contact with the photoresist. The wafer was baked on a hot plate at 65 C for 10 min and at 95 C for 1 hr. Once cooled to 25 C, the wafer was positioned in a custom exposure system that utilizes a Blak-Ray B-100 series UV lamp (UVP, LLC; Upland, CA, USA), and exposed through a transparency photomask (designed with AutoCAD software and printed by CAD Art Services Inc., Bandon, OR, USA) to a 365-nm Ultraviolet light with an intensity of 2 mW/cm^2. The wafer was exposed to an effective dose of 123 mJ/cm^2. The mold was baked on a hot plate at 65 C for 10 min and at 95 C for 1 hr, and cooled to 25 C. The spin coating, exposure, and baking steps were repeated with a second transparency photomask to produce a 10-μm height difference, △h, between the posts and platform (Figure 3).

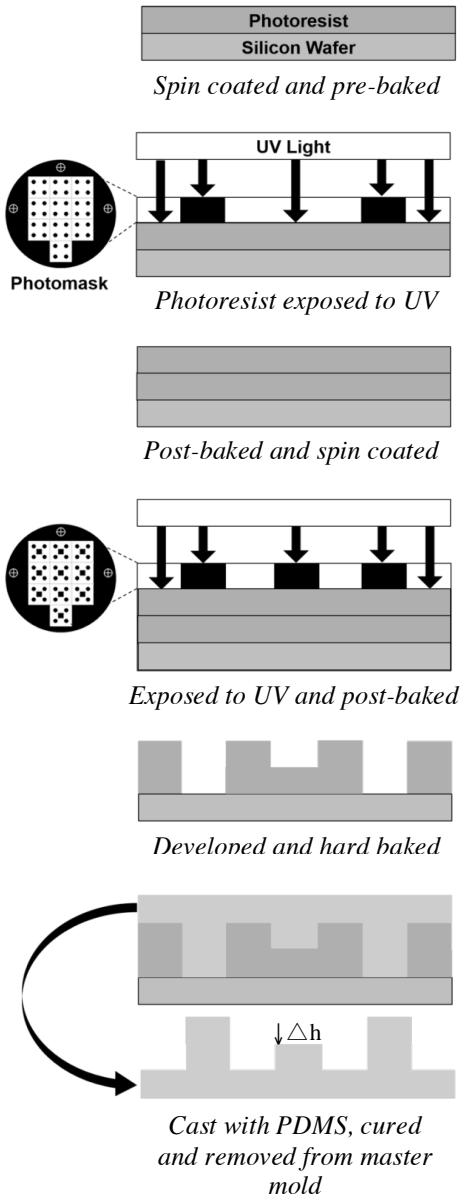

Figure 3. Microfabrication procedure for the counting chamber base

To selectively dissolve the photoresist not exposed to UV light, the wafer was immersed in SU8 developer solution (Microchem Corp., Newton, MA, USA), and developed for 2 min. The wafer was rinsed for 1 min with fresh developer and for 10 s with isopropyl alcohol, and dried with nitrogen gas. The master mold was hard baked at 150 C for 90 min. Once cooled, the master mold was placed into a vacuum chamber with a glass petri dish for the purpose of silanization via vapor deposition. A Pasteur pipette was used to dispense 5 drops of trichlorosilane into the glass petri dish, and the chamber was placed under vacuum for 10 min before the master molds were removed, hard baked at 150 C for 90 min, and cooled to room temperature. This process altered the surface chemistry of the wafer to allow for PDMS replica to be removed evenly, with no residue.

To create a replica of the master mold, polydimethylsiloxane (PDMS, 184 Sylgard Silicone, Dow Corning, Midland, MI, USA) was mixed at a ratio of 10:1 (base: curing agent), placed in a vacuum chamber at -600 mm Hg for 15 min, cast on the master mold, and cured in an oven at 75 C for 90 min. Once cooled, the PDMS replica was carefully peeled from the master mold, and a 20 mm x 20 mm section containing the post and platform microfeatures was cut from the replica. The PDMS and a clean glass slide (76.2 x 25.4 x 1mm, Fisher Scientific) were each treated with a plasma cleaner (PDC-32G, Harric Plasma, Ithica, NY, USA) for 30 s at 1.8 W, and the PDMS was irreversibly bonded to the cover slip. To test reproducibility of fabrication methods, the height difference between the posts and platform of three counting chambers was measured with a Nikon Measurescope (Vision Engineering Dynascope Inspection Microscope X-Y Stage).

2.3 Testing Precision and Accuracy of Sperm Concentration Estimates

The counting chamber was tested for accuracy and precision of concentration measurements with samples of zebrafish (*Danio rerio*) sperm. Manual counts were performed in triplicate using three PDMS counting chambers and Computer Assisted Sperm Analysis (CEROS model, Hamilton Thorne, Beverly, MA, USA) under tungsten filament illumination using a light microscope (CX41 Olympus America Corp Center Valley, CA, USA) with a 200-x total magnification. For each replicate, 10 µL of sperm suspension were pipetted on the center of the platform and the base was covered immediately with the coverslip. The microscope was focused on the coverslip grid, and sperm cells within were manually counted. The number of sperm counted within 10 squares of the grid was multiplied by a factor of 10^6 to obtain the number of sperm cells per mL. Precision of concentration estimates was tested through comparison of estimates obtained with the three PDMS counting chambers using a two-tailed T test. Accuracy of concentration estimates was tested through comparison to estimates obtained using a Makler® counting chamber using a two-tailed T test.

3. Results and Discussion

3.1 High Resolution 3-D Printing

The height difference between the posts and platform of two counting chambers fabricated using a 3-D printed mold was 16.1 ± 1.6 µm (Figure 4). Thus, the desired 10-µm height difference for a uniform cell monolayer was not achieved with this method.

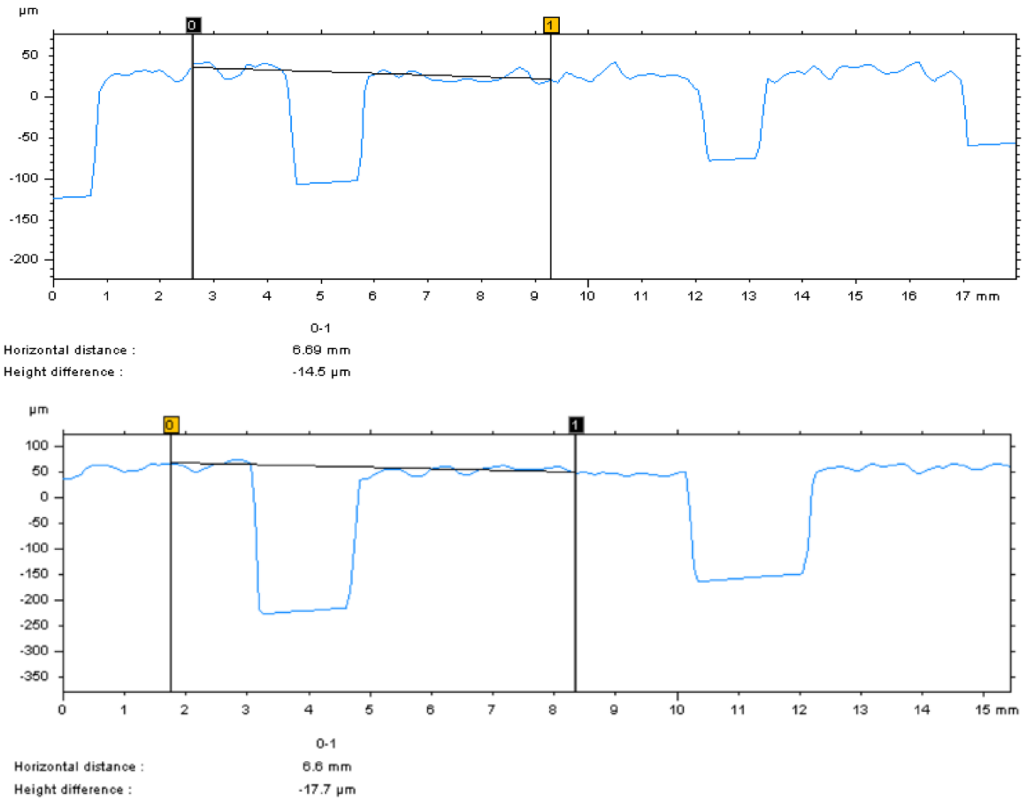

Figure 4. Non-contact optical profile of height difference between the platform and posts of two base components

Additionally, surface topography caused by the texture of the 3-D printed extruder lines was observed in the counting chambers fabricated using the 3-D printed master mold (Figure 5). The non-uniform surface of the mold created a reflective PDMS replica surface that inhibited imaging and accurate manual acquisition of concentration estimates. Thus, high resolution 3-D printing was not selected as the preferable master mold fabrication method.

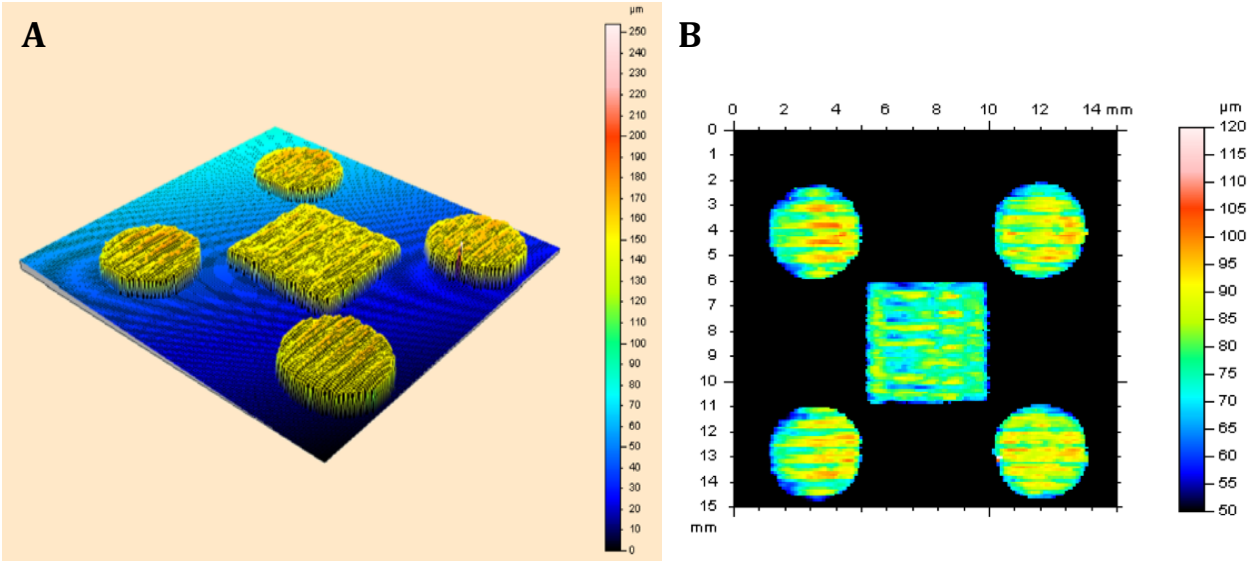

Figure 5. Base component surface topography observed with A) 3-D and B) 2-D profilometry

3.2 Two-step Photolithography

The height difference between the posts and platform of three counting chambers fabricated using two-step photolithography was 10.000 ± 0.015 µm, verifying device reproducibility and fabrication precision using this method. Additionally, during testing, the surface of the PDMS replica was not found to be reflective. Thus, two-step photolithography was selected as the preferable master mold fabrication method to fabricate the final device, the Microfabricated Enumeration Grid Chamber (MEGC) (Figure 6.)

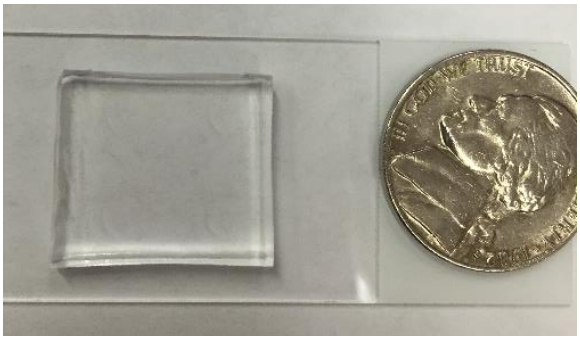

Figure 6. Base component of the microfabricated enumeration grid chamber (MEGC)

3.3 Precision of Concentration Estimates

No significant ($p \geq 0.05$) difference was found between sperm concentration counted using three PDMS counting chambers, establishing the precision of concentration measurements made with the microdevice (Figure 7).

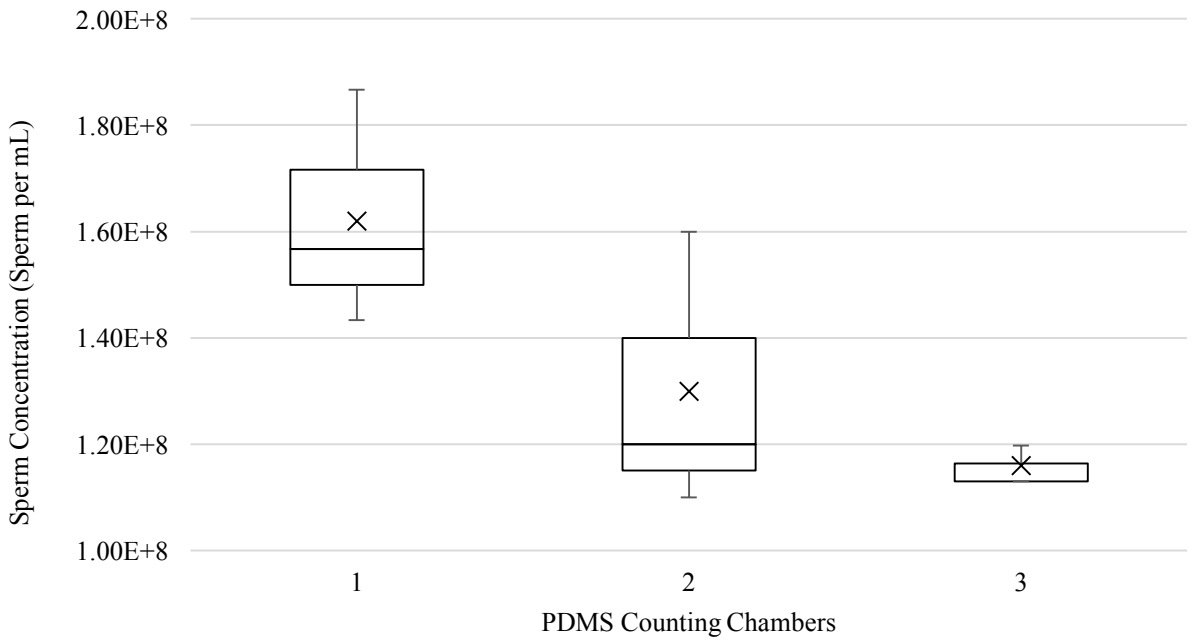

Figure 7. Calculated sperm concentration using three separate PDMS counting chambers. Boxes represent the distribution of concentration data obtained, where the top line represents the 75th percentile, the central line represents the median value, the lower line represents the 25th percentile, and the mean is shown as an X. Error bars indicate standard error, with caps indicating maximum and minimum values of concentration.

3.4 Accuracy of Concentration Estimates

No significant ($p \geq 0.05$) difference was found between sperm concentration estimated with the Makler® counting chamber and the concentration calculated using PDMS counting chamber, establishing the accuracy of concentration measurements made with the microdevice (Figure 8).

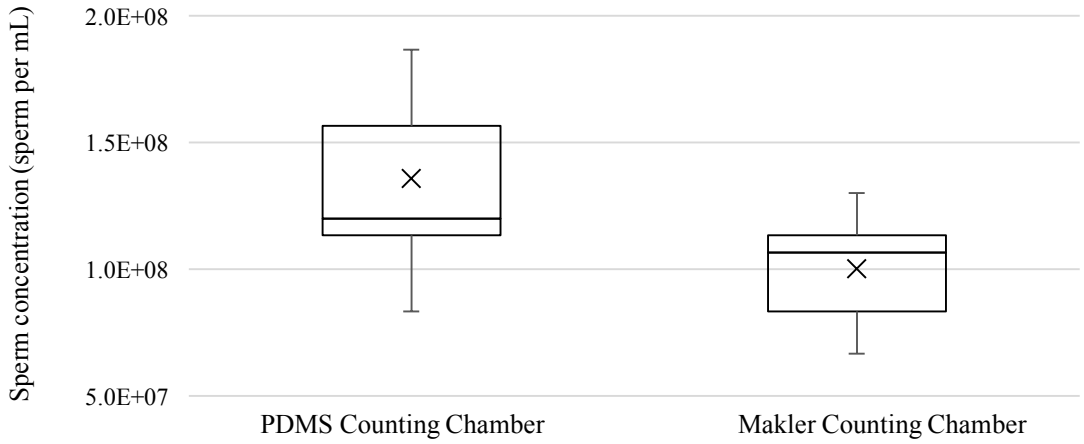

Figure 8. Comparison of concentration measurement. Boxes represent the distribution of concentration data obtained, where the top line represents the 75th percentile, the central line represents the median value, the lower line represents the 25th percentile, and the mean is shown as an X. Error bars indicate standard error, with caps indicating maximum and minimum values of concentration.

4. Conclusions

A disposable counting chamber to measure aquatic species sperm concentration was fabricated inexpensively using PDMS and was accurate, reproducible, and precise. In future work, the PDMS counting chamber will be optimized to improve utility. This technology will play an essential role in successful on-site cryopreservation and will influence the development of comprehensive aquatic germplasm repositories with on-site cryopreservation capabilities, which can lead to the protection of gene pools belonging to unique, declining, or endangered populations, restoration of the gene pools of these populations, and advances in aquaculture and fisheries management through standardization of reproduction and cryopreservation.

Acknowledgements

This research was supported by the Undergraduate Research Opportunity Program of the Louisiana Sea Grant College Program. We thank D. Park for access to the Nikon Measurescope and L. Torres for sperm sample preparation. We also acknowledge support from the National Science Foundation ARI-R^2 program grant CMMI-096348 and National Institutes of Health grant 5R24OD010441.

References

[1] Tiersch TR, Green CC. *Cryopreservation in Aquatic Species*. 2nd ed. Baton Rouge: World Aquaculture Society. 2011.

[2] Dong Q, Huang C, Tiersch TR. Control of sperm concentration is nececessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters. Cryobiology 10.1016/j.cryobiol.2006.11.007.

[3] Tiersch TR, Yang H, Hu E. Outlook for development of high-throughput cryopreservation for small-bodied biomedical model fishes. Comp Biochem Physiol C Toxicol Pharmacol 10.1016/j.cbpc.2011.08.007.

[4] Bobe J, Labbé C. Egg and sperm quality in fish. Gen Comp Endocrinol 10.1016/j.ygcen.2009.02.011

[5] Rurangwa E, Kime DE, Ollevier F, Nash JP. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 10.1016/j.aquaculture.2003.12.006

[6] Christensen P, Stryhn H, Hansen C. Discrepancies in the determination of sperm concentration using bürker-türk, thoma and makler counting chambers. Theriogenology. 10.1016/j.theriogenology.2004.05.026

[7] Seaman EK, Goluboff E, Barchama N, Fisch H. Accuracy of semen counting chambers as determined by the use of latex beads. Fertil Steril 10.2164/jandrol.111.013045

[8] World Health Organization. (2010). WHO Laboratory Manual for the Examination and Processing of Human Semen.

[9] Mahmoud AM, Depoorter B, Piens N, Comhaire FH. The performance of 10 different methods for the estimation of sperm concentration. Fertil Steril 10.1016/S0015-0282(97)81526-9

[10] Makler A. A new chamber for rapid sperm count and motility estimation. Fertil Steril 10.1111/j.2042-3306.2011.00523

[11] Imade GE, Towobola OA, Sagay AS, Otubu JA. Discrepancies in sperm count using improved neubauer, makler, and horwells counting chambers. Syst Biol Reprod Med 10.3109/01485019308988375

[12] Makler A, Murillo O, Huszar G, Tarlatzis B, DeCherney A, Naftolin F. Improved techniques for collecting motile spermatozoa from human semen. Int J Androl 10.1111/j.1365-2605.1984.tb00760.x

[13] Sia SK, Whitesides GM. Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies. Electrophoresis 10.1002/elps.200305584