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Rethinking gamete/embryo isolation and culture with microfluidics

Ronald S.Suh[1], Nandita Phadke[2], Dana A.Ohl[1], Shuichi Takayama[2] and Gary D.Smith[1,3,4,5]

[1]Department of Urology, [2]Department of Biomedical Engineering, [3]Department of Obstetrics and Gynecology and [4]Department of Molecular and Integrated Physiology, University of Michigan, Ann Arbor, MI 48109, USA

[5]To whom correspondence should be addressed at: 1150 W. Medical Center Drive, Medical Science I, Room 6428, Ann Arbor, MI 48109-0617, USA. E-mail: smithgd@med.umich.edu

IVF remains one of the most exciting modern scientific developments and continues to have a tremendous impact on people's lives. Since its beginnings, scientists have studied and critically analysed the techniques in order to find ways to improve outcomes; however, little has changed with the actual technology and equipment of IVF. Semen is still processed in test tubes and fertilization and culture still occurs in culture dishes. New technological possibilities exist with the burgeoning advancement of microfluidic technology. Microfluidics is based on the behaviour of liquids in a microenvironment. Although a young field, many developments have occurred which demonstrate the potential of this technology for IVF. In this review, we briefly discuss the physical principles of microfluidics and highlight some previous utilizations of this technology, ranging from chemical analysis to cell sorting. We then present the designs and outcomes for microfluidic devices utilized thus far for each step in IVF: gamete isolation and processing, fertilization, and embryo culture. Finally, we discuss and speculate on the ultimate goal of this technology—development of a single, integrated unit for in-vitro assisted reproduction techniques.

Key words: embryo culture/gamete isolation/IVF/microfluidics/semen processing

Introduction

Perhaps one of the most exciting and revolutionary scientific developments of the past 25 years has been the development of IVF to treat human infertility. It is impossible to quantify its impact on numerous families since the first IVF birth in 1978 in Oldham, UK (Steptoe and Edwards, 1978). Utilization of IVF and related medical procedures, categorized as assisted reproductive technology, has increased through the years, with an estimated 20 143 live-birth deliveries in the USA resulting from assisted reproductive technology in 1998 (Centers for Disease Control and Prevention, 2002). The following year, there were a reported 399 assisted reproductive technology clinics in the USA conducting a total of 86 822 assisted reproduction cycles with 30 285 babies born (Fountain and Krulewitch, 2002).

With increasing medical utilization of assisted reproductive technology, scientists and clinicians have been able to study extensively the process of IVF. Critical analysis of each step has improved outcomes and led to a greater understanding of reproductive physiology. Careful attention to sperm processing and isolation to increase recovery of motile sperm and reduce sperm damage has improved fertilization rates and embryo development (Mortimer, 1994). Use and refinement of ICSI has allowed fertilization even in severe cases of compromised sperm quality or number (Bonduelle *et al*., 1999). Finally, refinement of embryo culture has led to greater care spent on this aspect of assisted reproduction (Gardner and Lane, 1998; Mahadevan, 1998). Most scientific attention, however, has focused on methodologies rather than technology and equipment. Semen is still processed in test tubes regardless of technique, sperm are physically placed with oocytes after processing, and fertilization and embryo culture occur in culture dishes with variations in volume and distribution only (Trounson and Gardner, 2000). With the exception of ICSI and micromanipulation of gametes, no technological advancements in IVF have reached widespread use. Nevertheless, it is precisely those technological advancements, rather than procedural or methodology changes, that have had the greatest impact on assisted reproduction.

A promising new technology, microfluidics, does exist and is becoming increasingly studied; this technology shows promise as an alternative for each step in the process of IVF previously mentioned (Figure 1). Microfluidics, based on physical principles of fluid behaviour in a microenvironment, has been used extensively in chemical (Tomlinson *et al*., 1995) and molecular biology (Beebe *et al*., 2002a) applications. Although not termed microfluidics, Willadsen and colleagues first reported on the importance of microenvironment and embryo handling/culture in the 1970s (Willadsen, 1979). Currently, microfluidics is gaining interest in studies of cellular behaviour and interactions (Shim *et al*., 2003).

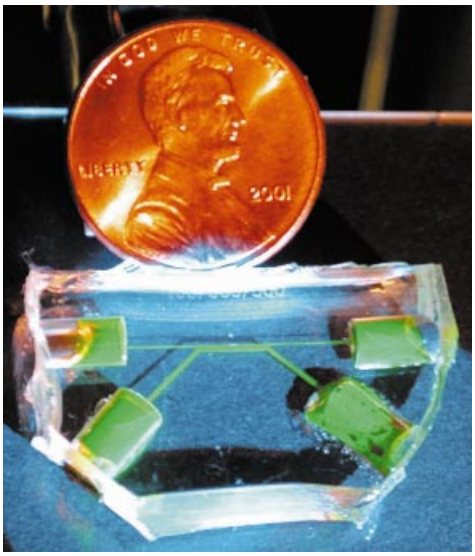


Figure 1. Currently used microfabricated devices utilizing microfluidics. Note the size of the device and its channels in comparison to a US penny.

With advancements in microtechnologies, a resurgence in investigating microfluidics and assisted reproductive technology has occurred (Glasgow *et al*., 2001; Schuster *et al*., 2003). In this review, we briefly introduce basics of fluid behaviour at the microscopic scale and highlight previous utilizations of this technology outside of the reproductive sciences. We then describe fabrication of devices and outline developments and outcomes in IVF using microfluidics. Finally, we provide speculation on future directions and potential of this technology.

Fluid behaviour in a microenvironment

Fluid mechanics is a complex physical science; therefore, an extensive technical description and review of fluid physics is well beyond the scope and intent of this manuscript. We will instead discuss basic physical principles which govern fluid behaviour in a microenvironment, especially those aspects with specific applicability to devices and technology currently being developed for IVF. We have purposely avoided including mathematical details, choosing instead to convey a general conceptual sense of fluid mechanics present within microchannels. Interested readers wishing to gain a more complete technical and mathematical understanding of microfluidic physics should consult reviews from Beebe *et al*. (2002a) and Brody *et al*. (1996), or references therein.

Fluids at the microscale are subject to forces typically not important at scales present in our everyday lives. Fluid at the scale of our normal environment is turbulent, meaning that a particle within a stream of fluid moves in an unpredictable pattern. Turbulent flow depends on certain fluid characteristics (viscosity, density, and velocity) and the geometry and size of the channel, leading to calculation of a value known as the Reynold's number. As the scale of the channel reaches micrometer levels, the Reynold's number decreases. Decrease of the Reynold's number below a threshold value leads to fluid flow in a laminar fashion. In other words, the flow within the channel becomes streamlined and predictable (Figure 2). The behaviour of fluid becomes increasingly governed by viscous forces and surface tension, which can be described as the cohesiveness of the liquid's molecules.

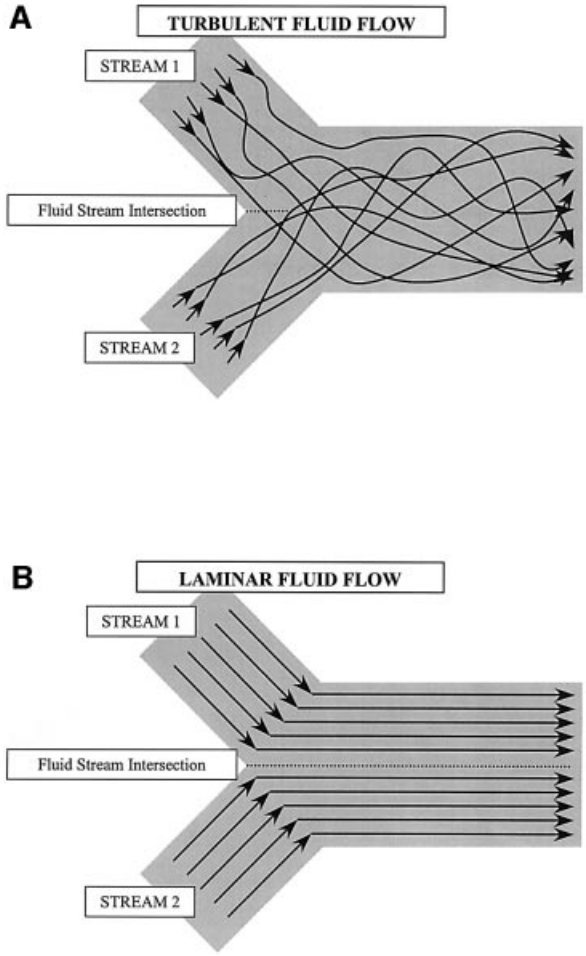


Figure 2. Schematic representation illustrating fluid interaction in turbulent (**A**) and laminar (**B**) flow patterns.

This dominance by viscous forces results in several interesting phenomena. Low Reynolds number flow possesses little to no momentum, and therefore fluid within a microchannel responds quickly and reliably to changes in external forces. In addition, at the microscale, two or more streams of laminar flow in contact with each other do not mix except by diffusion of molecules within the fluid across the interface of the streams. Diffusion is the process by which a concentrated group of particles in a volume will distribute themselves throughout the volume over time so that the average concentration in the total volume is uniform. The rate of diffusion between the contacting surfaces at the microscale may be very quick, partially due to the relatively short distances needed to cross fluid volumes. As the environment shrinks to microscale, the surface area-to-volume (SAV) ratio also increases. This increase in SAV ratio limits turbulence, ultimately affecting the microenvironment.

Many of these fluid characteristics at the microscale form the principles driving the interest in utilizing microchannels for gamete/embryo manipulation and in-vitro insemination. In general, a microenvironment more closely resembles the in-vivo

conditions of fertilization and development when compared with a culture dish or drop of media. Movement and rotation of the oocyte is believed to increase fertilization (Nir, 2002) and provide physiological benefits (Croxatto, 2002). In addition, maintenance of oocytes and subsequent embryos in microchannels with fluid flow allows contact of media on all surfaces of gametes/embryos and provides for fresh media without cell manipulation. Further explanation of the advantages of fluid behaviour at the microscale will be addressed later in each specific application of microfluidics.

Non-reproductive applications of microfluidics

Interest in microfluidics began with attempts to miniaturize chemical and biological analysis devices in the laboratory and has resulted in an ever-growing list of designs and uses (Kricka, 1998). An all-language survey of numerous publication and patent databases conducted by Kricka and Fortina (2002) almost 2 years ago resulted in >140 references specifically utilizing microfluidics for analytical purposes. Current designs are often referred to as 'laboratory-on-a-chip', or micro-total analysis systems (μTAS), and function by allowing a variety of chemical processes and interactions to occur as fluid flows within their miniature channels and chambers (Weigl and Yager, 1999). These devices perform all the analytical functions necessary for their purpose, including sample handling, mixing, incubation, sorting, transport, interaction, and detection or signalling within an integrated microfluidic 'chip'.

One example of a working, commercially available microfluidic device is the Biosite Triage® Cardiac System (USA) (Apple *et al*., 1999). Within this device is a microfluidic protein chip which accepts a blood sample, separates the plasma and directs it via capillary flow through microfluidic channels to a reaction chamber (Schulte *et al*., 2002). Presence of the compound of interest in the plasma results in a fluorescent reaction which is detected by proprietary fluorescent technology. Additional designs abound and have been used for sample purification (Footz *et al*., 2001; Jandik *et al*., 2002), chemical analysis and synthesis (Teuschel, 2001; Jones *et al*., 2002) (so-called microreactors), and a variety of molecular biology techniques. These include immunoassays for antibodies present in serum (Linder *et al*., 2002), performance of DNA PCR and subsequent separation steps (Regnier *et al*., 1999; Wabuyele *et al*., 2001), and assays determining enzyme reaction kinetics (Xue *et al*., 2001; Yakovleva *et al*., 2002).

In addition, applications in cellular analysis have emerged, such as integrated cell-sorting devices working at the microscale (Fu *et al*., 2002) and microfluidic devices which allow for the study of cellular interactions with substrates or other cells (Shim *et al*., 2003). An illustration of the potential of cell analysis with microfluidics utilizes the principle of laminar flow, allowing for selective exposure of subcellular areas of interest to membrane-permeable molecules (Takayama *et al*., 2001). This process, dubbed PARTCELL (partial treatment of cells using laminar flow), has been used to study subcellular processes without perturbation or influence on areas of non-interest. Parallel laminar flow streams have been applied to opposite poles of a live bovine endothelial cell. One stream was used to deliver a compound which interferes with actin, resulting in disruption of the cytoskeleton only on the exposed portion of the cell. Such precise delivery of molecules to cellular subdomains illustrates the precision with which microfluidic regulation of fluid flow is capable.

Advantages of such 'laboratory-on-a-chip' technology are multiple. First, once designed and tested, the manufacture of such devices is straightforward and inexpensive, allowing them to be disposable (McDonald *et al*., 2000). This avoids the possibility of cross-contamination by chemicals or cells, especially when used for highly sensitive detection assays. Microfluidic analysis devices utilize very low volumes of samples and reagents and provide for faster reactions and response times (Weigl and Yager, 1999). Miniaturization not only allows for portability, but more importantly allows for integration of multiple processes within a small, self-contained unit (Kricka, 1998). This can be translated into either multiple parallel analyses, consecutive serial processes, or both. Whether these advantages will result in future acceptance of microfluidics in assisted reproductive technology is difficult to predict.

The brief overview given here is only intended to familiarize readers with the variety of capabilities of microfluidic technology and is by no means a comprehensive listing of microfluidic applications in the sciences. Readers are encouraged to consult more thorough reviews by Verpoorte (2002) and Khandurina and Guttman (2002).

Fabrication of microfluidic devices

Most initial microfluidics systems were fabricated using materials and techniques common in the industry that inspired them—microelectronics (McDonald *et al*., 2000). Photolithography and etching of silicon and glass was a highly developed technology also readily available to researchers interested in miniaturizing analytical systems. However, the cost of these materials was significantly higher for the much larger-sized typical microfluidic 'chip' versus a microelectronic chip (Boone *et al*., 2002). In addition, the production processes for these devices proved to be complex for large-scale production. Cleanroom environments are necessary and sealing processes are time-consuming, require special equipment, and often result in low yields (McDonald and Whitesides, 2002). Dr Beebe's laboratory initially used a silicon-glass design in their embryo culture microfluidic device, but ultimately abandoned it due to difficulty with the fabrication process and problems visualizing embryos through the opaque silicon.

In the search for a suitable alternative, polymers have quickly emerged as good candidate materials for microfluidic device fabrication, especially for biological applications (McDonald *et al*., 2000). Compounds such as poly(methylmethacrylate) (Martynova *et al*., 1997), fluorinated ethylene propylene (Sahlin *et al*., 2002), and poly(dimethylsiloxane) (PDMS) (McDonald and Whitesides, 2002) are cheaper and easier to manipulate than silicon-glass alternatives (Martynova *et al*., 1997). They can be formed by molding rather than etching and can be easily sealed thermally or with adhesive compounds (McDonald *et al*., 2000). PDMS in particular has become one of the most actively explored and promising materials thus far, possessing numerous characteristics specifically suitable for biologic use. It is non-toxic, transparent, insulating, and permeable to gases (McDonald and Whitesides, 2002). From a fabrication standpoint, PDMS permits sub-

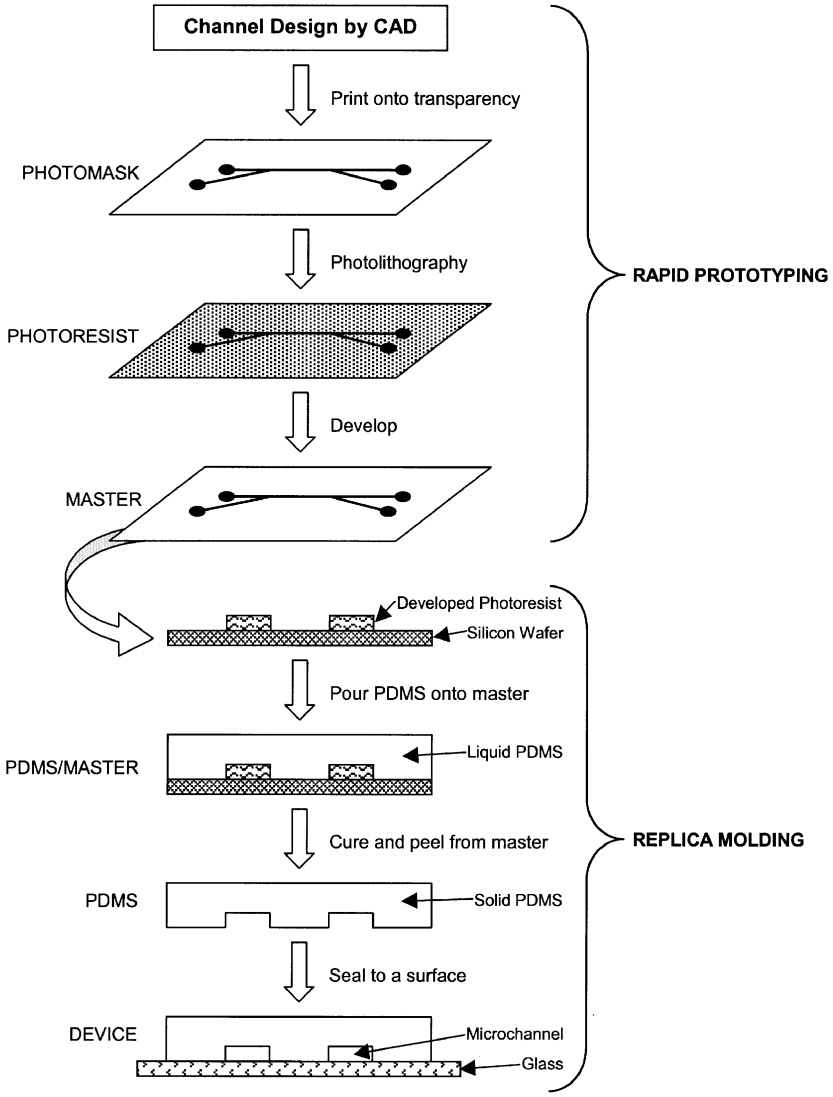


Figure 3. Scheme for soft lithography.

micrometer fidelity with molding, cures at low temperatures, and can easily seal reversibly to itself and a host of other materials (McDonald *et al*., 2000)

Microfluidic devices used in gamete or embryo handling have been constructed using PDMS with or without glass components. Fabrication is done using techniques based on soft lithography (Figure 3). The first step, rapid prototyping, generates a master on a silicon wafer which is used as the pattern mold for the actual device (McDonald *et al*., 2000). A computer-assisted design (CAD) program is used to generate the structure and size of the microchannels, which are then printed onto a transparency using a high-resolution commercial image setter. This transparency is used as the photomask for subsequent photolithography, and allows for generation of features and channels as small as 20 μm (McDonald and Whitesides, 2002). Photolithography using the transparency is conducted on a photocurable epoxy bonded onto a silicon wafer at the desired channel height. Once exposed and developed, this master can be used indefinitely, limited only by the fragility of the silicon wafer. Liquid pre-polymer PDMS is then poured onto the wafer and allowed to cure, generating a negative pattern of the master in PDMS. In other words, ridges on the silicon wafer form the microchannels within the PDMS device.

The network of channels within a microfluidic device can range from very simple to highly complex. Interactions between streams of fluid can be rendered in three dimensions. Two layers of PDMS microchannels can be overlaid, allowing for placement of membranes or other mediators between interacting fluid streams at intersections (McDonald and Whitesides, 2002). Final processing involves affixing the PDMS device to additional PDMS or glass with plasma oxidation, which also makes channel surfaces hydrophilic, allowing for improved fluid flow (Cho *et al*., 2003).

Although PDMS is generally regarded as non-toxic, special consideration must be given to its use in IVF because gametes and embryos can be particularly sensitive to their environment in comparison with somatic cell lines. Prior to use of microfluidic devices with embryos and sperm, testing confirmed that no

negative effects resulted from prolonged exposure to materials used in their fabrication. Glasgow *et al*. (2001) found that development of 2-cell mouse embryos to the blastocyst stage was unchanged by continuous exposure to numerous photolithography compounds in comparison with controls. Subsequent tests on embryos with PDMS alone showed no difference compared with traditional microdrop controls (Beebe *et al*., 2002b). Schuster *et al*. (2003) reported that 30 min exposure of PDMS did not alter sperm survival. Thus it appears that PDMS-composed microchannels or the materials used in their construction do not confer deleterious effects onto gametes or embryos.

Sperm isolation with microfluidics

Since the onset of IVF, significant efforts have improved methods of semen processing and sperm isolation. Currently, the swim-up technique or density gradient separation are the methods of choice (Trounson and Gardner, 2000). Both result in adequate recovery of motile sperm, although additional steps may be necessary in poor quality semen samples (Bourne *et al*., 1995a,b). Some researchers, however, have criticized these methods because these techniques may damage DNA and produce oxygen free radicals (Aitken and Clarkson, 1988; Zini *et al*., 1999). In addition, these techniques can be labour- and/or time-intensive. Ideal sperm isolation would involve a simple, rapid and atraumatic method to obtain sufficient motile sperm for use in either IVF or ICSI, depending on need and the quality of the original semen sample.

Attempts have been made to develop devices for such a purpose. The Wang tube (Wang *et al*., 1992), a uniquely configured glass tube, allows motile sperm to progress to an upper arm which is then separated for sperm use in intrauterine insemination or IVF. Wang reported using this device to recover sperm for IVF in 1992 with a clinical pregnancy rate of 31.6% (18/57). Comparison testing with swim-up and density gradient separation for normozoospermic samples revealed greater motility and morphology with the device (Wang, 1995). More recently, Lih *et al*. (1996) developed and tested a Lucite microchamber. The chamber consisted of a central loading well surrounded by slightly depressed sidewells, and was conceived on the observation that motile sperm migrate to the periphery of microdrops. The device was shown to concentrate motile sperm up to 13-fold in the sidewells, yielding a sufficient number for use in ICSI. In addition, hamster oocytes placed within the sidewells, serving as oocyte repositories, resulted in hamster oocyte sperm penetration in 12 of 19 cases (64%) (Gordon and Chen, 1995).

Microfluidics has been explored for sperm diagnostic purposes. In 1993, Kricka *et al*. (1993) designed and fabricated silicon and glass microfluidic devices for sperm motility evaluation. They evaluated sperm progression along the length of a microchannel (80 μm wide × 20 μm deep), and navigation through a network of branching channels. In their initial study, they demonstrated the feasibility of such a device and hypothesized that it could replace conventional methods of motility assessment and semen analysis. Following that, they demonstrated that sperm movement within microchannels, judged by the time needed to reach the end of the channel, correlated with forward progression scores (Kricka *et al*., 1997) However, the design of the device did not give any reliable information regarding sperm concentration or percentage motility, and therefore could only serve as an adjunctive test of motility and forward progression rather than a comprehensive semen analysis tool.

Schuster *et al*. (2003) developed a microfluidic device taking advantage of parallel laminar flow streams present at the microscale. In this device, a flowing stream of semen was placed in parallel to a flowing stream of media within a microchannel. Flow within microchannels was maintained by a novel gravity-driven, horizontally oriented pumping system developed specifically for the device (Cho *et al*., 2003). This allows for passive, not active, pumping of fluids. Such a system removes the need for external pumping, pump regulation, and problems associated with active pumping. As discussed, these two parallel laminar flow streams mix only by diffusion. Motile sperm diffused across contacting surface areas and deviated from the initial streamline into the media stream for collection, whereas non-motile sperm and cellular debris remained in the initial stream and exited the device (Figure 4).

Testing of this laminar flow sorting system was performed using 40 μl of unprocessed human semen, followed by semen samples artificially filled with debris using a stock solution of round immature germ cells to simulate poor quality samples. For unprocessed semen, the device consistently produced a sorted fraction with increased motility (mean 98% motile) and improved strict sperm morphology (mean 22% normal forms) versus the initial specimen (mean 44 and 10% respectively). For debris-filled samples meant to simulate very low quality specimens, the device not only concentrated motile sperm (mean 98% motile) within the collected fraction, but was also able to produce a round cell:sperm ratio of 1:33 compared with a 10:1 ratio in the starting specimen (Schuster *et al*., 2003).

This device provided a simple, atraumatic method of obtaining motile sperm of increased normal morphology from both unprocessed normal semen and poor quality specimens containing significant debris. This concurrent isolation of sperm with increased normal morphology with increased percentage motility following sorting is not surprising considering past reports of sperm motility and morphology correlations (Katz *et al*., 1982). A limitation of the device regards the flow rate, estimated at ~20–40 μl/h. In its current form, it is not capable of processing an entire semen specimen; however, it does provide a means of quickly and easily isolating a small sample of motile, normal morphology sperm for ICSI, insemination in microdrops under oil, or, as we will discuss shortly, IVF in a microfluidic environment. It is important to recognize that this device does not isolate every motile sperm, and studies are ongoing to make the isolation process more efficient. However, it is imperative to appreciate that future applications/integrations of the sperm sorter may not require absolute efficiency. In addition, modifications and improvements in the design are in progress, which may allow for large-scale processing in parallel and increased efficiency of flow and sorting (Schuster *et al*., 2003).

Oocyte handling with microfluidics

For human IVF, there is little need for significant manipulation of mature oocytes retrieved by ovulation induction and ultrasound-guided transvaginal oocyte retrieval (Trounson and Gardner, 2000). Oocytes are typically incubated for up to 4 h prior to insemination. Approximately 16 h later, it is necessary to denude

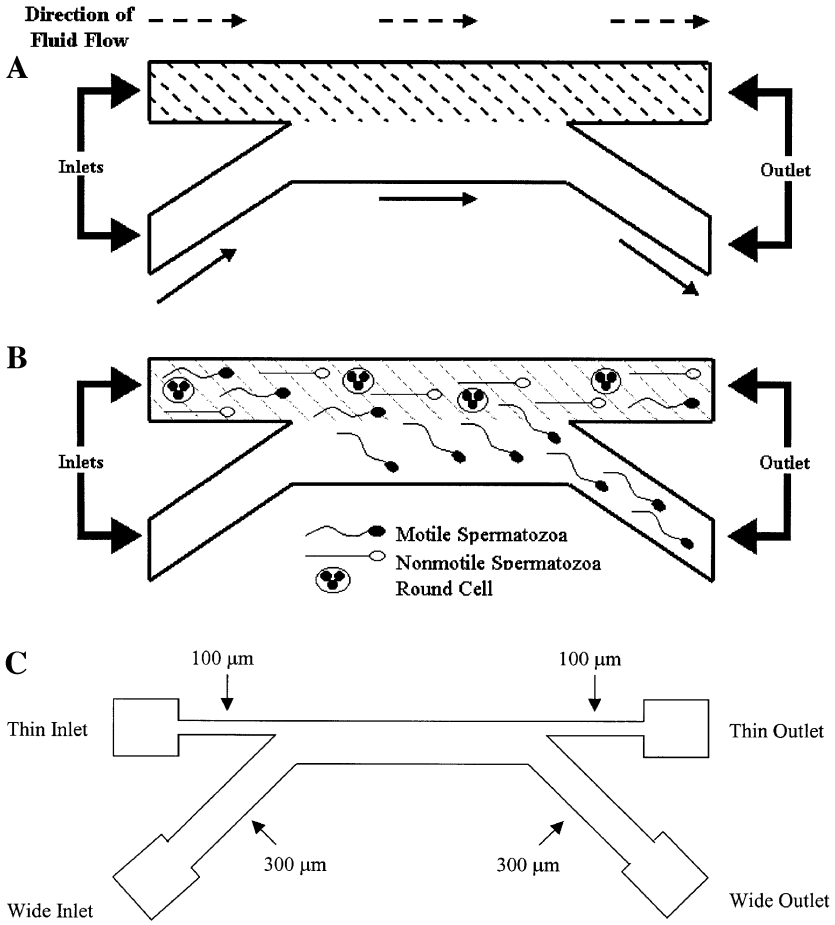


Figure 4. Microfluidic sperm sorter. (**A**) Medium flows from left to right. The initial sample is loaded into the upper stream inlet, while fresh medium is placed in the lower stream inlet. (**B**) Motile sperm are able to deviate from the initial streamline and cross the interface of the laminar flow streams, exiting into the lower stream outlet for recovery. Debris, non-motile sperm, and some motile sperm are collected in the upper stream outlet. (**C**) Dimensions of the channels are at the micrometer scale.

the enveloping cumulus cells in order to assess for the presence of two pronuclei and two polar bodies, signifying appropriate fertilization. This can be carried out by gentle repeated aspiration through a glass pipette tip, which mechanically removes surrounding cumulus and/or corona radiata cells. Such minimal handling probably has few consequences on subsequent embryo development; similar processes have been developed for, and carried out within, microfluidic devices with good results.

It has been hypothesized that in-vitro maturation of immature oocytes in a microfluidic environment may more closely resemble in-vivo conditions than traditional techniques and thereby confer some benefit. A comparison of cleavage rates in immature porcine oocytes matured in silicon-glass or PDMS microchannels, 500 μl culture dishes, and 8 μl microdrops over 44 h was performed. The 8 μl microdrops served as controls for media volume. A significant increase in percentage cleavage for oocytes that were initially matured in microchannels (67%) versus the controls (49%) was reported. Exact configuration and dimensions of the microchannels were not given (Beebe *et al*., 2002b).

Beebe *et al*. (for review see Beebe *et al*., 2002b) have developed microfluidic techniques for mechanical manipulation of the oocyte/embryo. Aspirated bovine oocytes were co-incubated with sperm for up to 22 h and subsequently transferred into the device. Loading and unloading was carried out with relative ease via a funnel-shaped inlet well and standard pipetting techniques. The microfluidic channel measured 500 μm wide × 400 μm deep, except at two narrowings within the channel referred to as conditioning areas, which were slightly wider than the oocyte itself. As the oocyte flowed along the microchannel, it passed through these conditioning areas, forcing the attached cumulus cells to the poles of the cell. Following this, the oocyte was manoeuvered to removal ports, two thin channels placed at 90° to each other. Gentle suction applied to these ports removed the cumulus cells from the oocyte, allowing it to be viewed and checked for fertilization. The placement of the removal ports at right angles allowed straightforward removal of the cumulus sequentially, once the cumulus cells had been forced to the poles. Ninety per cent of these zygotes cleaved following cumulus cell removal using the microfluidic device. The process of cumulus removal was reported to take 15–20 s per oocyte and did not appear to adversely affect subsequent development.

IVF with microfluidics

The actual process of insemination and fertilization *in vitro* can be carried out in a number of ways and essentially involves co-incubation of oocytes with an appropriate number of motile sperm in an easily controllable environment. Culture dishes with one or four wells, 5 ml tubes, and microdrops under oil are the most commonly utilized (Trounson and Gardner, 2000). With these methods, volumes of media ranging from 20 µl for microdrops to 1000 µl in tubes and dishes are used. Sperm is added at a concentration of ~1×10^5 motile sperm per ml, which translates into a total of 2000 to 50 000 motile sperm necessary for insemination per oocyte, depending on the system and volume used.

Although use of this technology has only recently been explored, there have been a number of early successes achieving fertilization within microfluidic channels. Dr Wheeler's group has conducted in-vitro oocyte maturation and subsequent fertilization in pigs within an integrated PDMS microfluidic device (channel dimensions 1000 µm wide × 250 µm deep; unpublished data). Controls were performed using traditional methods in 500 µl of media. Immature porcine oocytes were matured for 44 h and subsequently inseminated with a concentration of 3×10^5 sperm per ml in each system. The overall total number of sperm used for IVF within the microfluidic device was not reported, but can be estimated to be ⩽1500 based on the volume of the microchannel. After 6 h of co-incubation using both techniques, all putative embryos were cultured with standard methods and cleavage rates were checked on day 2 of development. No significant difference was found between the two methods (92/180, 51.1% for controls; 91/185, 49.2% for microfluidics).

We have conducted experiments utilizing the microfluidic sperm sorter developed by Schuster *et al*. (2003) for IVF. Mouse sperm were sorted by the device as previously described, with motile sperm deviating from the initial into the parallel media stream. This stream of sorted, motile sperm accumulated within a collection well (volume ~30–40 µl) containing a mouse cumulus mass (20–30 oocytes). Successful fertilization was noted following 24 h of co-incubation within the collection well. Metaphase II oocytes inseminated in collection wells of sperm sorters had similar rates of fertilization (56.7% + 5.1; $n = 245$) compared with oocytes inseminated with unsorted sperm in 0.5 ml centre-well dishes (55% + 5.0; $n = 196$; $P > 0.05$; unpublished data). This illustrates that sperm selected using the microfluidic sperm sorting device are able to subsequently fertilize an oocyte.

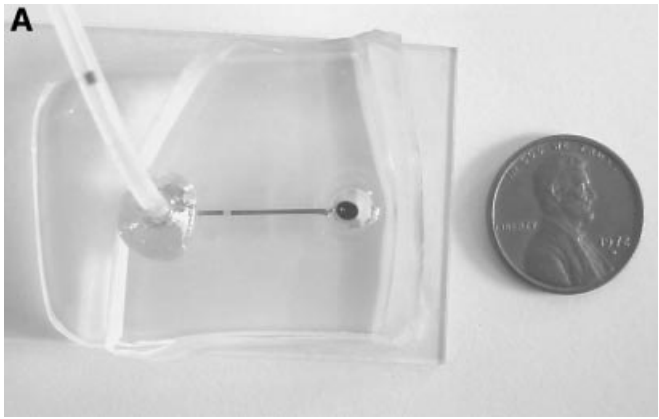
We have also fabricated a microfluidic insemination channel consisting of a single microchannel (500 µm wide × 180 µm deep) separated by a barrier grate manufactured in three dimensions. This grate permits unimpeded flow of sperm and media through the microchannel, but prevents migration of the oocyte (Figure 5). Fertilization of mouse oocytes has been achieved within this microfluidic insemination device at a sperm concentration of 1×10^6 sperm/ml (standard recommendation of mouse IVF; Hogan *et al*., 1994). However, it is important to note that percentage of oocytes fertilized at this concentration is significantly less than following conventional centre-well insemination ($P < 0.05$). This is probably due to sperm utilization of limited metabolic substrates in small amounts of media within microchannels. Preliminary experiments indicated that as total number of sperm inseminated in microchannels decreases, fertilization rate increases, both in comparison to 1×10^6 sperm/ml in microchannels, and similar decreasing sperm numbers in 0.5 ml centre-well dish insemination (unpublished data). In addition, fertilization occurred when sorted mouse sperm obtained from the microfluidic sperm sorter was used in the microfluidic insemination device, demonstrating the integration of the two systems. Ongoing studies continue to indicate the feasibility of this technology and improve device design.

Ultimately, one of the most significant benefits of performing insemination within a microchannel may be a reduction in absolute numbers of sperm necessary while maintaining the same concentration of sperm. Notably, it has been demonstrated that only a few hundred sperm eventually reach the ampulla of the oviduct for fertilization in humans (Settlage *et al*., 1973; Ahlgren, 1975); therefore, the concentrations used currently are *in vitro*-specific needs, probably dependent on the volume of media for co-incubation. The volume of the microenvironment within microfluidic devices can be as low as 1 µl, depending on the channel dimensions. Therefore, to achieve a concentration of 1×10^5 motile sperm per ml, only 100 sperm are required at such low volumes.

Encouraging studies based on this theory have been conducted, although not with microfluidic devices. Van der Ven *et al*. (1989) tested the use of sterile, non-heparinized haematocrit capillary tubes (75 mm length, 0.9 mm inner diameter) for IVF in humans. Normospermic samples were used with standard culture tubes as controls. Volumes of 5–10 µl containing a range of 500–4000 sperm per oocyte were used in the capillary tubes. Overall fertilization rates between controls and capillary tubes were similar (78 and 66% respectively), although slightly lower for sperm totals of 500–1000 (56%) compared with 2000–4000 (79%). Ranoux and Seibel (1990) used embryo cryopreservation straws in volumes up to 200 µl (Ranoux *et al*., 1988) with 2000–4000 motile sperm. Results compared favourably with controls, with 167 of 322 oocytes (51.8%) fertilized using the straw technique.

Hammitt *et al*. (1991) hypothesized that inseminating with dramatically elevated concentrations of sperm within a small volume would improve fertilization when poor quality sperm were used for IVF. Concentrations up to 9×10^6 motile sperm per ml were prepared for insemination of oocytes within straws. Volumes ranging from 10 to 150 µl were aspirated into the straws for co-incubation. Controls were not performed, and overall 80 of 277 oocytes (28.9%) were fertilized. Although the fertilization rate was quite low, at the time these findings represented a hopeful alternative for patients with severe male factor infertility.

Shortly thereafter, the first successful report using ICSI was published (Palermo *et al*., 1992), eventually overtaking traditional IVF techniques in the treatment of severe male factor infertility. Concerns still exist due to use of sperm otherwise unable to fertilize oocytes naturally. This raises the issue of potential genetic aberrations in offspring conceived by this technique. Currently, active debate exists on this issue, but one can appreciate that technology development alleviating use of ICSI in some cases would be beneficial. Microfluidics may ultimately provide an alternative to ICSI for oligospermic males. By mimicking in-vivo conditions of fertilization, decreasing volumes for insemination, and allowing delivery of high concentrations of sperm but low absolute numbers, males with insufficient sperm for traditional IVF may be treatable with insemination in a microenvironment.



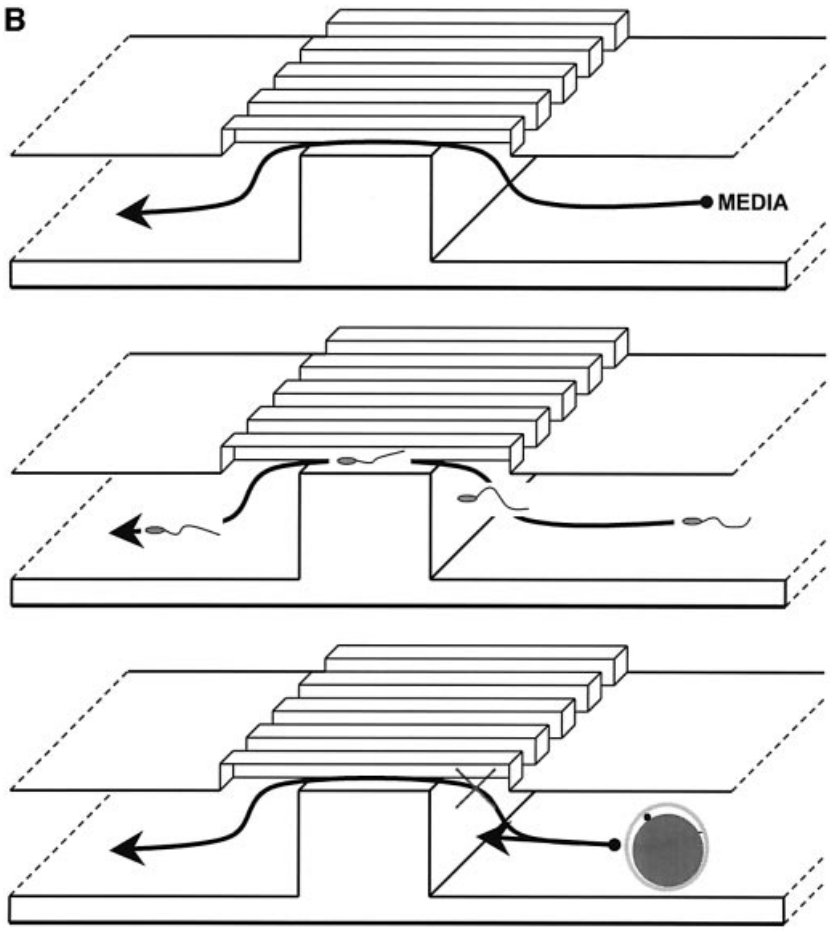


Figure 5. A microfluidic device consisting of a microchannel for co-incubation of sperm and oocytes for IVF. (**A**) Top view. Oocytes are loaded into the channel via the well on the right, then manipulated into the body of the channel for co-incubation within the microenvironment. A grate in the centre of the channel prevents migration of the oocyte. (**B**) Schematic angle view. The three-dimensional construct of the channel prevents movement of the oocyte beyond the grate, but allows for flow of medium and sperm through the channel.

Embryo manipulation and culture with microfluidics

Success rates for IVF may be compromised by limitations of current embryo culture methods, resulting in impaired embryo development and decreased viability (Trounson and Gardner, 2000). Improvements in embryo culture methods have been based on simulating in-vivo conditions and meeting the ever-changing needs of the embryo. Research into sequential media methods of embryo culture takes this hypothesis one step further (Gardner and Lane, 1998). Conventional embryo culture systems employ a single medium from the time of insemination until embryo transfer. *In vivo*, however, the embryo is bathed in a constantly changing environment as it moves through the oviduct to the uterus. Conditions required by the embryo at one point of development may be detrimental at later stages. Special media have been tested using sequential media embryo culture. Cleavage stage embryos are transferred to fresh media on day 3 rather than undergoing embryo transfer. Using this embryo culture technique (Gardner *et al*., 1998a,b), implantation rates of 50% have been reported for human blastocysts.

Current IVF technologies are not ideal for sequential media embryo culture systems. Embryos must be handled during media changes and environments are voluminous compared to in-vivo conditions which may temper the presence of embryotrophic factors. Microfluidics is well-suited to meet the needs of embryo culture. Changing of media is straightforward, and no manipulation of the embryo is necessary. The media can be gradually changed around the embryo, rather than subjecting it to sudden changes in environment. The microenvironment that exists within the device simulates physiological conditions and therefore also may provide some beneficial influence on development (Beebe *et al*., 2002b).

Thus far, there have been encouraging outcomes with microfluidic embryo culture technology. Glasgow *et al*. (2001) first established that manipulation and movement of an embryo in a microfluidic environment is possible at low flow rates. Microchannels were tested and designed with silicon-glass (350 μm wide × 200 μm deep) with constriction areas which disallow movement of the embryo down the channel while allowing for flow of media around and past the embryo, similar to the grate used in the microfluidic insemination channel. These constriction areas were either narrowings of the channel or elevations of the channel. No deformation or injury to the embryo was noted at the low flow rates used. Because of the embryo's size and weight, it sank to the bottom of the channels and would stick at average fluid velocities of <50 μm/s. However, a minimal pressure gradient of 0.16 Pascal/mm was adequate to maintain average fluid velocity >350 μm/s, allowing for embryo velocity of up to 250 μm/s. In addition, average flows of 100 nl/s, which correspond to fluid velocities of up to 2000 μm/s, did not noticeably deform the embryos at the constriction areas. Due to lack of fluid momentum in microfluidics, reverse of the flow immediately releases the embryo from constriction.

Once the feasibility of embryo handling and media flow within a microchannel was established, mouse embryos were cultured and development rates compared (Beebe *et al*., 2002b). Two-cell embryos were collected from superovulated mice. Controls were conducted in culture dishes with M16 media and 0.4% bovine serum albumin (BSA) in 30 μl microdrops, and the remainder was loaded into the microfluidic device pre-filled with the same media. The rate of flow and timing of media replenishment within the channel was not given. A higher percentage of embryos within the microfluidic channels reached the morula, blastocyst, and hatched blastocyst stage compared with controls.

E.M.Walters, D.J.Beebe and M.Wheeler (unpublished data) surgically collected 4-cell embryos from mated pigs and cultured them in North Carolina State University-23 medium with 1% BSA from the donor gilts. Controls were cultured in 1 ml of media within a 4-well Nunc dish. No difference in blastocyst formation was seen (79 versus 84%; microchannels versus controls). Blastocysts cultured in microchannels (n = 16) were then surgically transferred to the uterus of an asynchronous recipient, with resultant pregnancy and delivery of five live piglets (three females and two males). No developmental or reproductive abnormalities have been noted in the offspring. Though preliminary, this study suggests the ability of embryo culture in microfluidic environments to produce embryos capable of transfer, implantation, and production of live births.

Hickman *et al*. (2002) conducted a comparative study of mouse embryo development in various microfluidic environments. In-vitro culture using M16 media was conducted within microchannels (1000 μm wide × 250 μm deep) at continuous flow rates of 0.5 and 0.1 μl/h, and no flow for 72 h. Controls were performed in 35 μl microdrops and embryos were examined at 24 h intervals. Blastocyst and morula development was similar to controls within the no-flow microfluidic environment; however, at the continuous flow rates used, embryo development to blastocyst and morula was significantly decreased. It was hypothesized that at the tested rates of continuous media flow, rapid clearing of needed factors led to detrimental effects on development. It is unclear whether slower rates of flow, or pulsatile flow, would have the same or opposite effects. Nevertheless, current evidence regarding embryo culture in microfluidics is promising and further study may lead to optimization of conditions and improved results.

The future of microfluidic IVF

Although much of the work using microfluidics in IVF has been performed in a stepwise fashion, the ultimate goal of process miniaturization and microfluidic technology is integration. Use of microfluidic technology for sperm processing ultimately results in a small volume and fraction of motile sperm. Such volumes are difficult to subsequently utilize and translate into a macroscale environment. However, laminar flow-sorted sperm have been used for subsequent fertilization within a microenvironment. Integration of a microfluidic channel for the oocyte and the collection stream of sorted sperm would result in automatic co-incubation of the oocyte with these motile sperm. Following insemination, the oocyte can be directed to a secondary site for cumulus removal, evaluation for fertilization, and embryo culture. Sequential media can be provided for ideal embryo development. Each step logically follows the other, with no cell manipulation other than directing flow along a variety of channels. Miniaturization allows the entire system to be small and self-contained. Decreased intervention by laboratory personnel not only decreases gamete and embryo manipulation, but also provides for greater consistency of incubation conditions.

Obviously, multiple hurdles exist in engineering integration of these processes. The development of reliable methods for directing flow through a network of channels is necessary. Currently, a number of active and passive valves (Beebe *et al*., 2002a) or switches (McDonald *et al*., 2000) have been designed for microfluidic devices, but the applicability to embryo manipulation must be demonstrated. Automated delivery of fluid at precise rates is important for sperm sorting, culture media exchange, and embryo manipulation. Thus far, a passive gravity-driven fluid pump has been employed for microfluidic sperm sorting (Cho *et al*., 2003) and active, hands-on regulation of fluid flow using syringes is necessary for insemination and embryo handling (Beebe *et al*., 2002b). Alternatives, such as multiport flow-control systems (Chien and Parce, 2001), will need to be applied to current designs. Methods of fabrication and packaging of microfluidic devices will need to be refined prior to widespread acceptance of this technology for human applications. Current devices have been for research purposes only and therefore are not ergonomically sufficient for general laboratory use. However, development of an 'IVF-laboratory-on-a-chip' is a realistic and exciting goal.

Conclusions

Microfluidics shows significant promise as a revolutionary technology for IVF. Parallel laminar flow has been used to sort human sperm for motility and morphology. Microfluidic devices have been shown to adequately mature porcine and bovine oocytes and remove cumulus cells. Insemination and fertilization in minute volumes has been conducted and may require lower numbers of total sperm. Microenvironments have provided improved development of mouse and porcine embryos and live offspring have subsequently been born from these cultured blastocysts after embryo transfer. Conditions within microfluidic devices may more closely resemble those seen *in vivo*, resulting in improved efficiency and better outcomes with fewer gametes. Integration of each process in microfluidics is the ultimate goal of miniaturization. Development of an integrated system would allow for minimal interaction with the IVF process once semen and oocytes are introduced into the device. Continued development is necessary, but microfluidics appears poised to have a big impact on the practice of IVF.

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