Open-Channel Droplet Microfluidic Platform for Passive Generation of Human Sperm Microdroplets

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ABSTRACT: Sperm cryopreservation is important for individuals undergoing infertility treatment, and for those who wish to preserve fertility potential, prior to treatments like chemotherapy, radiation therapy, gender-affirming medical interventions, elective fertility delay, or individuals in high-risk professions such as the military. Current methods for sperm cryopreservation result in approximately 30-50% decrease in sperm motility. However, recent studies have shown that ultra-rapid freezing (vitrification) is a valuable approach for maintaining sperm quality after freeze-thawing processes in the clinical laboratory setting and requires sub-microliter to microliter volumes. A major challenge for the adoption of vitrification in fertility laboratories is the ability to pipette small volumes of sample. Here, we present a method that leverages open-channel droplet microfluidics to autonomously generate sub-microliter to microliter volumes of purified human sperm samples. Using a novel, open-channel droplet generator, we found no change in sperm movement and kinematic data after exposure to device and reagents in our platform. We conclude that our platform is compatible with human sperm, an important foundation for future implementation of vitrification in fertility laboratories.

Infertility is defined as the inability to conceive after 12 months of unprotected intercourse, or 6 months for couples with female partner over the age of 35.[1] Infertility is common, affecting 12.7% of couples in the US or one in six individuals worldwide; a male factor is identified in up to half of couples.[2–5] Sperm cryopreservation (freezing of sperm for later use) is a component of treatment for many patients with infertility, and also benefits individuals facing military service, individuals planning treatment for cancer, and transgender or nonbinary individuals prior to therapies that impact fertility potential. Traditional sperm cryopreservation techniques (slow and rapid freezing) are associated with adverse impacts on sperm motility, which may decline by 30-50% post-thaw.[6,7] Thus, there is a clinical need for improvement in sperm cryopreservation to maintain sperm quality. [8]

Ultra-rapid freezing, or vitrification, is an approach to reducing ice crystal formation which is the standard approach to cryopreservation of oocytes and embryos.[9,10] Prior studies have established the benefits of vitrification for sperm preservation[11,12] and proposed devices that can be used in the vitrification process.[13–15] A major barrier that has prevented the adoption of vitrification for sperm is the need for generation and manipulation of small fluid volumes (< 1 microliter), which is needed to achieve rapid cooling rates required for vitrification.[16–19] In standard vitrification workflows, sperm samples are portioned into sub-microliter to microliter volumes using a pipette, which can be time-consuming and challenging to achieve, thus resulting in a need for generating small volumes in an autonomous fashion.[20]

Towards this need, we present a platform for autonomous generation of microdroplets of purified sperm which leverages hydrostatic and interfacial surface tensions and autonomously generates microliter to nanoliter droplets[21] without the need for peripheral equipment such as syringe pumps or actuators. On a broad scale, microfluidics has been shown to be valuable and fitting for sperm preparation, selection, and analysis for the ability to achieve high-throughput, mimic the female reproductive tract, and enhance assisted reproductive technology (ART) workflows.[22–25] Open droplet microfluidic devices (including this platform) utilize an air-liquid interface via removal of the device ceiling, allowing for direct access to the droplets within the device which enables transferring, sorting, moving, and patterning of droplets.[26–31] Our previously established droplet generator[21] was adapted to include four rectangular wells in the outlet reservoir, enabling the seating of droplet holders and the movement of the droplets from the reservoir to the droplet holder via a stylus or polytetrafluoroethylene (PTFE)-coated forceps. In this study, we have generated sub-microliter to microliter sized droplets of purified sperm samples from three healthy sperm donors and demonstrate preservation of sperm motility and kinematics pre- and post- sample preparation and droplet generation.

The open-platform droplets generator used in this study was developed from Khor *et al.* [21] The device with a 0.2 mm wide constriction region was chosen since the expected droplet volumes were less than or equal to 1 microliter (Figure 1). Detailed device design and fabrication procedures are in supplemental material. Carrier fluids were prepared from pure HFE-7500

engineered fluid (The 3M Co., St. Paul, Minnesota) with 2 wt% FS-008 fluorosurfactant (RAN Biotechnologies Inc., Beverly, Massachusetts). HEPES buffered formulation with 0.5% Human Serum Albumin sperm wash (#2003, InVitro Care Inc., Frederick, Maryland) was used as the aqueous phase, which was tinted with red dye (McCormick & Co., Hunt Valley, Maryland) at a concentration of 1:500 (v/v) for visualization of the droplets in the device.

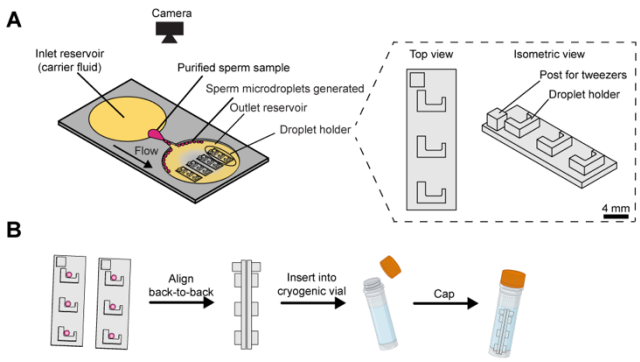

Figure 1. *Schematic of open-channel droplet generator and insertion of droplet holder into a cryovial.* This device (A) enables the encapsulation of human spermatozoa in microdroplets and the movement from the device to a droplet holder. This holder contains three slots that can hold one droplet each and, after vitrification (B) when placed back-to-back, the holder is able to be placed into a commercial cryogenic vial for long term storage. Created with BioRender.com.

Four droplet holders were then inserted into the slots in the outlet region. A 180 µL aliquot of the dyed aqueous plug was added to the inlet platform of the constriction region. Afterwards, 1.5 mL of carrier fluid was pipetted to the larger inlet portion of the device. Once droplet generation began, 0.5 mL of carrier fluid was added to maintain hydrostatic pressure. Devices were then observed for passive droplet generation. Video in supplemental materials shows passive droplet formation.

A stylette or PTFE-coated straight-tip forceps (#2A-SA-SE-TC15, Excelta One Star, Buellton, CA) were used to move the droplets from the device to the holder. A single droplet was transferred to each of the droplet holder slots. After vitrification is performed, the droplet holders are designed to be placed back-to-back in a commercial 2 mL polypropylene cryogenic vial (#03374059, Corning Inc., Corning, NY). A video of droplet transfer can be found in the supplementary materials.

Under an approved Institutional Review Board (STUDY00009510) protocol, healthy subjects provided informed consent and semen samples via masturbation following 2-5 days of abstinence.[20] The semen samples were purified using a direct swim-out procedure.[20] Motile sperm preparation was added to the device according to Khor *et al.* [21] and sperm kinematics were measured for different conditions (with and without dye and passage through the device) using the Hamilton Thorne Research (Beverly, MA) Integrated Visual Optical System (IVOS). A detailed protocol for the swim-out and the device operation can be found in Supporting Information section SI1.

We have adapted an established droplet generator[21] (Figure 1A) to be able to seat droplet holders (Figure 1A) for the storage of sperm microdroplets in commercial cryogenic vials (Figure 1B). For ultra-rapid freezing methods such as vitrification via liquid nitrogen, sperm specimens are fractioned into small volumes (~1 µL) using a pipette; however, obtaining the correct volume is challenging at low quantities. Thus, we are leveraging open-channel droplet microfluidics to aliquot rare sperm samples into 0.5-1 µL-sized droplets autonomously. The workflow for device operation can be found in Figure 2. Our device will be able to address the need for autonomously aliquoting small volumes of purified sperm samples for the purposes of sperm cryopreservation in andrological and urological laboratories. This device requires no additional tools or equipment - necessitating only a pipette – thus, the device can be implemented in laboratories with varying access to resources.

Within the device, there are four rectangular wells in the outlet reservoir for the seating of the droplet holders. The droplet holder measures 7 mm wide by 20 mm long and 3.92 mm in maximum height and is fabricated out of polystyrene, which is a known biocompatible material. Each slot has a height of 1.50 mm. We designed the holder to be placed back-to-back into a commercial cryogenic vial (Figure 1B and SI video). Moreover, each droplet holder can hold three individual droplets and contains a rectangular shaft to allow the holder to be picked up by forceps vitrification and placement into the cryogenic vial.

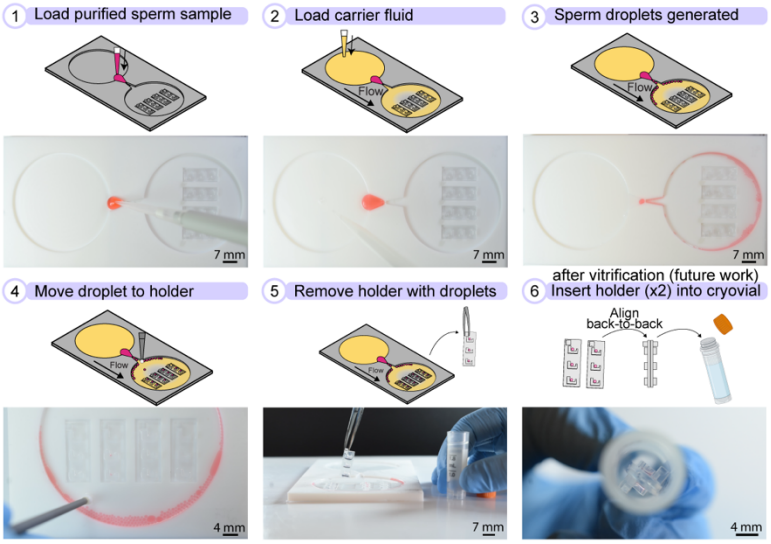

Figure 2. *Operation of open-channel droplet generator.* A 180-µL sample of purified sperm is loaded with a pipette into the inlet region and carrier fluid consisting of 2% fluorosurfactant in HFE-7500 is subsequently added to the inlet reservoir to initiate droplet generation. Droplets are then autonomously generated, which can be moved to the droplet holder using a stylette or PTFE-coated forceps. Created with BioRender.com.

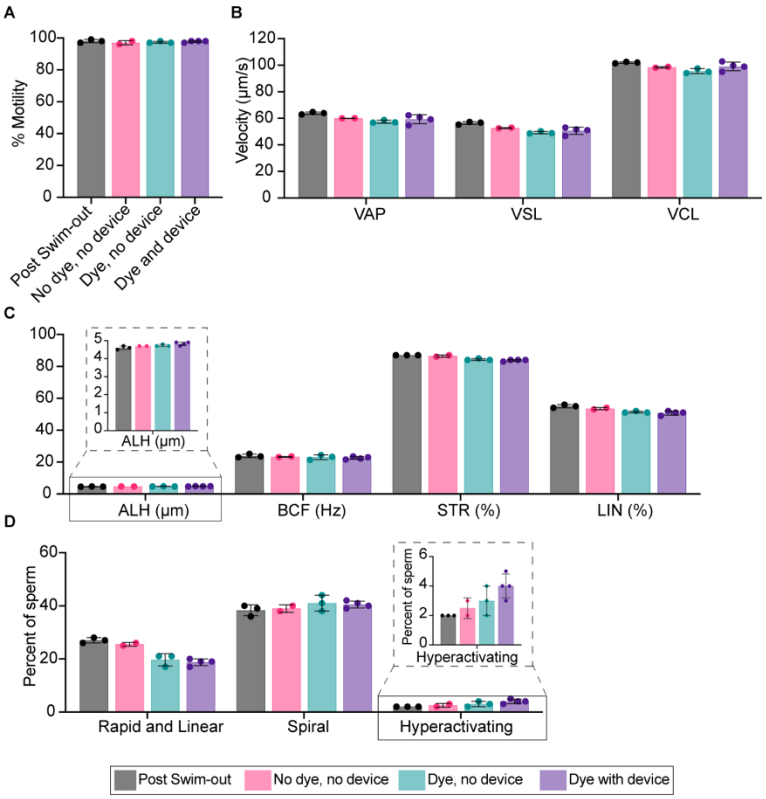

Figure 3. *Sperm (A) motilities, (B) velocities, (C) amplitude lateral height (ALH), beat/cross frequency (BCF), straightness (STR) and linearity (LIN) measurements, and (D) movements from one representative participant.* The sperm data were collected from post swim-out samples, no dye and no device samples (No dye, no device), with dye (1:500, v/v) without device samples (dye, no device) and with both dye (1:500, v/v) and device samples (dye with device). Three individual participant experiments were conducted with different devices; data from one participant are shown here, and all data are included in the SI. Each data point represents an individual device and measurement; the bar graph represents the mean ± SD of n = 2-4 devices/measurements.

In this study, the use of dye in sperm samples is crucial for visualization during droplet generation and manipulation. To address this, we investigated sperm motility and kinematic parameters before and after exposure to the dye and the PTFE

device. We assessed sperm motility parameters in three sperm donors using an automated sperm analyzer platform, Integrated Visual Optic System (IVOS) (Figure 3 and SI2.1). A representative participant is presented in Figure 3 and additional participant data can be found in SI section 2. In these graphs, we present sperm motility data (Figure 3A) as well as additional kinematic measures including average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), (Figure 3B) amplitude of lateral head displacement (ALH), beat/cross frequency (BCF), straightness (STR), linearity (LIN), (Figure 3C) as well as percentage of sperm that are rapid and linear, spiraling, and hyperactivating (Figure 3D).[32,33] We found no notable decline in motilities parameters when comparing the sperm samples exposed to dye and the PTFE or when one or both of those conditions are absent. We have included the post swim-out parameters here, which are measured immediately after swim-out, to account for differences in sperm motilities and kinematics over time, but no apparent differences were found.

Here, we have sustained sperm motility and kinematics using healthy male participants with normal sperm parameters with our adapted open-channel droplet generator. A novel droplet holder feature is also presented, allowing for the storage of droplets in cryogenic vials, which is a highly adaptable feature of our platform. Moreover, our device requires only a pipette, the sperm sample, droplet generation reagents, the device, and droplet holders to operate. With no peripheral equipment necessary and no specialized training is needed to generate the droplets, there is a strong potential to implement this platform in labs across a broad range of resource levels. Most importantly, our device enables direct access to the droplets for selection and manipulation, which is only afforded through open channels. To our knowledge, our device is one of the few to autonomously generate small volumes for the purposes of sperm vitrification. The device and workflow presented here lays the foundation for a new approach to sample preparation for vitrification of sperm, where maintaining sperm motility after passage through the device is crucial in the successful implementation of this device in the clinical setting. In this study, we used semen samples from individuals with normal sperm parameters, but we aim to apply this method to samples with rare sperm (<100k sperm/mL) in future studies. Sperm quality will also be assessed when coupling our method with vitrification, which currently faces integration barriers in fertility laboratories. Facilitating the adoption of vitrification for sperm could benefit individuals with low sperm counts, fertility laboratories and sperm banks.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental section and additional participants data (PDF), video of droplet generation (.mp4), and video of droplet transfer (.mp4), design file of droplet generator with holder slots (.STL), design file of droplet holder (.STL)

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Author Contributions

All authors have given approval to the final version of the manuscript.
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Notes

T.M.N., J.W.K., U.N.L., E.B., and A.B.T. filed patent Open Microfluidic Channel Design for Passive Droplet Formation and Manipulation May 23, 2022 through the University of Washington. A.B.T. and U.N.L. report filing multiple patents through the University of Washington and A.B.T. received a gift to support research outside the submitted work from Ionis Pharmaceuticals. E.B. is an inventor on multiple patents filed by Tasso, Inc., the University of Washington, and the University of Wisconsin-Madison. T.M.N. has ownership in Tasso, Inc.; E.B. has ownership in Tasso, Inc., Salus Discovery, LLC, and Seabright, LLC and is employed by Tasso, Inc.; and A.B.T. has ownership in Seabright, LLC; however, this research is not related to these companies. The terms of this arrangement have been reviewed and approved by the University of Washington in accordance with its policies governing outside work and financial conflicts of interest in research. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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