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Advances in Microfluidics-Based Assisted Reproductive Technology: From Sperm Sorter to Reproductive System-on-a-Chip

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The fields of assisted reproductive technology (ART) and in vitro fertilization (IVF) have progressed rapidly, yet still need further improvements. Microfluidic technology can incorporate various ART procedures such as embryo/gamete (sperm/oocyte) analysis, sorting, manipulation, culture, and monitoring. The introduction of paper-based and droplet-based microfluidics further improves the commercialization potential of this technology. The progress in 3D printing technology allows for the integration of microfluidics with tissue engineering that may revolutionize current practices in biology and medicine. This review categorizes ART methods according to continuous-flow microfluidics, paper-based microfluidics, droplet-based microfluidics, and organ-on-a-chip. The advances are summarized and potential opportunities in infertility diagnosis, sperm selection, sperm guidance, oocyte selection, insemination, embryo culture, embryo monitoring, and cryopreservation are identified. While some advances of continuous-flow microfluidics for ART have already been reviewed, other microfluidic techniques are still in their early stages. It is envisioned that advances in droplet-based microfluidics, especially digital microfluidics, will allow for more progress in human IVF, particularly single embryo transfer. Droplet-based microfluidics may also lead to fully integrated and high-throughput platforms for animal IVF. Recent advances in organ-on-a-chip including ovary/uterus/oviduct-on-chip platforms hold promise for the integration of the whole human reproductive system-on-a-chip for clinical applications.

1. Introduction

The development of mammalian reproductive technologies, in particular assisted reproductive technology (ART), has been one of the greatest achievements of medicine and life science. ART and its various subcategories such as ovulation induction, in vitro fertilization (IVF), and intrauterine insemination (IUI) provide an excellent opportunity for infertile couples to have their own children. Once considered as a science fiction, IVF and intracytoplasmic sperm injection (ICSI) are now routinely used in fertility clinics. ART is not necessarily only for biologically infertile couples but also for induced infertility. ART techniques such as cryopreservation of gamete/embryo can be used before undergoing treatments such as chemotherapy, which may adversely affect the fertility. ART also has a significant impact on livestock industry,[1] animal husbandry,[2] and world food production.[3] The field has been progressed rapidly in the past 40 years since its inception. Combining ART with tissue engineering and regenerative medicine can lead to the development of artificial uterus, stem cell-derived gametes, and even human cloning. Despite these advances, common ART techniques such as IVF and ICSI still suffer from a variety of limitations. First, as the name of the technology implies, i.e., ART needs to be performed by highly skilled personnel, and the outcome may vary from one expert to another. Second, the whole procedure is still invasive and may potentially cause some complications such as ovarian hyperstimulation syndrome and others.[4] Most importantly, IVF/ICSI bypasses natural selection and in vivo reproductive tract environment. There has been an outstanding improvement in culture medium for gamete handling and sequential replacement of culture media based on embryo needs, mimicking in vivo conditions. However, current physical tools and environment of IVF for gamete manipulation and embryo culture are far from in vivo conditions. In particular, test tubes, Petri dishes, microdroplets, and microwells poorly mimic the dynamic microenvironment, which in vivo embryo experiences during its journey toward the uterus. Such unrealistic conditions not only may affect the quality of the preimplantation embryo and the pregnancy rate but also may influence the epigenetics of the offspring. Immediate effects include low birth weight, high blood pressure, a higher rate of obesity, cardiovascular disease, and increased susceptibility to carcinogens.[5] Future health consequences on the next generation still need to be evaluated in more follow-up studies. Although these complications

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are not common, an increasing number of ART procedures may increase the frequency rate of these complications. Therefore, optimization works for this technology are needed.

Similar to ART, microfluidics has also rapidly evolved over the last three decades. Some unique characteristics of microfluidics include micrometer-sized length scale, high surface-to-volume ratio, ability to handle a small volume of fluids (microliter to picoliter), requiring low reagent/waste, fast response, the dominance of laminar flow, surface forces, molecular diffusion, and Brownian motion.[6] These characteristics make microfluidics an excellent tool for a variety of fields such as chemical analysis, cell biology, and medicine. The advantages of microfluidics, such as portability, automation, high-throughput, and the ability to integrate multiple components on a single chip,[7] lab-on-a-chip, have opened up new avenues for drug delivery,[8] drug discovery,[9] and cancer treatment.[10]

Microfluidics has been successfully incorporated in various fields of ART. These fields can be categorized as 1) infertility diagnosis, 2) sperm selection, 3) sperm guidance, 4) oocyte analysis, 5) insemination, 6) embryo culture, 7) embryo selection, and 8) cryopreservation and vitrification. The literature clearly shows that during 1990s silicon photolithography with cleanroom facilities had been the dominant manufacturing microtechnology. This trend had been shifted toward replication molding using polydimethylsiloxane (PDMS) soft lithography for most 2000s microfluidic platforms used for ART. The translation toward commercialization is now replacing PDMS soft lithography with hot embossing of thermoplastic materials such as cyclic olefin polymer (COP). However, most of these microfluidic platforms are based on single-phase continuous flow. **Table 1** summarizes the relevant review papers, which mainly discussed single-phase continuous-flow microfluidics (CFM) mostly fabricated by PDMS soft lithography.

However, single-phase CFM has some limitations, e.g., it needs external equipment to drive the flow. These technical challenges affect its portability, commercialization potential, and clinical acceptance. Paper-based microfluidics is an inexpensive, portable, and equipment-free alternative for point-of-care diagnostics.[11] Paper-based microfluidics can be an excellent platform for semen analysis in ART. Paper-based microfluidics has also been used for 3D cell culture and cryopreservation.[12] Furthermore, droplet-based microfluidics is another emerging technology. Droplet-based microfluidics offers a well-controlled environment for the species encapsulated in the microdroplets. The science of manipulating discrete droplets with noncontact forces, digital microfluidics is another subcategory of droplet-based microfluidics. Digital microfluidics may become more popular for embryologists since it shares many similarities with standard IVF microdroplet technique. Therefore, droplet-based microfluidics and digital microfluidics can have applications in various fields of ART. Finally, with advances in 3D cell culture and fabrication techniques, the combination of microfluidics with tissue engineering and regenerative medicine can revolutionize every aspect of ART. **Figure 1** illustrates the contribution made by each of these microfluidic approaches to the fields of ART from the published works.

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Table 1. Summary of the review articles addressing the applications of single-phase CFM in ART in chronological order.

Main focus of the review	Year
The authors reviewed mostly their works on cumulus-oocyte complex (COC) denudation and embryo culture[62b]	2002
The authors reviewed few works reporting motile sperm separation, oocyte handling, microfluidic IVF and embryo culture[155]	2003
The authors reviewed few works in oocyte maturation, insemination, and embryo culture[156]	2007
The authors discussed static and dynamic microenvironment for embryo culture[157]	2010
Comprehensive review comparing static and dynamic culture platforms with an introduction to specialized surface coatings[86a]	2011
An insightful review describing the integration and automation of whole IVF procedure with microfluidics[21]	2012
Mainly focused on nonmicrofluidic aspects of ART in farm animals[158]	2012
A comprehensive and systematic review paper on microfluidic advances in ART[86b]	2013
A comprehensive review paper focusing on microfluidics in sperm analysis[26]	2015
A special review focusing on mechanical properties of sperm/oocyte/embryo[68a]	2015
A variety of single oocyte analysis techniques including simple perfusion apparatus, Raman microspectroscopy and microfluidic platforms were reviewed[61]	2016
The topics covered were similar to ref. [158] with little emphasis on microfluidics[159]	2016
A short and brief review on microfluidic advances in sperm counting, sorting, fertilization and forensic medicine[160]	2016
A comprehensive review of the microfluidic platforms for single/group culture of embryo in static/dynamic conditions and best quality embryo selection[79a]	2016
A concise review on microfluidic advances on semen/oocyte/embryo manipulation and embryo culture[161]	2016
A brief review of continuous-flow microfluidic advances in human ART mainly focusing on clinical needs[74]	2017
Based on 12 research articles, the authors categorized microfluidic dynamic embryo culture in three groups: "microdroplet dynamic bioreactor, micro-channel based culture and microcontainers"[79b]	2017

While the majority of past review articles confined their scopes to the applications of CFM in ART, a comprehensive review paper covering all microfluidic approaches in this field is lacking. This article will first briefly review the application of CFM in the various areas of ART. We will introduce the seminal work and the most recent advances in each area. Subsequently, the potential research gaps will be identified. Research areas such as sperm guidance, which received less attention in the past, will be discussed in more details, while the others will be summarized and referred to the relevant review articles. Also, the limitations and potential solutions toward commercialization of CFM in ART will be explored. Next, paper-based microfluidic platforms in ART, particularly those used for semen analysis and infertility diagnosis, will be discussed. Subsequently, the works that employed droplet-based microfluidics and digital microfluidics will be analyzed. Finally, we will discuss the applications of microfluidics in tissue engineering, which led to the development of the relevant organ-on-a-chip platforms such as ovary-on-a-chip, uterus-on-a-chip, and oviduct-on-a-chip.

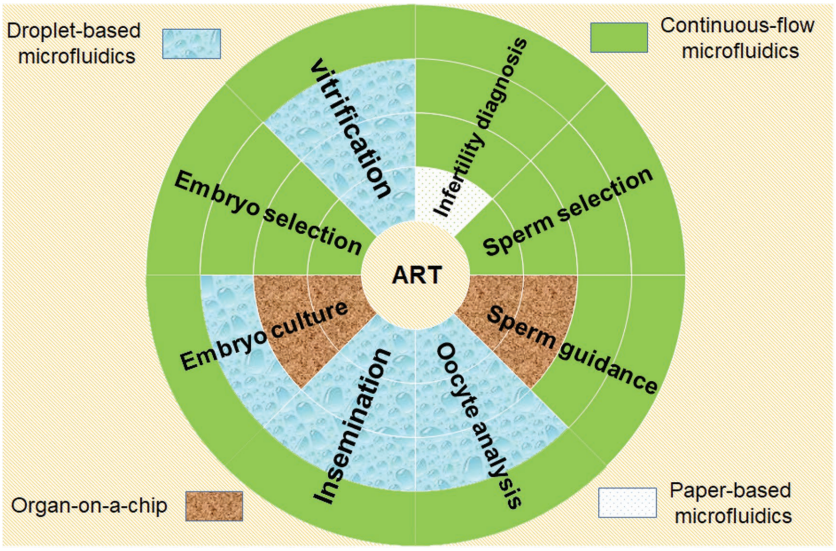

Figure 1. The scope of the present review article. The diagram shows various areas of ART and the contribution made by four different microfluidic approaches to each field.

2. Continuous-Flow Microfluidics in ART

2.1. Semen Analysis for Male Infertility Diagnosis

To evaluate the capability of sperms to fertilize a mature oocyte, semen analysis needs to be performed to characterize both sperm quantity and sperm quality. The sperm quantity is measured based on its concentration. According to World Health Organization (fifth edition, WHO 2010), the cutoff value for sperm concentration is 15×10^6 sperms per milliliter (15 M mL^{-1}) of the ejaculated semen. A concentration lower than this value is referred to as low sperm count (oligospermia) and attributes to male infertility. Of equal importance for infertility diagnosis is the quality of the ejaculated sperms. Sperm quality can be evaluated by measuring

one or more parameters including sperm morphology, motility, maturity, liquefaction time, DNA integrity, and levels of pH and fructose. Following the conventional clinical approach, counting chambers and computer-assisted sperm analysis (CASA) are used to respectively evaluate the quantity and quality of sperms. However, these techniques are expensive, labor-intensive, subjective (accuracy may vary from one laboratory to another), and require trained personnel. In addition, even advanced clinical sperm analyzers such as CASA need manual dilution and mixing. To automate and shorten the semen liquefaction time, Park et al. reported the fabrication of an efficient microfluidic mixer capable of diluting sperm in a short time period, which was followed by precise positioning the diluted sperm in CASA for further analysis.[13]

Coulter principle of sizing and counting particles was incorporated in microfluidics for sperm analysis and first reported by Sogerink et al.[14] The authors showed that differences in electrical impedance could distinguish the spermatozoa from polystyrene beads and HL-60 cells. Schaff et al. reported the first FDA (US Food and Drug Administration) approved microfluidic-based home test kit, Trak, determining a linear spectrum of sperm concentration.[15] Following the semen liquefaction in an enzyme-coated cup, 0.25 mL of the sample is transferred to the microchannels inlet. With a rotation speed of 7000 rpm within 7 min, centrifugal force separates and transfers the sperms to the metered reading chamber (**Figure 2**A). Recently, Kanakasabapathy et al. fabricated a home-based semen analyzer to quantify sperm concentration and motility using a disposable microfluidic platform[16] (Figure 2B). The disposable microchip and the optical part are attached to a smartphone to display the final result. Further comparison with lab-based semen analyzer showed 98% accuracy using 350 clinical semen samples. This device illustrates how microfluidic technology can be integrated with the off-the-shelf equipment to produce home-based, point-of-care diagnostic tools.

2.2. Sperm Selection

There are many advances in selecting the high-quality sperms for subsequent IVF/ICSI/IUI. Based on sperm morphology, Bartoov et al. developed a new method called motile sperm organelle morphology examination (MSOME) using high-magnification (6300X) with an inverted microscope and high-power Nomarski optics.[17] The authors subsequently developed the technique of intracytoplasmic morphologically selected sperm injection (IMSI) by combining MSOME with clinical ICSI. However, more recent clinical trials[18] proved that MSOME and subsequent IMSI could not significantly improve the clinical outcome of pregnancy rate and live birth compared to conventional ICSI. Sperm sorting based on dielectrophoresis (DEP) is another versatile sorting technique to preselect the sex of offspring and omit the sperms with X-linked genetic disorders. For instance, DEP had been successfully used to separate X-bearing from Y-bearing sperms.[19] A commercially patented sperm sorter, Microflow, based on DEP was later developed by Atiken and co-workers.[20] The working principle of such sperm sorter is based on the fact that high-quality sperms have normal head size (i.e., 5 µm filtered by a membrane) and high electronegativity (induced by DEP).[21] The authors further showed the clinical efficacy of DEP separation following ICSI.[22] Nasr-Esfahani and Tavalaee[23] provided a comprehensive review of other noninvasive sperm sorting techniques based on the surface electric charge.

2.2.1. Sperm Selection Based on Motility

Since only motile sperms have the potential to swim up the oviduct and inseminate the oocyte in vivo, they are appropriate candidates for IVF/ICSI/IUI. Conventional clinical methods such as swim-up and density gradient involve centrifugation and have

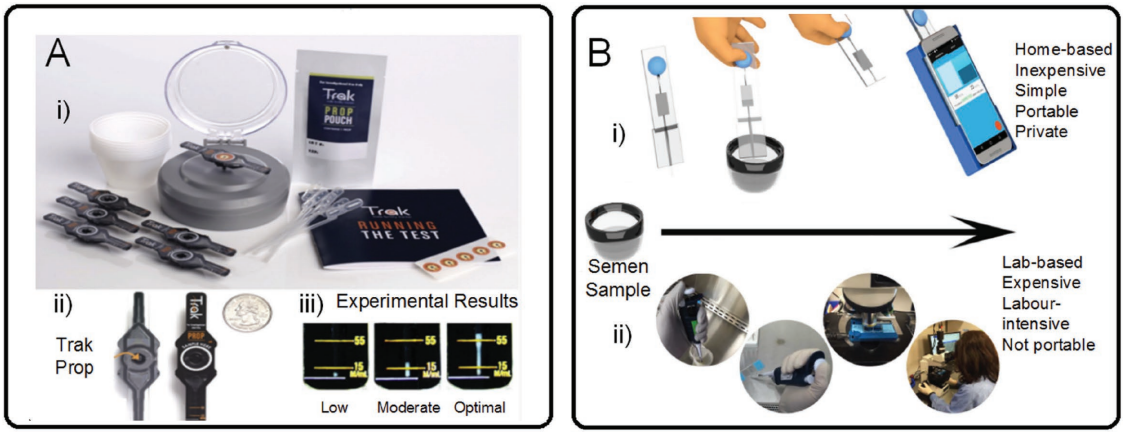

Figure 2. Two commercialized microfluidic-based infertility diagnosis systems for a male. A) FDA approved microfluidic test kit to determine the sperm concentration: i) Different components of the device; ii) disposable props with sample inlet and collection channels; iii) final results showing the range of sperm concentration in a semen sample: Sperm concentration below 15 M mL^{-1} is considered as "low" while it is "optimal" if it is higher than 55 M mL^{-1}. Reproduced with permission.[15] Copyright 2017, Elsevier. B) A home-based microfluidic device which can be attached to a smartphone for readout: i) The steps required for the device operation: The disposable microchannels and detachable tip are placed into the semen sample. The microchannels filled with the semen will be attached to the optical part connected to a smartphone. Finally, a customized application in the smartphone will process the sample in less than 5 s; ii) The steps required to process the semen sample in a laboratory-based method. Reproduced with permission.[16] Copyright 2017, American Association for the Advancement of Science.

a long processing time up to 2 h. Centrifugal force can damage sperm DNA, and a long processing time exposes sperms to intensive reactive oxygen species.[24] Therefore, more efficient sorting techniques for motile sperms are needed. Accordingly, various microfluidic motile sperm sorting devices have been developed. Specifically, they can be beneficial for selecting motile sperms in oligozoospermic sample. Matsuura et al. showed that microfluidic motile sperm sorter could significantly decrease the processing time for ICSI when a sample with low sperm concentrations was used.[25] Depending on the driving force, Knowlton et al. categorized microfluidic sperm sorting techniques as either active or passive ones.[26] In active sorting techniques, semen sample and media are driven by external forces including hydrostatic pressure, chemical and thermal gradients. Passive sorting techniques rely on intrinsic nature of motile sperms which swim toward collecting chambers.

Cho et al. were among the first researchers who reported a microfluidic sperm sorter based on laminar flow.[27] The sorting mechanism was based on the combination of both intrinsic characteristics of sperms and unique features of microfluidics. Intrinsically, motile sperms have a higher diffusion rate which allows them to swim across the laminar streamlines. Thus, microfluidic devices, where laminar flow with diffusive mixing is dominant, can be perfectly exploited to sort motile sperms. The follow-up works based on this idea are summarized in **Table 2**. Made by hot embossing using COP, the devices received FDA approval in 2016.[28]

Seo et al. fabricated a hydrostatically driven microfluidic platform to sort the progressively motile sperms based on positive sperm rheotaxis.[29] The authors further combined the cell counting concept using electrical impedance with laminar flow sperm sorting described by Cho et al.[27] to fabricate a simple but efficient microfluidic sperm counter.[30] Subsequently, Chen et al. combined the concept of electric impedance with positive sperm rheotaxis to analyze both the concentration of motile sperms and their motility.[31] The authors further expanded the device to the measurement of other sperm characteristics such as swimming velocity and tail-beat frequency.[32] Other designs such as microdiffusers[33] have also been used to sort progressive motile sperms based on positive sperm rheotaxis. To capture the image of a wider portion of motile sperms, Zhang et al. devised a microfluidic device with an integrated lens less charge-coupled device.[34]

Table 2. Various microfluidic sperm sorters based on the mechanism described by Cho et al.[27] It took around 13 years for such system to get FDA approval, QUALIS.

Fabrication technique	Added benefits	Year
PDMS soft lithography (SL)	No centrifugation, high-throughput, portable and disposable[162]	2003
PDMS SL+ coating with PEGMA	Surface modification of PDMS with PEGMA prolonged stability[163]	2006
Quartz photolithography	MS percentage increased from 21.1% to 67.1% which facilities sperm selection for ICSI[164]	2007
COP micromachining	COP outperforms PDMS for clinical safety and is cheaper than silicon[24]	2012
PDMS SL+ PEGMA	Multiple channels could increase the volume of processing semen[165]	2014
PDMS	Three inputs/outputs sorter increased the efficiency[166]	2015
QUALIS (FDA approved)	Higher MS sorting and lower DFI compared to clinical techniques[28]	2016

SL: soft lithography; PEGMA: poly(ethylene glycol) methacrylate; MS: motile sperm; COP: cyclic olefin polymer; DFI: DNA fragmentation index.

2.2.2. Motility-Independent Sperm Selection

Although sperm motility is the gold-standard for a selection assay, there are several instances, where it cannot be used. For example, to treat asthenospermia (the infertile male with poor sperm motility) or retrieve cryopreserved sperms (which had been frozen before the patient underwent chemotherapy), very limited viable sperms are available. Conventional lab-based viability tests such as LIVE/DEAD viability kit, dye exclusion, and hypotonic swelling are invasive, low-throughput and potentially toxic for sperm. Alternatively, Garcia et al. fabricated a microfluidic platform to generate the DEP field using optoelectronic tweezers to noninvasively sort viable sperms from nonmotile sperm sample.[35] Termed as optoelectrical microfluidics, Mishra et al. reviewed the fundamentals and other applications of this emerging field in cell trapping and cell sorting.[36]

Another interesting particle separation technique in microfluidics is pinched flow fractionation (PFF) as described by Yamada et al.[37] PFF is an efficient method for label-free sorting a mixture of particles with different dimensions by regulating the flow rate. The key separation principle in PFF is based on hydrodynamic flow at low Reynolds number. Depending on the channel geometry and the flow rate ratio, particles with various sizes and shapes can be efficiently separated. Therefore, this method does not rely on sperm motility and can be used for selecting nonmotile sperms from other species. Liu et al. used a modified version of PFF to separate a mixture of epithelial cells (with 40–60 μm diameter representing the victim's cells) from human sperm heads (with 4–6 μm diameter representing the perpetrator's cells) with the potential application in sexual assault and forensic medicine.[38] However, numerical and experimental results conducted by Berendsen et al.[39] demonstrated that PFF alone could not be used to separate a mixture of sperms with flagellar (tails) from 11 μm microbeads. It was mainly related to the oblong shape of spermatozoon whose tail is almost 10 times larger than its head (≈50 μm (tail) × 5 μm (head)). To overcome this problem, the authors combined PFF with hydrodynamic filtration technique and incorporated a pillar array with 4 μm spacing in the PFF system, as shown in **Figure 3**A.

Inertial microfluidics can also be used as an efficient passive mechanism for cell sorting.[40] At a relatively high Reynolds number, the inertia of the fluid flow in microchannels becomes necessary. Therefore, particles in the flow with different sizes can be separated using inertial migration.[40] Son et al. used such technique in spiral microchannels to separate nonmotile sperms from red blood cells[41] (Figure 3B). This microfluidic

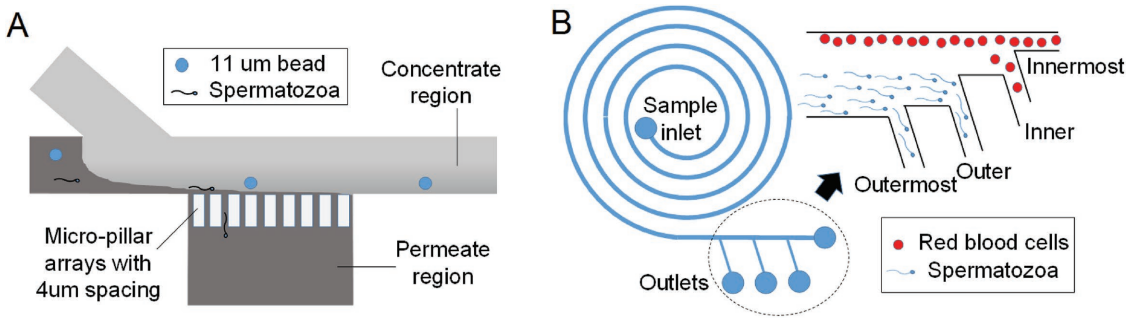

Figure 3. Two passive microfluidic sorting techniques for the selection of nonmotile sperms. A) The schematic of modified pinched flow fraction (PFF) technique developed by Berendsen et al. They coupled the PFF with pillar arrays (4 µm spacing) as a filter for hydrodynamic trapping. As shown in the figure, sperms can pass through the pillar while the microbeads with 11 µm diameter cannot pass the filter. B) The inertial microfluidic sorting technique used to sort nonmotile sperms from red blood cells. Following a testicular sperm extraction procedure, the sample is mainly composed of sperm cells and other cells (mainly red blood cells). The sample is injected at the inlet of the spiral microchannel. The lift and drag forces cause the sperm cells to be separated from other cells and will be collected from outlets. The figures were redrawn with some changes from ref. [39] and ref. [41], respectively.

sperm sorter can be used as an alternative to expensive clinical counterparts, especially for azoospermic patients. Limitations of current clinical practice in treating nonobstructive azoospermia and the possibilities offered by microfluidic technology are evaluated by Samuel et al.[42]

2.3. Sperm Guidance by Mimicking the Environment of Female Reproductive Tract

Once considered as a passive canal, it is now well known that female reproductive tract plays an important role in selecting and guiding sperms for fertilization. Before storing in oviduct reservoir, only morphologically normal and motile sperms can pass through highly viscous cervical mucus.[43] At the time of ovulation, the stored sperms become capacitated and hyperactive. These capacitated sperms travel through the oviduct via three guiding mechanisms including chemotaxis (movement of sperms due to chemical gradient in oviduct surface), thermotaxis (movement of sperms due to temperature difference on oviduct surface), and rheotaxis (movement of sperms due to intrinsic property of sperms that swim against the flow). Therefore, mimicking the natural environment of female reproductive tract is of great importance for a successful IVF. Suarez and Wu reviewed various microfluidic platforms for studying sperm migration toward oocyte in the female reproductive tract.[44] In the following sections, we will briefly review the microfluidic devices which sort sperm inspired by natural conditions of female tractive tract for sperm guidance.

2.3.1. Sperm Motility Based on Geometry and Viscosity Conditions in Oviduct

Inspired by studies in bacterial migration, Denissenkoa et al. studied sperm behavior in curved microchannels with cross sections similar to an oviduct (100 × 100 µm).[45] The authors showed that motile sperms follow the boundaries of the channel. Sperms tend to swim in one direction rather than scattering in 3D. Utilizing this unique feature of motile sperms, Nosrati et al. fabricated a network of 500 radial microchannels capable of processing the largest sperm volume (1 mL) among reported microfluidic sperm sorters.[46] The device mimicked the high viscosity stagnant condition of oviduct whereby sperms spontaneously swim through the outlet. This biomimetic sperm sorter also improved DNA integrity of the selected sperms to more than 80%. Recently, the same group fabricated three versions of this sperm sorter, but this time restricted the motion paths in only three directions.[47] The authors showed that motile sperms that swam along the corners of the microchannels (right and left swimmers) had a higher DNA integrity compared to straight swimmers. The same research group also compared the sperm viability in hyaluronic acid (HA) and methyl cellulose (MC) to demonstrate the effect of viscosity on the performance of sorting.[48] The authors showed that MC, which had a higher viscosity similar to that of the cervical mucus, outperformed the lower viscosity one (i.e., HA).This experiment confirms that the highly viscous environment of the reproductive tract promotes sperm motility. The authors later suggested that in a similar microenvironment of the oviduct, i.e., highly viscous medium (viscosity larger than 20 mPa s) and short distance (within 1 µm from the surface), human sperms adopt a distinct 2D swimming mode that enables them to swim faster.[49]

2.3.2. Sperm Chemotaxis

Sperm chemotaxis, movement of sperms due to a gradient of the chemoattractant concentration, is one of the most important factors facilitating the navigation of sperms toward oocyte in a female reproductive tract. The chemoattractant is the progesterone secreted by cumulus cells of an ovulated oocyte which attracts selected sperms. There are a number of microfluidic platforms utilizing a gradient of chemicals to sort the motile sperms selectively[50] and had already been reviewed elsewhere.[26,51]

Using a microfluidic chemotaxis sorter coupled with IVF, Hussain et al. recently showed that chemotactic sperms subsequently increased the fertility rate in sea urchin.[52] Zhang et al. devised a hexagonal shape microchamber connected to six microchannels to simultaneously study the effect of sperm chemotaxis at three concentration gradients of progesterone.[53] The merit of their system was the use of precise concentration

gradient generator (CGG) to solely study sperm chemoattractant behaviour. This design could also include three parallel CGGs for better comparison.

2.3.3. Sperm Thermotaxis

Movement of sperms toward high concentration of progesterone, chemoattractant, can take place within the vicinity of an oocyte. Therefore, long-distance navigation of sperms in mammalian oviduct is justified by sperm thermotaxis, the ability of selected sperm to change their swimming direction at a higher temperature. Though not measured for human, the surgically removed rabbit oviduct showed 1.6° temperature difference along its 10 cm length. Following the initial work of Ko et al. the only completed microfluidic device to study sperm thermotaxis was reported by Li et al.[54] In their design, motile sperms are first selected in a straight microchannel using the standard screening protocol. Then, the thermotactic ability of previously selected motile sperms is evaluated in a chamber, where the temperature is varied in 1.3° intervals using an external thermal controller. Moreover, reverse air flow enabled motile sperms being trapped in the thermal gradient channels. Using this design, they successfully showed sperm thermotaxis in ≈10% of motile sperms through 36.0–37.3 °C temperature range. Therefore, an integrated microfluidic platform that sorts the most motile, chemotactic and thermotactic sperms better mimics the in vivo natural sperm selection and consequently can significantly improve the fertility rate and embryo quality.

2.3.4. Sperm Rheotaxis

Some aqueous swimmers can orient themselves and swim against (positive rheotaxis) or toward (negative rheotaxis) a fluid flow. Sperms also show positive rheotaxis by aligning themselves opposing the flow direction, which is from oviduct to uterus, and they swim upstream toward the oocyte. Studying the wavelike-motion of worms against the flow, Yuan et al. showed that this behavior could be justified merely by hydrodynamic nature of a flow field near a surface.[55]

Similarly, Zhang et al. proved that sperm rheotaxis is a passive mechanism, and no sensory or active process is required.[56] However, Miki et al. had already quantified a unique parameter of sperm motion termed as "head's rolling rate" which can provide the necessary mechanical force for positive sperm rheotaxis.[57] Their obtained results also suggested that among all other factors, long-distance sperm traveling toward oocyte is related to its rheotactic behavior. Kantsler et al. conducted the very first microfluidic assay to thoroughly characterize the sperm rheotaxis based on flow rate and fluid viscosity.[58] Using a microfluidic platform, El-Sherry et al. confirmed the importance of sperm rheotaxis for its guidance in mammalian reproductive tract.[59] The authors evaluated the effect of shear stress and geometrical conditions on sperm rheotactic behavior.[59] They observed that more than 80% of sperms show rheotactic behavior if the flow velocity is less than 130 μm s^{-1}. Recently, the same research group investigated the effect of pH variation on bull sperm rheotaxis and motility using a microfluidic setup coupled with CASA.[60] The authors showed that while pH variation did not affect sperm motility, it significantly affected the rheotactic behavior. Specifically, pH values in the range of 6.4–6.6 resulted in best sperm rheotaxis.[60]

2.4. Oocyte Analysis and Selection

Unlike the abundance of sperms that are produced daily, the total number of mammalian oocytes are fixed and limited throughout the lifetime. Even with hormone stimulation, the maximum number of retrievable oocytes from a human is 8–10.[61] Nonetheless, it is one of the largest cells among human cells (100–150 μm). Therefore, it is well suited to the size scale of microfluidics, where single-cell analysis can also be performed precisely. Early research in this field reported successful chemical (removal of zona pellucida) and mechanical (removal of cumulus cells) manipulations of the oocyte.[62]

Selecting oocyte of the highest quality prior to fertilization is as important as selecting high-quality sperms. Oocyte quality can determine the fate of fertilization and subsequent embryo health. However, main features of "high-quality" oocyte are subjective criteria. While morphological analysis based on image processing can reveal valuable information about the quality of oocyte, its application is time-consuming, subjective and does not fit for automation purposes. Microfluidic platforms can provide more quantitative analysis of oocyte quality. Known as simple perfusion apparatus (SPA), the microfluidic system devised by Angione et al. is a perfect example of such platforms[63] (**Figure 4**A). The hydrodynamic trap of SPA coupled with temperature control stage and media perfusion chambers provided an excellent platform for image analysis of viable oocyte. Using inverted/polarization microscopy to detect the meiotic spindle, the oocyte quality can be assessed in such a system. The integrated optical sensor had also been used to noninvasively determine the maturity of the oocyte in microchannels[64] (Figure 4B).

Similar to sperm selection, DEP can be applied to differentiate healthy oocyte as reported by Choi et al.[65] To automate the manipulation and alignment process of oocyte, the same group modified the DEP separation by an optoelectrofluidic platform.[66] As shown in Figure 4C, a photoconductive layer was applied to an electrode while the other end was the ground electrode. When the light was focused on a liquid crystal display (LCD) and directed to a region of the photoconductive layer, it became transparent, and the electrode underneath became active. This optically induced electrical gradient causes the oocyte to move the photoconductive layer (positive DEP force) upward. Under the influence of positive DEP, the authors observed that healthy oocytes moved faster than the unhealthy ones. Mechanical stiffness of oocyte can also be used as a biomarker for maturity and fertilization. Microfluidic devices were also utilized to measure the biomechanical properties of the oocyte[67] (Figure 4D). In various stages of oocyte meiotic maturation and subsequently after its fertilization, the stiffness of its zona pellucida changes accordingly. The microfluidic approach can provide a minimally invasive platform to quantify the zona pellucida softening/hardening based on Young's modulus of elasticity. The advances in this part are extensively reviewed.[68] Once considered

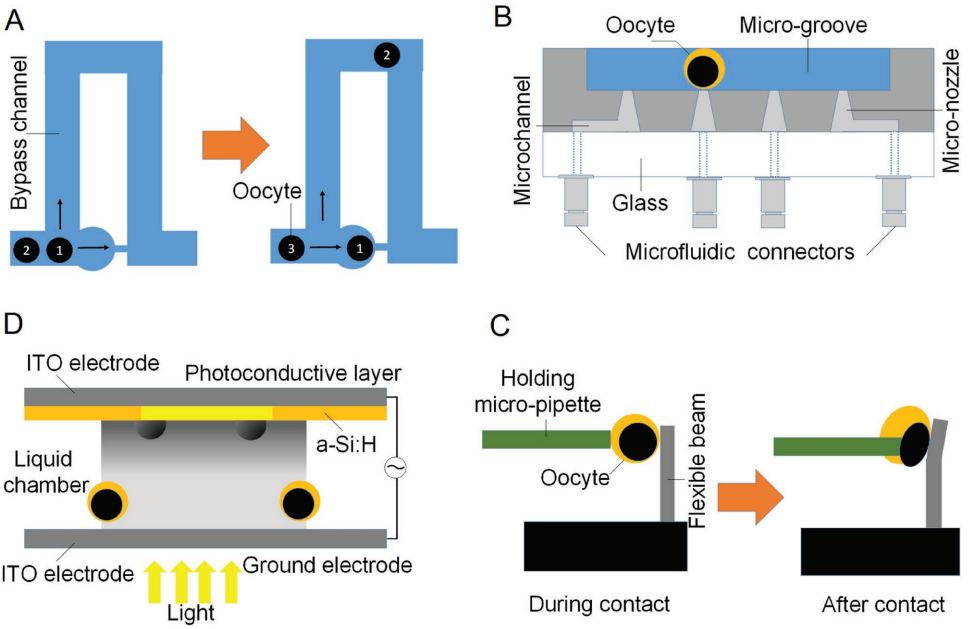

Figure 4. Different mechanisms reported in the literature to select high-quality and healthy oocyte. A) Schematic representation of simple perfusion apparatus (SPA) approach developed by Angione et al. It is based on a hydrodynamic trap coupled with a temperature control stage for long-term monitoring. Depending on the ratio of the trap resistance (R_t) to the bypass channel resistance (R_b), oocyte trapping mechanism can be either direct ($R_t/R_b < 1$) or indirect ($R_t/R_b > 1$). B) The microfluidic oocyte handling device with integrated optical maturation sensor. C) Dielectrophoretic force (DEP) separation coupled with an optoelectrofluidic platform for automation. The photoconductive layer consisted both indium tin oxide (ITO) electrode and hydrogenated amorphous silicon (a-Si: H). Upon illumination, the exposed portions of the photoconductive layer become transparent, and nonuniform electric field will be generated. Subsequently, healthy oocytes move toward positive DEP force. D) Quantification of oocyte mechanical stiffness with deformation-load experiment. A micropipette is used to press the oocyte against a flexible beam. By measuring both the oocyte deformation and the beam deflection, the stiffness of the oocyte can be estimated. The figures were redrawn with some changes from refs. [63,64,66] and [67b], respectively.

redundant, cumulus cells surrounding the oocyte can also reveal valuable genomic information about the oocyte quality.[69] This approach is similar to cell-free circulating tumor DNA, which first had been considered as cell waste; yet, now is a promising cancer biomarker.[70] Selecting "high-quality" oocyte based on genomic biomarkers expressed in cumulus cells can subsequently lead to successful insemination and pregnancy.[21]

Table 3 summarizes other microfluidic techniques that had been used in oocyte manipulation and characterization. Most importantly, microfluidic platforms can be used to characterize

Table 3. Summary of microfluidics used for oocyte characterization and in vitro maturation.

Oocyte analysis	Measured parameter(s)	Fabrication Technology	Benefit(s)	Year
Osmolality Characterization	Oocyte temporal volume variation	PDMS SL	Decreased osmotic shock and toxicity[167]	2011
	Oocyte/zygote Shrinkage rate	PDMS SL	Automated gradual adding of CPA decreased shrinkage rate[94]	2015
	Oocyte temporal volume variation	Silica glass slides sealed with silicone rubber sheets	Oocyte transport properties can be quantified in a controllable and recyclable device[168]	2017
In vitro oocyte maturation (IVM)	% of oocytes advancing to metaphase II	PDMS SL	Microfluidic IVM resembled in vivo maturation+ denudation occurred without enzyme[169]	2016
	Maturation procedure followed by IVF	PDMS SL	Oocytes were matured in both dynamic and static microfluidic devices. Dynamic IVM was superior[170]	2016
	Maturation procedure followed by IVF	COP with combined hot-embossing & micromilling	The device has more commercial capability, yet requires further improvement[76]	2017

SL: soft lithography; CPA: cryoprotective agents; IVM: in vitro oocyte maturation; COP: cyclic olefin polymer.

the oocyte transport properties. It is particularly important in vitrification and drug administration where the media compositions surrounding the oocyte are subject to change. This change may induce excess osmotic pressure which can affect the oocyte volume. In vitro maturation (IVM) is also another important procedure which can be conducted in microfluidic devices. Rather than expensive enzymatic treatment, microfluidic platforms can successfully mature oocytes with improved cleavage rate.[71]

2.5. Insemination

Regarding insemination, CFM can be used for both IVF and real-time imaging of the fertilization process to reveal the underlying mechanism. For a successful IVF, proper oocyte manipulation, trapping and retrieval are necessary. Microfluidic IVF can be coupled with both sperm and oocyte sorters. One of the greatest advantages of microfluidic IVF is the reduction of polyspermy.[72] It is mainly because unlike static test tube or microdroplet, the oocyte can be exposed to few selected sperms in a microfluidic device. Therefore, a proper sperm sorter increases the probability of monospermic oocyte penetration. Microfluidic IVF also requires a lower sperm concentration. Suh et al. showed that with a sperm concentration as low as 2×10^4 Sperm mL^{-1}, the fertilization rate was significantly higher in a microfluidic platform compared to standard well dishes.[73] Therefore, microfluidic IVF can be considered as an alternative to clinical ICSI; yet, more clinical trials and comparison are required.[74] However, it is worth mentioning that none of the reported microfluidic IVF platforms had been equipped with a proper oocyte sorting mechanism to actively select high-quality and healthy oocytes for fertilization. **Figure 5** summarizes

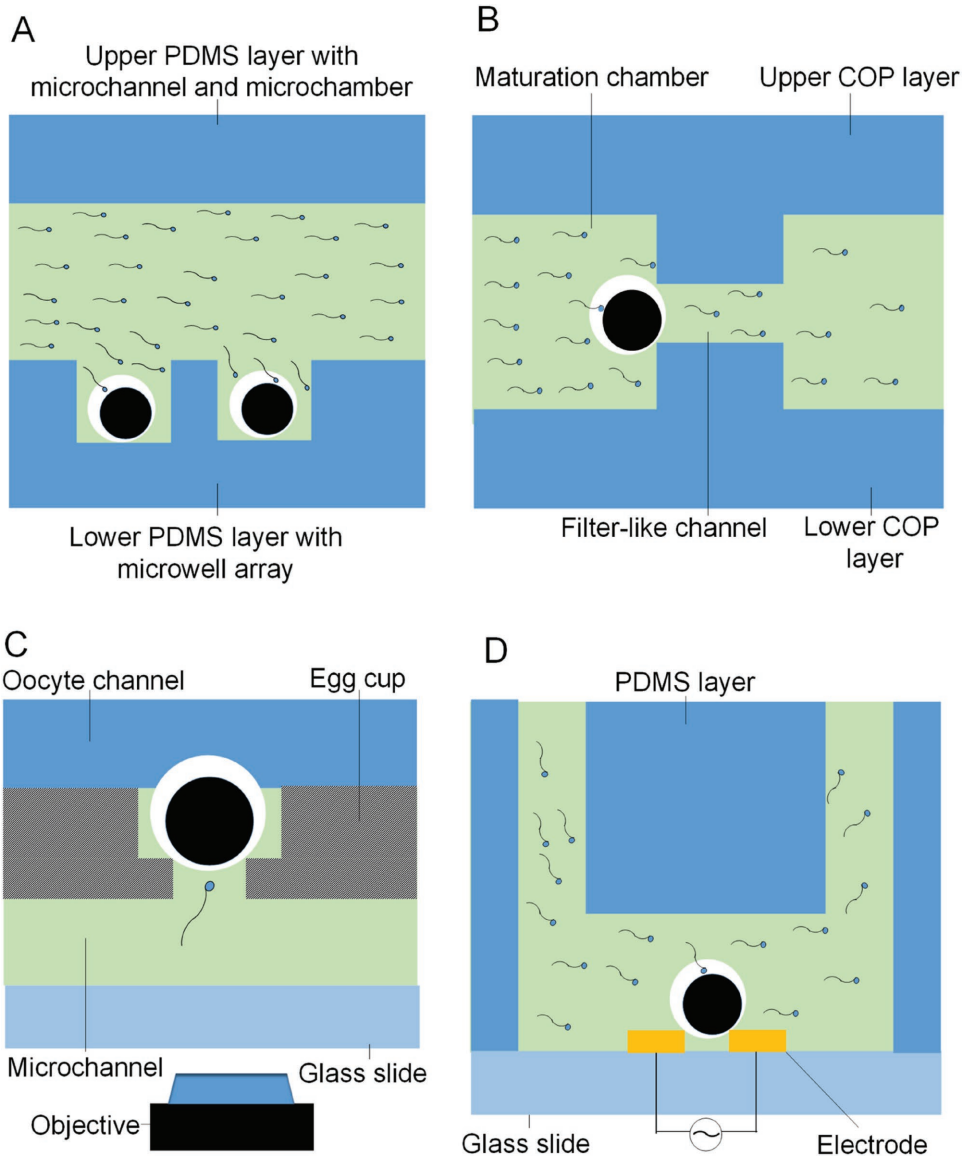

Figure 5. Various microfluidic mechanisms used for insemination. A) Microwell arrays for oocyte trapping[75]; B) filter-like microchannel design to confine the oocyte fabricated from cyclic olefin polymer (COP)[76]; C) egg cup oocyte trapping with on-chip imaging of insemination process[77]; D) oocyte trapping with positive DEP.[78] The figures were redrawn with some changes from refs. [75,77,76] and [78], respectively.

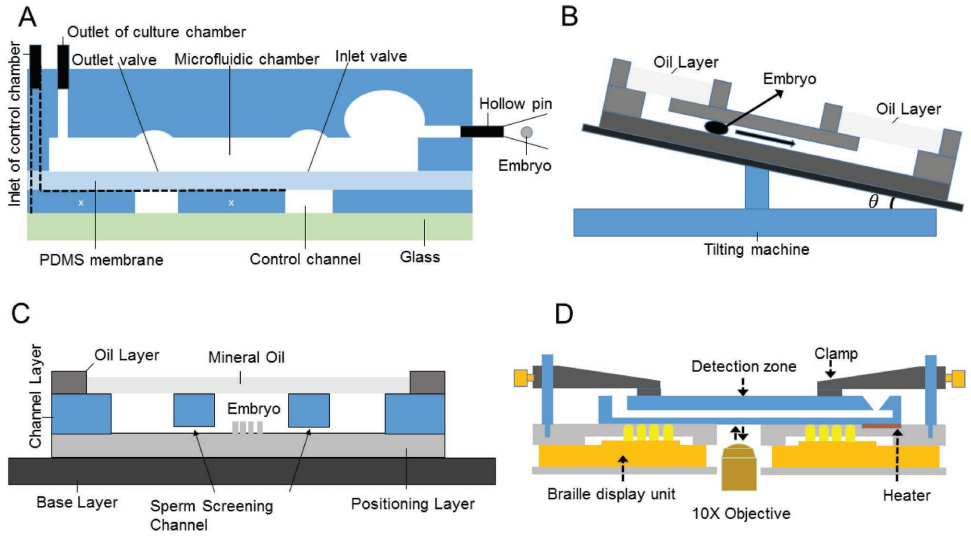

Figure 6. Different microfluidic device for embryo culture. A) Static culture with a group of two embryos per chamber[84]; B) dynamic culture of a group of embryos with mechanical stimulation (tilting machine)[89b]; C) single culture within a microfluidic device[50c]; D) dynamic culture with controlled shear stress (a piezoelectric actuator with Braille system).[97b] The figures were redrawn with some changes from refs. [84,89b,50c] and [97b], respectively.

different microfluidic mechanisms used in the literature to inseminate oocyte, i.e., microwell arrays,[75] filter-like microchannel,[76] egg-cup structure,[77] and noncontact positive DEP trapping.[78] Han et al. devised a two-layer microfluidic device for oocyte trapping and subsequent insemination and embryo culture[75] (Figure 5A). The first layer consisted of a microchannel and a microchamber, and the second layer included the microwell arrays to trap the oocyte. To facilitate oocyte/embryo retrieval, the first and the second layer were partially bonded with oxygen plasma. Recently, Berenguel-Alonso et al. used hot embossing with COP to fabricate a filter-like microfluidic structure[76] (Figure 5B). The design allows the sperms to pass through while restricts the motion of the oocyte. Ravaux et al. reported the fabrication of a microfluidic device with an egg cup structure[77] (Figure 5C). In such device, sperms can inseminate the entrapped oocyte from the bottom microchannel, and an optical microscope can take the images of the insemination process. Huang et al. devised a noncontact microfluidic platform to trap oocytes[78] (Figure 5D). The authors showed the response of sperms and oocytes to positive DEP under the applied bias conditions. The device also increased the concentration of sperms in the area near the oocyte. This increased concentration can increase the possibility of insemination. In addition, the trapping mechanism is based on the noncontact force which decreases the probability of oocyte/embryo damage. Therefore, such microfluidic device based on DEP can be an excellent candidate for clinical IVF; yet, clinical trials with the human sample and subsequent follow-up studies are required to evaluate the effectiveness of the device.

2.6. Embryo Culture

CFM can provide an in vivo-like microenvironment to facilitate embryo development from a zygote up to a blastocyst stage. In the last two decades, many microfluidics research groups have focused in this area of ART as recently reviewed.[74,79] These microfluidic platforms employed both static and dynamic culture conditions.

Static microfluidic platforms mainly consisted of microwells,[80] glass capillaries[81] and microchannels.[82] Here, the high surface-to-volume ratio of microfluidic devices offers a significant advantage. Embryo density (defined as the ratio between the number of embryos and the volume of culture medium) also plays an important role. Most studies confirmed the advantages of group culture (high embryo density) due to an increased rate of secreted growth factors. However, gene expression study conducted by de Oliveira et al. showed that increasing embryo density could compromise embryo development.[83] **Figure 6** summarizes the microfluidic systems capable of single/group embryo culture. Melin et al. devised a microfluidic device capable of culturing a group of two embryos up to blastocyst stage in each sub-microliter microwells.[84] Esteves et al. demonstrated that unlike clinical microdrops, single embryo cultured in a microfluidic device could successfully lead to normal birth.[85] CFM also provides a dynamic culture microenvironment similar to in vivo conditions embryo experiences in the oviduct. This dynamic environment may refer to shear stress produced as a result of flow rate or mechanical stimuli.[86] Flow rate can be controlled by hydrostatic pressure,[72c,73] syringe pump[87] or Braille actuator.[88] Mechanical stimuli also included tilting culture system,[89] vibration[90] and rotation.[91] Microfluidic devices can also be used to coculture the embryo with somatic cells. These systems will be further discussed in Section 5, organ-on-a-chip.

2.7. Embryo Selection

As a final step in a successful IVF process, selecting the "best embryo" is crucial. Rather than subjective observation, microfluidics can offer more objective-based platform. Categorized

based on oxygen and nutrient consumption rate as well as morphokinetics, the advances using CFM (mostly in microwell configuration) were reviewed recently.[79a] However, due to the invasiveness of the analysis and possible contamination of the culture media, such approach can be problematic. With the improvement in preimplantation genetic screening (PGS), future work may focus more on detecting embryo genomic biomarkers as noninvasive tools to select the "best embryo." Droplet-based microfluidics can be a promising approach in this field which will be discussed in Section 4.

2.8. Cryopreservation

Preserving the vital gamete/embryo at low temperature for future use is an important clinical procedure. A common problem of cryopreservation is an osmotic shock, membrane damage, and ice crystal formation.[92] To avoid ice crystallization and subsequent cell dehydration and damage, loading of cryoprotective agents (CPAs) is essential. However, long-term exposure or a high concentration of CPAs can be detrimental.[93] Accordingly, diffusion-based laminar flow in CFM can eliminate these problems.[94] For instance, Zhao and Fu[95] showed that microfluidic setup could reduce osmotic shock and increase postthaw survivability by precisely loading and unloading the cryoprotective agents in microchannels. This research group recently reviewed the state-of-the-art advances of microfluidics in cryopreservation and vitrification.[96]

2.9. Limitations and Technical Challenges of CFM in ART

As briefly reviewed above, the field of CFM has greatly contributed to ART. Some works demonstrated the potential of this field to integrate multiple IVF steps on a single chip.[50c,97] Despite the sophisticated proof of concept platforms, very few of these devices had translated into clinical settings. The reason can be mainly related to the inherent limitations of CFM. First, due to limited access of bonded microchannels, retrieval of gamete/embryo is problematic. To overcome this issue, most researchers used microfunnel for loading and unloading the gamete.[62b,88b] Others have used open channels with mineral oil overlay.[98] As shown by Han et al.,[75] reversible bonding of the cultured region can also be useful. The second limitation is related to the additional equipment required to drive the flow through or handle, manipulate and analyze the gamete/embryo in bulk flow. The external setup makes packaging and commercialization very difficult and renders the field ironically from lab-on-a-chip to chip-in-a-lab. One approach to address this problem could be using the idea of "Microfluidic Apps."[99] In this method, microfluidic technology can be used in conventional laboratory-based or home-based instruments. The recent work described by Kanakasabapathy et al. is a perfect example of using "Microfluidic Apps" for ART application.[16] Rather than requiring auxiliary optical/image analyzer platforms, the microfluidic device can be connected to user's smartphone for readout.

The common use of PDMS soft-lithography in academia is another obstacle in the commercialization of microfluidic platforms. Hot embossing and microinjection molding of thermoplastic polymers are superior mass production techniques. In ART application, the clinical safety of PDMS is questionable. PDMS is oxygen-permeable which is excellent for somatic cell culture application. Somatic cells require atmospheric oxygen concentration, 21%, and 5% CO_2 concentration. It can be accomplished by simply putting the whole PDMS-based cell culture chamber into the incubator. However, the mammalian embryo only needs 5% oxygen concentration[100] while its exposure to CO_2 could be harmful. Moreover, penetration of water from PDMS into the culture media may adversely alter the osmolality of the solution. As illustrated in Table 2, while PDMS soft-lithography is a good first-step for proof-of-concept fabrication, commercial microfluidic sperm sorters have been fabricated using COP hot embossing. Moreover, unlike PDMS, COP is gas impermeable. Recent work conducted by Berenguel-Alonso et al.[76] showed that this property could play two opposite roles. In their design, the insemination was significantly lower in the COP microchip compared to that in a conventional culture dish. However, COP microchip with an integrated heater does not require incubation, greatly reducing the risk of atmospheric contamination and eliminating the need for additional equipment such as an incubator.

The aforementioned limitations of CFM open rooms for other emerging branches of microfluidics such as paper-based, droplet-based and tissue engineering microfluidics for ART applications.

3. Paper-Based Microfluidics in ART

As discussed in the previous section, plastic/polymer-based CFM requires an external force to drive the fluid. This external force should be provided either passively, e.g., hydrostatic pressure (gravity), or actively, e.g., syringe pump, centrifugal force, and piezoelectric actuator. CFM devices need external equipment which is an obstacle for their commercialization. Therefore, low-cost and pump-free microfluidic devices have a better chance for commercialization success. To this aim, disposable capillary driven paper-based microfluidics has been evolved.[101] Paper is an excellent candidate for fabricating point-of-care diagnostic devices, where the low-cost and fast response from the small sample are crucial. Recently, its use has been extended to detect tumor biomarkers.[102] In the realm of ART, paper-based microfluidic devices are perfectly suitable for male infertility diagnosis. Their usage as a commercially available home-based diagnostic test to determine the sperm concentration is prevailing. FertilMARQ is a commercially available home-based test kit, which stains a specific protein in semen and compares it with the reference color to determine the user sperm concentration. SpermCHECK FERTILITY and Babystart SpermTest are also home-based sperm test kits that use the same principle to determine the range of sperm concentration in 10 min with 98% accuracy. However, these paper-based test kits are yes/no devices and cannot be used in clinical settings. Using wax printing followed by image analysis, Matsuura et al. measured both sperm concentration and sperm motility by a paper-based microfluidic device and a digital camera (**Figure 7**A).[103] Thiazine and tetrazolium-based colorimetric assays were

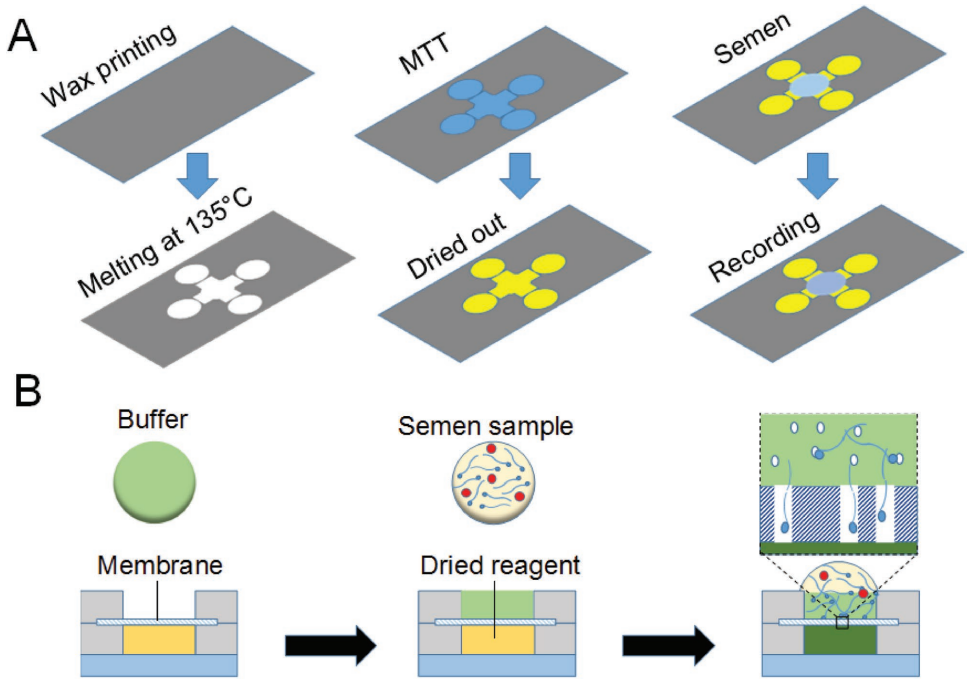

Figure 7. Various paper-based microfluidics for semen analysis. A) The fabrication process of the device designed by Matsuura et al. the analysis of both sperm concentration and sperm motility[103]; B) Detailed motile concentration assay of the device developed by Nosrati et al.[106] The figures were redrawn with some changes from refs. [103] and [106], respectively.

used to estimate the sperm concentration and sperm motility, respectively. Later, they successfully used the same device in an infertility treatment clinic to quantify the sperm concentration and motility of 120 patients.[104] Recently, the group used this device to investigate the relationship between porcine sperm motility and sperm enzymatic reactivity in the presence of sperm motility inhibitors.[105] Similarly, Nosrati et al. fabricated a three-layer paper-based microfluidic device to measure live sperm concentration, motile sperm concentration and sperm motility[106] (Figure 7B). Instead of a digital camera, which was used by Matsuura et al.,[103] a letter-sized scanner captured the images before and after semen sample. The authors have demonstrated that paper-based microfluidics can be used to measure sperm DNA integrity regarding DNA fragmentation index[107] as well as DNA packaging.[108]

4. Droplet-Based Microfluidics in ART

Due to the limitations of continuous and single-phase flow in microchannels, the recent research interest has been shifted toward droplet-based microfluidics. Droplet-based microfluidics is a promising strategy to control and manipulate the individual compartment of fluid, rather than the bulk flow. Study of micro-segmented flow is more analogous to and better mimics the fluidic compartmentalization in nature as thoroughly explained in ref. [109] While droplet-based microfluidics still benefits from advantages of homogenous and single-phase CFM, it eliminates some of its limitations. For instance, the velocity profile in laminar continuous-flow is parabolic, meaning that particles near the center line of channel experience higher velocity. Also, in CFM, mixing is dominated by molecular diffusion, and cross contamination may also occur. The main advantages of droplet-based microfluidics include a further reduction in sample/reagents, faster reaction time, high-throughput screening, contamination-free fluid handling, enhanced automation potential, and increased control over dispersion, mixing, and separation.[110] Based on the fabrication and handling technique, the field itself can be further divided into two groups: (i) emulsion-based droplet microfluidics and (ii) digital microfluidics.[110a]

4.1. Emulsion-Based Droplet Microfluidics

In emulsion-based droplet microfluidics, either droplet or plug can be formed from the interaction of two immiscible fluids (such as oil and water-in continuous and disperse phases, respectively).[111] This can be a very facile method to automatically generate individual microbioreactors from the cell suspension. For ART application, this branch of microfluidics is very similar to what embryologists perform in clinical IVF, i.e., encapsulating the gamete in microdroplet covered with oil overlay. Moreover, the technology can overcome many handling difficulties of microdroplet in such clinical settings. Unlike CFM, emulsion-based microfluidic platforms solving tasks of ART or IVF are very limited in the literature. Using this approach in a flow focusing geometry, Clausell-Tormos et al.[112] successfully showed the viability of full-life cycle of encapsulated (both in droplet and plug) human cells and multicellular organisms (*C. elegans*) (**Figure 8**A). Employing a nonplanar microfluidic flow-focusing platform, Agarwal et al. devised a core–shell encapsulation technique for 3D culture of embryonic

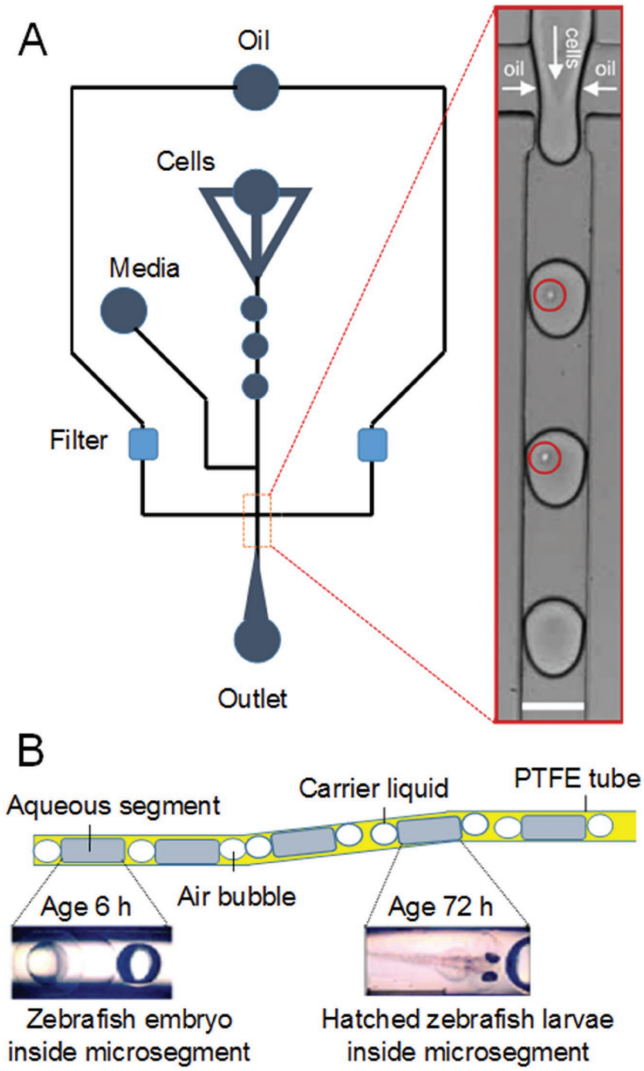

Figure 8. Application of emulsion-based droplet microfluidic in encapsulating: A) Human cells and multicellular organism are encapsulated using flow-focusing geometry; B) Illustration of the microfluid segment technique developed by Funfak et al.[115] The authors encapsulated zebrafish egg into the aqueous segment and monitored its development inside the system until it hatched. The figures were redrawn with some changes from refs. [112] and [115], respectively.

cells.[113] Subsequently, they used this technique to in vitro culture the ovary follicles with alginate and type I collagen as shell and core, respectively.[114] This method will be later discussed in the section covering microfluidics-based tissue engineering.

Microsegmented flow can be used for gamete encapsulation and culture. The most relevant work in this field was reported by Funfak et al.[115] (Figure 8B). The authors used the so called "microfluid segment technique" to encapsulate the eggs from zebrafish (*Danio rerio*) until hatching time in polytetrafluoroethylene (PTFE) tube with perfluoro methyl decalin (PP9) as the carrier liquid. To generate the fluid segment, a computer-controlled syringe pump was used to precisely control the flow rate. First, the tube was filled with the carrier liquid with a flow rate of 500 μL min^{-1}, and the suspension of the aqueous embryo was pumped in at a flow rate of 30 μL min^{-1}. Using a stop-flow method, ten series of segmented flow, each containing one zebrafish egg, was produced inside the tube and maintained for over 72 h until the fish hatched. Each segment was supported by air plug in a background of the carrier liquid. This work showed the feasibility of automatically controlled microsegmented flow in supporting the embryo viability in confined geometries.

4.2. Digital Microfluidics

Digital microfluidics is another emerging and very promising field of droplet-based microfluidics. Using this technology, individual droplets (either in open or confined channels) can be precisely manipulated by noncontact forces such as electrical, magnetic or thermal ones. Using noncontact forces is of great benefit especially for ART and IVF applications. Hydrodynamic flow in CFM and emulsion-based microfluidics is potentially detrimental to gamete/embryo development. Culturing preimplantation mouse embryo in microchannels under hydrodynamic flow, Hickman et al.[87b] observed abnormal mouse development at a flow rate as small as 0.5 μL h^{-1}. Subsequently, Luo et al. showed that murine oocyte entrapped in a microchannel subjected to hydrodynamic flow could be significantly deformed and its spindle structure might be severely damaged at flow rates higher than 10 μL min^{-1}.[116] Therefore, digital microfluidics is an excellent candidate for gamete manipulation and/or embryo culture. In this regard, two common digital microfluidics techniques have been used in the literature: electrowetting on dielectric (EWOD) and liquid marble (LM).

4.2.1. Electrowetting on Dielectric

EWOD is a facile method to manipulate discretized droplets on a solid (usually hydrophobic) surface in a programmable manner using electrodes made of a conductor such as indium tin oxide (ITO). At least one of the surfaces in contact with the liquid is insulated with a layer of dielectric, e.g., Parylene, and a layer of a hydrophobic material, e.g., Teflon (**Figure 9**A). The pattern of the electrodes then defines the desired droplet path. Therefore, by arranging the electrodes in the desired way, a variety of parallel or sequential analyses can be accomplished. Son and Garrell were among the first researchers who employed this technique to transport live yeast and zebrafish embryo.[117] They showed contamination-free transport of viable yeast using EWOD technique within 2 h of operation. The zebrafish embryo also remained viable until it hatched inside the two-plate digital microfluidic platform. By mixing a droplet containing a reagent with the droplet encapsulating the zebrafish embryo, the authors also showed the process of chorion removal using such approach. Li et al. demonstrated the use of EWOD-based digital microfluidics for the dynamic culture of the mammalian embryo.[118] The EWOD plates were patterned with ITO electrodes and coated with SU-8 as the dielectric layer and Teflon as the hydrophobic layer. The biocompatibility of these materials, e.g., ITO, SU-8, and Teflon, were experimentally verified. Mouse embryo was encapsulated in a culture media (3.5 μL) as a core and an oil droplet (1.5 μL) as

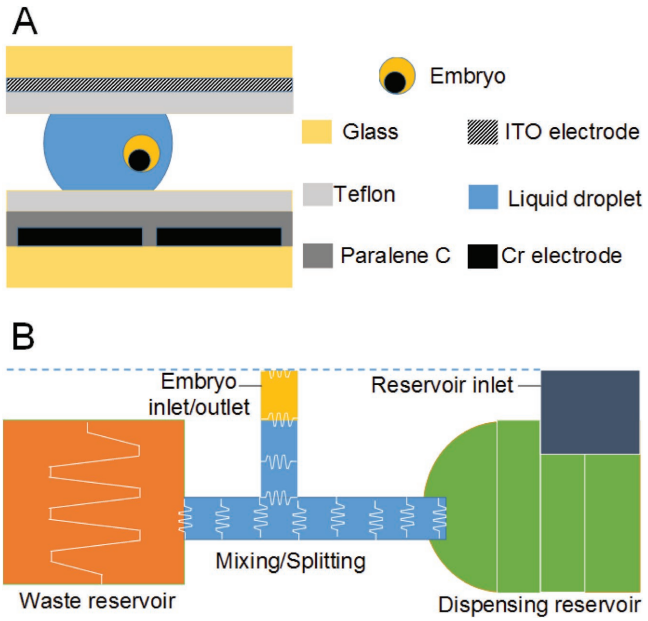

Figure 9. Illustration of EWOD technique. A) Various components of an EWOD system; B) automated mixing and gradual exposure to CPA solution for vitrification of mouse embryo. The figures were redrawn with some changes from ref. [121a].

a shell at the bottom of the EWOD plate. Following the safety assessment of Pluronic F12 as the surfactant, it was used to facilitate the droplet movement in the EWOD device.

The EWOD platform can dynamically culture mouse embryo with the blastocyst rate 62.5% compared to 55% with the control Petri dish method. Subsequently, the same research group further demonstrated the success rate of EWOD digital microfluidics in ART.[119] The group showed live births after transferring the mouse embryo cultured in the EWOD device to the uterus of a pseudopregnant female mouse. First, two-cell stage mouse embryo was encapsulated in the 1 µL culture medium and 4 µL mineral oil as core and shell, respectively. A 260 µm thick adhesive tape was used to bond the two plates. The core–shell encapsulated embryo was then manipulated over 100 times in three days in the EWOD device until it developed to blastocyst and preimplantation stage. The blastocyst rate in the dynamic EWOD device was significantly higher than that in a static counterpart. Ten pups with healthy appearance were born out of 19 implanted blastocysts cultured in the EWOD device, i.e., 50.6% live birth rate.

EWOD technique can also be used for both slow-freezing cryopreservation (SFC) and vitrification of cells, tissues, and embryos. In SFC, low concentration of CPA is sufficient to prevent the formation of intracellular ice crystallization. Park et al. showed that EWOD digital microfluidics could be successfully used to select the optimized mixture of CPAs for efficient SFC.[120] Following a sequential actuation of electrodes, a broad spectrum of individual droplets with different CPAs mixture concentrations was generated. This high-throughput screening platform was then used to characterize the freezing and thawing protocol of the wide range of CPAs mixture containing the desired cells.[120] Key benefits of such DFM platform over CFM counterpart are the simplicity, the ease of fabrication, high throughput and minute reagent requirement. In vitrification, a high concentration of CPA is used. The sample rapidly is then frozen in a liquid nitrogen bath. Therefore, in comparison with SFC, less sophisticated and expensive equipment is required. However, the exposure to a high concentration of CPA can be cytotoxic and post-thaw viability is subjective. Devising an EWOD platform, automated and gradual exposure of mouse embryo to the high level of CPA solution was demonstrated by Pyne et al.[121] In their EWOD device, the CPA solution, which was the combination of dimethyl sulfoxide and sucrose, was loaded into the reservoir electrode before bonding. Figure 9B shows a culture media droplet containing the embryo was then loaded into the device. The sequential actuation of electrodes transports the embryo droplet into the T-junction where mixing with the CPA solution takes place. After a given period, the electrodes split the merged droplet. The segregated droplet without the embryo leaves the device through the waste reservoir. This process of mixing and splitting is repeated until the desired concentration is obtained. The group further compared this automated mixing process with that used in conventional vitrification practice, where the embryo is first exposed to 50% concentration of CPA solution and then will be subjected to its full concentration. Postthaw survivability of the vitrified embryos was higher in the EWOD platform. However, the hydrophobicity of the device can be compromised by the introduction of serum in the culture media. This can potentially hinder the movement of embryo droplet in the instrument. Therefore, designing an EWOD setup with more robust hydrophobicity, e.g., with the superhydrophobic surface, can further extend the applicability of the device. Also, more sophisticated EWOD platforms which can automate the whole process of vitrification on a digital microfluidic chip is expected. screening (NGS) can be integrated and automated using EWOD platform as described by Rival et al.[122] Most importantly, unlike current detection techniques, DMF based on EWOD can process the minute amount of sample cells. It is particularly essential for PGS and preimplantation genetic diagnosis (PGD). Currently, the clinical applicability of PGS and PGD in human embryo is controversial. It can be partly related to technical difficulty as well as potential risks associated with extracting a lot of cells from developing fetus as required for conventional genetic screening techniques. Very recently, Lee et al.[123] used a DMF PCR to detect SEA-type α-thalassemia in blastomere biopsies. Using this method, the authors were able to identify the cultured embryos affected by this gene mutation successfully. This can particularly prevent implanting embryos that would otherwise develop Hemoglobin Bart's hydrops fetalis. Future work with EWOD based PGS and PGD coupled with digital PCR can revolutionize the IVF outcome, preventing severe pregnancy complications and significantly improving the offspring health.

4.2.2. Liquid Marble

Liquid marble can also be regarded as another emerging field of digital microfluidics. Aussillous and Quere[124] first introduced the concept of "liquid marble" as a straightforward yet facile method for manipulating a water droplet on a solid surface. As shown in **Figure 10**A, a liquid marble can be directly

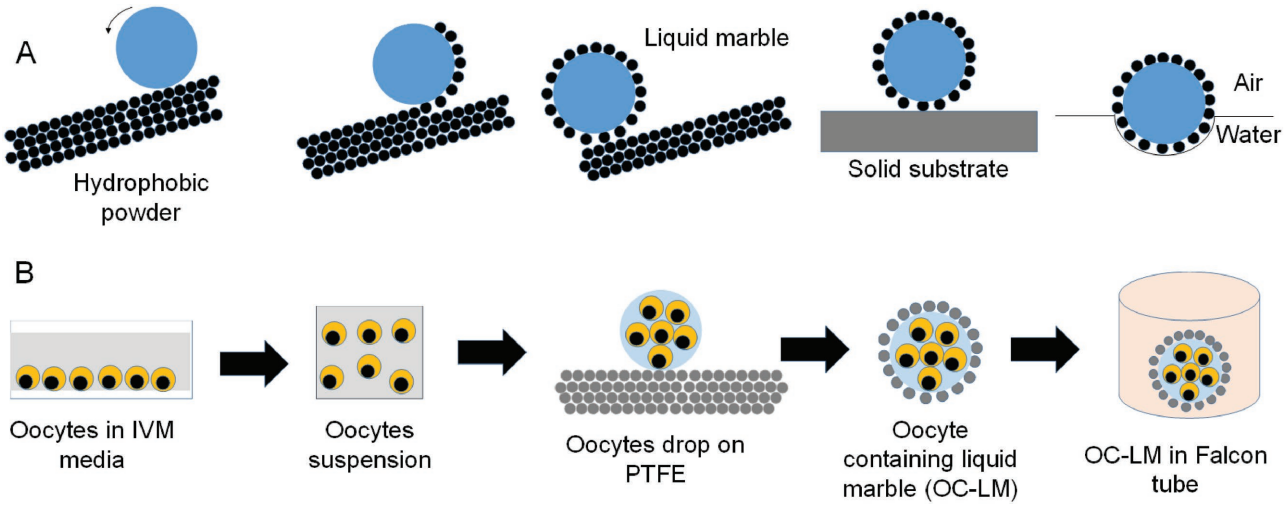

Figure 10. A) Preparation of liquid marble (LM) using PTFE particle; B) detailed process for the preparation of oocyte containing liquid marble (OC-LM) which was used for IVM. The figures were redrawn with some changes from refs. [171,133] and [172], respectively.

prepared by rolling water droplets on hydrophobic powders such as PTFE or lycopodium grains. Electrowetting and controlled motion of LM based on applied voltage were shown by Newton et al.[125] Subsequently, Bormashenko et al.[126] reported magnetically driven ferrofluid LM. Dynamic behavior of magnetically actuated LM in a carrier liquid with different concentrations of aqueous glycerol was also evaluated.[127] Therefore, LM actuated and manipulated with various external forces,[128] can be regarded as a new platform for digital microfluidics. Moreover, LM can be used as a bioreactor for 3D spheroid formation.[129] Sarvi et al. demonstrated the feasibility of formation of uniform embryonic bodies from an embryonic stem cell (Oct4B2-ESC) within 3 d culture time in LM.[130] They also showed that LM microenvironment could facilitate differentiation of these embryo bodies into cardiomyocytes. The application of LM as a minireactor[131] as well as its potential for biological applications[132] has been recently reviewed.

Using LM as an efficient microbioreactor for oocyte IVM, Ledda et al. reported the first application of LM in ART.[133] First, the 30 µL droplet was formed by encapsulating ten ovine cumulus-oocyte complexes (COCs) in an IVM media. IVM media contained tissue culture medium (TCM) 199 supplemented with 10% oestrus sheep serum (OSS) and 0.1 IU (international unit) Luteinizing Hormone (LH) and follicle stimulating hormone (FSH). LM bioreactor was formed by covering the droplet with PTFE particles (Figure 10B). In the control group, the same number of COCs were cultured and matured in four well-plates containing 600 µL IVM media. Although the results showed no significant difference between COCs matured in the LM and the control group, it signifies that LM requiring a minute volume of IVM media can be regarded as a reliable alternative for the conventional IVM platforms. LM bioreactor has also been used for cryopreservation of mammalian cells, murine L929 fibroblasts. Surprisingly, the authors reported that no CPA was required for both cryopreservation protocols, i.e., slow cooling and rapid immersion in liquid nitrogen bath. After the formation of LM containing cell lines and culture medium, it was placed in a cryotube for subsequent freezing (either slow or fast). Following the thawing process, the effects of droplet volume and cell density on viability, morphology, and proliferation of LM cryopreserved cells were evaluated. The authors showed that LM could be successfully used for cryopreservation of mammalian cells without CPA. This study can be further extended for cryopreservation of mammalian embryo and can open a new avenue for utilizing LM-based CPA-free vitrification in ART.

5. Organ-on-a-Chip

Tissue engineering is a highly interdisciplinary field requiring a broad knowledge of engineering, cell biology, material science and microfluidics. When stem cells are used, it can be synonymous with regenerative medicine. It can be used as an alternative to allogeneic tissue transplant.[134] Recently, the term "REPROTEN" (reproductive tissue engineering) has been suggested to introduce a new branch of tissue engineering in reproductive medicine.[135] The integration of microfluidics into tissue engineering can be regarded as an efficient way to vascularize engineered tissues.[136] The vascularization allows continuous perfusion of nutrients and oxygen to the tissue. Thus, it can lead to the fabrication of large-scale tissues more suitable for clinical applications.[137] With the rapid progress in microfabrication technique, especially 3D printing, as well as 3D cell culture scaffolds,[138] more physiologically relevant tissues can be engineered.[139] This organ-on-a-chip technology can be a useful platform for various pharmaceutical and tissue development studies.[140] For ART application, these organ-on-a-chip platforms can lead to functional and physiologically relevant organ-on-a-chip such as ovary-on-a-chip, uterus-on-a-chip, oviduct-on-a-chip, and female reproductive tract-on-a-chip.

5.1. Ovary-on-a-Chip

The ovary is responsible for the periodic production, store, and release of the oocytes. To this aim, ovarian follicles play a

major role in providing the necessary environment for oocyte production. Ovarian cancer, cysts, and aging are common problems affecting the ovarian follicles. In such cases, one versatile method to produce high-quality oocytes is in vitro culture of the ovarian follicles. Accordingly, hydrogel-based tissue engineering can be used by coculturing the somatic cells with the germ cells. Researchers have used an alginate hydrogel scaffold to 3D coculture follicular cells (somatic) and oocyte (germ cells).[141] Murine oocytes matured in 3D hydrogel matrix with mouse follicles had higher quality regarding successful fertilization and pregnancy rate.[142] However, the mechanical stiffness of the mammalian ovary varies. To incorporate this mechanical heterogeneity and extracellular matrix of the ovarian follicles, Choi et al. developed an emulsion-based microfluidic platform to encapsulate the preantral follicles of the deer mouse[143] (**Figure 11**A). This biomimetic core–shell encapsulation platform mainly used nonplanar microfluidic flow-focusing droplet generation to fabricate the ovarian microtissues. As shown in Figure 11A, the follicles suspended in collagen as a core and alginate solution as a shell flow into the device from inlets I-1 and I-2, respectively. Mineral oil with $CaCl_2$ enters from inlet I-3 as the emulsion phase. To further separate each ovarian follicle from each other, the same core solution was injected from I-4. In the meandering microchannels, the shell solution was crosslinked and hardened. An aqueous extracting solution enters from I-5 to separate the oil from the microtissue in the extraction channel with gradually increasing width. The ovarian follicles encapsulated with this technique developed to the antral stage and successfully ovulated to cumulus-oocyte complex. Other microfluidic platforms to culture ovarian follicles are recently reviewed.[144]

5.2. Uterus-on-a-Chip

For women suffering from absolute uterine factor infertility, either traditional/gestational surrogacy or uterine transplant had been the only option. While surrogacy has many legal and ethical issues, allogeneic uterus transplantation also suffers from various limitations. In this context, bioengineering the uterus from biocompatible materials and patient-specific stem cells can be an alternative option. Campo et al. recently reviewed the current advances in tissue engineering and regenerative medicine for the fabrication of artificial uterus.[145]

The present purpose of uterus/oviduct-on-a-chip platforms is to provide an in vivo-like physiochemical environment to enhance embryonic cleavage and develop high-quality preimplantation embryos. One strategy is to provide a cell culture environment similar to the uterus. This technique is analogous to the current expensive practice in some IVF clinics, where the embryos are cocultured with somatic cells, also called helper or feeder cells. In poor prognosis candidates such as those with repeated IVF failures, the embryo coculture can be helpful. These feeder cells can be obtained from the patient's oviduct, e.g., ampullary cells,[146] or through endometrial biopsy from the lining of the uterus.[147] The cocultures of human

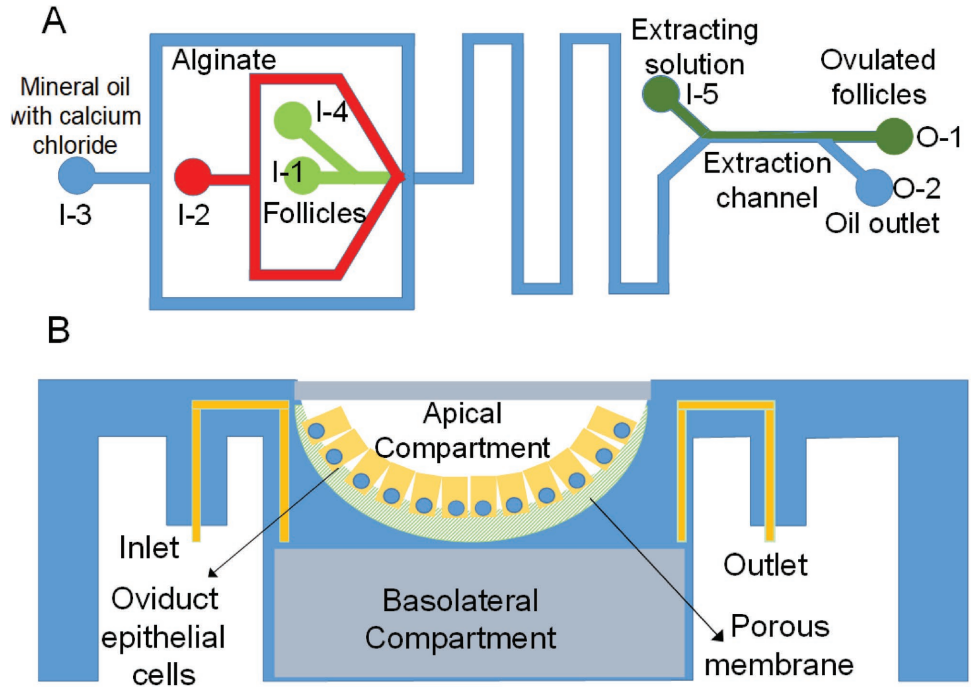

Figure 11. Various applications of biomicrofluidics in ART. A) Core–shell emulsion-based droplet microfluidic for in vitro culture of ovarian follicles described by Choi et al.[143] Using an extraction channel, the ovulated follicles were successfully separated from the mineral oil and retrieved from the outlet. B) Oviduct-on-a-chip developed by Ferraz et al.[152b] . They used a dynamic microenvironment to 3D culture oviduct epithelial cells. The porous membrane separated the two compartments of the system, i.e., an apical compartment and a basolateral compartment. The figures were redrawn with some changes from refs. [143] and [152b], respectively.

embryos with animal feeder such as the kidney epithelial cells obtained from Green monkey[148] is not recommended.[149] Mizuno et al. first utilized microfluidic technology to fabricate a preliminary uterus-on-a-chip platform.[150] By coculturing human endometrial cells with donated 2–4 cells human zygotes as well as using a pulsating micropump to perfuse the culture medium, their microfluidic system mimicked the physicochemical features of the uterus. Compared to microdrop coculture, they showed that percentage of zygotes reaching blastocyst stage was higher in the microfluidic platform, i.e., 14 out of 19 versus 9 out 20 human zygotes arrived at the blastocyst stage in the microfluidic platform and the microdrop, respectively. Further quality assessment of the blastocysts confirmed that embryos cocultured in the microfluidic platform had higher quality than those in the microdrop. Later, these researchers fabricated a microfluidic platform to individually coculture single embryos with endometrial cells.[98] The culture chamber was separated from the perfusion chamber with a commercially available polyester microporous membrane. They demonstrated that mouse embryo reached to the blastocyst stage in the microfluidic device faster than the conventional microdrop.

Recently, Chang et al. devised a more sophisticated 3D uterus-on-a-chip platform to enhance the embryo culture.[151] Three primary functions of the female reproductive system were incorporated into a single microfluidic system, namely the secreted hormones from ovaries, pulsating nature of the lining cilia of oviduct and coculture with uterus endometrial cells. To evaluate the effect of various levels of ovary hormones on the development of the embryo, the authors fabricated a CGG to produce six different concentrations of progesterone. Similar to the previously described device,[98] a membrane was used to support the endometrial cells and separate the culture chamber from the perfusion chamber. Here, the porous membrane was fabricated by PDMS soft lithography. Curved microstructures with the minimum flow velocity were incorporated inside the culture chamber to resemble the pulsating function of the lining cilia of the oviduct. A pneumatic channel controlled the flow through the culture chamber. This biomimetic uterus-on-a-chip improved the blastocyst rate of mouse embryos in a manner that they hatched within the 48 h of culture. One of the limitations of these uterus-on-a-chip devices is the monolayer coculture of the endometrial cells. 3D culture of these feeder cells with sophisticated cell culture scaffolds can better recapitulate the uterus microenvironment and subsequently enhance the embryo development.

5.3. Oviduct-on-a-Chip

Combining the transwell cell culture insert and the 3D printing technology with stereolithography, Ferraz et al. reported the fabrication of an oviduct-on-a-chip platform[152] (Figure 11B). The authors showed that tube-shaped structure of the device could support a long-term culture of oviduct epithelial cells (OECs). Using a perfusion chamber, the polarisation of OECs was also feasible. The OECs polarisation led to the formation of primary cilia as well as apical and basolateral compartments. Therefore, sperm can attach to the apical portion. At the same time, two separate perfusions from both apical and basolateral compartments are possible. Recently, these researchers used this oviduct-on-a-chip system for sperm capacitation and in vitro fertilization of bovine oocytes.[153] They showed that 3D culture and polarisation of bovine OECs contributed significantly to monospermic fertilization and avoided parthenogenesis. This 3D platform also allowed live imaging of the mechanism of sperm attachment and release as a result of its interaction with the 3D culture of bovine OECs.

Recently, Xiao et al. fabricated three microfluidic devices, which could ultimately simulate the hormonal profiles of female reproductive tract and evaluate its interactions with other tissues, respectively.[154] Using a single culture microfluidic platform with pneumatic control, the device supported the culture of the murine ovary, its maturation, and differentiation. The device also allowed for the secretion of steroid and peptide hormones for 28 d corresponding to human menstrual cycle. Subsequently, the group devised another microfluidic platform with electromagnetic actuation flow control to culture the reproductive tract tissues, i.e., ovary, oviduct, and uterus, and the secondary tissues, i.e., liver and cervix. Using this 3D multiculture platform, the effects of hormonal signals of the female reproductive tract at the cellular and multitissue levels were evaluated. Such principles hold promise for the next step integration of whole human reproductive system-on-a-chip for clinical ART application.

6. Conclusion and Future Direction

The present paper highlighted the significant contributions made by the microfluidic community in the various areas of ART. These areas included 1) infertility diagnosis; 2) sperm selection; 3) sperm guidance; 4) oocyte analysis; 5) insemination; 6) embryo culture; 7) embryo selection; and 8) cryopreservation and vitrification. To this aim, the ART applications of four different branches of microfluidics, namely CFM, paper-based microfluidics, droplet-based microfluidics, and organ-on-a-chip, were discussed. While a large number of previous works devised CFM for ART applications, two areas still demand further investigation, viz. sorting based on nonmotile sperms and devices based on noncontact forces. Passive CFM sorting mechanisms, e.g., pinched flow fraction and inertial microfluidics, can show significant advantages over conventional approaches to sort nonmotile sperms. Therefore, future CFM devices can consider other sorting mechanisms for nonmotile sperm selection. Hydrodynamic flow in CFM platforms seems to be problematic for both commercialization of the device and the health of the oocyte/embryo. Hence, clinical trials based on CFM with noncontact forces such as DEP are demanding.

One of the main goals of the present review paper was to draw the attention of the scientific community to the other branches of microfluidics beside CFM. While many sophisticated CFM platforms in ART had been introduced, very few of them translated to the real clinical settings. In Section 2.9, we briefly introduced these limitations and technical challenges of CFM platforms along with some recommended solutions. Other branches of microfluidics have more potential for real-world applications. For infertility analysis, paper-based microfluidics can offer disposable, fast responsive, and user-friendly

approach. However, the simplicity of the device further hinders its applications for other fields of ART. Embryo culture based on droplet-based microfluidics is an interesting field, which surprisingly attracted less attention. Droplet-based microfluidics was further divided into two groups: (i) emulsion-based droplet microfluidics and (ii) digital microfluidics. Accordingly, cutting-edge platforms using digital microfluidics, especially EWOD and liquid marble, in various fields of ART were evaluated. Droplet-based microfluidics and digital microfluidic platforms share many similarities with the current IVF clinical techniques. In particular, digital microfluidics based on liquid marble can be a promising approach for oocyte handling, insemination, embryo culture, and cryopreservation. However, further improvements in liquid marble manipulation and practical considerations to prevent its evaporation are required. Also, digital microfluidics based on EWOD can play a major role in embryo culture and selection. However, there are still many biological and technological challenges needed to be addressed. Further, the potential of EWOD for preimplantation genetic screening and diagnosis is yet to be evaluated. With the advances in 3D cell culture and microfabrication, organ-on-a-chip can offer great platforms to better mimic the in vivo mechanism of insemination. The combination of organ-on-a-chip and tissue engineering can automate the whole IVF procedure, and elective embryo transfer can become possible with such platforms.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

assisted reproduction technology, embryo culture, oviduct-on-a-chip, microfluidics, oocyte selection, sperm selection, womb-on-a-chip

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