New Chapter

Microfluidic Mixers for Standardization of Computer-Assisted Sperm Analysis

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Overview

Maintenance of rare or valuable genetic material is vital for preservation of biomedical research resources derived from model organisms such as zebrafish *Danio rerio* and medaka *Oryzias latipes*. However, the aquarium fish research community is becoming overwhelmed by the maintenance of thousands of research lines as live populations. Cryopreservation is a proven method for large-scale preservation and maintenance of important genetic material. It extends the reproductive potential of males, reduces the need to maintain live fish, and can prevent catastrophic loss of irreplaceable research lines. Cryopreservation is the most cost-effective alternative for maintaining genetic resources of aquatic organisms, because it can reduce costs for fish and facility maintenance, personnel, and space, and accelerate the development of new research lines.

A cryopreservation program requires post-thaw evaluation of sperm quality on a per sample basis. This challenge is magnified when working with a high-throughput system, when non-wild-type strains are being cryopreserved, and when standardization is required among laboratories. Currently the most commonly used method for estimating quality of fish sperm is evaluation of motility. This has been performed qualitatively by microscopic observation, but now increasingly is performed by use of computer-assisted semen analysis (CASA) systems originally designed for use with human and livestock sperm. Unfortunately, zebrafish sperm can lose peak motility rapidly after activation (within 10 sec) and existing CASA systems cannot begin to capture data rapidly enough to reliably monitor the peak motility phase. This problem is exacerbated by use of volumetric viewing chambers that are slow to fill and are subject to swirling currents that can interfere with the CASA analysis. These challenges have been addressed by performing CASA analysis with open glass slides that allow rapid initiation of data collection, but compromise standardization and eliminates features such as automated cell counting offered by the volumetric chamber. Therefore a need exists to develop microfluidic capabilities to enable rapid mixing of sperm cells with activation solution during transport into a volumetric viewing chamber for reliable and accurate assessment by CASA. In addition, microfluidics offers a new field of opportunity for application with aquatic species gametes.

Sperm Quality Analysis as a Critical Hurdle in Aquatic Germplasm Cryopreservation

In the mid-1990s CASA was first applied to use in aquatic species. Since then there have been 46 publications (including conference abstracts and review articles) addressing this topic (reviewed elsewhere in this volume by Yang and Tiersch). Of these, 26 are peer-reviewed primary research articles. The bulk of this research addresses demonstration of the feasibility of CASA application in fish, and as yet no standardization of methodology exists for aquatic species. This is important because there are a number of fundamental differences between the sperm of mammals and aquatic species including cell size, motility activation mechanisms, and swimming speed and duration. Moreover, very few of these publications address thawed sperm and most utilize fresh sperm collected by stripping. Because this early work was based on

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demonstrating the feasibility of CASA for aquatic species, it focused on the output parameters and showed that several were useful for evaluating gamete quality. This work shows great promise for CASA use, but routine application is limited by: 1) lack of clearly established instrument settings, especially for material other than fresh, stripped sperm of fish; 2) lack of standardized protocols, and 3) consequently because of these deficiencies, not taking advantage of the full range of analysis capabilities of these powerful instruments. These problems are compounded by a lack of instruments specifically configured for use with aquatic species, especially with respect to the need to rapidly mix sperm and activating solutions and make accurate readings during the peak phase of motility without interference from swirling or other incidental effects that simulate or obscure actual motility.

Microfluidics Technology for Meeting the Challenges of Sperm Quality Analysis

Microfluidics is the field of study involved with the flow and interaction of fluids on a small scale. Generally, the fluids are controlled in environments where the geometrical dimensions are less than 1 mm, power consumption is low, and sample volumes are below the microliter, and are often in the picoliter range. Most microfluidic devices have been utilized in research settings in conjunction with microscopes to visualize flow. Overall device sizes tend to be the size of a standard microscope slide as seen in Figure 1A. In a microfluidic environment, fluids generally move in strictly laminar flow. This is expressed by the dimensionless Reynolds number ($Re = \frac{VL}{v}$, where V is the fluid velocity, L is the characteristic length (or diameter) of the channel, and v is the kinematic viscosity of the fluid). At low Re the flow is classified as laminar, lacking turbulent flow which typically causes mixing of different fluids at a macroscopic scale. Due to the highly laminar flow (Figure 1B) that occurs in small diameter platforms resulting from low Reynolds number (<100), the physics of these platforms are primarily dominated by viscous forces rather than inertial forces. This means the mixing that does occur is strictly from molecular diffusion of the fluids, generally a much slower process than turbulent mixing. However, with the small scale of microfluidics, the gained benefit of low power consumption and the rapid completion of chemical reactions due to high surface-area-to-volume ratio, has gained prominence for the field

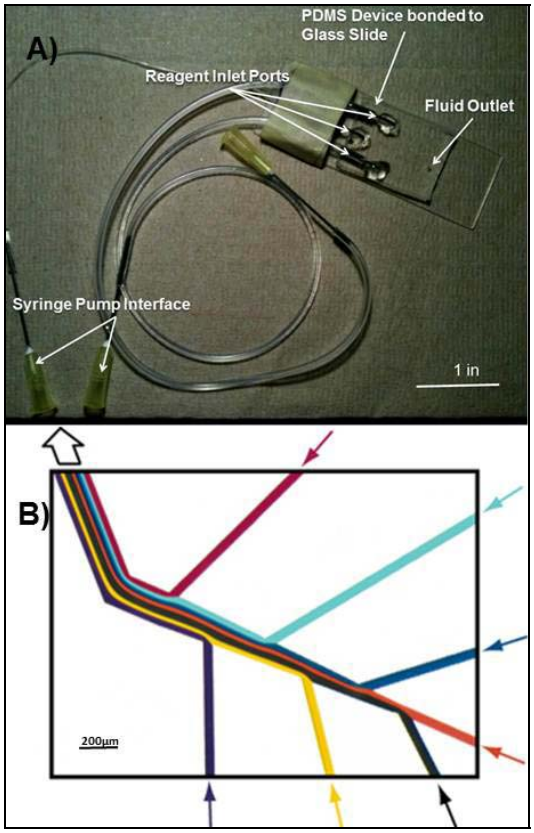

Figure 1. A) Example of a microfabricated chip for sperm analysis, whereby sperm samples are introduced into the microfluidic network through small-bore Tygon tubing. B) Example of laminar flow in a microfluidic network designed by Whitesides and colleagues, where inlet streams of dyed water do not mix. Image in (B) from *Science* cover (2 July 1999) reproduced with permission from AAAS.

in point-of-care diagnostic and care applications as well as processes that require many process steps. These devices, often called Lab-on-a-Chip (LOC) or micro-total-analysis-systems (μTAS) have the ability to integrate multiple laboratory techniques onto a single microfluidic device, potentially reducing the need for large-scale laboratory equipment for many applications such as pathogen detection and gene sequencing.

With respect to more basic and applied aquaculture, microfluidics has yet had little impact. These technologies have been used to shorten analysis times, reduce amount of reagents, and enable new discoveries in cell biology (Beebe et al. 2002), yet few have demonstrated applications for sperm physiology. The exceptions are *in vitro* fertilization studies in mammals, where microchannels have been used for more predictable delivery of sperm to oocytes (Suh et al. 2006), sperm sorting and qualitative selection (Lih et al. 1996, Schuster et al. 2002, Cho et al. 2003, Suh et al. 2005, Seo et al. 2007). A recent report has studied the swimming behavior of bovine sperm in microfluidics, suggesting the influence of side-wall and other microstructures on sperm motility (Lopez-Garcia et al. 2008). While there has been much progress on the mixing of simple solutions in microchannels (e.g., Hessel et al. 2005), no reports exist on the use of microscale fluidic elements to mix solutions containing sperm cells for their activation and assessment.

Design Concepts for Microfluidic Mixers

Microfluidics offers a novel environment to manipulate and study cell activity while reducing costs. With devices the size of a microscope slide, multiple tests can be used to replace larger bench-top analyses. Intricate networks can be created to serve a wide variety of analyses with channels on the order of 100 μm. Because of the absence of turbulence, as stated above, mixing occurs exclusively through molecular diffusion. Therefore, dedicated mixing structures are required to combine sperm samples with activating diluents. Because the physics of flow in these systems is dominated by viscous forces rather than inertial forces, mixing on the microscale has been a notoriously difficult aspect of these platforms, so a component in most microfluidic devices is the micromixer. There are numerous designs that attempt to mix fluids efficiently, and all micromixers are characterized as either active or passive (reviewed in Nguyen and Wu 2005). Active micromixers involve the recruitment of an external field or force to increase perturbation in fluids present in the device. These micromixers are typically more difficult to fabricate and as a result are more costly and less often achieve implementation beyond laboratory experimentation. Passive micromixers utilize specialized channel geometries to increase mixing efficiency by increasing the interface area between fluids with differing concentrations of solutes and solvents. This increases chaotic advection of fluid particles and promotes diffusion of solutions down their concentration gradients. While passive micromixers are typically the most accessible forms of mixers due to their comparable simplicity to active mixers, they tend to be less effective at fully mixing the desired reagents.

A compromise must be determined based on the desired application of mixing. For instance, when mixing aquatic sperm, “complete” mixing may not be required so long as the concentration passes the needed threshold for the desired reaction. Similarly, activated sperm may enhance mixing by the beating of their flagella. It is important to note that traditional active mixers such as electro-osmotic mixers may interfere with the normal activity of the characterized cells leading to artificial results that become difficult to interpret. Passive mixers could also interfere with normal cell activity by introducing a greater amount of shear stress through

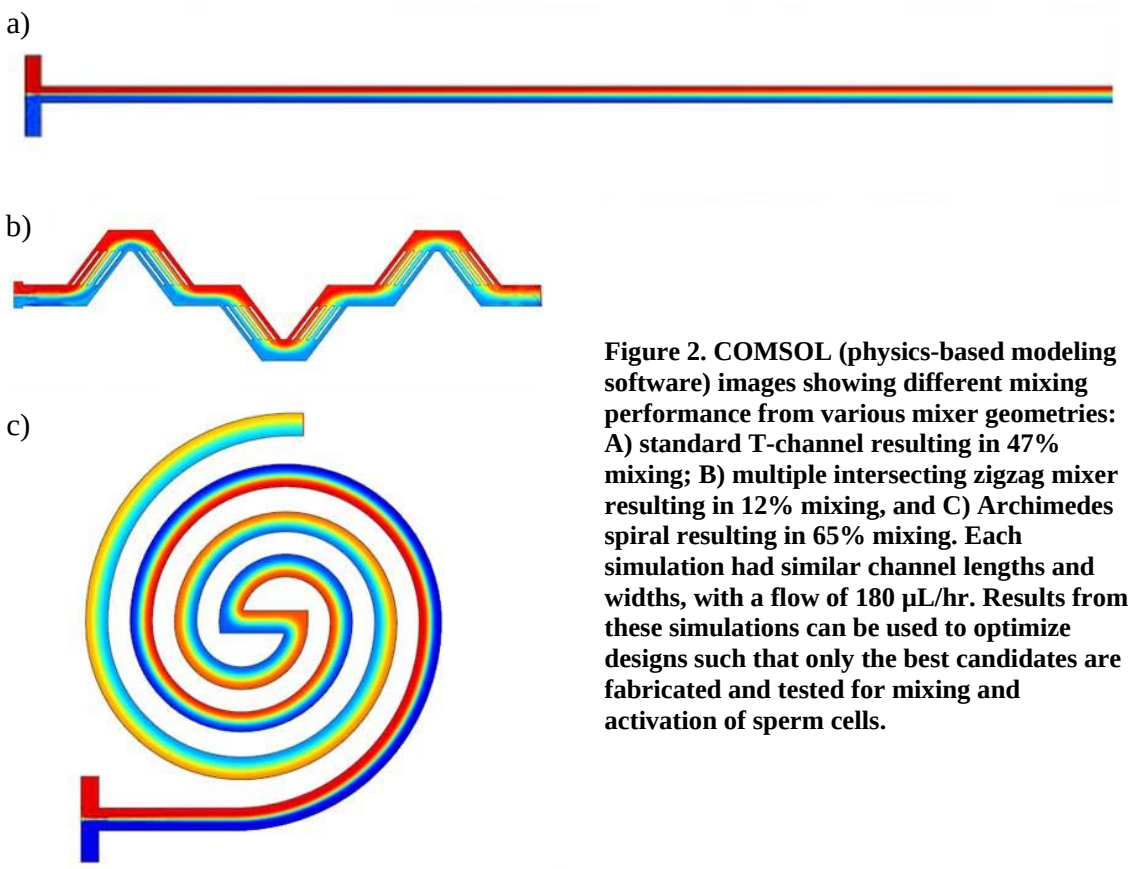

Figure 2. COMSOL (physics-based modeling software) images showing different mixing performance from various mixer geometries: A) standard T-channel resulting in 47% mixing; B) multiple intersecting zigzag mixer resulting in 12% mixing, and C) Archimedes spiral resulting in 65% mixing. Each simulation had similar channel lengths and widths, with a flow of 180 µL/hr. Results from these simulations can be used to optimize designs such that only the best candidates are fabricated and tested for mixing and activation of sperm cells.

complex channel geometries and increased fluid velocities. Constraints such as these require design of passive mixers with simple geometries and high mixing efficiency with a short residence time in the device.

As stated above, because the microfluidic environment is so different than the macrofluidic environments that we are typically exposed to, design of such a mixer can be difficult and expensive in time and materials. At the beginning of design, the required geometries for rapid, effective, non-destructive mixing are often unknown, and this typically requires much prototyping and preliminary testing. However, computing technology has now advanced enough to allow a broader population of researchers to use multiphysics modeling software packages (such as COMSOL, *www.comsol.com*) to predict various properties of microfluidic devices. A micromixer geometry for example can be drawn in a computer-aided design (CAD) software and imported into a modeling application where the fluidic properties (such as density, viscosity, and diffusion coefficient) of the expected reagents can be inputted along with various flow parameters. The final results from the modeling yield a fairly accurate simulation of the micromixer with information such as flow velocity, Reynolds number, and most importantly, concentration at any given point (Figure 2). A workflow of design and modeling can be utilized to design and redesign the micromixers to determine the optimal geometry for the micromixer before any prototype is fabricated. After several candidates have been shown to have effective mixing results in the simulations, these mixers can be fabricated and tested with dyes to evaluate mixing in a real world setting. The use of this modeling technique will not always yield perfect results, however; utilizing it can guide the development process to save resources and optimize the design of the microfluidic structures.

Microfabrication Methods of Microfluidic Devices for Sperm Analysis

The overall fabrication process of microfluidic devices consists of multiple steps including design, microfabrication, generation of through-holes, sealing, and incorporation of macro-to-micro interfaces (Figure 3A). Design of microfluidic devices based on performance simulation results is carried out using CAD tools prior to microfabrication processes. Generation of microstructures for microfluidic devices is preceded either by fabrication of a mask or fabrication of a master mold (Figure 3B). Masks are fabricated by either patterning of a thin metal layer on glass, or high-resolution printing on a transparency (Weibel et al. 2007). Fabrication of master molds are done typically by lithography of photoresist (Whitesides et al. 2001), high-precision micro-milling of metal such as brass (Hupert et al. 2007), or electroplating of nickel (Park et al. 2008).

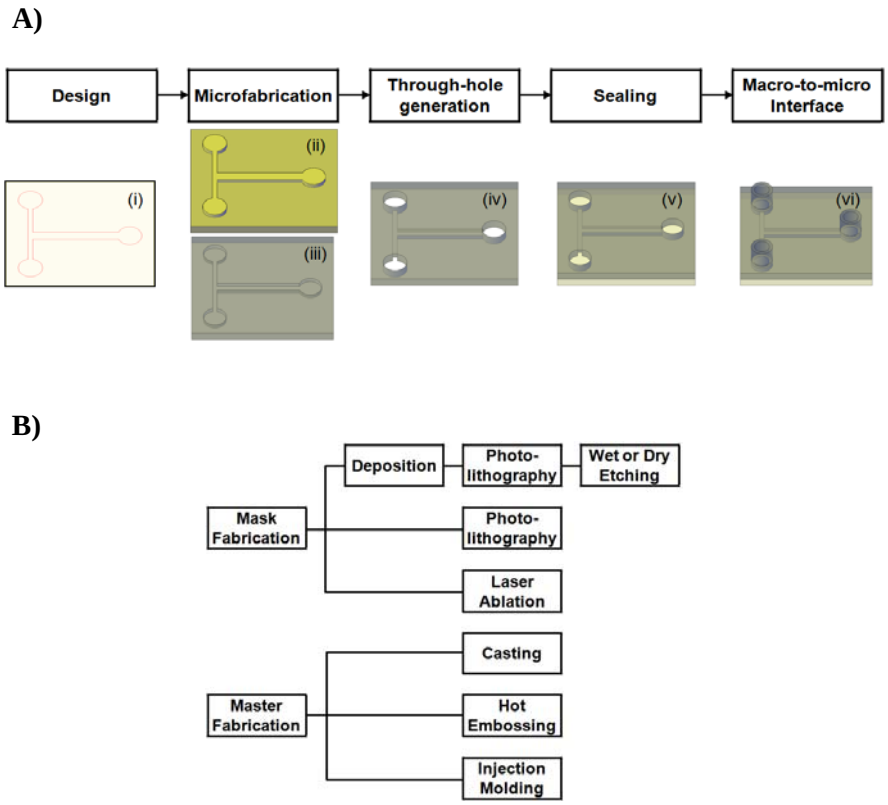

Figure 3. A) The overall fabrication process of microfluidic devices: i) design of a microfluidic device, ii) fabrication of a master mold, iii) fabrication of a microfluidic device, iv) generation of through-holes, v) sealing, and vi) incorporation of macro-to-micro interface. B) The steps in microfabrication processes commonly used for fabrication of microfluidic devices.

Methods of generating features and devices on scales from nanometers to micrometers have been put forth by the well-established semiconductor industry for purposes of creating integrated circuits, where smaller circuit features equates to faster microchips. These same techniques have enabled the fabrication of microfluidic devices on silicon or glass by use of deposition of metal or dielectric materials, lithography of photoresist materials through a

fabricated mask, and wet or dry etching (Beebe et al. 2002). Wet etching of silicon is commonly carried out with potassium hydroxide for anisotropic etching (Zou et al. 2005) and wet etching of glass with hydrofluoric acid for isotropic etching (Liu et al.2006). Dry etching of silicon is preferred for fabrication of high aspect ratio structures using tetrafluoromethane gas or sulfur hexafluoride gas chemistries (Nagrath et al. 2007). Those approaches have been applied to the study of gametes, such as manipulation of single oocytes in silicon microfluidic devices using wet and dry etching (Zeggari et al. 2007). With glass devices using wet etching, studies have been carried out for separation of mammalian sperm from epithelial cells (Horsman et al. 2005), on-chip cell lysis and DNA extraction from sperm cells (Bienvenue et al. 2006), and on-chip determination of spermatozoa concentration (Segerink et al. 2010). Glass was one of the first materials of choice because it allows for constant observation of on-chip processes via standard light microscopy. From a fabrication perspective, glass was chosen because of its well-established fabrication techniques, but it also carries the drawbacks of high material and process costs, and limited disposability for microfluidic devices (Fiorini and Chiu 2005). Other microfabrication methods are available for fabrication of microfluidic devices including direct patterning of thermoset polymers by photolithography (Sikanen et al. 2010) and laser ablation (Malek 2006a, Malek 2006b).

Microfabrication of polymer devices with fabricated master molds in the past decade has taken advantage of their low cost and disposability. Soft lithography of polydimethylsiloxane (PDMS) casting against a photoresist master mold has become a typical method due to its low material costs, simple microfabrication process, and disposability. Examples include human applications such as: *in vitro* fertilization (Suh et al. 2006), gamete and embryo isolation and culture (Suh et al. 2003, Smith et al. 2007), sperm sorting and separation (Cho et al. 2003, Suh et al. 2005, Wu et al. 2006, Seo et al. 2007), and study of swimming behavior with bovine sperm (Lopez-Garcia et al. 2008). Micro-molding of thermoplastics such as polymethyl methacrylate (PMMA), polycarbonate (PC), and cycloolefin copolymers (COC) can be utilized via hot embossing or injection molding (Becker and Gärtner 2008) to overcome the limited compatibility of PDMS to organic solvents and low microfabrication throughput and to open new opportunities for study of sperm physiology. Table 1 shows the summary of microfabrication methods and materials for microfluidic devices, providing general guidelines for choice of methods and materials of microfluidic devices for sperm analysis.

Table 1. Summary of microfabrication methods and materials for microfluidic devices.

Materials	Silicon	Glass	PDMS	Thermoplastics	
Microstructure generation	Wet or dry etching	Wet etching	Casting	Hot embossing	Injection molding
Material cost	Medium	Medium-high	Low	Low	Low
Process difficulty	Medium	Medium	Easy	Easy-medium	Medium
Process cost	High	Medium-high	Low	Medium	Medium-high
Fabrication throughput	Low	Low	Low	Medium	High
Solvent resistance	High	High	Low	Low-medium	Low-medium

Post-microfabrication processes including generating through-holes, sealing, and incorporating macro-to-micro interfaces are used to complete functional microfluidic devices. Through-holes for fluidic access are commonly generated by drilling or punching of holes to which macro tubing is connected (Liu et al. 2006, Christensen et al. 2005). Sealing of

microfluidic devices is carried out by a variety of methods such as anodic bonding, thermal fusion bonding, adhesive bonding, solvent-assisted bonding, welding, surface modification, and lamination (Zhang et al. 2006, Tsao et al. 2009). For fluidic handling, a connection between a microfluidic device and the macro-environment such as a syringe pump is required to minimize pressure drop, dead volume, and leakage. This present challenges in utilizing microfluidic devices in real world settings and are often referred to as “macro-to-micro interfaces”, interconnects, or “world-to-chip interface” (Fredrickson and Fan 2004). Common methods of macro-to-micro interface are direct tubing with epoxy (Park et al. 2010), PDMS interconnects (Li and Chen, 2003), pressure-fit of a reusable needle with a luer connector (Christensen et al. 2005), integrated interface using self-aligned interconnects with flanged tubing (Puntambekar and Ahn 2002), and embedded interconnecting ports in polymer chips (Lee and Kwon 2010). Modular interfaces with a housing including capillaries, tubes, or other macrofluidic components as a separate module can be used for high-throughput analysis (Nittis et al. 2001, Yang and Maeda 2003).

Preliminary Studies of Microfluidic Devices for Fish Sperm Activation and Analysis

During the spring of 2009 we worked with an undergraduate design team to create a micromixer capable of activating zebrafish sperm. The goal of the project was to create a microfluidic device that could mix a sperm sample with water for activation and analysis via CASA (Figure 4). Our initial design criteria were to evaluate a variety of micro-architectural designs and geometries for mixing capability, and to deliver a 1 µl sperm sample to a defined volume for analysis by CASA within 15 sec of activation. We evaluated two mixing designs, a herringbone depression chaotic mixer (Stroock et al. 2002) and a combination of multiple intersecting and a zigzag microchannel design (Bing et al. 2001, Mengeaud et al. 2002). Molds of microfluidic channels with both features were prototyped using a micromill. A positive brass master was hot-embossed into a PMMA blank which was used as a negative mold. Advantages of this approach were the reduction in machining marks at the surface of channels and chambers, which can occlude transparency. A feature resolution of 0.1 µm, precision of ±1 µm, and aspect ratio of 10:1 (depth:width) was produced with this system, and the mold was filled with PDMS which, following curing, was bonded to a glass slide. Both designs contained two inlets so that dye could be injected through one and water through the other to evaluate mixing based on the design features. Syringe pumps (NE-500, New Era Pump Systems, Inc.) were used to drive matched flows between the two inlets at rates from 50 to 250 µl/hr.

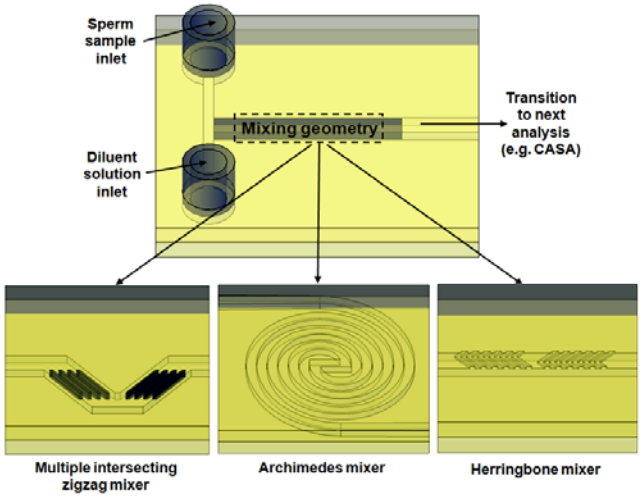

Figure 4. Schematic diagrams of a microfluidic device and three candidate geometries of micromixers (multiple intersecting zigzag, Archimedes, and herringbone) for mixing of sperm with a diluent solution for activation and delivery to CASA.

Light microscopy was used to visualize mixing (Figure 5) where the herringbone features showed superior performance over the multiple intersecting zigzag microchannel design. Inspection of the inlets of both designs showed the challenge of mixing in these microchannels where flow is essentially laminar (with Reynold's numbers of <10). A boundary of sharp contrast can be seen between the neighboring dye and water flow streams, with no mixing until subjected to mixing geometries.

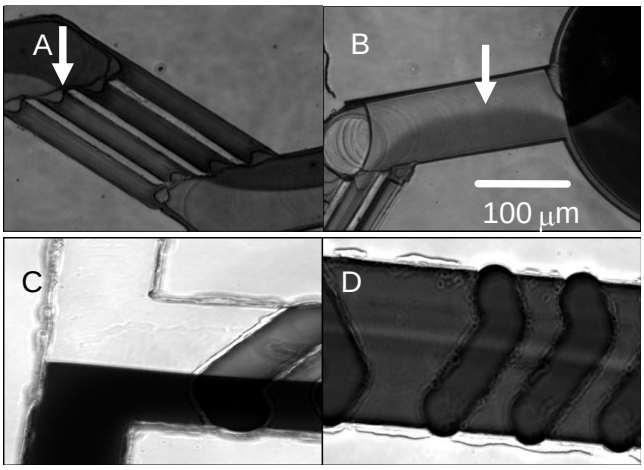

Figure 5. Multiple intersecting zigzag microchannels (A-B) and herringbone mixers (C-D) for mixing in a microfluidic channel. The herringbone design provided more complete mixing by the end of the microchannel (D) than did the multiple intersecting zigzag design (arrow indicates unmixed fluid in B).

Based on the preliminary success of the herringbone mixer adapted for use at these scales, we performed a more quantitative analysis of mixing using fluorescein dye (1 mM) in one inlet which enabled more sensitive probing of mixing over several series of mixing elements in a microchannel (Figure 6). To quantify mixing as a function of length along the channel (or residence time at a given flowrate,) *Equation 1* (Johnson et al. 2002) was used, where N represents the number of measurements taken for a distance, I_i represents the intensity recorded for event *i*, I_i^0 represents the intensity with no fluorescence, and $I_i^{perf\,mix}$ represents the intensity of a perfectly mixed solution. The equation relates the difference of a recorded intensity to a perfectly mixed solution and one with no fluorescence. The results of this design indicate that 95% mixing efficiency can be achieved in less than 2 sec with 100 µl/hr flow rates in the herringbone mixer (Figure 7).

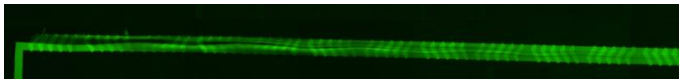

Figure 6. Collage of fluorescent images showing water (top left inlet) mixing with fluorescein (bottom left inlet) while flowing (100 µl/hr) along a series of herringbone elements.

$$Eqn\ 1:\ \text{Mixing Efficiency} = \left(1 - \frac{\sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(I_i - I_i^{perf\,mix}\right)^2}}{\sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(I_i^0 - I_i^{perf\,mix}\right)^2}}\right) \times 100$$

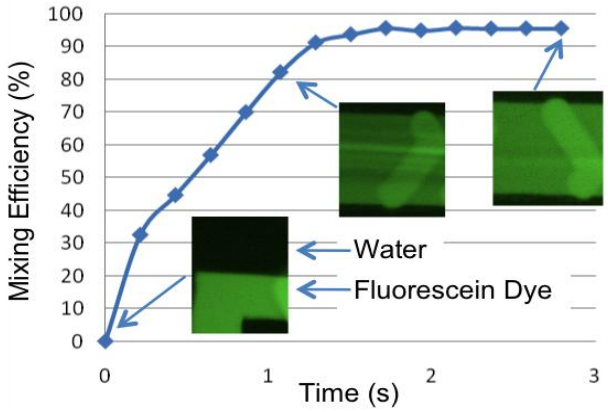

Figure 7. Mixing of fluorescein and water across herringbone mixing elements occurred in less than 2 sec (at 100 µl/hr) in the prototype design.

We evaluated the ability of the herringbone device to activate zebrafish sperm by mixing with water to induce activation. In this case, the PDMS mixing device was bonded to a standard CASA slide and evaluated on a CASA system

(CEROS model, Hamilton-Thorne). Sperm samples mixed with isotonic HBSS showed no motility, negating shear or other device effects on activating sperm. Sperm samples from the same stock were activated by hand (48% motile) and in the device (52% motile) to evaluate if zebrafish sperm could be activated by the device and analyzed by CASA. It was also noted that delivery of the sample into the chamber was accomplished within 10 sec after cessation of flow, with minimal drift within the chamber. The lack of drift achieved with this system may overcome the significant problem of swirling currents that occur often in hand-mixing and complicate accurate CASA measurements. These preliminary results show the promise of microfluidics for sperm analysis. Further design optimization is necessary to obtain effective mixing elements that protect sperm from damage. Expansion of microfluidic devices to include new functionalities beyond mixing and CASA will be necessary to accomplish more robust processing and testing of sperm physiology.

Future Application of Microfluidics to Assessment of Sperm Motility

Subjective estimation of sperm motility is the most frequently used measure of sperm quality in aquatic species (Terner 1986, Tiersch and Mazik 2000). Motility estimates are most reliably performed using darkfield microscopy. The use of CASA instruments specifically configured for use with aquatic species will significantly advance study and banking of aquatic germplasm. Acitivities such as the routine assessment of sperm quality for fresh and post-thaw samples, and determination of sperm activation curves as a function of osmolality will be accelerated by the availability of microfluidic devices, where successive solutions can be rapidly mixed, analyzed by CASA, and rinsed through the device to increase throughput. Similarly, the effects of cryoprotectants and other potential toxins on sperm viability could be studied in shorter time frames following exposure compared to the time scales of current protocols. It is hoped that the application these types of design principles to marine sperm analysis will also enable higher throughput analysis in order to meet the demands of the increasing numbers of model organisms requiring cryopreservation. It is important to note that work of this type is only possible through interdisciplinary collaboration combining engineering, microfabrication, and biology.

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