Published in final edited form as:

Reprod Fertil Dev. 2020 June ; 32(9): 873–878. doi:10.1071/RD19154.

Microfabrication of low-cost customisable counting chambers for standardised estimation of sperm concentration

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Abstract

Evaluation of sperm concentration is essential for research and procedures involving AI, cryopreservation and sperm quality assessment. Microfabrication technologies have shown tremendous potential for rapid prototyping and fabrication of devices to assist reproduction and fertility research, but such utility has not yet been made available for most reproduction laboratories. The aim of this study was to evaluate the feasibility of using microfabrication techniques to produce counting chambers for estimation of sperm concentration. Zebrafish (*Danio rerio*) spermatozoa were used as a model for evaluation of functionality of the chambers. These microfabricated enumeration grid chambers (MEGC) were composed of a polydimethylsiloxane (PDMS) coverslip with grid patterns (100 $\mu\text{m} \times 100 \mu\text{m}$) and a PDMS base platform to create a known volume with a 10- μm height to restrict the cells to a single layer. The results of cell counts estimated by two of three prototype MEGC devices tested were not significantly different from the control device, a commercially available Makler chamber. The material cost for a MEGC was less than US\$0.10 compared with product costs of approximately US\$100 for a standard haemocytometer and US\$700 for a Makler counting chamber. This study demonstrates the feasibility of microfabrication in creating low-cost counting chambers to enhance standardisation and strengthen interdisciplinary collaborations.

Keywords

aquatic models; cryopreservation; zebrafish

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Conflicts of interest

No competing financial interests exist.

Introduction

Evaluation of sperm concentration is essential for assessment of sperm quality, AI, and cryopreservation in research activities and practical applications (Liu *et al.* 2018). However, most aquatic research laboratories neglect standardised estimation of sperm concentration because specialised hardware can be expensive, inaccurate or require extensive training to use (Hagedorn *et al.* 2019). For example, automated instrumentation, such as flow cytometry, microvolume spectrophotometry, computer-aided sperm analysis (CASA) and microfluidic sperm-counting devices with electric components (Kjelland *et al.* 2014) are cost prohibitive to many laboratories. Standard haemocytometers are relatively inexpensive (~US\$100), but can lead to erroneous sperm cell counts due to a relatively tall (100 µm) standard chamber height that may cause the under-counting of cells outside the focal plane, requiring procedures (such as fixation to drop the cells to the bottom of the chamber) or training of technicians (to manually adjust focus to include cells outside the focal plane; Seaman *et al.* 1996; Christensen *et al.* 2005; World Health Organization (WHO) 2010). Specialised counting chambers, such as commercially available Makler counting chambers (Sefi-Medical Instruments), with constrained chamber heights (10 µm) avoid some of these drawbacks, yet can be cost prohibitive, currently costing US\$700.

Microfabrication techniques provide great potential to produce standardisable, low-cost and disposable devices for reproducible analysis of sperm cells (Park *et al.* 2012; Beckham *et al.* 2018). The need for this was identified by the recent Cryopreservation of Aquatic Biomedical Models Workshop (7–8 January 2017; Birmingham, AL, USA) hosted by the Office of Research Infrastructure Programs of the National Institutes of Health (NIH). One of the three major recommendations in the workshop report (Hagedorn *et al.* 2019) specifically suggested:

...provide mechanisms to support innovative technical advancements that will increase the reliability, reproducibility, simplicity, throughput, and efficiency of the cryopreservation process. This recommendation encompasses basic cryopreservation knowledge and engineering technology, such as microfluidics and automated processing technologies.

To date, no low-cost microfabricated counting chambers have been reported for evaluation of sperm concentration. The goal of this study was to evaluate the feasibility of producing a microfabricated enumeration grid chamber (MEGC) by soft lithography for estimation of sperm concentration using zebrafish as a model. With feasibility demonstrated, reproducibility can be investigated in further studies.

Materials and methods

Design

In preliminary trials, high-resolution three-dimensional (3D) printing by fused deposition modelling (FDM) was evaluated for fabricating the base part mould (Fig. 1a), but this did not meet quality expectations because of irregular surface geometries that resulted from the deposition process (Fig. 1b). Although it is anticipated that as this technology matures (e.g. high-resolution stereolithography) 3D printing will eventually be used to create moulds

or even microdevices for direct use, the current commercially available FDM 3D printers lack the required resolution for these applications. For comparison, optical profilometry was also used to characterise the surfaces of the moulds fabricated via photolithography methods (described below), which yielded much more accurate geometries and smooth surfaces (Fig. 1c, d). For example, the variation in height, as measured across the full width of the central platform square fabricated from photolithography, was 0.19 μm . Because of its higher precision that produced optically clear features and an accurate chamber volume, photolithography was chosen to fabricate the MEGC.

The MEGC described herein was composed of two components: a polydimethylsiloxane (PDMS) coverslip with grid patterns, and a PDMS base piece with pillars at the four corners and a central platform (Fig. 2a). Moulds for casting the PDMS pieces were prepared by photolithography (Fig. 2b). The PDMS coverslip was designed with 100 $\mu\text{m} \times 100 \mu\text{m}$ grid patterns to guide visual counting. The base of the counting chamber was composed of a central platform on which sperm samples were loaded and four pillars at the corners, which were designed to support the coverslip at 10 μm above the platform. This restricted sample volume to produce a monolayer of sperm cells.

Counting chamber coverslip fabrication

To fabricate the coverslip for the counting chamber, a master mould was made by standard single-layer photolithography and a permanent epoxy-based negative photoresist (SU-8 2025; MicroChem; Beckham *et al.* 2018).

Briefly, to prepare the coverslip master mould, a 100-mm N-type silicon wafer was spin coated with 4 mL SU-8 2025 photoresist using a spin coater (WS-650-23B; Laurell) at 500 r.p.m. for 10 s at 100 r.p.m. s^{-1} followed by 3000 r.p.m. for 30 s at 300 r.p.m. s^{-1} to achieve a thickness of 25 μm . The wafer was baked on a hotplate at 65°C for 10 min followed by 95°C for 1 h. When cooled to 25°C, the wafer was positioned inside a customised chamber and exposed to a 365-nm ultraviolet light (Blak-Ray B-100 series UV lamp; UVP) at an irradiance intensity of 2 mW cm^{-2} through a chrome photomask with the desired design modelled with AutoCAD software (Autodesk, Inc.) and printed by Front Range Photomask. The mould was exposed to an effective dose of 175 mJ cm^{-2} , baked on a hotplate at 65°C for 10 min at 95°C for 1 h and cooled to 25°C.

To selectively dissolve the photoresist, the wafer was immersed in SU-8 developer solution (Microchem) for 4 min, rinsed with fresh developer for 1 min, rinsed with isopropyl alcohol for 10 s and dried with nitrogen gas. The master mould was hard-baked at 150°C for 90 min, cooled to 25°C and placed in a vacuum chamber within a glass Petri dish (with 150 μL dichlorosilane) for 10 min for silanisation via vapour deposition. The master mould was removed, hard-baked at 150°C for 90 min and cooled to 25°C.

To create casts from the master mould, PDMS (Sylgard 184 silicone; Dow Silicones Corporation) was mixed at a ratio of 10 : 1 (base : curing agent), placed in a vacuum chamber at -600 mmHg for 15 min, cast on the master mould and cured in an oven at 75°C for 90 min. When cooled to 25°C, the PDMS casts were carefully peeled from the master mould. A 20 mm $\times$ 20 mm section containing the coverslip grid features was cut from the

replica. The PDMS and a clean glass slide (76.2 mm × 25.4 mm × 1 mm) were treated with a plasma cleaner (PDC-32G; Harrick Plasma) for 30 s at 1.8 W, and the PDMS was irreversibly bonded to the coverslip.

Counting chamber base fabrication

To create a uniform 10-μm height difference between the central platform and pillars, a two-step photolithography process was used. The fabrication procedure was as described above with the following exceptions. In the first coating, the wafer was spin coated with SU-8 2010 photoresist at 500 r.p.m. for 10 s at 100 r.p.m. s⁻¹ followed by 3500 r.p.m. for 30 s at 300 r.p.m. s⁻¹ to achieve a thickness of 10 μm. After UV exposure and baking, the spin coating, exposure and baking steps were repeated with a second transparency photomask to produce a 10-μm height difference between the posts and platform. The wafer was developed for 2 min and hard-baked using the procedures described above, and cast with PDMS. The fabrication precision of the base pieces was evaluated by measurement of height differences between the pillars and platform of three representative MEGC using a MeasureScope (Nikon). The mean (±s.d.) height difference between the corner pillars and central platform was 10.00 ± 0.02 μm, indicating the feasibility of these methods to produce the required chamber height for sperm analysis. Surface profiles of moulds were evaluated using a non-contact optical profilometer (Nanovea ST400; Micro Photonics).

Zebrafish husbandry and sperm collection

Zebrafish spermatozoa were used as a model to investigate the functionality of the MEGC. Protocols for the use of animals in this study were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee. Fish were maintained within a recirculating system at 28.5°C, pH 8.5, under a 12-h light–dark photoperiod. Fish were fed to satiation twice daily with adult zebrafish diet (Zeigler Bros).

Male fish were anaesthetised with 0.01% tricaine methane-sulfonate (MS-222; Western Chemical), placed ventral side up on a moist sponge and stripped by gently pressing the abdominal area with the fingers. Spermatozoa were collected into a 10-μL glass capillary tube (Drummond Scientific) and immediately released into a 1.5-mL microcentrifuge tube containing Hanks' balanced salt solution with osmolality of 300 mOsmol kg⁻¹ (functionally isotonic to zebrafish plasma).

Estimation of sperm concentration with MEGC

Fish spermatozoa are typically immotile when diluted in isotonic solutions, and thus no fixation is required during sperm counting. Alternatively, mammalian spermatozoa are typically motile during counting, and thus a 10-μm chamber depth enables counting a single layer of cells without fixation. Sperm suspensions (10 μL) were pipetted onto the central platform of the PDMS base piece (Fig. 2c), after which the PDMS coverslip was placed on top with the grid-side of the coverslip facing downwards (Fig. 2d). Using this sample volume (10 μL) allowed excess sample to spill over the platform edges to the bottom of the chamber, which did not interfere with measurement. This volume also permitted flush mounting of the coverslip to the posts of the bottom chamber, ensuring a consistent chamber volume. Spermatozoa were viewed with a dark-field microscope at a magnification of ×200

(CX41; Olympus), and images were captured with the charge-coupled device camera of the CASA system (HTM-CEROS, version 14 Build 013; Hamilton Thorne Biosciences). (Image capture was for evaluation purposes, and thus could be optional for practical operation.) Three MEGC devices (MEGC1–3) were used to estimate sperm concentrations as number of cells within one square $\times 10^7$ cells mL⁻¹ (counting a total of 10 squares in a row for each MEGC with a total area of 0.1 mm² and volume of 1×10^{-3} μ L). One Makler chamber (Sefi-Medical Instruments) was used for comparisons in triplicate (counting of 10 squares from one chamber) and comparisons were made using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test in SAS 9.4 (SAS Institute).

Results

Grids and sperm cells were distinctly visible in the MEGC when observed with a microscope at a magnification of $\times 200$ (Fig. 2e). The grids featured bright lines with perpendicular crossings that formed distinct squares. The sperm cells appeared as white spots with round or oval shapes. In each chamber, 115, 132 and 174 sperm cells were counted with an estimated sampling error of 8–9% (calculated as $(100 \times (N^{1/2}/N))$ based on the number of cells (N) counted; WHO 2010). Estimations by the MEGCs and the Makler had a normal distribution (PROC UNIVARIATE, $0.2093 < P < 0.7319$) and homogeneity of variances (PROC GLM, $P = 0.9474$). No significant differences ($P > 0.05$) were found in sperm concentrations among estimates made with the Makler chamber and MEGC2 and MEGC3 (Fig. 2f), but a significant difference ($P < 0.0001$) was found between MEGC1 and the Makler chamber and MEGC2 and MEGC-3, with estimations ~73% higher with MEGC-1 than with the Makler chamber.

Discussion

This study demonstrated the feasibility of constructing custom designs of novel devices for estimation of sperm concentration using microfabrication techniques to create standardised, low-cost and disposable chambers such as the MEGC. The use of PDMS soft lithography was ideal because it produces transparent, waterproof, inexpensive, non-toxic and easily replicated casts from a master mould, allowing for systematic prototyping and development of standardised devices (Sia and Whitesides 2003). The estimated material cost for a single MEGC device is less than US\$0.10, which would facilitate adoption of the technology and standardised counting practices compared with the single-unit product cost of US\$700 for the Makler chamber. The MEGC can be customised by changing the chamber depth during the spin coating process (a modification in the control software) to accommodate different sizes of spermatozoa or cell types.

Cell counts estimated by two of three MEGC devices were not significantly different (difference was less than a factor of two) from those estimated using the control method. The single MEGC outlier could have been due to improper moulding or demoulding of the base piece, or non-union of it with the coverslip during assembly, which could yield a greater chamber height and higher counting values (we included data from this device to reflect the realities of microfabrication prototyping). The goal of the present study was to demonstrate the feasibility of design and production of microfabricated sperm counting

chambers. The eventual functionality would be improved with standard quality control and quality assurance for commercial-scale production, but even prototype devices such as this MEGC have potential to provide low-cost standardised tools for the reproduction community to enhance standardisation and reproducibility of research procedures. Based on the fabrication technology of these prototypes, reproducibility (e.g. feature dimensions), precision, accuracy and reliability can be further investigated and improved.

Although microfabrication technologies have shown potential to provide rapid prototyping capability and fabricate devices to assist reproduction and fertility research, such utility has not yet been realised by most biological and clinical science laboratories. However, it would not be difficult for motivated laboratories to gain access to such technologies and devices through a variety of approaches. For example, microfabrication can be made available by establishing interdisciplinary collaborations with engineering facilities at many academic campuses to utilise open-source design files. Moreover, such technologies can be accessible through commercially available sources that provide microfabrication services. For example, moulds fabricated by high-resolution computer numeric control machining or photolithography can be ordered online and shipped to customers within days. Prototyped devices and well-developed products (e.g. PDMS chips) could be ordered or distributed through community-based resources, such as research or technology development centres. In addition to enhancing the standardisation of research and procedures, funding opportunities can be strengthened by interdisciplinary collaborations between reproduction science and engineering laboratories. The NIH, as indicated above, and the National Science Foundation have many programs that emphasise the importance of interdisciplinary approaches.

Acknowledgments

This research was supported, in part, by the Undergraduate Research Opportunity Program of the Louisiana Sea Grant College Program and the National Institutes of Health, Office of Research Infrastructure Programs (R24-OD010441 and R24-OD011120), the National Institute of Food and Agriculture, United States Department of Agriculture (Hatch projects LAB94231 and NC1194) and the Louisiana State University-Audubon Center for Research of Endangered Species Collaborative Projects. The authors thank D. Park for access to equipment for evaluation of fabrication quality and L. Torres for sperm sample preparation. This report was approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript number 2020-241-34712.

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Fig. 1.

Evaluation of preliminary fabrication trials of three-dimensional (3D) printed moulds by fused deposition modelling (FDM) for the base piece of the microfabricated enumeration grid chamber (MEGC). This method, although promising, was not used for further fabrication of MEGC because it did not meet quality expectations compared with photolithography fabrication methods. (a) A mould fabricated with high-resolution 3D printing. (b) Irregular surface properties were identified by optical scanning profilometry ($>50\ \mu\text{m}$ in surface height variation). (c) Profilometry scan of the MEGC base mould showing the smooth surface of the platform ($0.19\ \mu\text{m}$ height variation) produced by photolithography. (d) Profilometry scan of the MEGC base at same scaling as the 3D printed mould in (b).

Fig. 2.

Fabrication and use of a microfabricated enumeration grid chamber (MEGC) for concentration estimation of zebrafish spermatozoa. (a) Overview of the grid-patterned coverslip with $100\ \mu\text{m} \times 100\ \mu\text{m}$ grids placed on top of posts that create a $10\text{-}\mu\text{m}$ tall chamber to create a known volume ($1 \times 10^{-4}\ \mu\text{L}$ for $100\ \mu\text{m} \times 100\ \mu\text{m} \times 10\ \mu\text{m}$) for accurate estimation of sperm concentration. Δh , height difference. (b) The microfabrication process for the MEGC was photolithography and polydimethylsiloxane (PDMS) casting. The mould for the coverslip was prepared by exposing a spun negative photoresist masked by a $100\text{-}\mu\text{m} \times 100\text{-}\mu\text{m}$ grid pattern. The mould for the base piece was prepared by two-step photolithography (PL), yielding a $10\text{-}\mu\text{m}$ height difference between the central platform and the adjacent pillars. UV, ultraviolet. (c, d) For use of the MEGC, zebrafish sperm samples were pipetted onto the central platform of the base piece (c), followed by placing of a coverslip on top (d). (e) To estimate sperm concentration, sperm samples were observed with a dark-field microscope ($\times 200$ magnification) and the number of cells (white dots; arrow) within each square ($100\ \mu\text{m} \times 100\ \mu\text{m}$) was recorded. Only a portion of the grids squares is shown in the figure because of limitations of image acquisition. (f) Box plots showing

estimates of sperm concentrations made using three MEGC and one Makler counting chamber as the current technology control. The boxes show the interquartile range, with the median value indicated by the horizontal line; mean values are indicated by the 'X' and whiskers show the range. The same letters above boxes indicate no significant differences between estimates ($n = 10$; $P > 0.05$).