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NEW RESEARCH HORIZON *Review*

Integration of microfluidics in animal *in vitro* embryo production

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ABSTRACT: The *in vitro* production of livestock embryos is central to several areas of animal biotechnology. Further, the use of *in vitro* embryo manipulation is expanding as new applications emerge. ARTs find direct applications in increasing genetic quality of livestock, producing transgenic animals, cloning, artificial insemination, reducing disease transmission, preserving endangered germplasm, producing chimeric animals for disease research, and treating infertility. Whereas new techniques such as nuclear transfer and intracytoplasmic sperm injection are now commonly used, basic embryo culture procedures remain the limiting step to the development of these techniques. Research over the past 2 decades focusing on improving the culture medium has greatly improved *in vitro* development of embryos. However, cleavage rates and viability of these embryos is reduced compared with *in vivo* indicating that present *in vitro* systems are still not optimal. Furthermore, the methods of handling mammalian oocytes and embryos have changed little in recent decades. While pipetting techniques have served embryology well in the past, advanced handling and manipulation technologies will be required to efficiently implement and commercialize the basic biological advances made in recent years. Microfluidic systems can be used to handle gametes, mature oocytes, culture embryos, and perform other basic procedures in a microenvironment that more closely mimic *in vivo* conditions. The use of microfluidic technologies to fabricate microscale devices has being investigated to overcome this obstacle. In this review, we summarize the development and testing of microfabricated fluidic systems with feature sizes similar to the diameter of an embryo for *in vitro* production of pre-implantation mammalian embryos.

Key words: animals / microfluidics / oocyte maturation / embryo / sperm

Introduction

Assisted reproductive technologies

The use of biotechnology for assisted reproduction in livestock has increased each year for the past ~40 years. The practice of *in vitro* production of bovine embryos has dramatically increased in recent years, representing ~66% of embryos transferred in the world (Guimarães *et al.*, 2014). In 2011, the number of the *in vitro* production (IVP) bovine embryos produced and transferred were 453 471 and 373 836, respectively, worldwide (Parrish, 2014). The efficiency of IVP of mammalian embryos is relatively low. The data for embryo production for livestock species, from both *in vivo* and *in vitro* produced embryos, is published each year by the Data Retrieval Committee of the International Embryo Technology Society [IETS; www.iets.org]. This low efficiency is due primarily to artificial stressors including gamete and embryo handling and environmental changes. For example, during embryo production, oocytes/embryos are manipulated more than twenty times. This continued stress has effects both directly on the gametes/embryos and indirectly through the environment (culture medium), which is sensitive to changes in temperature and gas concentration. Because of the manipulations during IVP, the gametes and embryos are exposed to changes in pH, osmolarity and mechanical stress, which can be detrimental to successful blastocyst production. Most of the research efforts to reduce these types of stresses during IVP have been focused on the media modulation rather than finding systems that could reduce the stress introduced by the operator. Further, the physical environment and physical aspects of that environment in which, aspects, oocyte maturation, fertilization and embryo culture, of IVP occur have largely gone ignored by much of the IVP community.

One possible solution to reduce many of these types of stresses on the gametes/embryos may be the application of micro- and nano-technologies to these IVP systems, particularly microfluidic technologies. Microfluidic technologies first emerged in the late 1980s and early 1990s and initially reported the fusion of individual cells in a silicone fluidic device (Masuda *et al.*, 1987) and transport of animal cells (Fuhr and Shirley, 1995). Based on these previous observations our original idea was that microfluidics could allow oocytes and embryos to be handled in a much gentler manner than traditional pipetting techniques allow. Instead of moving embryos from one microdrop to another, the embryo is gently moved into a specified location and 'parked' or placed for further

manipulation including environmental, chemical or mechanical manipulation but without further gamete or embryo handling (Fig. 1). Different medium can then moved to the embryo, e.g. changing oocyte maturation medium to fertilization medium. Media changes can be achieved gradually, reducing environmental stress (Glasgow *et al.*, 2001).

Some of our first studies in the late 1990s, concerning the application of microfluidic technology in the IVP field were performed to evaluate the real biocompatibility of the materials needed to build a device (Choi *et al.*, 1998; Chan *et al.*, 1999, 2001; Glasgow *et al.*, 2001). These initial studies were performed using mouse embryos (Glasgow *et al.*, 2001) and pig oocytes (Hester *et al.*, 2002). The results showed that there are many materials like silicon nitrate, silicon oxide, borosilicate glass chromium, gold, titanium and polydimethylsiloxane (PDMS) that have no detrimental effect on embryo development. Another study to verify biocompatibility with sensitive gametes was performed using porcine sperm, targeting sperm motility, and in this case, Clark *et al.* (2002) did not find any difference between the control group and the PDMS group. These results provided evidence of the positive properties (easy to use, low cost and high compliance) of PDMS and suggested this material may be among the best for microfluidic device fabrication. Similar positive aspects of PDMS were suggested by at least two other research groups (Regehr *et al.*, 2009; Su *et al.*, 2013). However, along with the positive aspects there at least five possible adverse characteristics that affect microscale cell culture. These adverse affects include absorption of media or components, deformation of the channels/device features, evaporation (increase of the osmolarity), hydrophobic recovery of small molecular weight chain molecules and leaching of mis-crosslinked PDMS oligomers into the channels (Heo *et al.*, 2007; Regehr *et al.*, 2009; Berthier *et al.*, 2012). These issues can be mitigated by addition of specific coatings and processing methods of PDMS devices. However, these limitations have prompted research to find new materials that can potentially replace PDMS, but it is important to realize that in spite of the negative effects of PDMS cited in the literature, the use of PDMS depending on the experimental design, length of culture or cell type may or may not be a serious issue (Regehr *et al.*, 2009).

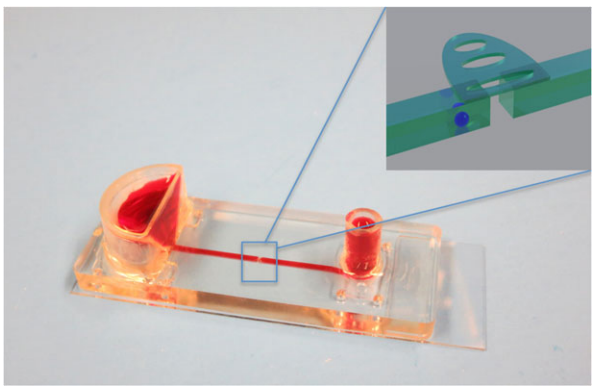

Figure 1 Shown is a photograph of a prototype microfluidic embryo culture device. Upper right corner panel shows a close-up illustration of the 'parking place' region of microchannel. The embryo/oocyte is held stationary while fluid can flow around and past the embryo/oocyte. The construction of the microchannel allows for easy visualization during IVP and facilitates gradual media changes and chemical treatments. (Illustration courtesy of Henry Zeringue and Vitae, LLC, Madison WI, reprinted from Wheeler *et al.* (2007), with permission from Elsevier).

Depending on the device designs the loading and unloading of the oocytes/embryo can be a challenge. A simple straight microchannel device with a small loading port makes it difficult to load and unload oocytes or embryos. One effective solution is to incorporate a loading/unloading funnel in the device design that allows the oocyte/embryos to be placed into the funnel, with a pipette, and loaded into the microchannel by gravity. Unloading of the oocyte/embryos is achieved by simple tilting of the device so the oocyte/embryos 'roll' back to the funnel where they can be retrieved by the pipette (Beebe *et al.*, 2002). Other microfluidic devices may be more or less problematic for oocyte/embryo loading/unloading depending on the device design.

New developments

Oocyte manipulation

The process of oocyte maturation is a complex coordination of molecular, cytoplasmic, and nuclear events that must occur in a synchronized manner for successful fertilization. *In vivo*, mammalian oocytes acquire cytoplasmic maturity (capacitation) and the competence to resume meiosis (maturation) during follicle and oocyte growth (Gosden *et al.*, 1997; Hyttel *et al.*, 1997, 2001). Thus, both nuclear and cytoplasmic maturation are required to ensure normal fertilization and embryo development. In the last few decades, many studies have been performed trying to improve oocyte maturation. It is known that maturation has an important impact on fertilization, embryo quality, blastocyst rate and pregnancy rates.

It has been hypothesized that microfluidic environment may look more like the *in vivo* system compared to the static system in a Petri dish. Culture systems that are static do not permit real-time changes in the culture media that surround the embryo as it develops. Beginning in 2001, a series of different experiments were performed to evaluate the impact of the microchannel/microfluidics on the maturation of oocytes. Walters at al. showed that oocytes maturated in microchannel had the same nuclear maturation of those maturated in standard drops (Walters *et al.*, 2003). This study followed a previous study that showed that the embryo cleavage rate was higher in the microchannel than in the standard procedure (67 vs 49%, respectively) (Hester *et al.*, 2002). In 2014, Yuan *et al.* compared two different types of microchannels and each one of these had a subgroup: static or rocking. That paper showed that microchannels used in a static system, can be used for successful maturation of 'individual oocytes and culture individual embryos without compromising, and perhaps enhancing, developmental potential'. (Yuan *et al.*, 2014) Furthermore, that report found that the rocking movement (in this particular device) was detrimental for both oocyte and embryo development (Yuan *et al.*, 2014). In a similar type of study, a French group designed and built a device that transported the oocytes in the microchannel up to a specific point (trap) where the fertilization was carried out. In this case the channel was made of silicon instead of PDMS and the movement of the oocyte was support by a pump that pumped the medium over the oocyte (Sadani *et al.*, 2005).

In parallel, other researchers in our group were trying to exploit microfluidic technology for another important step during gamete manipulation: cumulus cell removal from oocytes. Cumulus removal is routinely necessary before (mouse and porcine) or after IVF (bovine). There are two principal methods used to remove the cumulus cells, (i) mechanically by vortexing or pipetting and (ii) chemically with hyaluronidase. The first papers that used microfluidics to remove cumulus cells date back to 2000 when Zeringue *et al.* started to determine optimal device designs and materials for efficient device performance. In these studies, cumulus cells were removed from bovine oocytes after IVF (Zeringue *et al.*, 2000a, 2002). Following these pioneering experiments in 2004, Zeringue *et al.* (2005a) evaluated the impact of this manipulation on the embryo production. They went on to compare two cumulus cells removal protocols: standard (vortexing) vs a microchannel device. The microfluidic device showed very favorable results. Beginning on Day 2 (post-fertilization), the percentage of development to more advanced stages was higher in the microfluidic device than in the control treatment (35 vs 20%). This developmental gap grew larger when blastocyst formation was evaluated; the microfluidic group produced 57% blastocysts compared with 30% blastocysts in the control group. The second analysis performed was an RNA evaluation; results showed that oocytes treated in the microfluidic device had essentially no transcriptional activity while the vortexed (control) group showed some transcription at 2 h post processing. These results are suggestive of the production of repair proteins, which is consistent with the concept of vortex-induced oocyte damage. This suggested that the microfluidic procedure was less stressful that the standard protocol for cumulus removal (Zeringue *et al.*, 2005a).

Since 1997 there has been an increased demand for cloned embryos both in the commercial livestock and biotechnology research sectors. This has encouraged several research groups to develop new techniques to improve the various steps required by embryo cloning protocols. In 2011, Hagiwara *et al.* tried to take advantage of microchannels for the enucleation of oocytes (Hagiwara *et al.*, 2011). Their idea was to use magnetic charge to cut the oocyte into two pieces. To achieve this, they put two opposing magnetic metal strips sharpened at the ends to form two micro-blades in the microchannel. They then put the oocyte in the center of the device and activated the magnetic attraction and the two metal blades worked together in opposition to cut the oocyte quickly into two pieces (Hagiwara *et al.*, 2011).

Heo *et al.* (2011) suggested another possible use of microchannels, this group proposed the use of microchannels to vitrify the oocytes. It has been known for some time that one of the biggest problems during cryopreservation is the interaction between gametes/embryos and the cryoprotectants. This group used the flow of the fluids in the microchannel to reduce the interaction between the gametes/embryos and the cryoprotectant. Their results suggest that it is possible cryopreserve oocytes using a microchannel. In this instance it was important to design the channel while taking into consideration the high viscosity of the cryoprotectant, which is characteristic of most of the molecules (glycerol, ethlylene glycol) used in the cryopreservation process. This device was specifically designed to cryopreserve one oocyte at time (Heo *et al.*, 2011).

Semen manipulation

Sperm selection is essential to assisted reproductive technology (Morrell and Rodriguez-Martinez, 2011). The presence of non-motile or damaged sperm, sperm fragments and other debris negatively affects semen quality. For use in IVF, motile, morphologically normal sperm must be separated from seminal plasma components, semen extender components and/or undesired cells. A number of techniques have been developed to recover and purify homogeneous populations of highly motile sperm cells. These techniques are used for removing seminal plasma, dead cells, abnormal sperm, cryoprotective agents and other factors (Xie *et al.*, 2006). The techniques include Percoll density gradient centrifugation, swim-up migration, washing by centrifugation, glass wool filtration and several others (Flesch and Gadella, 2000; Trentalance and Beorlegui, 2002; Ainsworth *et al.*, 2005; Cesari *et al.*, 2006; Machado *et al.*, 2009; Petyim *et al.*, 2009; Morrell and Rodriguez-Martinez, 2011). The impact of poor quality sperm on IVF outcomes is similar to that of poor quality oocytes. Several groups have studied sperm selection using microfluidics as a possible sperm enrichment methodology.

The first microfluidic sperm separation device was developed by Kricka *et al.* (1993). This paper showed a direct correlation between the intervals that the sperm needed to transverse the channel and the pre-determined sperm progression score, which was determined before the sperm were put into the channel. This device can give an accurate evaluation by using the time it takes for the sperm to swim a prescribed distance, which was then correlated with progressive motility of the identical samples counted in a Makler chamber (Kricka *et al.*, 1997). However, it gave no direct measurement of sperm motility or concentration. A subsequent study, by Cho *et al.* (2003) developed a gravity-based passive pumping system to sort motile sperm from immotile sperm and other debris. The construction of this device was based on the typical movement of parallel laminar flow at the microscale (Schuster *et al.*, 2003). Non-motile sperm, cellular debris, and seminal plasma do not cross this barrier and are shuttled into a waste reservoir. The biggest problem with this device is the amount of sperm that can be processed. The concentration is too low for IVF but otherwise it produces a very high quality sperm sample that can be used for ICSI.

In vitro fertilization

The female reproductive tract has a number of functions, including the facilitation of sperm migration to the site of fertilization, where oocytes are waiting. Another important function of the tract is the selection of healthy, motile sperm for fertilization (Suarez, 2016). The interactions between the gametes and the tract are both chemical and physical. The lumen of the tract contains fluid, which assists with gamete transport, removes waste products and nurtures the gametes and developing embryos. The fluid flow in the reproductive tract is mediated by secretion and smooth muscle contraction. Recently, mouse and human sperm have been shown to exhibit rheotaxis in this fluid flow in the reproductive tract (Miki and Clapham, 2013). We know that many of these characteristics of the reproductive tract are impossible to reproduce during *in vitro* fertilization, but some aspects, such as unidirectional, laminar or gradient flow, containment in a 3D physical environment and changing the chemical composition in the medium can be achieved in a microfluidic environment.

If we consider the gamete preparation the first step of a robust *in vitro* embryo production (IVEP) routine, we can define the IVF as step number two. In general, when we talk about IVF we mean the co-incubation of the gametes. The ratio between number of oocytes and

sperm changes between protocols, but it is typically the ratio of one oocyte to 10 000 sperm. Unfortunately, this ratio is not very healthy for the future embryos because the excess sperm produce potentially harmful waste products in the IVF media (Dietl and Rauth, 1989; Hickman *et al.*, 2002). Taking these aspects together and taking into account the natural fluid flow that surrounds the gametes during fertilization, some research groups attempted to design devises that take advantage of the natural microchannel idea. Funahashi *et al.* conducted one of the first experiments in this regard with their so-called climbing-over-a-wall (COW) method (Funahashi and Nagai, 2000). This experiment was designed for porcine IVF and their idea was to put a 'wall' between oocytes and sperm. Using this method they hypothesized that only motile sperm could swim up over the wall and arrive at the oocytes for fertilization. The results showed that when 5.0×10^5 sperm/ml were used, the oocyte penetration rate was similar in both treatments. However, with the COW system, the monospermic penetration rate was higher ($P < 0.05$) than in the control treatment. When the investigators varied the sperm concentration in the COW device from 0.5 to 10×10^5 sperm/ml, oocyte penetration rate was directly correlated to sperm concentration and monospermic oocyte penetration was inversely correlated to sperm concentration.

Funahashi and Romar (2004) subsequently developed the 'Straw IVF' system in order to try to take advantage of fluidic principles. This method consisted of a 0.25 ml semen straw ~5 cm length, which is used as the IVF channel. The sperm solution was deposited at one end of the straw and the oocytes were placed at the other end. The results showed that the number of sperm that interacted with oocytes was less than the standard drop protocol, and they speculated that only the high-quality sperm swam toward and interacted with the female gametes (Funahashi and Romar, 2004). Both experiments had as their goal to reduce the sperm concentration around oocytes and obtain sperm: oocyte ratios closer to those in *in vivo*.

Following a similar train of thought, our group evaluated the potential of a PDMS/borosilicate microchannel for IVF (Clark *et al.*, 2002). This study showed that the microchannel system had similar fertilization rates to the control group providing evidence that microchannel device could be used for porcine *in vitro* fertilization. These encouraging results lead Clark *et al.* to evaluate different outcomes of the system including the percentage of polyspermic fertilization. The results showed that incidence of polyspermy was higher in the control group compared with microfluidic device ($P < 0.05$) (Clark *et al.*, 2005). Consequently, the percentage of monospermic oocyte penetration was higher ($P < 0.05$) in the microfluidic device compared to the control (Clark *et al.*, 2005). Similar results were observed regarding low polyspermy rates and high embryo rates by Sano *et al.* (2010) using a different microfluidic device design with porcine oocytes.

These results gave rise to a new series of experiments from other groups that showed the power of microfluidics for IVF procedures. Suh *et al.* (2006) developed and tested a microchannel for mouse IVF. In this experiment the microchannel was 500 μm wide × 180 μm deep and had an integral 3D barrier. The barrier permitted unimpeded flow of sperm and media through the microchannel but prevented migration of the oocyte (Suh *et al.*, 2006). Using the standard mouse sperm concentration for IVF of 1×10^6/ml the fertilization rates were low (12%) in the microchannel. However, when the sperm concentration in the microchannel was reduced, the IVF rates were significantly higher ($P < 0.001$) than the control drops (Suh *et al.*, 2006). The results showed that they could increase fertilization rates in the microchannel while reducing the sperm concentrations. The authors thought that this increased fertilization was due to an increase in the local sperm concentration and the interaction between sperm and oocytes, which may result in optimization of sperm and oocyte cofactors. The possibility of using less sperm for animal IVF could be very helpful if we want to use gender's elected (sexed) sperm or if the semen samples have compromised sperm concentrations (i.e. oligospermia, frozen-thawed sperm or sperm from aged males) (Palermo *et al.*, 1992). One such study, designed with this idea in mind, was conducted to increase natural sperm selection, such as the swimming quality of the sperm, utilized different microchannel shapes and oocyte traps designs to select the fertilizing sperm (Ma *et al.*, 2011).

Embryo culture

The pre-implantation period for livestock embryos *in vivo* ranges from 5 to 7 days depending on the species. This pre-implantation period includes the time from gamete fusion until embryo attachment in the uterus. During this time the embryos are not in direct contact with the reproductive tract and all their nutrients are taken from the luminal secretions (oviductal and uterine) into the tract. These luminal secretions can be thought of as the '*in vivo*' embryo culture medium. *In vitro*, embryo culture media and physical embryo culture conditions (gas environment, temperature, containment system (drops, microdrops, dishes, microfluidics, static or dynamic flow) and humidity) are two of the most studied topics in the embryo production arena. Currently, all of these methods are a poor substitute for the oviduct with regard to 'normal' embryo development.

None of the attempts to mimic the *in vivo* system have resulted in the same quality of embryos as those produced *in vivo*. Many of the differences between embryos produced *in vitro* or *in vivo* are known, including speed of development, metabolism, lipid concentration, gene expression, resistance to freezing and embryonic mortality after transfer (Wrenzycki *et al.*, 1996; Boni *et al.*, 1999; Hasler, 2000; Khurana and Niemann, 2000; Thompson, 2000; Niemann *et al.*, 2002; Rubessa *et al.*, 2011). The *in vitro* environment becomes more important if we remember that embryos are held in media for up to 7 or 8 days (Thompson, 1997). Standard *in vitro* protocols consist of one or two media being used during the embryo culture. This is in contrast to the *in vivo* situation in which, embryos grow in fluid (medium) with a constantly changing environment. This changing environment likely occurs because some metabolites necessary during one phase of development may not be needed during others stages. One of the first dynamic embryo culture systems was the formulation of sequential media where each phase of embryo development had specific substrate concentrations. The disadvantages of these systems are (i) the number of times that the operator has to handle the embryos and (ii) the volume of media (higher compared with standard protocol) reduces the positive effects of embryo trophic factors. In such a scenario, microfluidic embryo culture devices may be a better method to deliver sequential media in a dynamic situation.

One of the first studies regarding microfluidic embryo culture was performed by Glasgow *et al.* (2001). This study showed that a slow flow was enough to maintain a suitable embryo environment (Glasgow *et al.*, 2001). Further, it showed that 82% of mouse embryos placed into the device exhibited blastocyst development similar to that seen

in microdrop culture. In this prototype device, only 200 nl of culture medium was within a 2 mm hemispherical radius of the embryo, where as in the conventional drop 17 µl or ~85 times the amount of medium is in that same 2 mm hemispherical radius. The microchannel clearly has the ability to establish microenvironments for the embryo similar to what occurs *in vivo*. This study also evaluated different types of microchannels and different ways to prevent embryo movement while flowing the media past it. They did not find any damage to the embryos and they were able to confirm the minimum flow power required to overcome the embryos from sticking to the microchannel (Glasgow *et al.*, 2001). In 2004, experiments were conducted to evaluate the impact of a microchannel on mouse embryo development. Two-cell embryos were collected from superovulated mice. Three different kinds of device were designed: (i) one composed of entirely PDMS (complete PDMS), (ii) a PDMS channel and borosilicate glass bottom and (iii) a silicon wafer/borosilicate sandwich. All three systems supported high levels of embryo development (Raty *et al.*, 2004). Using PDMS:borosilicate glass microchannels during a culture period of 72 h, the researchers showed a higher number of blastocysts developing ($P > 0.01$) in the microchannels (Fig. 2) compared to the controls (Raty *et al.*, 2004). Further, with the third device (the silicon wafer/borosilicate sandwich) they had more blastocysts than the control and the time for development to the blastocyst stage was similar to that seen *in vivo*. Another group, Cabrera *et al.* (2006), showed similar results using a microfluidic device with dynamic media flow. Other microfluidic devices were developed that included computer-controlled pumps and valves (Gu *et al.*, 2004). This system took advantage of the elastic nature of the PDMS microchannels combined with Braille pins to help 'squeeze' fluid through the microchannel. The Braille pins are able to generate a forward or backward flow of media in the channels depending on the pattern of the valves in the device. This allows flow to be precisely controlled by the operator. In a subsequent study, Heo *et al.* incorporated the Braille pins into a device that was a fusion of a microfunnel and a microchannel. These authors showed an increase in the number hatched blastocysts and improved embryo implantation, in mice, compared with a static system (Heo *et al.*, 2010).

The microchannel has also been used to culture porcine embryos resulting in the birth of five normal piglets (Walters *et al.*, 2003) (Fig. 3). In cattle, Bormann *et al.* (2007), showed that embryos cultured in a microfluidic device resulted in a higher number of embryos than with in the static control group. All of these experiments combined indicate the feasibility and utility of microchannel devices in mammalian embryo culture. Further, they support the notion that the physical surroundings in the microchannel allow the environment to more closely mimic in vivo embryo growth conditions.

One of the most important benefits of microchannel is the reduction of the ratio between media and embryos, which may act to increase the effect of embryo trophic factors. To better understand the role of volume between control and microchannels we can easily

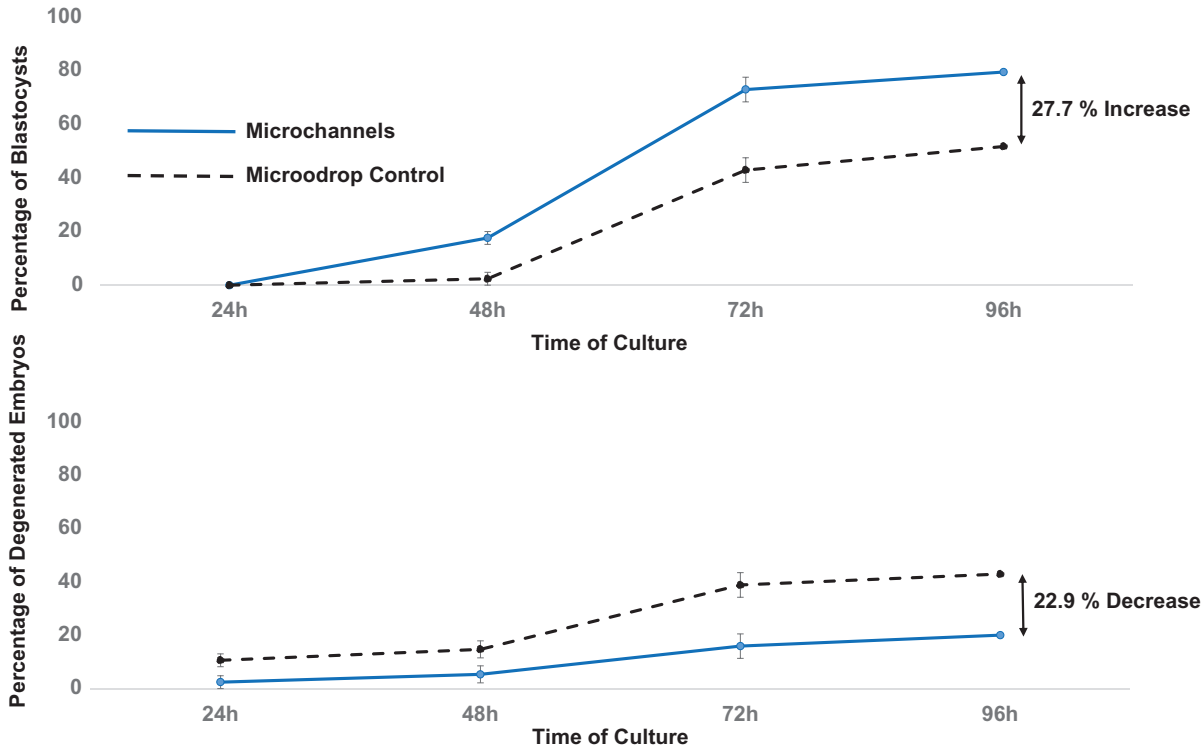

Figure 2 Developmental efficiencies of ICRXB6SJL/F1 mouse blastocysts (top) and degenerated embryos (bottom) in the PDMS/borosilicate microchannels and the control over the 96 h culture period. Microchannels significantly (top) improve blastocyst rates and (bottom) decrease the number of degenerate embryos. (Adapted from Beebe *et al.* (2002), with permission from Elsevier).

Figure 3 A litter of piglets and a litter of mice (inset) produced from 2-cell embryos cultured to the blastocyst stage a microchannel device.

compare the volumes of the two systems: microdrops range from 50 to 950 µl (Kouba *et al.*, 2000; Raty *et al.*, 2001) while the microchannels average ~0.125 µl (Hickman *et al.*, 2002) at least 400 times lower. This lower fluid volume is comparable to the fluid volume found in the reproductive tract (Wheeler *et al.*, 2002). Following this idea, Melin and colleagues, investigated mouse embryo culture in precisely designed microfluidic system that enabled culture in sub-microliter volumes of between 5 and 500 nl. They we able to show that mouse embryos cultured in pairs in this device had development rates to the blastocyst stage in excess of 80% (Melin *et al.*, 2009). Practically, we would like to culture single embryos to advanced stages (blastocyst, expanded blastocyst and possibly hatched blastocyst). Further, we would like to be able to separate an individual animal's embryos and check them individually on a daily basis for embryo development. It is well known that the speed of embryo development is a significant and accurate predictor of embryo quality (Sattar *et al.*, 2011). In this particular aspect, microfluidics can definitely assist in the evaluation of the speed of embryo development because of its ability to culture embryos individually. One example of a microfluidic device to culture mouse embryos individually was reported by Esteves and co-workers. They showed that development of mouse embryos grown singly in a microchannel system achieved blastocyst rates of between 93 and 97%. Further, they showed that these embryos cultured individually produced pregnancy rates of 29–33%, which is similar to the pregnancy rate of embryos cultured in groups (Esteves *et al.*, 2013). In summary, Esteves *et al.* (2013) found that it was possible to culture single embryos without altering the pregnancy rate of those embryos.

Embryo manipulation

Embryo manipulation has become an important step for ART in the last 30 years, especially for ICSI and PEGD in humans, biopsies and the production of clones, chimeras and transgenics in livestock, which require robust techniques for manipulation. This is particularly important when we consider the genetic engineering of livestock. For many of these embryo manipulations we need to remove the zona pellucida. The zona is a glycoprotein matrix that envelops the oocytes, helping with sperm binding at fertilization and also preventing polyspermy. It further acts as a shield and filter after fertilization protecting the developing embryo until the embryo becomes too large and 'hatches' from inside the zona pellucida. Currently, the common methods to remove the zona pellucida include the use of proteolytic enzymes, which can be detrimental to both oocytes and embryos if exposed too long.

In this regard, the microchannel can play an important role; it is possible to use a dynamic microchannel system to reduce the negative effect caused by enzymes. Zeringue *et al.* (2000b) published the first report showing how a microfluidic device could be used to remove the zona pellucida from an oocyte/embryo. They designed a microchannel system from PDMS, with an inner diameter of ~120 µm, that used pressure driven flow to move the embryos into the channel. The results showed that it was possible to precisely control the embryos in the channel during chemical removal of the zona pellucida (Zeringue *et al.*, 2000b, 2005b).

Integrated IVP microfluidic devices

Integrating the whole process of IVP was the goal of Clark *et al.* using the pig as a model (Clark *et al.*, 2003). This research was the first report regarding the combination of two IVP steps, *in vitro* maturation and followed by *in vitro* fertilization. The results showed that it was indeed possible to integrate two different phases of embryo production efficiently onto a single microfluidic chip (Clark *et al.*, 2003). The oocytes were loaded into a microchannel or a microdrop (control) with maturation medium then following oocyte maturation, the medium removed and replaced with fresh IVF medium for fertilization. Following fertilization all the presumptive zygotes from both treatments were washed and cultured for 48 h. The results indicated no difference ($P > 0.05$) in the cleavage rates on Day 2, between the control and microchannel groups, 51.1 vs 49.2%, respectively. These results provided evidence all phases of the IVP process could be integrated on a single microfluidic channel. Similar studies are currently ongoing with other livestock species, primarily cattle and small ruminants

Discussion

The full potential of microfluidic technology has yet to be realized for assisted reproduction in livestock. There have been many exciting developments and demonstrations of the different aspects of *in vitro* embryo production and of embryo/oocyte manipulation using microfluidic principals and devices. Major advances in automation and robotics combined with microfluidics have the potential to revolutionize livestock IVEP and animal breeding. New technologies in 3D printing may vastly improve our ability to develop physical systems that closely mimic the *in vivo* environment. Sophisticated pumping and fluid handling methods will allow for the alteration of the fluid milieu that surrounds the gametes and embryos. Subtle changes in media composition delivered at specific time frames are achievable using microfluidic devices.

The use of microfluidic devices in assisted reproduction has been shown to improve manipulation processes and developmental efficiencies while decreasing human error. Such improvements will lead to lower costs, increased access and decreased physical stress on the gametes and embryos. Microfluidics allows oocytes and embryos to be handled in a much gentler manner than traditional pipetting techniques allow. Instead of moving embryos from one microdrop to another the

embryo is gently moved into a specified location and 'parked'. Then different media (maturation, fertilization and/or embryo culture) can be moved to the embryo in either in static or dynamic flow paradigms. These media changes can be achieved gradually reducing environmental stress. Furthermore, microchannel devices can contain much smaller volumes of media than traditional microdrop culture systems allowing the addition of expensive growth factors at a fraction of the cost of microdrop systems. In addition to providing a more *in-vivo* like culture environment, microfluidic technology is ideally suited for complex embryo manipulations, such as removal of the zona pellucida, stripping of cumulus cells and potentially manipulations such as pronuclear injection and nuclear transfer.

With all the potential of microfluidic technology for assisted reproduction, there are currently no micro devices being used for livestock embryo production other that the 'Well-of-the Well' device developed by Vajta *et al.* (2000). However, the WOW is in essence a small microwell and not a microfluidic device. Some of the difficulties have been in large scale manufacturing of devices with such small (micron scale) features. Many of the devices described in this review were produced by soft-lithography, which is largely done by hand. A few up to a dozen devices may be fabricated by these methods but not the thousands or hundreds of thousands that are required for commercial ART for livestock. New microfabrication methods may solve this issue in the near future.

The potential of microfluidic technology for IVEP in livestock is tremendous. Future research needs to focus on optimizing media formulation, handling protocols including robotics and culture conditions to take full advantage of this great potential. We are only at the beginning but based on the results already obtained, the future of this technology will be exciting to see unfold.

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Conflict of interest

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References

Ainsworth C, Nixon B, Aitken RJ. Development of a novel electrophoretic system for the isolation of human spermatozoa. *Hum Reprod* 2005;**20**: 2261–2270.

Beebe D, Wheeler M, Zeringue H, Walters E, Raty S. Microfluidic technology for assisted reproduction. *Theriogenology* 2002;**57**:125–135.

Berthier E, Young EW, Beebe D. Engineers are from PDMS-land, Biologists are from Polystyrenia. *Lab Chip* 2012;**12**:1224–1237.

Boni R, Tosti E, Roviello S, Dale B. Intercellular communication in in vivo- and in vitro-produced bovine embryos. *Biol Reprod* 1999;**61**:1050–1055.

Bormann C, Cabrera L, Heo YS, Takayama S, Smith G. Dynamic microfluidic embryo culture enhances blastocyst development of murine and bovine embryos. *Biol Reprod* 2007;**77**:89–90.

Cabrera LM, Heo YS, Ding J, Takayama S, Smith DG. Improved blastocyst development with microfluidics and braille pin actuator enabled dynamic culture. *Fertil Steril* 2006;**86**:S43.

Cesari A, Kaiser GG, Mucci N, Mutto A, Vincenti A, Fornés MW, Alberio RH. Integrated morphophysiological assessment of two methods for sperm selection in bovine embryo production in vitro. *Theriogenology* 2006;**66**:1185–1193.

Chan NG, Lyman JT, Choi S-J, Zeringue HC, Glasgow IK, Beebe DJ, Wheeler MB. Development of an embryo transport and analysis system: material biocompatibility. *Theriogenology* 1999;**51**:234.

Chan NG, Raty S, Zeringue HC, Beebe DJ, Wheeler MB. Development of microfabricated devices for embryo production: embryo biocompatibility. *Theriogenology* 2001;**55**:332.

Cho BS, Schuster TG, Zhu X, Chang D, Smith GD, Takayama S. Passively driven integrated microfluidic system for separation of motile sperm. *Anal Chem* 2003;**75**:1671–1675.

Choi S-J, Glasgow IK, Zeringue HC, Beebe DJ, Wheeler MB. Development of microelectromechanical systems to analyze individual mammalian embryos: embryo biocompatibility. *Biol Reprod* 1998;**58**:96.

Clark SG, Haubert K, Beebe DJ, Ferguson CE, Wheeler MB. Reduction of polyspermic penetration using biomimetic microfluidic technology during in vitro fertilization. *Lab Chip* 2005;**5**:1229–1232.

Clark SG, Walters EM, Beebe DJ, Wheeler MB. In vitro fertilization of porcine oocytes in polydimethylsiloxane (PDMS)-glass microchannels. *Biol Reprod* 2002;**66**:528.

Clark SG, Walters EM, Beebe DJ, Wheeler MB. A novel integrated in vitro maturation and in vitro fertilization system for swine. *Theriogenology* 2003;**59**:441.

Dietl JA, Rauth G. Molecular aspects of mammalian fertilization. *Hum Reprod* 1989;**4**:869–875.

Esteves TC, van Rossem F, Nordhoff V, Schlatt S, Boiani M, Le Gac S. A microfluidic system supports single mouse embryo culture leading to full-term development. *RSC Adv* 2013;**3**:26451–26458.

Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim Biophys Acta* 2000;**1469**: 197–235.

Fuhr G, Shirley SG. Cell handling and characterization using micron and submicron electrode arrays: state of the art and perspectives of semiconductor microtools. *J Micromech Microeng* 1995;**5**:77–85.

Funahashi H, Nagai T. Sperm selection by a climbing-over-a-wall IVF method reduces the incidence of polyspermic penetration of porcine oocytes. *J Reprod Dev* 2000;**46**:319–324.

Funahashi H, Romar R. Reduction of the incidence of polyspermic penetration into porcine oocytes by pretreatment of fresh spermatozoa with adenosine and a transient co-incubation of the gametes with caffeine. *Reproduction* 2004;**128**:789–800.

Glasgow IK, Zeringue HC, Beebe DJ, Choi SJ, Lyman JT, Chan NG, Wheeler MB. Handling individual mammalian embryos using microfluidics. *IEEE Trans Biomed Eng* 2001;**48**:570–578.

Gosden R, Krapez J, Briggs D. Growth and development of the mammalian oocyte. *Bioessays* 1997;**19**:875–882.

Gu W, Zhu X, Futai N, Cho BS, Takayama S. Computerized microfluidic cell culture using elastomeric channels and Braille displays. *Proc Natl Acad Sci USA* 2004;**101**:15861–15866.

Guimarães ACG, Leivas FG, Santos FW, Schwengber EB, Giotto AB, Machado CIU, Gonçalves CGM, Folchini NP, Brum DS. Reduction of centrifugation force in discontinuous percoll gradients increases in vitro fertilization rates without reducing bovine sperm recovery. *Anim Reprod Sci* 2014;**146**:103–110.

Hagiwara M, Kawahara T, Yamanishi Y, Arai F. Precise control of magnetically driven microtools for enucleation of oocytes in a microfluidic chip. *Adv Robot* 2011;**25**:991–1005.

Hasler JF. In vitro culture of bovine embryos in Menezo's B2 medium with or without coculture and serum: the normalcy of pregnancies and calves resulting from transferred embryos. *Anim Reprod Sci* 2000;**60–61**:81–91.

Heo YS, Cabrera LM, Bormann CL, Shah CT, Takayama S, Smith GD. Dynamic microfunnel culture enhances mouse embryo development and pregnancy rates. *Hum Reprod* 2010;**25**:613–622.

Heo YS, Cabrera LM, Song JW, Futai N, Tung YC, Smith GD, Takayama S. Characterization and resolution of evaporation-mediated osmolality shifts that constrain microfluidic cell culture in poly(dimethylsiloxane) devices. *Anal Chem* 2007;**79**:1126–1134.

Heo YS, Lee HJ, Hassell BA, Irimia D, Toth TL, Elmoazzen H, Toner M. Controlled loading of cryoprotectants (CPAs) to oocyte with linear and complex CPA profiles on a microfluidic platform. *Lab Chip* 2011;**11**:3530–3537.

Hester PN, Roseman HM, Clark SG, Walters EM, Beebe DJ, Wheeler MB. Enhanced cleavage rates following in vitro maturation of pig oocytes within polydimethylsiloxane-borosilicate microchannels. *Theriogenology* 2002;**57**:723.

Hickman DL, Beebe DJ, Rodriguez-Zas SL, Wheeler MB. Comparison of static and dynamic medium environments for culturing of preimplantation mouse embryos. *Comp Med* 2002;**52**:122–126.

Hyttel P, Fair T, Callesen H, Greve T. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* 1997;**47**:23–32.

Hyttel P, Viuff D, Fair T, Laurincik J, Thomsen PD, Callesen H, Vos PL, Hendriksen PJ, Dieleman SJ, Schellander K *et al*. Ribosomal RNA gene expression and chromosome aberrations in bovine oocytes and preimplantation embryos. *Reproduction* 2001;**122**:21–30.

Khurana NK, Niemann H. Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. *Biol Reprod* 2000;**62**:847–856.

Kouba AJ, Abeydeera LR, Alvarez IM, Day BN, Buhi WC. Effects of the porcine oviduct-specific glycoprotein on fertilization, polyspermy and embryonic development in vitro. *Biol Reprod* 2000;**63**:242–250.

Kricka LJ, Faro I, Heyner S, Garside WT, Fitzpatrick G, McKinnon G, Ho J, Wilding P. Micromachined analytical devices: microchips for semen testing. *J Pharm Biomed Anal* 1997;**15**:1443–1447.

Kricka LJ, Nozaki O, Heyner S, Garside WT, Wilding P. Applications of a microfabricated device for evaluating sperm function. *Clin Chem* 1993;**39**:1944–1947.

Ma R, Xie L, Han C, Su K, Qiu T, Wang L, Huang G, Xing W, Qiao J, Wang J *et al*. In vitro fertilization on a single-oocyte positioning system integrated with motile sperm selection and early embryo development. *Anal Chem* 2011;**83**:2964–2970.

Machado GM, Carvalho JO, Filho ES, Caixeta ES, Franco MM, Rumpf R, Dode MAN. Effect of Percoll volume, duration and force of centrifugation, on in vitro production and sex ratio of bovine embryos. *Theriogenology* 2009;**71**:1289–1297.

Masuda, S, Washizu, M, and Nanba, T Novel methods of cell fusion and handling using fluid integrating circuit. In *'Seventh International Conference on Electrostatic Phenomena (Electrostatics' 87)'*, 1987. Oxford, UK, pp. 69–74.

Melin J, Lee A, Foygel K, Leong DE, Quake SR, Yao MW. In vitro embryo culture in defined, sub-microliter volumes. *Dev Dyn* 2009;**238**:950–955.

Miki K, Clapham DE. Rheotaxis guides mammalian sperm. *Curr Biol* 2013;**23**:443–452.

Morrell JM, Rodriguez-Martinez H. Practical applications of sperm selection techniques as a tool for improving reproductive efficiency. *Vet Med Int* 2011;**2011**:1–9.

Niemann H, Wrenzycki C, Lucas-Hahn A, Brambrink T, Kues WA, Carnwath JW. Gene expression patterns in bovine in vitro-produced and nuclear transfer-derived embryos and their implications for early development. *Cloning Stem Cells* 2002;**4**:29–38.

Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;**340**:17–18.

Parrish JJ. Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology* 2014;**81**:67–73.

Petyim S, Choavaratana R, Suksompong S, Laokirkkiat P, Makemaharn O. Outcome of sperm preparation using double-gradients technique study in Siriraj Hospital. *J Med Assoc Thai* 2009;**92**:878–884.

Raty S, Beebe DJ, Rodriguez-Zas SL, Wheeler MB. Culture in microchannels enhances in vitro embryonic development of preimplantation mouse embryos. *Theriogenology* 2001;**55**:241.

Raty S, Walters EM, Davis J, Zeringue H, Beebe DJ, Rodriguez-Zas SL, Wheeler MB. Embryonic development in the mouse is enhanced via microchannel culture. *Lab Chip* 2004;**4**:186–190.

Regehr KJ, Domenech M, Koepsel JT, Carver KC, Ellison-Zelski SJ, Murphy WL, Schuler LA, Alarid ET, Beebe DJ. Biological implications of polydimethylsiloxane-based microfluidic cell culture. *Lab Chip* 2009;**9**:2132–2139.

Rubessa M, Boccia L, Campanile G, Longobardi V, Albarella S, Tateo A, Zicarelli L, Gasparrini B. Effect of energy source during culture on in vitro embryo development, resistance to cryopreservation and sex ratio. *Theriogenology* 2011;**76**:1347–1355.

Sadani Z, Wacogne B, Pieralli C, Roux C, Gharbi T. Microsystems and microfluidic device for single oocyte transportation and trapping: toward the automation of in vitro fertilising. *Sens Actuators A: Phys* 2005;**121**:364–372.

Sano H, Matsuura K, Naruse K, Funahashi H. Application of a microfluidic sperm sorter to the in-vitro fertilization of porcine oocytes reduced the incidence of polyspermic penetration. *Theriogenology* 2010;**74**:863–870.

Sattar A, Rubessa M, Di Francesco S, Longobardi V, Di Palo R, Zicarelli L, Campanile G, Gasparrini B. The influence of gamete co-incubation length on the in vitro fertility and sex ratio of bovine bulls with different penetration speed. *Reprod Domest Anim* 2011;**46**:1090–1097.

Schuster TG, Cho B, Keller LM, Takayama S, Smith GD. Isolation of motile spermatozoa from semen samples using microfluidics. *Reprod Biomed Online* 2003;**7**:75–81.

Su X, Theberge AB, January CT, Beebe DJ. Effect of microculture on cell metabolism and biochemistry: do cells get stressed in microchannels? *Anal Chem* 2013;**85**:1562–1570.

Suarez SS. Mammalian sperm interactions with the female reproductive tract. *Cell Tissue Res* 2016;**363**:185–194.

Suh RS, Zhu X, Phadke N, Ohl DA, Takayama S, Smith GD. IVF within microfluidic channels requires lower total numbers and lower concentrations of sperm. *Hum Reprod* 2006;**21**:477–483.

Thompson JG. Comparison between in vivo-derived and in vitro-produced pre-elongation embryos from domestic ruminants. *Reprod Fertil Dev* 1997;**9**:341–354.

Thompson JG. In vitro culture and embryo metabolism of cattle and sheep embryos—a decade of achievement. *Anim Reprod Sci* 2000;**60–61**:263–275.

Trentalance GM, Beorlegui NB. Sperm evaluation in cryopreserved bovine semen recovered by two selection methods. *Andrologia* 2002; **34**:397–403.

Vajta G, Peura TT, Holm P, Páldi A, Greve T, Trounson AO, Callesen H. New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. *Mol Reprod Dev* 2000;**55**:256–264.

Walters EM, Clark S, Roseman HM, Beebe DJ, Wheeler MB. Production of live piglets following in vitro embryo culture in a microfluidic environment. *Theriogenology* 2003;**59**:353.

Wheeler MB, Beebe DJ, Walters EM, Raty S. Microfluidic technology for in vitro embryo production. *Med Biol Proc* 2002;**2**:104–108.

Wheeler MB, Walters EM, Beebe DJ. Toward culture of single gametes: the development of microfluidic platforms for assisted reproduction. *Theriogenology* 2007;**68**:S178–S189.

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H. Expression of the gap junction gene connexin43 (Cx43) in preimplantation bovine embryos derived in vitro or in vivo. *J Reprod Fertil* 1996;**108**:17–24.

Xie Y, Wang F, Zhong W, Puscheck E, Shen H, Rappolee DA. Shear stress induces preimplantation embryo death that is delayed by the zona pellucida and associated with stress-activated protein kinase-mediated apoptosis. *Biol Reprod* 2006;**75**:45–55.

Yuan Y, Paczkowski M, Wheeler MB, Krisher RL. Use of a novel polydimethylsiloxane well insert to successfully mature, culture and identify single porcine oocytes and embryos. *Reprod Fertil Dev* 2014; **26**:375–384.

Zeringue HC, Beebe DJ, Wheeler MB. Removal of cumulus from mammalian zygotes using microfluidic techniques. *Biomed Microdevices* 2000a;**3**: 219–224.

Zeringue, HC, King, KR, Glasgow, IK, Raty, S, Wheeler, MB, and Beebe, DJ (2000b) Zona pellucida removal of mammalian embryos in a microfluidic system. In *'Micro Total Analysis Systems 2000: Proceedings of the μTAS 2000 Symposium, held in Enschede, The Netherlands, 14–18 May 2000.'* (Eds. A Berg, W Olthuis and P Bergveld). Springer Netherlands: Dordrecht, pp. 435–438.

Zeringue HC, Rutledge JJ, Beebe DJ. Early mammalian embryo development depends on cumulus removal technique. *Lab Chip* 2005a;**5**:86–90.

Zeringue HC, Walters EM, Leibfried-Rutledge LL, Wheeler MB, Beebe DJ. Development of bovine in vitro–produced embryos after cumulus cell removal with a microfluidic microchannel device. *Theriogenology* 2002; **57**:533.

Zeringue HC, Wheeler MB, Beebe DJ. A microfluidic method for removal of the zona pellucida from mammalian embryos. *Lab Chip* 2005b;**5**:108–110.