

# Improving the outcome of in vitro embryo production through microfluidic technology

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## Abstract

*Infertility is one of the main problems of the 21st century facing both the veterinary and human medicine. A series of assisted reproductive technologies such as artificial insemination, hormone regulation of ovulation, in vitro fertilization and embryo transfer in order to overcome problems of reproductive health. Unfortunately, the outcome of such reproductive biotechnologies, is still below expectations, therefore, new state of art technologies such as microfluidics can revolutionize human and animal assisted reproduction.*

*Development of an integrated microfluidic system for assisted reproduction, which may manipulate female and male gametes, embryos and culture media, and additionally, incorporate biomarker analysis on the same platform, could have a decisive impact on the future of assisted reproduction technologies. Microfluidics has the potential of making assisted reproduction techniques more accessible and most of all improving the success rate in patients experiencing infertility problems.*

**Keywords:** microfluidics, semen sorting, embryoculture, IVF

## 1. Introduction

The term microfluidics was originally introduced in the late 1980s and refers to a new area that includes notions of physics, engineering, biotechnology, nanotechnology and chemistry (WHITESIDES [1]). This new technology has numerous applications in various fields such as printing industry, engineering, molecular biology and nonetheless medicine (Table 1) and its basic principle lie in the handling of liquids and gases through microchannels with dimensions smaller than 100  $\mu\text{m}$  (TSAI [2]).

Because of its sensitivity, precision, low costs for producing the microfluidics chips as well as automation, this technology has a massive potential in the future and will decisively contribute in improving the knowledge in the area of biomedicine (GORKIN & al. [3], SACKMANN & al. [4]).

Numerous studies in this field have focused on embedding several tests in one single chip, the so-called lab-on-chip concept, aiming to obtain a device that allows the rapid, low-cost and automatic analysis of multiple parameters simultaneously (ABGRALL and GUE [5], TEMIZ & al. [6]).

**Table 1. Applications of microfluidics in different fields (MCDONALD & al.[7])**

<b>FIELD</b>	<b>APPLICATIONS</b>
Genomics	Improvements in DNA sequencing, analysis Elaborations of different DNA arrays
Science and protection of the environment	Analysis of environmental pollutants and contaminants
Engineering	Printing industry
Biology and chemistry	Identification and qualifications of toxins and pathogen agents Elaboration of devices that mimic a biological function Development of lab-on-a-chip technology
Medicine	Toxicological assays Drug delivery devices Assisted reproductive technologies Embryo development Identification of samples in forensic medicine

This review aims to summarize the current ongoing research carried out with the purpose of standardizing different stages of assisted reproductive programs by the introduction of new state of the art technologies such as microfluidics.

## **2. History background**

Infertility is one of the main problems of the 21st century facing both the veterinary and human medicine, one in 4 couples being affected by it. Therefore, many researches have been focused on developing and implementing a series of assisted reproductive technologies such as artificial insemination, hormone regulation of ovulation, in vitro fertilization and embryo transfer in order to overcome problems of reproductive health (INHORN and PATRIZIO [8]) and obtain the offspring of superior genetic potential (VERMA & al. [9]).

Due to increasing need for food globally, assisted reproductive biotechnologies have become an essential tool for improving milk, meat and wool production, especially in the industrialized countries. Reproductive biotechnologies are intended for routinely use to shorten the generational intervals and to propagate the genetic material among breeding animal populations (RATH & al. [10]).

The constant need to improve assisted reproduction outcomes led to the intensive research and development of new generation biotechnologies for the objective sorting of sperm cells, micromanipulation of oocytes and embryo culture. The purpose of implementing such technologies at different stages of the in vitro fertilization (IVF) is to standardize the entire process of assisted reproduction.

## **3. Microfluidics for in vitro embryo production**

The in vitro embryo production is an assisted reproduction biotechnology that consists in the recovery and maturation of female gametes from donors, in vitro fertilization (IVF) of the

matured gametes using male gametes and subsequently the in vitro culture of the embryos obtained after fertilization (SIRARD and COENEN [11]).

In order to improve the outcomes of in vitro embryo production, efforts must be made for the improvement of all the IVF stages, from female and male gametes sorting to embryo culture.

Applications of microfluidics could be integrated at all stages of embryo production, starting with a qualitative and sorting of gametes and continuing with embryo culture and vitrification, in order to miniaturize and include several laborious lab procedures in one compact device, so called lab-on-a-chip.

### **3.1. Sperm sorting using microfluidics**

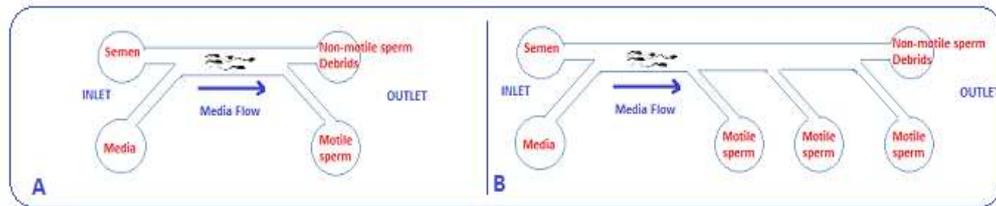
A study conducted by Enciso and collaborators (ENCISO & al. [12]) showed that sperm DNA has a decisive role in embryo production (IVP), sperm with fragmented DNA being able of fertilizing the female gamete (CARRELL & al. [13], BANSAL and BILASPURI, [14]), but may adversely affect the quality of the embryo causing embryonic development interruption (often in the first phase of gestation) (EVENSON [15], MACIUC & al. [16]) and subsequently determining low pregnancy rates after IVF or IA (VIRRO & al. [17], WATERHOUSE & al. [18], SAKKAS and ALVAREZ [19]).

Additionally, a study led by Wdowiak and Bojar (WDOWIAK and BOJAR [20]) has showed that the intracytoplasmic injection of sperms with a higher degree of DNA fragmentation may negatively influence the morphokinetic parameters of embryos, thus, the sperm DNA fragmentation index (SDF) may be used as a predictive tool for estimating pregnancy rates after ICSI.

One of the main objectives of an assisted reproduction program is the selection of high quality cells in order to improve the fertilization rates of IVF, ICSI. Some sperm selection technologies such as swim-up, filtration or gradient centrifugation have proven efficient for sorting sperm cells based on a specific propriety such as motility or a group of characteristics such as normal chromatin, motility and morphology (VALEANU & al. [21]), but yet, the outcomes of FIV or ICSI are still lower than expected, one key factor being the subjective approach when it comes to choosing the best sperm cell for fertilization of the egg (SAID and LAND [22]).

The introduction of molecular markers for sperm quality analysis opened new pathways into research of objective methods for retrieving high quality spermatozoa for fertilization in vitro. Some of the microfluidics based devices use the sperm motility as the main mechanism of cell sorting, since non-motile sperm cell and cellular debris, lack the capability of traversing streamlines in a laminar flow circuit determined by the hydrostatic pressure (CHO & al. [23]).

Due to the existing correlations between motility and normal morphology, these devices are able to eliminate abnormal cells from the samples, concomitant with imotile ones. Other passively driven microfluidics devices use gravity as a mechanism for creating several microchannels (Figure 1.B), relying on the capacity of motile sperm to move across the laminar streamlines towards the collection reservoir (XIE & al. [24], KO & al. [25]). Both types of devices are equipped with the different chambers for the introduction of sperm sample and media, collection of motile spermatozoa, as well as non-motile cells and cellular debris (Figure 1 A).



**Figure 1. The Passively Driven Integrated Microfluidic System for sperm sorting (A- single reservoir for collection of motile sperm; B- multichannel reservoir for the collection of motile sperm)**

A study conducted by Seo (SEO [26]) showed that the movement of the sperm cells associated with the hydrostatic pressure may facilitate the sorting and alignment of bull, mouse and human spermatozoa through the micro-channel.

By integrating a charge-coupled device (CCD) to the microfluidic device, Zhang and collaborators (ZHANG & al. [27]) elaborated an automated algorithm capable of sorting sperm populations based on their motility pattern by determining several kinetic parameters such as sperm average velocity, straight line velocity, straightness of swimming path and average acceleration. This algorithm may facilitate a more objective selection of sperm intended for ICSI.

More recently, a study conducted by Chen and collaborators (CHEN & al. [28]), proposed the use of microfluidic resistive pulse technique (RPT) for the development of a device that may enable the quantification of both sperm concentration and motility, thus making this technique an economical alternative to sperm analysis systems such as CASA.

Furthermore, microfluidics might also prove helpful in improving the sexed semen technology, which enables livestock owners to obtain offspring of a desire sex. Among the methods currently used for obtaining sexed semen are included FISH, PCR (CENARIU & al. [29]), density gradient centrifugation and FACS, the latter being the most effective (90% accuracy) (RATH & al. [30]). When spermatozoa are sorted using FACS, the sperm DNA must be stained with a dye called Hoechst 33342, which enables the differentiation between chromosome X and Y. Despite the high efficiency that this method has, the main drawback resides in the fact that Hoechst 33342 (Bis-benzimidazole) has a toxic effect on sperm cells and the sexed sorted semen which is obtained, usually has a much lower fertility compared to semen which is not sex-sorted (WHEELER & al. [31]).

### **3.2. Selection of oocytes using microfluidic systems**

The quality of oocytes is a factor of great importance, which has a direct impact on both fertilization and embryo production. The selection of oocytes for in vitro fertilization is carried out subjectively, based on morpho-structural properties of the cells (RIENZI & al. [32]), BUKOWSKA & al. [33]).

The challenges in terms of implementation of a more sophisticated method for assessing the quality of oocytes, consists in developing an objectivity-based technique that would not jeopardize the integrity of cells, which then may be used for fertilization. This goal was achieved by Choi and collaborators (CHOI & al. [34]), by developing a microfluidic device that may establish the maturity degree of the female gamete by analyzing the index of refraction and optical absorption. Significant differences were observed between mature and immature oocytes regarding the optical absorption spectrum, thus this type of device may be a keystone in making the selection of oocytes for IVF less subjective.

Furthermore, in a study conducted in 2009, Szczepanska and collaborators (SZCZEPANSKA & al. [35]), assessed the micro-spectro-photometric features of bovine and porcine oocytes using a Lab-on-Chip platform in order to establish their potency. Another spectrophotometric methodology utilizing lab-on-a chip device for assessing the quality of bovine oocytes was developed by Walczak and collaborators (WALCZAK & al. [36]).

Besides the selection of the oocytes, the denudation of the oocytes represents another essential step during in vitro maturation (IVM) respectively, in vitro fertilization (IVF).

This process implies the removal of the cumulus oocyte complex (COC) by repeatedly aspiration and flushing with the tip of a narrow Pasteur pipette, thus causing a mechanical stress to the cells (LAI [37]).

### **3.3. In Vitro Fertilization on a chip (IVF-on-a-chip)**

Conventionally, during in vitro fertilization process, the mature oocytes are co-incubated with the washed semen in a culture dish using a fertilization media, thus facilitating the interaction between spermatozoa and zona-pellucida of the oocyte. Since abnormal sperm function is the principle cause of human infertility, alternative procedures for IVF, such as ICSI (Intracytoplasmic sperm injection) or IMSI (Intracytoplasmic morphologically selected sperm injection) are used to overcome severe male infertility in patients with a history of repeated conventional IVF failure.

Accordingly, the principle of both ICSI and IMSI is the assisted penetration of the oocytes, by injecting the sperm into the egg cytoplasm. Unlike ICSI, IMSI is performed with an inverted microscope, 400 times more powerful than the one used for ICSI, the internal morphology of sperm cell selected for injection being evaluated in detail (LAI, [37]).

A series of microfluidics device destined with weir-type trap design were elaborated for IVF. Clark and collaborators (CLARK & al. [38]) elaborated a device that reduces the polyspermic penetration by limiting the period of time in which the oocytes interact with the spermatozoa, while Suhand and collaborators (SUH & al. [39]) focus on developing a device that significantly increased the fertilization rates with a low total number and concentration of sperm. Even more, in 2013, a group of researchers (GIGLIO & al. [40]) concentrated their efforts in developing a fully automated microfluidic system that may be integrated in ICSI procedure. Spermatozoa are selected based on their motility and the oocytes are denuded in a separate compartment through a chemical and mechanical process.

Nevertheless, the revolutionary aspect of this device consists in the fact that the processing of both sexual gametes (sperm and oocytes) are fully integrated with the same device. Additionally, the system presents individual compartment for the culture and monitoring of a single embryo.

### **3.4. Dynamic embryo culture**

Since the achievement of the first successful in vitro fertilization, researchers and clinicians have made constant efforts to understand aspects of basic biology regarding gametes and embryos in order to improve the success rates in the assisted reproduction programs. Up to this moment, most of the research has been focused on improving the chemical composition of different media used for fertilization and embryo culture, as well as optimization and simplifications of protocols.

Unfortunately, the protocols used presently are extremely laborious and require repeated washing and replacement of culture media, thus generating changes in temperature, osmolarity, pH and most important, mechanical stress to the embryos (LAI [37]). However, the major drawback of the classical protocols for embryo culture is the lack of resemblance to

what occurs in vivo. Naturally, after fertilization, the embryos are transported through the fallopian tubes by a continuous flow of fluid that has an extremely complex composition (CROXATTO [41]).

Because the success rate in assisted reproduction programs is below expectations and new revolutionary technologies such as biomimetics and microfluidics are emerging, scientists started to explore the possibility of implementing the advantages of these new technologies in ART.

A dynamic platform, based on microfluidic technology mimics the in vivo process of embryo culture, studies conducted in 2013 by Hao and collaborators, (HAO & al. [42]) showing that the quality of the embryos cultured using a microfluidic platform was superior as compared to the control group, cultured in standard media.

### **3.5. Automatization of Vitrification**

Cryopreservation is a key technology in biology and constant efforts are made to minimize the osmotic stress effects and to improve survivability of the cells that are cryopreserved. The cryopreservation of oocytes and embryos may be achieved either by slow freezing or vitrification, though recent studies are highlighting that vitrification is superior since it provides a higher cleavage percentage, as well as higher survival and pregnancy rates (SMITH & al. [43]).

Microfluidic systems may facilitate a precise control of fluid osmolarity, this aspect being of great importance in the process of vitrification, where different concentrations of cryoprotectant agents (CPA) are used (LAI [37]). The exposure to various concentrations of CPA during the vitrification process increases cryosurvival and development in the bovine oocytes and embryos, however, the application of such a practice is limited since performing numerous precise pipetting steps in a short amount of time it is unlikely to be achieved by manual operation (PYNE & al. [44]).

A microfluidic device for vitrification can provide a continuous, sequentially flow, using various concentrations of cryoprotectants agents, simultaneously being able to provide real-time images of cells that undergo the process of cryopreservation, thus in the future, the entire process of human and mammalian oocyte and embryo vitrification may be automatized (PYNE & al. [44], MENG & al. [45]).

Dai concluded in the thesis published in 2014 that microfluidic systems are able to perform certain protocols that could not be made by conventional methods, thus reducing both the disadvantages of manual pipetting and chemical stress to which the cells were subjected.

## **4. Future challenges in reproductive biotechnologies**

Microfluidics-based lab-on-a-chip devices enable the selection of progressive sperm, along with the analysis of a wide range of sperm parameters. Magdanz and collaborators (MAGDANZ & al. [46]) made a breakthrough towards assisted fertilization by developing sperm micro-bio-robots, also called sperm-boots. Bull spermatozoa were trapped inside magnetic micro-tubes and the motility of the sperm was used as driving force. The trajectory of the spermbots can be controlled remotely by applying an external magnetic field. These nano-robots might prove their efficiency for in vitro fertilization, although further studies are necessary. Other research groups have focused their efforts on embryo culture, this stage being widely accepted as a critical point in the in vitro fertilization process (SWAINE & al.

[47]). Hence, sustained efforts are made constantly in finding solutions that could determine the improvement of embryo development.

Studies conducted on mouse embryos showed that a digitized microfluidic device powered with electro-wetting on a dielectric (EWOD) enhanced the rate of embryo cleavage by mimicking the in vivo environment (LI & al. [48], HUANG & al. [49]). Further extensive research might lead, eventually, to the elaboration of a new protocol for embryo production in IVF clinical practice.

Since we live in an era of technological innovation, the great challenge of the future will be to combine microfluidics technology-based platforms with intelligent technology such as smartphones and tablets (GALLEGOS & al. [50], CHEN & al. [51], ERICKSON & al. [52], ONCESCU & al. [53], BARBOSA & al. [54]) to create a complex system for continuous monitoring of male reproductive health at home (FRANCO [55]).

These type of systems could be an affordable alternative to current technologies and nonetheless, would offer a series of advantages such as privacy, intimacy and accessibility to patients.

## 5. Conclusions

Development of an integrated microfluidic system for assisted reproduction, which may manipulate female and male gametes, embryos and culture media, and additionally, incorporate biomarker analysis on the same platform, could have a decisive impact on the future of assisted reproduction technologies.

The recent state of art research in microfluidics has paved the way for the emergence of compact devices, characterized by simplicity and economy, which have the potential of making these procedures more accessible and most of all improving the success rate in patients experiencing infertility problems, in which assisted reproduction is the only solution for conceiving a child.

## References

1. G.M. WHITESIDES, The origins and the future of microfluidics. *Nature*, 442, 368, 373 (2006).
2. S.Q. TSAI, Application of microfluidic technologies to the quantification and manipulation of sperm. *Urol. Sci.*, In Press, available online 18 september (2014).
3. R. GORKIN, L. CLIME, M. MADOU, H. KIDO, Pneumatic pumping in centrifugal microfluidic platforms. *MicrofluidNanofluid.*, 9, 541, 549 (2010).
4. E.K. SACKMANN, A.L. FULTON, D.J. BEEBE, The present and future role of microfluidics in biomedical research. *Nature*, 507 (7491), 181, 190 (2014).
5. P. ABGRALL and A.M. GUE, Lab-on-chip technologies: making a microfluidic network and coupling it into a complete microsystem—a review. *J. Micromech. Microeng.*, 17, 15, 49 (2007).
6. Y. TEMIZ, R.D. LOVCHIK, G.V. KAIGALA, E. DELAMARCHE, Lab-on-a-chip devices: How to close and plug the lab?. *Microelectron. Eng.*, 132 (25), 156, 175 (2015).
7. J.C. MCDONALD, D.C. DUFFY, J.R. ANDERSON, D.T. CHIU, H. WU, O.J. SCHUELLER, G.M. WHITESIDES, Fabrication of microfluidic systems in poly (dimethylsiloxane). *Electrophoresis*, 21 (1), 27, 40 (2000).
8. M.C. INHORNE and P. PATRIZIO, Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century. *Hum. Reprod. Update*, 21 (4), 411, 426 (2015).
9. O.P. VERMA, R. KUMAR, A. KUMAR, S. CHAND, Assisted Reproductive Techniques in Farm Animal - From Artificial Insemination to Nanobiotechnology. *Vet. World*, 5 (5), 301, 310 (2012).

10. D. RATH, S. BARCIKOWSKI, S. DE GRAAF, W. GARRELS, R. GROSSFELD, S. KLEIN, W. KNABE, C. KNORR, W. KUES, H. MEYER, J. MICHL, G. MOENCH-TEGEDER, C. REHBOCK, U. TAYLOR, S. WASHAUSEN, Sex selection of sperm in farm animals: status report and developmental prospects. *Reproduction*, 1 (145), 15, 30 (2013).
11. M.A. SIRARD and K. COENEN, In vitro maturation and embryo production in cattle. *Methods Mol. Biol.*, 348, 35, 42 (2006).
12. M. ENCISO, L. MURIEL, J.L. FERNANDEZ, Infertile men with varicocele show a high relative proportion of sperm cells with intense nuclear damage level, evidenced by the sperm chromatin dispersion test. *J. Androl.*, 27, 106,111 (2006)
13. D.T. CARRELL, B.R. EMERY, S. HAMMOUD, The aetiology of sperm protamine abnormalities and their potential impact on the sperm epigenome. *Int. J. Androl.*, 31(6), 537-45, (2008).
14. A.K. BANSAL and G.S. BILASPURI, Impacts of Oxidative Stress and Antioxidants on Semen Functions. *Vet Med Int.*, Volume 2011, Article ID686137, 7 pages (2011).
15. D.P. EVENSON, Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum. Reprod.*, 14 (4), 1039, 1049 (1999).
16. V. MACIUC, Ş. CREANGĂ. S.S. CHELMU, The reproductive capacity and specific applied biotechnologies in rendering cows reproduction efficient. *Rom. Biotech. Lett.*, 19, (2), 9155, 9161 (2014).
17. M.R. VIRRO, L. KJERSTEN, K.L. LARSON-COOK, D.P. EVENSON, Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertil. Steril.*, 81, 1289, 1295 (2004).
18. K.E. WATERHOUSE, T. HAUGAN, E. KOMMISRU, A. TVERDAL, G. FLATBERG, W. FARSTAD, D.P. EVENSON, P.M. DE ANGELIS, Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. *Reprod. Fertil. Dev.*, 18, 781,788 (2006).
19. D. SAKKAS and J.G. ALVAREZ, Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *FertilSteril.*, 93 (4), 1027, 1036 (2010).
20. A. WDOVIK and I. BOJAR, Relationship between pregnancy, embryo development, and sperm deoxyribonucleic acid fragmentation dynamics. *Saudi. J. Biol. Sci.*, In Press, Available online 10 August (2015).
21. S. VALEANU, D. DRUGOCIU, P. ROSCA, Effect of Single layer centrifugation using Bovipure on frozen-thawed bovine sperm during a 6 hours survival test. *Rom. Biotech. Lett.*, 20 (2), 10327, 10333 (2015).
22. T.M. SAID and J.A. LAND, Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. *Hum. Reprod. Update.*, 17 (6), 719, 733 (2011).
23. B.S. CHO, T.G. SCHUSTER, X. ZHU, D. CHANG, G.D. SMITH, S. TAKAYAMA, Passively driven integrated microfluidic system for separation of motile sperm. *Anal. Chem.*, 75 (7), 1671-1675 (2003).
24. L. XIE, R. M.A., C. HAN, K. SU, Q. ZHANG, T. QIU, L. WANG, G HUANG, J. QIAO, J. WANG, J. CHENG, Integration of sperm motility and chemotaxis screening with a microchannel-based device. *Clin. Chem.*, 56, 1270, 1278 (2010).
25. Y. KO, J.H. MAENG, B.C. LEE, S. LEE, S.Y. HWANG, Y. AHN, Separation of progressive motile sperm from mouse semen using on-chip chemotaxis. *Anal. Sci.*, 28, 27, 32 (2012),
26. D. Seo, A development of the motile sperm sorting microfluidic devices (Dissertation Thesis) (2007).
27. X. ZHANG, I. KHIMJI, U.A. GURKAN, H. SAFAEE, P.N. CATALANO, H.O. KELES, E. KAYAALP, U. DEMIRCI, Lensless imaging for simultaneous microfluidic sperm monitoring and sorting. *Lab Chip.*, 11, 2535, 2540 (2011).
28. W. CHEN, N.T. HUANG, X. LI, Z.T. YU, K. KURABAYASHI, J. FU, Emerging Microfluidic Tools for Functional Cellular Immunophenotyping: A New Potential Paradigm for Immune Status Characterization. *Front. Oncol.*, Apr 22;3:98. doi: 10.3389/fonc.2013.00098. eCollection (2013).
29. M. CENARIU, I. GROZA, P. EMOKE, L. BOGDAN, I. MORAR, S. CIUPE, R. POP, Sexing of Bovine Embryos Using Polymerase Chain Reaction (PCR) and Fluorescent In Situ Hybridization (FISH). *Rom. Biotech. Lett.*, 16, (2), 6055, 60611 (2011).
30. D. RATH, S. BARCIKOWSKI, S. DE GRAAF, W. GARRELS, R. GROSSFELD, S. KLEIN, W. KNABE, C. KNORR, W. KUES, H. MEYER, J. MICHL, G. MOENCH-TEGEDER, C. REHBOCK, U. TAYLOR, S. WASHAUSEN, Sex selection of sperm in farm animals: status report and developmental prospects. *Reprod.*, 145, 15, 30, (2013).

31. M.B. WHEELER, J.J. RUTLEDGE, A. FISCHER-BROWN, T. VANETTEN, S. MALUSKY, D.J. BEEBE, Application of sexed semen technology to in vitro embryo production in cattle. *Theriogenology*. Jan 7; 65 (1): 219-27. Epub 2005 Nov. 2, (2006).
32. L. RIENZI, G. VAJTA, F UBALDI, Predictive value of oocyte morphology in human IVF: a systematic review of the literature. *Hum. Reprod. Update.*, 17 (1), 34, 45 (2011).
33. D. BUKOWSKA, B. KEMPISTY, H. PIOTROWSKA, R. WALCZAK, P. SNIADEK, K.P. BRUSSOW, J.M. JASKOWSKI, M. NOWICKI, The invasive and new non-invasive methods of mammalian oocyte and embryo quality assessment: a review. *Vet. Med.*, 57, (4), 169, 176 (2012).
34. W. CHOI, J.S. KIM, D.H. LEE, K.K. LEE, D.B. KOO, J.K. PARK, Dielectrophoretic oocyte selection chip for in vitro fertilization. *Biomed. Microdevices.*, 10 (3):337-45 (2008).
35. P. SZCZEPANSKA, R. WALCZAK, J. DZIUBAN, M. JACKOWSKA, B. KEMPISTY, J.M. JASKOWSKI, S. BARGIEL, Lab-on-chip quality classification of porcine/bovine oocytes. *Procedia Chem.*, 1, 341–344 (2009).
36. R. WALCZAK, P. SZCZEPANSKA, J. DZIUBAN, B. KEMPISTY, M. JACKOWSKA, P. ANTOSIK, J. JASKOWSKI, A. CHELMONSKA-SOYTA, Lab-on-a-chip for developmental competence assessment of bovine oocytes. *Elektronika* 11, 30–33 (2010).
37. D. LAI, Microfluidic reduction of osmotic stress in oocyte and zygote vitrification (Dissertation Thesis), The University of Michigan, (2014).
38. S.G. CLARK, K. HAUBERT, D.J. BEEBE, C.E. FERGUSON, M.B. WHEELER, Reduction of polyspermic penetration using biomimetic microfluidic technology during in vitro fertilization. *Lab Chip.*, 5:1229–1232 (2005);
39. R.S. SUH, X. ZHU, N. PHADKE, D.A. OHL, S. TAKAYAMA, G.D. SMITH, IVF within microfluidic channels requires lower total numbers and lower concentrations of sperm. *Hum. Reprod.* 21:477–483, (2006).
40. A. GIGLIO, S.H. CHEONG, Q.V. NERI, Z. ROSENWAKS, G.D. PALERMO, ICSI-on-a-chip. *Fertility and Sterility Supp.*, 100, (3), Supplement, S1-S572, (2013).
41. H.B. CROXATTO, Physiology of gamete and embryo transport through the fallopian tube. *Reprod. Biomed.* 4(2):160-9 (2002).
42. Z. HAO, C. KIESLINGER, C. VERGOUW, H. KOSTELIJK, B. LAMBALK, Microfluidic protocol for in vitro culture of human embryos. 17th international conference on miniaturized systems for chemistry and life sciences 27-31 October, Freiburg, Germany, (2013) pp. 536-538.
43. R.L. SMITH, C.J. DEMERS, S.D. COLLINS, Microfluidic device for the combinatorial application and maintenance of dynamically imposed diffusional gradients. *Microfluid. Nanofluid.*, 9(4):613-622 (2010).
44. D.G. PYNE, J.L. MOHAMED ABDELGAWAD, Y. SUN, Digital Microfluidic Processing of Mammalian Embryos for Vitrification. *PLoS One*, 9 (9), e108128 (2014).
45. L. MENG, X. HUEZO, B.A. STONE, K. BAEK, G. RINGLER, R.P. MARRS, Development of a microfluidic device for automated vitrification human embryo. *Fertility. Sterility. Suppl.*, 96, (3), S207, (2011).
46. V. MAGDANZ, M. GUIX, O.G. SCHMIDT, Tubular micromotors: from microjets to spermboats. *Robotics Biomim.*, 1:11 (2014).
47. J.E. SWAIN, D. LAI, S. TAKAYAMA, G.D. SMITH, Thinking big by thinking small: application of microfluidic technology to improve ART. *Lab Chip*, 13, 1213-1224 (2013).
48. X. LI, D.R. BALLERINI, W. SHEN. A perspective on paper-based microfluidics: Current status and future trends. *Biomicrofluidics*, 6(1): 011301–011301-13, (2012).
49. H.Y HUANG, H.H. SHEN, C.H. TIEN, C.J. LI, S.K. FAN, C.H. LIU, W.S. HSU, D.J. YAO, Digital Microfluidic Dynamic Culture of Mammalian Embryos on an Electrowetting on Dielectric (EWOD) Chip. *Plos One*, <http://dx.doi.org/10.1371/journal.pone.0124196>, (2015).
50. D. GALLEGOS, K.D. LONG, H. YU, P.P. CLARK, Y. LIN, S. GEORGE, P. NATHA, B.T. CUNNINGHAM, Label-free biodetection using a smartphone. *Lab Chip.*, 13, 2124-2132 (2013).
51. Y. CHEN, P. LI, P.H. HUANG, Y. XIE, J.D. MAI, L. WANG, N.T. NGUYEN, T.J. HUANG, Rare cell isolation and analysis in microfluidics. *Lab Chip* 14, 626–45 (2014).
52. D. ERICKSON, D. O'DELL, L. JIANG, V. ONCESCU, A. GUMUS, S. LEE, M. MANCUSO, S. MEHTA, *Lab Chip*. Sep 7;14(17):3159-64. doi: 10.1039/c4lc00142g, Smartphone technology can be transformative to the deployment of lab-on-chip diagnostics, (2014).

53. V. ONCESCU, S. LEE, A. GUMUS, K. KARLSSON, D. ERICKSON, Autonomous Device for Application in Late-Phase Hemorrhagic Shock Prevention. PLOS ONE, <http://dx.doi.org/10.1371/journal.pone.0089903>, (2014).
54. A.I. BARBOSA, P. GEHLOT, K. SIDAPRA, A.D. EDWARDS, N.M. REIS, Biosensors and Bioelectronics70, 2015, pp. 5–14.
55. M. FRANCO, iSperm lets your iPad analyze your swimmers at home, [www.cnet.com](http://www.cnet.com), (2015).