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Thinking big by thinking small: application of microfluidic technology to improve ART

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In Vitro Fertilization (IVF) laboratories often carry a penchant to resist change while in the pursuit of maintaining consistency in laboratory conditions. However, implementation of new technology is often critical to expand scientific discoveries and to improve upon prior successes to advance the field. Microfluidic platforms represent a technology that has the potential to revolutionize the fundamental processes of IVF. While the focus of microfluidic application in IVF has centered on embryo culture, the innovative platforms carry tremendous potential to improve other procedural steps and represents a possible paradigm shift in how we handle gametes and embryos. The following review will highlight application of various microfluidic platforms in IVF for use in maturation, manipulation, culture, cryopreservation and non-invasive quality assessment; pointing out new insights gained into functions of sperm, oocytes and embryos. Platform design and function will also be discussed, focusing on limitations, advancements and future refinements that can further aid in their clinical implementation.

Introduction

Within the field of assisted reproductive technologies (ART), like many other fields, there is an ongoing endeavor to improve outcomes and optimize efficiency. Achieving this goal is facilitated by various scientific discoveries regarding the development and function of gametes and preimplantation embryos, of which more are revealed each day. Importantly, technological innovations, several of which are developed with entirely different disciplines in mind, permit many of these new discoveries. Furthermore, in addition to novel information gained from their application, these new technologies may be adapted to change the fundamental approaches used for everyday laboratory procedures, like cell culture. Microfluidics represents such a technological advancement.

While the focus of microfluidic application in ART has recently been centered on embryo culture,[1,2] the innovative platforms carry tremendous potential to revolutionize all aspects of ART and represents a possible paradigm shift in how we handle gametes and embryos (Fig. 1). The following review will highlight applications of various microfluidic platforms in ART, pointing out new insights gained into functions of sperm, oocytes and embryos, while also highlighting the platforms themselves; specifically focusing on limitations, advancements and future refinements that can further aid in their widespread clinical implementation .

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Andrology

One of the earliest applications of microfluidic platforms in ART entailed assessment of spermatozoa. The constrictive nature of microfluidic platforms and ability to create complex networks of channels and reservoirs proved a useful combination in assessing characteristics of motile sperm.

As early as 1993, simple microchannel devices made of silicone were used to evaluate rudimentary aspects of sperm function *via* interactions with cervical mucus, hyaluronan, spermicide and anti-sperm antibody beads as motile sperm traversed various network layouts.[3] Follow-up studies demonstrated the ability to perform quantitative sperm counts and motility assessments on etched glass microchannel devices.[4] While these early devices did not integrate active fluid flow or utilize the unique physical aspect of fluids during flow through microfluidic channels, they did provide insight into the potential applications of the platforms. As design and fabrication processes have advanced, application of microfluidics in andrology has grown.

Sperm counts

A microfluidic chip was recently developed consisting of channels etched on a glass wafer fabricated to perform sperm counts. Directing sperm movement at varying velocity by adjusting height of fluid columns from two media reservoirs, cells were able to pass along a microfluidic channel flanked on

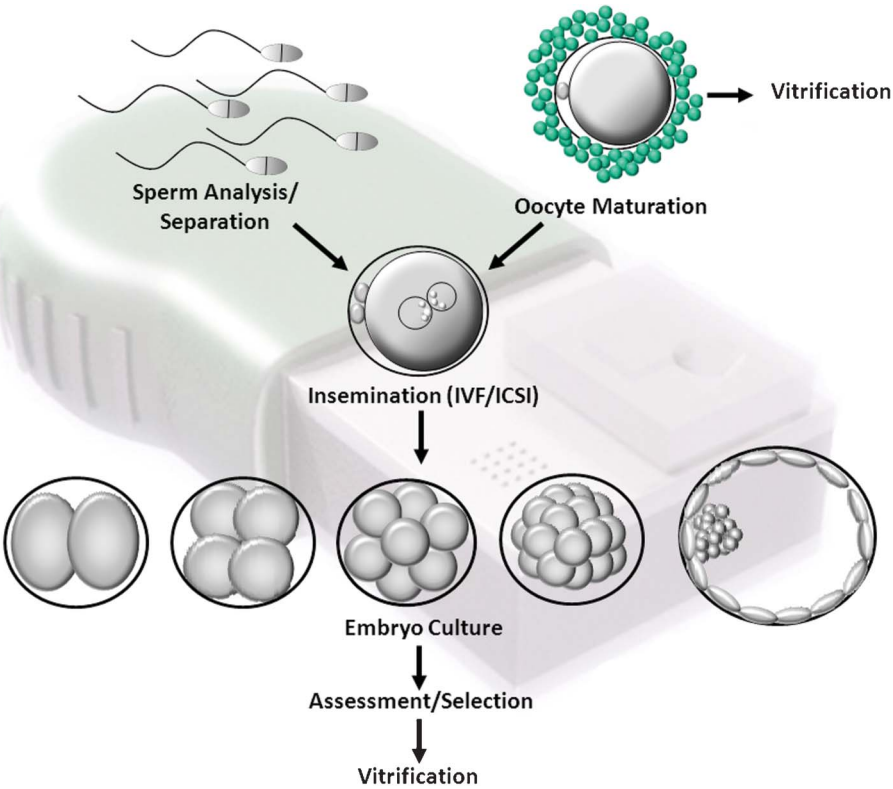

Fig. 1 Schematic of basic steps involved in the *in vitro* fertilization process that have utilized microfluidic devices.

either side with planar electrodes. The resulting electrical impedance measurements recorded as sperm passed the electrodes permitted estimation of sperm concentration.[5] While the simple media flow generated by gravity and hydrostatic pressure is easy-to-use, careful regulation is required to ensure proper speed of sperm for accurate assessment. Importantly, the approach was able to distinguish sperm from polystyrene beads and HL-60 cells, which is important for clinical application due to other debris in seminal plasma. With refinements and considerations to production costs, a similar system could be envisioned for use for routine semen analysis to streamline and standardize sperm counts.

Sperm separation

Perhaps the most widespread application of microfluidic technology in andrology entails use for sperm separation. Many of these approaches rely on devices that generate fluid flow from media reservoirs through some sort of microchannel. Devices designed using microfluidic channels for sperm separation are highly dependent upon variables affecting the laminar flow and velocity of the fluid streams in motion. Factors like channel width, height, depth, as well as fluid velocity and viscosity are all critical parameters that must be considered when trying to optimize separation of a specific sperm population. This task is further complicated by the varying viscosity of semen and presence of cellular debris in addition to sperm. Despite these sometimes difficult variables, several modern devices have addressed some of these issues.

One of the earlier reports using microfluidic technology for separation of motile sperm from semen samples utilized a polydimethylsiloxane (PDMS) passive gravity-driven device where the hydrostatic pressure of two separate inlet reservoirs drove media flow down a converging microfluidic channel[6] (Fig. 2) The principle of the device took advantage of the fact that only motile sperm can traverse the border that separates the parallel streams of diluted semen and fresh medium. Thus, the laminar flow properties exhibited by media in microchannels allowed motile sperm to swim away from non-motile sperm, debris, and seminal plasma and collect in a separate outlet reservoir. Follow-up experiments demonstrated this microfluidic device design was not only biocompatible with human sperm, but that it could isolate motile, morphologically normal cells.[7] The novel approach appeared to offer a feasible alternative to isolate sperm from oligozoospermic patients for use in intra-cytoplasmic sperm injection (ICSI).

Another approach employing microfluidics for sperm sorting utilized mouse sperm placed into a PDMS/glass device, to isolate sperm based on motility, but also *via* chemotaxis towards cumulus cells.[8] Sperm were placed into an inlet reservoir and allowed to swim down a straight channel, whose dimensions were optimized for motile sperm recovery. Sperm were then collected in a small central reservoir, where video imaging could occur, before swimming onward into 1 of 2 branching channels, each leading to separate collection reservoir. Other microfluidic devices to explore sperm chemotaxis also exist and offer further unique tools to explore sperm function.[9]

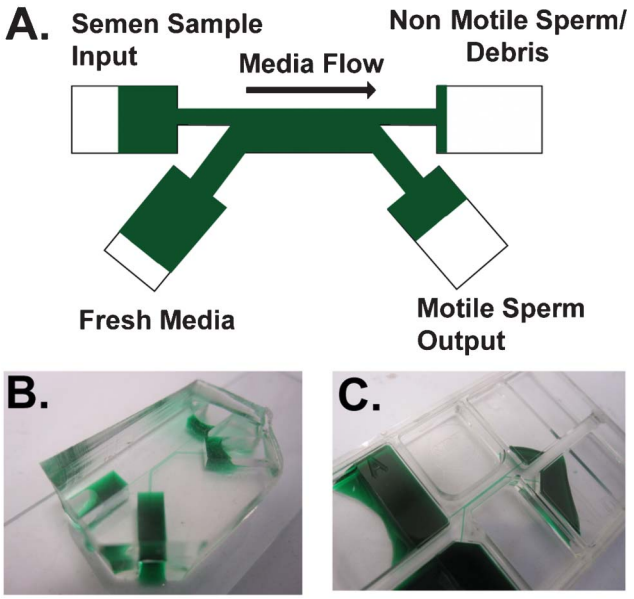

Fig. 2 A) Schematic of a microfluidic device created for sperm sorting. Passively driven by a difference in surface tension, the separation of motile sperm relies on laminar flow of the merging fluid streams, and motile sperm can swim across the interface to collect in a reservoir.[7] B) Sperm sorter device made of PDMS used in initial design and testing. C) Modification of the microfluidic sperm sorting device made of polystyrene suitable for clinical use.

In another use of microfluidic technology for andrology, a PDMS/glass device was constructed that directed sperm flow within microchannels to separate, align and orient sperm of mouse, bull and human,[10] providing potential applications for ICSI. Utilizing the fact that motile sperm orient themselves against media flow within these devices, and that motile sperm can swim against media flow of certain velocity, a series of three reservoirs and four microfluidic channels allow processing of sperm *via* hydrostatic media flow. Of note, this device requires very precise regulation of media volumes within the reservoirs to regulate hydrostatic pressure.

Utilizing these advantages of a microfluidic device for sperm isolation, implementation of a microfluidic sperm sorter manufactured out of quartz has begun in clinical IVF. A preliminary report indicates human semen can be processed rapidly and isolated motile sperm can be used to successfully fertilize human oocytes following ICSI[11] (Fig. 2). Follow up studies by the same group have since produced PDMS versions of the sperm separation device that have widened microfluidic channels to help isolate sperm with higher velocity and are more cost-effective to permit clinical application.[12]

Due to the limitations inherent in a microfluidic sperm sorting device, utilization of the technology must provide some added benefit over conventional processing methods. Conventional sperm preparation methods, such as serial centrifugation, density gradient separation or swim-up, are reported to induce sperm DNA damage, perhaps to exposure to reaction oxygen species (ROS).[13–15] Preliminary data indicate that sperm isolated using a microfluidic sperm sorting device had significantly lower levels of DNA damage and higher motility compared to these more conventional approaches.[16] Thus, microfluidic sperm sorting may allow for selection of higher quality sperm, potentially leading to improved embryo quality. With the above mentioned applications, one can envision a microfluidic device that assesses sperm concentration, motility, progression and then separates sperm based on various qualities or characteristics. This isolated sperm could then be manipulated in a fashion to make it suitable for ICSI within the same microfluidic platform to streamline the current practice of moving sperm from dish-to-dish. This approach could prove a powerful tool in reducing variability in andrology laboratory testing and processing and help improving ART outcomes. However, it should be noted that, despite the ability of microfluidic devices to perform high-speed and high-throughput sperm sorting, selection is based solely on motility. Thus, the approach is unable to identify non-motile but viable sperm for ICSI, which is relevant for patients suffering from severe or complete asthenozoospermia.

Embryology

Employing microfluidic technology for embryology requires considerations unique from microfluidic devices utilized with adherent cells, or even those used for sperm assessment. The main obstacle to consider is reliability of cell recovery. While it may be acceptable to recover only a portion of an initial cell population when dealing with hundreds of thousands or millions of cells, 100% recovery is essential for applications involving oocytes and embryos. Thus, platform design must make accessibility and recovery of paramount importance, and it must do so without limiting the inherent properties of microfluidics that make it advantageous to cell biology, namely the constrictive nature. In addition, the added effort and cost of applying microfluidics must be offset by a measurable benefit on gametes or embryos to justify implementation of the technology. Fortunately, many of these issues are actively being addressed, and, as a result, platforms have begun to receive initial testing in all aspects of embryology, including *in vitro* oocyte maturation (IVM), *in vitro* fertilization (IVF) and embryo culture.

Oocyte maturation

In vitro oocyte maturation is an especially appealing approach for human ART considering the tremendous advantages offered, including reduced cost and reduced health risk to disorders such as ovarian hyperstimulation syndrome (OHSS).[17] However, IVM is still an inefficient practice. Fortunately, microfluidic approaches offer the potential to improve current IVM success rates. In the first report of oocyte maturation using microfluidics, a microfluidic channel flanked by two media reservoirs with no regulated media flow, did not result in porcine oocytes maturing efficiently in silicone devices (2% Metaphase II; MII). However, when these oocytes were matured in PDMS devices, they matured at a significantly higher rate compared to control microdrops (2% *vs.* 71% to MII, respectively).[18] Interestingly, cumulus cell expansion was noticeably diminished in oocytes matured in microchannels compared to larger microdrops. This observa-

tion may be indicative of quality oocyte cytoplasmic maturation, as oocytes regulate cumulus cell development and function.[19] Thus, while the size and/or volume limitations of microchannels may improve nuclear maturation, they may also have some limiting effect on porcine oocyte cytoplasmic properties, or physically restrict cumulus expansion. In contrast, subsequent follow-up experiments by Walters and coworkers from the same research group suggest oocyte cytoplasmic maturation may actually be enhanced in static microchannels. Pig oocytes matured in 250 μm wide PDMS/borosilicate glass microchannels produced significantly higher numbers of 2-cell embryos following IVF and embryo culture in microdrops compared to oocytes matured in 500 μl drops (67 *vs.* 49%).[20] Unfortunately, pronuclear formation, embryo development past the maternal-zygotic transition, or blastocyst cell numbers as measures of improved oocyte developmental competence were not reported. The differential results from these studies in similar designed devices of differing materials suggest that material selection is of paramount importance for the sensitive oocyte.

Interestingly, preliminary studies indicate bovine oocytes matured in PDMS microfunnels with Braille pin regulated media flow actually yields improved blastocyst development following IVM compared to static matured oocytes (unpublished results). Despite these preliminary findings, the field awaits peer reviewed publications examining effects of fluid flow in microfluidic devices on more informative markers of oocyte developmental competence. Toward this end, a microfluidic device does exist that utilizes magnetically actuated manipulators in conjunction with ultrasonic vibration for single cell manipulation.[21] This device can move and orient porcine oocytes and may be modified for potential micromanipulation purposes. Interestingly, a vibrating culture platform was previously shown to enhance porcine oocyte developmental competence when applied to standard culture dishes with larger volumes.[22] Thus, the vibrational aspect of the previously mentioned microfluidic device used to move oocytes may also offer some benefit to oocyte cytoplasmic maturation and could be potentially modified to improve IVM.

In vitro fertilization

Another demonstration of microfluidic application in ART can be seen in insemination performed ''on chip''. Insemination is a particularly difficult procedural step in ART, as sperm motility characteristics and interactions with oocytes in microfluidic channels are highly dependent upon fluid flow rate. The speed of fluid movement influences sperm motion and a threshold exists where sperm are no longer capable of independent movement.[23] Furthermore, sperm motion paths can be influenced by the contours of the device. These qualities have immense implications for success of fertilization within microfluidic platforms.

The first attempt at the procedural step of insemination was performed using porcine oocytes placed into PDMS/borosilicate microchannels (Fig. 3). Sperm were added in a manner where hydrostatic pressure differences created from differing volume of media added to reservoirs at either end of the microchannel resulted in gravity-driven fluid flow of sperm past oocytes, which were immobilized at a constriction point within the microchannel. Fertilization in this device resulted in significantly lower rates of polyspermic penetration compared to fertilization in control microdrops.[24] Reduced polyspermic penetration rates were attributed to the physical characteristics of the microfluidic device, mimicking the environment *in vivo*. It was hypothesized that microfluidic devices served to limit time of oocyte exposure to sperm, as sperm were not confined to the vicinity of the oocytes, but allowed to flow past the eggs.

Subsequently, Suh and colleagues demonstrated successful fertilization of mouse oocytes on a more complex microfluidic device that consisted of a series of open slots within a microchannel[25] (Fig. 3). Although initial experiments utilizing high concentrations of sperm revealed that overall fertilization rates were decreased on the microfluidic device compared to control microdrops, subsequent experiments demonstrated that, by lowering sperm concentration, fertilization rates in microfluidic devices were actually higher than controls. These results appear to be the result of chip design, as authors observed increased concentration of sperm in the vicinity of the oocyte in microfluidic devices.

More recently, fertilization has been performed on a microfluidic microwell device[26,27] (Fig. 3). Similar to previous devices, platforms were made of PDMS and constructed with an inlet and outlet reservoir, which generated media flow using gravity. Microchannels leading from each reservoir connect to a larger microchamber that housed individual square microwells containing individual oocytes. Microwell depth was optimized to permit retention of oocytes while allowing adequate debris removal and sperm interaction with oocytes. Media and sperm could then be flowed over the microwells housing the oocytes. Using this approach, similar rates of mouse oocyte fertilization were obtained compared to controls (69.0 *vs.* 71.4%).[26] An alternate device from the same research group with differing design to incorporate multiple procedural steps also yielded promising fertilization results.[27] Additional microfluidic devices incorporating insemination with other procedural steps have also been developed and are discussed later.[26]

Other oocyte manipulations

In addition to maturation and insemination, other oocyte manipulations are common during routine IVF procedures. Microfluidic devices have been developed that can perform some of these tasks. Cumulus cell removal can be performed in a series of microchannels.[28,29] Utilizing fluid flow driven manually *via* attached syringes, exposing oocytes to chemical and physical manipulation, cumulus cells could be removed, leaving the oocyte intact. A similar approach has also been successful in removing the zona pellucida.[30] More recently, a system combining microfluidic channels and an optical ablation laser was used to manipulate clam oocytes through a series of channels, which permitted media exchange and positioning of cells for laser ablation.[31] Application of a similar device that could rotate cells[32] could be envisioned as useful for clinical procedures like assisted hatching.

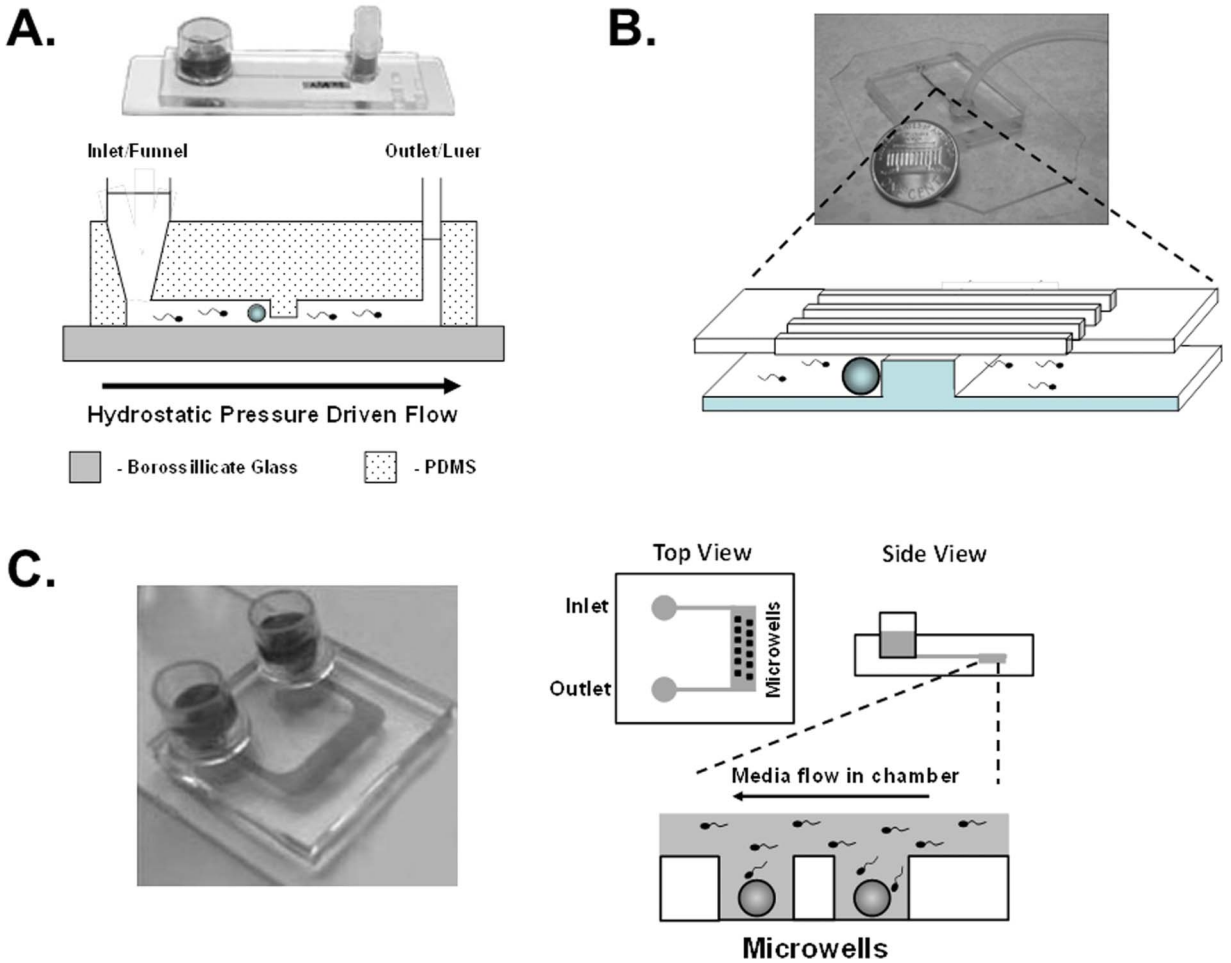

Fig. 3 A) First device design used to successfully perform fertilization of pig oocytes using microfluidics.[24,69] This device utilized a straight microchannel with a small constriction point to prevent oocytes from rolling the length of the channel, while permitting sperm to flow past the oocyte. This particular device utilized gravity driven media flow and was not engineered to sustain controlled fluid flow over time. A similar device was used for oocyte maturation.[69] B) A modified microfluidic platform used to successfully perform fertilization of mouse oocytes.[25] C) Alternate fertilization device using microwells.[26,27] This device may create a more suitable microenvironment, while also permitting individual identification of zygotes following fertilization. All of the aforementioned fertilization microdevices are designed with controlled width/depth/height of microwells or channels tailored accordingly to account for oocyte size of each respective species.

Embryo culture

Exhaustive studies have been conducted aimed at optimizing preimplantation embryo culture system *in vitro*, and, similar to IVM and IVF, a microfluidic platform may aid in this endeavor. The initial report on embryo culture using microfluidics by Raty and colleagues indicated that 2-cell mouse embryos could be cultured to the blastocyst stage within static microchannels[33,34] (Fig. 4A). Compared to 30 μl control microdrops, culture within these microchannels containing about 500 μl of media resulted in significantly greater 16-cell/morula formation at 24 h, greater blastocyst formation at 48 h and 72 h, and a greater portion of hatched blastocysts at 72 and 96 h. However, impact on implantation or live birth was not evaluated. Subsequent experiments utilizing a similar device by Walters and coworkers from the same research group showed that *in vivo* derived 4-cell porcine embryos could be cultured to blastocyst and transferred, resulting in live birth.[35] However, in these follow-up experiments, no observable beneficial effects on embryo development were seen when compared to culture in control organ-well dishes. Furthermore, cell recovery issues were apparent when culturing cells in microchannels.

Building upon their initial microchannel static embryo culture studies, Hickman *et al.* examined mouse embryo development in microchannels with media flow, controlled *via* an external large syringe infusion pump.[36] Flow rates examined (0.1 and 0.5 μl h^{-1}) did not enhance development compared to static culture. In fact, a flow-rate of 0.5 μl h^{-1} resulted in significantly lower development of 2-cell mouse embryos to morula and blastocyst stages, while producing higher numbers of abnormal embryos compared to controls. Thus, flow rate and manner of flow delivery appeared to be important variables for embryo culture in microfluidic devices. Indeed, embryos can sense sheer stress, which can induce apoptosis and be detrimental to embryo development.[37] However, it is questionable if flow rates necessary for dynamic fluid flow in microfluidic channels would approach velocities

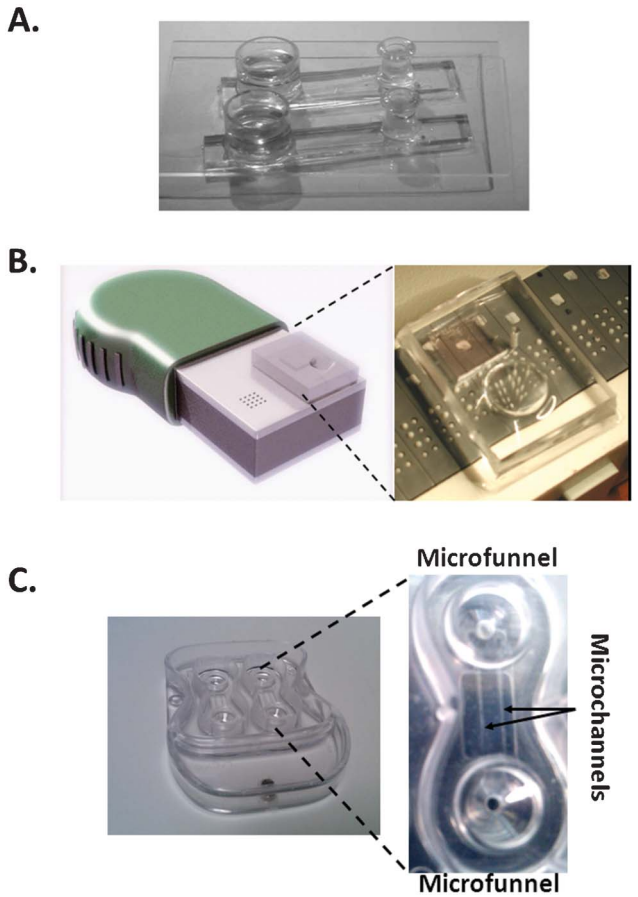

Fig. 4 A) First microfluidic channel used to culture mammalian embryos.[33,34] Microchannels are problematic with regard to cell access and consistent recovery. B) First microfluidic platform using microfunnels formed in PDMS with actively controlled media flow *via* use of Braille pin actuators for culture of mouse embryos that was shown to improve implantation and live birth outcomes.[45,47] C) Modified microfunnel platform made of polystyrene to permit automated media flow suitable for clinical use.[48] The microfunnel design for embryo culture provided ease of access to the embryo for clinical purposes, while maintaining effective fluidic stimulation for enhanced outcomes.

high enough to cause concern. Additionally, it should be noted that these data on the impact of media flow and flow-rate on embryo development should be re-assessed, as culture conditions may have been suboptimal. In this particular study, control mouse embryos cultured in control static microchannels did not improve embryo development as previously reported by Raty and colleagues from the same research group.[33] One possible source of variation requiring future study was the increased number of embryos cultured in each device, a factor which undoubtedly would have an impact due to influence of group embryo culture,[38,39] especially within the confines of a microfluidic platform.

Yet another approach to culturing embryos within microfluidic device employed not only dynamic media flow, but also co-culture. Mizuno and coworkers developed a ''womb-on-a-chip'', where endometrial cells can be grown in a lower chamber, while embryos are cultured in an upper chamber, separated from the lower by a thin membrane[40,41] (Fig. 5A). The design permits embryo exposure to secreted factors from the endometrial cells, while avoiding direct cell contact. In their preliminary report, authors demonstrated that mouse ova fertilized on and resulting embryos cultured in these devices showed similar cleavage to 2-cell and similar blastocyst formation rates compared to 50 ul control microdrops.[40] Furthermore, cell number was significantly higher in blastocysts fertilized/cultured in microfluidic devices. Subsequently, blastocysts obtained from microfluidic devices were transferred to recipient female mice and resulted in live offspring at rates similar to embryo cultured in static microdrops. A similar co-culture approach was taken by the same group, culturing 2-cell mouse embryos to blastocyst stage on the refined OptiCell microfluidic device. OptiCell microfluidic co-culture culture yielded chromosomally normal embryos, capable of yielding live offspring.[42] Mizuno and colleagues later published an abstract reporting the first instance of human embryo culture within their co-culture microfluidic devices. Donated 2–4 stage frozen human embryos were cultured to the blastocyst stage, resulting in significantly higher rates of blastocyst development from microfluidic devices compared to control microdrops.[41] Additionally, visual scoring of microfluidic-derived blastocyst development revealed higher quality blastocysts with significantly higher cell numbers compared to static controls. A similar device was later constructed using a microporous membrane to separate mouse embryos from endometrial cells[43] (Fig. 5B). Unfortunately, co-culture confounds interpretation of results obtained, as it is impossible to discern if beneficial effects are attributed to co-culture or the physical properties of the microfluidic device/design.

Notably, a braille pumping system using tiny electric piezo actuators has been used successfully to grow embryos by peristaltic media motion along microchannels formed in PDMS through a microfunnel housing the cells[44–47] (Fig. 4B) This approach is unique from other microfluidic devices used in ART, in that it permits precise computerized regulation of speed and flow patterns, rather than passive media flow due to gravity, or the more course media flow provided by external syringes. It was demonstrated that regardless of media flow pattern (back and forth *vs.* flow-through) or speed (fast *vs.* slow), 1-cell mouse embryos cultured in these microfluidic devices showed greater hatching of blastocysts and significantly higher cell number than static controls, yielding numbers similar to those obtained from *in vivo* derived blastocysts.[45,47] It was also shown that this approach produced greater number of mouse embryos reaching morula stage at 48 h, blastocyst at 72 h and hatched blastocyst at 96 h compared to control static chips, while significantly more bovine embryos reached the blastocyst stage at 144 h in microfluidic devices compared to control static devices.[44] Follow up experiments indicated that beneficial effects of embryo culture in the dynamic culture device are additive and require a minimum 48 h of culture at the beginning or end of 96 h culture periods.[46,47] Importantly, this device was the first report that a microfluidic dynamic culture platform could not only improve preimplantation embryo development, but that quality of embryos cultured in a microfluidic device with media flow are superior to those grown in static systems, as

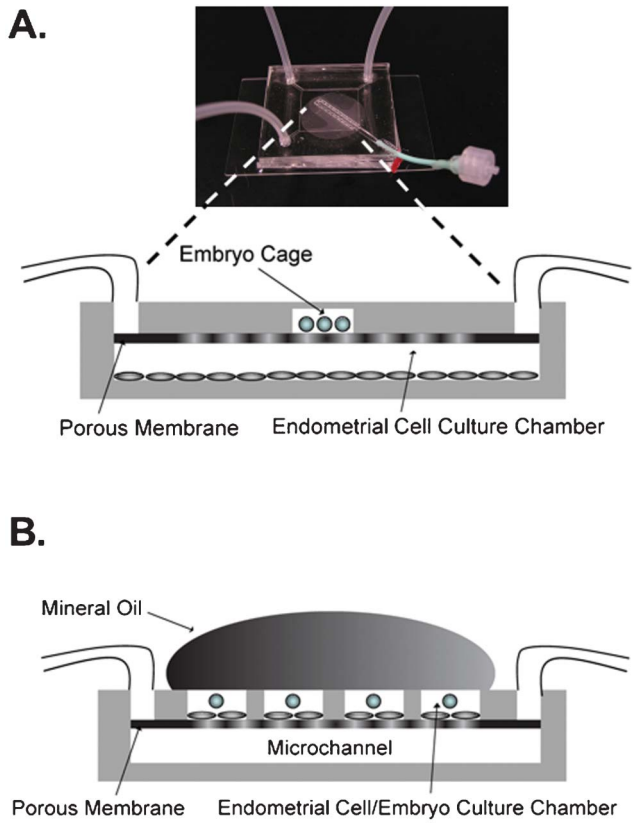

Fig. 5 A) Microfluidic platform used for endometrial co-culture of mouse and human embryos.[40,41] B) Alternate microfluidic device using a microporous membrane to separate mouse embryos during co-culture with endometrial cells.[43] Both devices have porous membranes as an essential part of design. However, within the field of IVF, there has been a movement away from co-culture towards use of more define culture systems

evidenced by increased implantation rates, lower rates of absorption and higher ongoing pregnancy rates in mouse.[47] Also important to note, the studies were eloquently controlled using static controls within the microfunnel platform, which permitted the conclusion that the media flow as responsible for beneficial effects, rather than simply an effect of the material or microfunnel design. Subsequently, a modified microfunnel constructed for clinical trials was able to support human blastocyst formation, while producing higher quality embryos on day 3 with lower levels of fragmentation[48] (Fig. 4C)

More recently, at least one study has used a tilting culture system in conjunction with microfluidic channels to gently agitate embryos during culture. Bovine embryos were tilted 10° over 1 min and cultured inside straight microchannels or microchannels with a 150–160 μm constriction. Though no difference in blastocyst formation was observed, and the influence of the tilting system alone was not examined, authors suggest that combining an embryo tilting system with a 169 um constricted microchannel may offer a means of improving bovine embryo cleavage, yielding higher rates of 8-cell development after 44 h or culture compared to straight channels (56.7 *vs.* 23.9%).[49] It should be pointed out, however, that the tilting approach is simply agitation and doesn't necessary remove or replenish the existing media like perfusion systems. A similar microchannel system from the same group also examined use of a micromodulated syringe pump for culturing bovine embryos to supply mechanical stimulation.[50] Similar methods of combining a simplified microchannel or microfluidic devices with other dynamic methods of inducing fluid movement, generally used at the macro-level, such as vibration or agitation, may be helpful, as gentle agitation appears to beneficial for human embryos.[51,52] Though the more recent dynamic culture platforms that agitate media and embryos by simply placing traditional culture dishes with microdrops or larger volumes of media on a moving or vibrating platform have been examined and appear promising,[51–54,22,49] in the context of this review, these aren't considered microfluidic culture platforms and may not utilize the full potential of the constrictive microenvironments offered by microfluidic approaches.

Regardless, microfluidic systems with perfusion, or some other means of agitation, may benefit embryo development for a variety of reasons. One intriguing possibility is the idea that gentle mechanical stimulation, either through fluid flow or gentle movement of the embryos, could be beneficial. This ''Active Embryo Hypothesis'', theorizes that gentle physical stimulation of embryos can activate embryotrophic signaling pathways and promote embryo development.[55] However, excessive forces can be detrimental. Indeed, it is well known that various sensory mechanotranduction systems are evident in a variety of cells types, usually manifested by regulation of ion channels.[56] The same may hold true in embryos, or even oocytes, as it is known that embryos can sense sheer forces for rotating embryo culture or other pipetting, which activates various signaling cascades.[37,57] Whether beneficial signaling pathways are activated in mammalian oocytes or embryos within these dynamic culture platforms, what these pathways may be, or whether they are responsible for observed benefits, remains unknown.

Finally, another aspect of novel microfluidic culture devices that may convey an unexpected benefit to developing embryos is the use of novel materials. While these materials may be initially selected due to ease of manufacturing intricate designs using techniques such as photolithography, the platforms surfaces themselves may improve embryo development. Indeed, it has been postulated that coating of platform surfaces with specific molecules may benefit embryo development, either through direct physical effects, or through modifications of the chemical environment.[2,55] Thus, the actual material used in device construction may also impact the culture microenvironment. This was found to be the case when using PDMS for culture device construction, as the material is absorptive and caused detrimental osmolality shifts unless a specialized parylene coating was applied.[58] It was later reported that PDMS embryo culture devices that were ''softer'' supported better embryo development.[59] In comparing polystyrene of high stiffness, with a softer PDMS or collagen, it was found that softer surfaces improved embryo and placental development. However, due to the zona pellucida as a barrier preventing direct contact to the embryo, the stiffness of the polymer or substrate likely played no role. Rather, the absorptive nature of the varying surfaces likely

differed and differently impacted media characteristics. Interestingly, it was recently reported that standard polystyrene petri dishes, as most commonly used to culture embryos, may weaken cells, as evidenced by reduced proliferative capacity along a vertical wall of adherent cell lines[60] Specifically, it found a subtle softening and swelling of the polystyrene surface due to interactions with water that created nanoscopic layers of hydroxide ions that altered localized pH and induced a layer of ROS. This may have ramifications for optimizing embryo culture and use of more appropriate surfaces or materials may be warranted.

Cryopreservation

Expanding upon the application of microfluidic devices for specific procedural steps within ART, the precise fluid control offered by these dynamic platforms may be especially well-suited to exposure to cryoprotectants. Conditions of cryoprotectant exposure can impact cell function and survival, with factors like concentration and time of exposure as crucial variables.[61] This is especially true for vitrification approaches, which utilize very high concentrations of cryoprotectant agent (CPA) and require very low exposure times to prevent toxicity. Detrimental effects of CPA exposure on genotoxicity, even without cryopreservation, have been shown.[62] Even sub-lethal genotoxicity such as DNA fragmentation[63] can still cause catastrophic outcomes.[64] Thus, given the fact that oocyte vitrification procedure successes to date have been achieved despite the lack of a quantitative understanding of the mechanical and chemical effects different CPAs and CPA exchange procedures can have on gametes and embryos, microfluidics may permit optimizing CPA exchange procedures and reduce sub-lethal damages.

Importantly, practical issues exist with current manual cryoprotectant exposure regimes that limit preciseness and repeatability. There is inherent variability in timing of manual procedures between individuals. Also, discrete manual dilution steps are required to make cryoprotectant exposure feasible. While logistically important for the embryologist, these approaches may not be ideal for the cell. Thus, it is clear that currently, laboratories lack the tools needed to perform CPA exchange precisely at the cellular level.

Although the two or three step CPA exchange concept is now relatively broadly accepted for embryo or oocyte vitrification, the exact procedure differs from lab to lab contributing to the variability in cryopreservation success rates. More fundamentally, there is a problem that there are more potential combinations of CPAs and CPA exchange procedures that could be utilized than can be efficiently and reliably tested for by trial and error experiments alone. The approach of using specialized bioengineered devices to design vitrification CPA exchange protocols, where manual dilutions do not limit efficacy, might help alleviate both the osmotic shock (mechanical effects) and CPA toxicity (chemical effects). A more gradual, rather than sudden stepwise increases in CPA concentration, is known to increase viability.[65] There is a practical and capability limit, however, in terms of how many exposure steps and in what time the CPA exchange can be performed using the current gold standard; manual pipetting. Furthermore, the transfer of cells from solution to solution for each step involves rapid pipetting that can introduce mechanical stress.[57]

To address these current limitations with manual approaches, microfluidic devices have been designed where, rather than moving the oocyte from one CPA solution to another by pipetting, the oocyte will be stationary and the CPA solutions will be pumped over it by computer control. This permits continuous and gradual, rather than discrete step increases, in CPA concentrations that the oocyte is exposed to. Preliminary reports indicate potential of this approach. Controlled CPA exposure with a microfluidic platform reduced the size change of oocytes compared to traditional approaches, which may be important for cell viability[66] (Fig. 6A). However, the platform design yields limited access to cells for loading and removal, which is problematic in terms of clinical practicality. A similar approach for using computerized control of media exposure to permit gradual cryoprotectant exchange to limit large osmolality changes have been used with embryos[67] (Fig. 6B). Perhaps more importantly, use of a microfluidic platform for gradual CPA removal during oocyte/embryo warming may be beneficial in further optimizing cryosurvival. This application remains to be explored.

Another obstacle that has hindered rapid and wide-spread acceptance of vitrification approaches has centered on ease-of-use issues of vitrification devices. There are numerous commercially available and in-house devices to contain oocytes or embryos for low temperature storage following vitrification.[68] These utilize very small volumes of vitrification solution to permit the vitrification process and avoid damaging ice crystal formation. While both open and close containers can be used successfully, the combination of the low volume and rapid time needed to load devices to prevent toxicity from cryoprotectant exposure can be problematic, especially compared to 0.25 cc straws used with slow-rate approaches. A novel microfluidic platform regulating CPA solution exposure to oocytes, that integrates a novel vitrification device, could simply bypass the current step-wise procedure and eliminate errors with manual dilutions and movement of cells through various media and loading of complicated devices.

Future directions

Though incremental advances in application of microfluidic technology to enhance current ART protocols should now be readily apparent, the full potential of the approach has yet to be recognized. Much of this is due to the limitations in design and function, both perceived and actual. However, emerging studies are pushing the envelope to harness the capability of these novel devices. Three main areas in microfluidic refinement that hold tremendous promise for revolutionizing efficiency of IVF include ability to implement multiple IVF procedural steps on a single device, automation of procedural steps and implementation of diagnostic inline assays. If implemented in a clinically feasible platform, these advancements may lead to improved culture conditions, reduction of

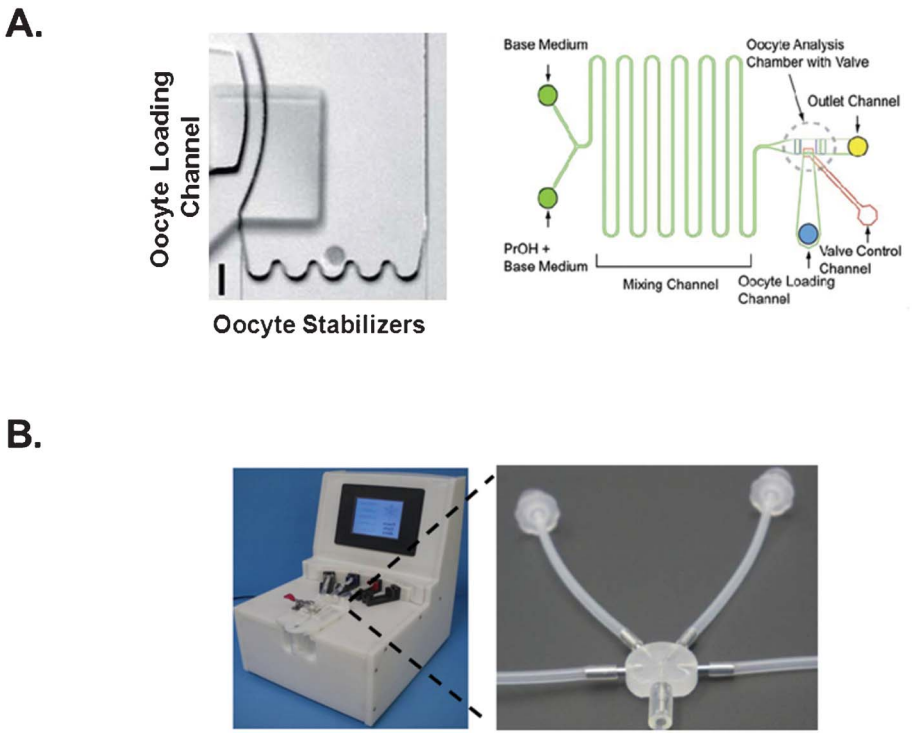

Fig. 6 A) Microfluidic device used to optimize cryoprotectant exposure schemes for oocytes by measuring volume changes.[66] Serpentine channels were used to effectively mix two solutions of temporally changing flow rates to produce a gradual cryoprotectant exposure scheme. A valving mechanism was also introduced to lock the oocyte inside the analysis chamber during flow. B) Alternate device used to automate gradual cryoprotectant exposure for embryos.[67]

in vitro stressors, as well as improved embryo selection; ultimately culmination in improvement in ART outcomes.

Multiple ART procedural steps on a single device

Many of the above mentioned studies implement a single procedural step on a microfluidic device. However, implementation of multiple steps on the same device would be advantageous, as it would reduce cell handling and the associated environmental stressors. Keeping the delicate cells in place, while gradually changing media, or gently rolling the cell to a new location may produce a less stressful environment and help optimize the *in vitro* culture system.

One of the first examples of integration of multiple procedural steps on a single microfluidic platform was presented by Clark and coworkers. Their preliminary data demonstrated that both IVM and IVF of porcine oocytes could be performed on the same microfluidic device without removal of the cells between procedures.[69] Media within devices were changed by withdrawing from the 2 reservoirs and sperm added *via* manual pipetting without disturbing oocytes, which were housing in a connecting microchannel. Although there were no observable benefits achieving cleavage to 2-cell compared to control treatments (49% *vs.* 51%), this demonstrated that multiple tasks of *in vitro* embryo production could be performed upon the same microfluidic platform.

Another example of integrating multiple IVF steps on a single platforms entails separation of porcine sperm using laminar flow in a microfluidic device, and then fertilizing oocytes that were then placed in a chamber near the exit point of the separated, motile sperm.[70] Rates of monospermic penetration varied depending on oocyte location in the exit chamber. Subsequent analysis revealed that the microfluidic device yielded higher rates of normal fertilization and subsequent blastocyst development compared to standard insemination protocols in petri dishes.

More recently, mouse embryos have been cultured successfully in microwells housed on the same microfluidic device where fertilization occurred. Han *et al.*[26] demonstrated that oocytes could be fertilized in microwells using media and sperm movement *via* gravity driven hydrostatic pressure . Fertilization media was then replaced with embryo culture media by flowing media though the channels on the same device and embryos cultured. Though there was no active media flow during the 96 h of embryo development, similar high rates of blastocyst formation were obtained following culture in microwells compared to controls (87.5 *vs.* 87.8%). Similarly, using a revised device design employing the ''octacolumn'' to house oocytes/embryos rather than microwells, the same group again demonstrated mouse oocyte fertilization and subsequent embryo development on the same microfluidic platform, yielding high rates of blastocyst formation, similar to controls (86.9% *vs.* 85.3%).[27] Another device also exists that performs IVF and embryo culture without removing the cells.[40]

More ambitious and complex devices are emerging. One report details a device that incorporates a variety of procedural steps from oocyte manipulation, insemination and embryo culture.[27] Similarly, another ''chip'' has been developed that

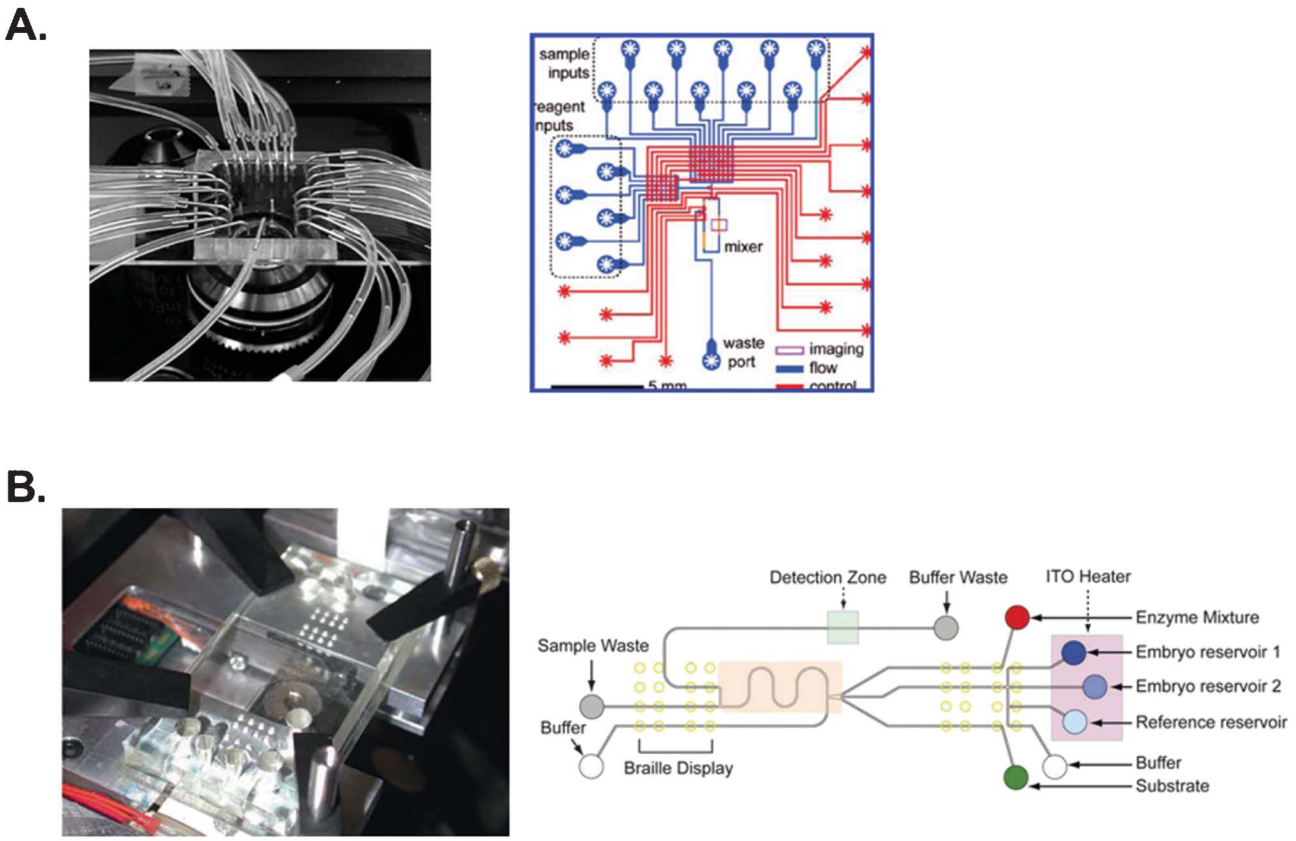

Fig. 7 A) Microfluidic device used to analyze embryo metabolism.[82] External tubing used for pneumatics or syringe driven inlets can be cumbersome and problematic for clinical application B) Microfluidic device used to culture embryos, while simultaneously permitting assessment of glucose utilization as a means of non-invasive embryo assessment on the same device.[81] Clinical usability is preserved by using a preprogrammed Braille display to drive flow.

attempts to integrate each step of the IVF process including oocyte positioning, sperm screening, fertilization and embryo culture with media replacement.[26]

Most recently, a miniaturized embryo array has been developed that can trap multiple zebrafish embryos, holding them in individual locations while utilizing microfluidic perfusion to supply fresh media or pharmacologic compounds to examine effects over time.[71] Integrated with a perfusion pump, stage heater and valves to help regulate flow and remove waste, this platform is also combined with real-time video analysis. A similar approach could be envisioned for use with mammalian embryo culture, where real-time embryo imaging devices placed in incubators has received widespread attention.[72,73] Using a miniaturized approach, each microfluidic "chip" could in theory have its own thermal heat source below the embryos, contain a miniaturized camera and self-contained LED light source for observation, and even maintain their individual atmosphere in isolated chambers. This would be a large departure from multiple dishes/patients sharing a large common incubator, where dishes are removed on a regulator basis for microscopic observation and subject to changes in temperature and pH.

Automation

Automation of intricate step-wise procedures performed on microfluidic platforms within the ART laboratory is a potential method to reduce variability and perhaps improve ease-of-use. Others have already conjectured that some level of automation may be the future of the IVF laboratory.[74] As an example of the promise of this approach, automation of blastomere visualization/tracking during mitosis and use of a predictive algorithum for automatic embryo selection has been utilized.[72,73] Additionally, more complex procedures that could utilize a microfluidic platform, such as automated microinjection have been explored.[75,76] Toward this end, an automated microfluidic device aimed at sorting, collecting, orienting *xenopus* oocytes for subsequent microinjection has been developed.[77] Authors claimed a dramatically improved efficiency, reducing the time needed to process 400 oocytes from 1 day to ~4 h. While this may have application for large scale animal production purposes, the relevance to human oocytes, which are limited in number for each case, may not be as significant. It does, however, introduce the ability to reduce variability and stress in the culture system. Automation of vitrification procedures has also been examined as a way to reduce variability in this highly sensitive approach,[67] and may prove to be an instrumental tool in further improving success.

Inline diagnostic assays

Implementation of inline diagnostic assays to aid in selection of viable gametes for use or embryos for transfer would further help realize the immense potential of microfluidics in ART.[78] Microfluidic devices have been developed to assess embryo metabolism[79] (Fig. 7A) and oxygen consumption,[80] though these devices were not developed with extended culture or cell recovery for subsequent use in mind. More recently, a promising approach of measuring glucose consumption in the same microfluidic device where embryos are cultured has been developed[78,81] (Fig. 7B). This approach may permit real-time metabolic assessment of embryos, ultimately providing a profile of metabolism over time. Such a profile could be used in conjunction with similar profiles collected over time, such as those collected using emerging morphokinetic time-lapse analyses. Combining multiple real-time analytic profiles would undoubtedly provide greater insight into embryo development and permit better selection for transfer.

Conclusion

It is clear that microfluidics offer many opportunities to revolutionize the ART laboratory as we currently know it. Importantly, these technologies would not negate the need for an embryologist. Rather, they would serve to streamline processes, act as an adjunct to reduce stress imposed upon gametes and embryos, reduce variability between personnel/lab, and serve to help optimize the culture environment. Though personnel would be required to change their current procedures and likely undergo extensive training to learn to operate and troubleshoot new microfluidic or automated systems, this should be viewed as enhanced job training; a necessity in any position that attempts to constantly improve upon past successes. That being said, it is acknowledged that microfluidic devices are generally designed with functionality first and foremost, with little or no regard to ease of clinical applications. In this respect, future designs should consider simplicity and usability to facilitate the adoption of microfluidics in the clinical ART laboratory.

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