

Vinod Kumar Yata

# Microfluidics for Assisted Reproduction in Animals



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## Foreword

Microfluidic methods offer new approaches and solutions to assisted reproductive technologies for animal applications. This book, “*Microfluidics for Assisted Reproduction in Animals*,” assembles the major aspects of microfluidics and animal assisted reproductive technologies. It represents an essential reference to a broad scientific community, including physico-chemists, biologists, and animal scientists in general. I hope that this contribution to the animal reproduction field will give rise to positive discussions between scientists of diverse research fields. Animal applications of microfluidic methods are still in the early stage of research and development. I hope that this book will give the reader a resource for keeping pace with the fast development in this emerging field. I congratulate Dr Vinod Kumar Yata for his efforts in bringing out this book to interdisciplinary researchers.

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## Preface

Animal reproductive technologies augmented and expanded greatly in terms of its technological advancements. Assisted reproductive technology (ART) protocols have continued to advance with efforts to get better benefits to society. Research is heading in the direction of improving the reproduction of food-producing animals. Animal reproduction research has been growing at a faster pace than in humans. The economic interests have also stimulated the research on safe and efficient animal reproduction methods. Microfluidics is an exciting topic of the specialized area that is considered one of the hot topics of the current research. Microfluidic methods have exhibited successful applications in several other fields, and it has begun to explore in the area of animal reproduction. The main intention of this book is to build up a theoretical and technical aspect of ARTs with emphasis on microfluidic methods. There are prospective benefits and synergies between microfluidics and reproductive technologies to improve animal breeding programs.

Chapter 1 provides a brief overview and applications of microfluidics. This chapter also provides a brief introduction to assisted reproductive techniques.

Chapter 2 discusses the basic principles of microfluidics with a focus on transport mechanisms of fluids. This chapter provides a discussion on various materials being used in the fabrication of microfluidics, and this information is useful for biologists to choose the biocompatible materials for fabrication. The final part of this chapter focused on the detailed discussion of the fabrication methods of microfluidics.

Chapter 3 provides a discussion on microfluidics and non-microfluidic methods of sperm sorting and sperm analysis. This chapter aims to provide information on existing sperm sorting and analysis methods with recent progress on microfluidics sperm research. This chapter helps to offer a new direction to design and develop new approaches in sperm research.

Chapter 4 is a worthy compilation of applications of microfluidics in in vitro fertilization and embryo culture. This chapter presents a brief discussion on existing methods and a detailed discussion on microfluidic methods.

Chapter 5 deals with principles of cryopreservation, cryoprotectants, and recent developments in microfluidic methods for oocyte, embryo, and sperm cryopreservation. Existing methods of cryopreservation and microfluidic methods of cryopreservation were discussed to highlight the gaps in the microfluidics-based cryopreservation research.

Chapter 6 is a worthy compilation of existing knowledge on sperm sexing and future directions for the development of a novel microfluidic method of sperm sexing. This chapter provides a discussion on possible clues for sperm sexing which could form the basis for design and development of new microfluidic-based sperm sexing methods.

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I would like to extend my thanks to Dr Bhavik Sawhney, Editor-Biomedicine, Springer Nature and Mr. Beracah John Martyn, Production Editor, Springer Nature, for their generous assistance, constant support, and patience in initializing and publication of this book. I would like to acknowledge Dr. Suneel Kumar Onteru and Dr. Venkata Satish Kumar Mattaparthi for the critical reading of this book and subsequent valuable suggestions. I also thank Mr. G. Vinay Bhaskar and Mr. Amit Kumar for their assistance while developing this book.

Finally, I would like to express my sincere gratitude to my beloved wife, Dr. Smitha, for her endless support. Special thanks to my son, Rudransh, for interrupting and rejuvenating me several times while writing this book.

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## About the Book

The aim of this book is to disseminate the knowledge to interdisciplinary researchers working on microfluidics and animal reproduction. This book describes the fundamentals of microfluidics and fabrication methods of microfluidic devices which could be well adopted for animal reproductive applications. This book comprehensively covers sperm sorting methods by using microfluidic methods which offer isolation of dynamic spermatozoa with high potential for fertility. Applications of microfluidics in in vitro fertilization and embryo culture were discussed in a separate chapter. Sperm sexing has gained momentum in the production of desired sex livestock, and the role of microfluidics in sperm sexing is also discussed in this book. This book is ideal for interdisciplinary researchers who are interested in developing novel, inexpensive, and efficient methods for animal assisted reproduction.

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## About the Author



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## Abstract

Microfluidics enabled a wide variety of disciplines with its applications by implying mechanical, optical, chemical, and electrical and bio-interfacial principles. Recent advances in micro- and nanofabrication make possible to implicate the microfluidics in chemical assays, electronic systems, diagnostics approaches, single-cell analysis, and pharmaceuticals. Portability, low sample or reagent volumes, biocompatibility, tissue engineering are some of the advantages that attracted the biologists to imply the microfluidics for biological applications.

Sustainability cannot be achieved with natural reproductive methods in livestock production. Demand driven livestock growth necessitated the research towards the assisted reproductive technology (ART) for multiplication of animals at a much faster pace. Existing ART methods need to be improved with the help of interdisciplinary approaches. Miniaturized devices attracted the reproductive biologists due to their ease in handling of single cells and small sample volumes. Use of microfluidics in animal reproduction may revolutionize the assisted reproductive techniques in the near future.

## Keywords

Microfluidics · Assisted reproductive technology · Animal reproduction · Livestock · fabrication · Miniaturized devices

## 1.1 What Is Microfluidics?

The term microfluidics refers to manipulation and precise control of liquids or gases inside micrometer-sized channel (Whitesides 2006). Microfluidics offers performing reactions with fewer volumes in shorter time (Sackmann et al. 2014). It also allows performing multiple reactions simultaneously in a single device. The better

separation efficiency and shorter transport time can be achieved by using microfluidics (Weibel and Whitesides 2006). Chemical analysis, mixing with minute volumes, continuous flow of fluids, temperature control, pH manipulation, cell culture, and imaging of microchannels are some of the basic applications of microfluidic devices (Bohr et al. 2019). Selection of fabrication material and design of microfluidic devices play a pivotal role in the successful application of microfluidic devices (Hou et al. 2017).

## 1.2 History of Microfluidics

Microfluidics development involves the combined contribution of multidisciplinary researchers. Manipulation of fluids at microscale has gained attention of several researchers due to its commercial applicability (Bohr et al. 2019). Jack Kilby and Robert Noyce were pioneers of the invention of integrated circuits. This invention led to the creation of computer and electronic devices (O'Regan 2016). Initially integrated circuits worked on Germanium circuit, and later replaced the Germanium with silicone materials to overcome material limitations. Tuckerman and Pease (1981) proposed a microchannel system for forced cooling of silicone integrated circuits. The combination of electrical and mechanical systems was introduced in the accelerometers by Petersen et al. (1982) and Chen et al. (1984). This invention led to the coining of the term “Microelectromechanical systems” (MEMS) (Dean Jr and Luque 2009). Later, “optical” term was included to MEMS and it is termed as “Micro-optoelectromechanical systems (MOEMS)” (Gauthier et al. 2002). Photolithographic technique was developed to make patterns on printed circuit boards. This method involves photosensitive coating or “resist” on the layer, irradiation of resist through UV light through optical mask, and producing desired designs or patterns (Andrus and Bond 1958). A milestone was achieved by the development of inkjet printing nozzles in the year 1997. In this study, truncated pyramidal holes etched on silicone to achieve uniformity of direction, velocity, and drop formation of fluids (Bassous et al. 1977). Origin of microfluidics has started in the year 1980 with the invention of miniaturized air analyzer on silicon wafer. In this study, photolithography and chemical etching methods were used on silicon to build miniaturized gas analysis system that worked on gas chromatography principles (Terry et al. 1979). Despite the success of photolithography in micro- and nanofabrication, the use of photolithography methods was limited due to high cost and clean room environment. Moreover, photolithography was restricted to semiconductors. Silicon was the most commonly used semiconductor in the most of the photolithography methods and its use was limited due to its undesired properties such as opaqueness, low gas penetration. Therefore, soft lithography was introduced and it is based on self-assembly and molding. Soft lithography differed from conventional methods by the use of polymers with optical transparency, rapid prototyping, and replica molding (Xia and Whitesides 1998). One such polymer is poly (dimethylsiloxane), popularly known as PDMS and it was first introduced for the microfabrication by Whitesides (Duffy et al. 1998). Soft lithography emerged as low

cost and convenient method for micro- and nanofabrication. Zhao et al. (1997) introduced four methods of soft lithography, namely, microcontact printing, replica molding, micro-molding in capillaries, and microtransfer molding. A concept of miniaturized “total chemical analysis system” ( $\mu$ TAS) was introduced by Manz et al. (1990). This term refers to the periodic transformation of chemical information into electronic information by using miniaturized device. In recent years, microfluidics techniques have been developed and designed exclusively to contribute in the most diversified way.

### 1.3 Applications of Microfluidics

Microfluidics enabled applications in chemical, engineering, biological, and medical fields. Applications of microfluidics were summarized in Fig. 1.1. The reagents and gases can be pumped into the microfluidic devices and it allows performing the reactions and separation of products. Furthermore, microfluidics can be integrated with detection systems for the separation of chemicals (Livak-Dahl et al. 2011). Use of transparent material in the fabrication of microfluidic devices allows monitoring the reactions throughout the process. Microfluidic devices have proven to reduce the mass transfer limitations by mixing and dispersing the small volumes of the fluids in a phased or continuous manner (Losey et al. 2002). Biosensors and biochemical assays such as enzyme-linked immunosorbent assay and nucleic acid hybridization

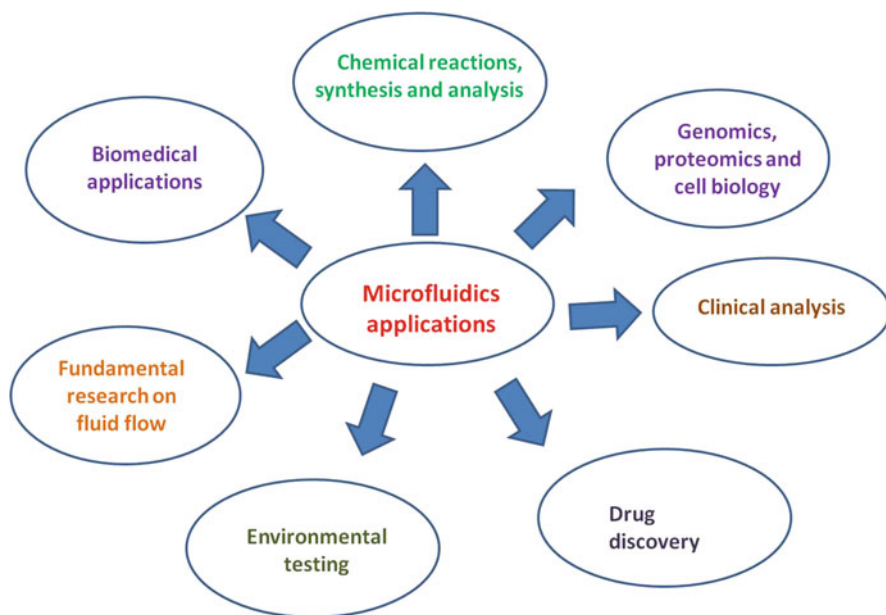


**Fig. 1.1** Summary of applications of microfluidics in various fields

can be performed in microsystems with reduced reagent volumes in a short period of times (Shi et al. 2019). Integrated microfluidic devices greatly reduce the reagent volumes and reaction times in DNA sequencing (Paegel et al. 2003). Sample transportation, mixing, and DNA amplification are the important steps in polymerase chain reaction, and first microfluidics application in PCR was started in 1998 (Ahrberg et al. 2016). Owing to the advantages of microfluidics in PCR, several researchers have gained interest in implication of microfluidics in PCR for small samples handling and efficient nucleic acid amplification (Roper et al. 2005). Microfluidic-based point-of-care devices have increasingly gained the attention of several researchers due to rapid testing with low volumes of patient at samples (Nasseri et al. 2018). Recently, microfluidics platforms have been used increasingly for drug discovery and development (Pihl et al. 2005). Reduced analysis time and high throughput screening with minimum reagents, microfluidics-based in vitro testing are some of the advantages of microfluidic devices in drug discovery and development (Kang et al. 2008). On-site environmental monitoring and detection of pollutants can be achieved by implications of the microfluidics (Yew et al. 2019). Flow of fluid concepts can be studied in microfluidic devices and it leads to the development of novel principles of fluid management in various applications (Ballerini et al. 2011).

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## 1.4 Assisted Reproduction in Animals

Assisted Reproductive Technology (ART) refers to the handling of gametes and embryos for successful fertilization (De Geyter 2019). The International Committee for Monitoring Assisted Reproductive Technology and the World Health Organization Revised Glossary on ART Terminology in the year 2009 and excluded the artificial insemination from ART (Zegers-Hochschild et al. 2009). The ART procedures were summarized in Fig. 1.1. Recently, dependency on assisted reproduction techniques has been greatly increased in the livestock sector to overcome the difficulties associated with natural mating of animals (Chakravarthi and Sri Balaji 2010). Productivity of animals can be increased many fold by using assisted reproductive techniques for animal breeding (Evans 1991). ART offers the production of genetically elite animals and also facilitates the genetic manipulation (Solti et al. 2000). Animal infertility is one of the problems in reproduction, and it can be addressed by ARTs (Sinclair 2008). Extracorporeal fertilization and placing the embryos in uterus or fallopian tubes are challenging tasks in assisted reproduction (Verma et al. 2012). Preservation of gametes, zygotes, embryos, or gonadal tissue plays a key role in success of assisted reproduction in animals. Isolation of spermatozoa with potential to fertilize the egg and analysis of the semen quality are prerequisites for assisted reproduction (Nawroth et al. 2005). Sperm sexing refers to the separation of X and Y chromosome bearing spermatozoa from semen samples (Maxwell et al. 2004). Sexed semen is in high demand for the production of either male or female animals (Johnson 2000). The available methods of ARTs are either

cost effective or low efficient and success of an ART depends on the skill of the personnel involved in it (Rodriguez-Martinez 2012).

### 1.4.1 Sperm Preparation for ART

Raw semen is not suitable for the most of the ART methods and sperm preparation is required for the selection of spermatozoa that can be able to fertilize the eggs (Yang et al. 2015). The fertilization capability of semen samples can be improved by the selection of the intact, viable, and motile spermatozoa with high DNA integrity (Paasch et al. 2007). Isolation of dead, non-motile, and defective sperm from the semen samples is the main challenge in the sperm preparation methods (Henkel and Schill 2003). Presence of incompetent sperms in the semen samples limits the success of ART methods. Conventional methods of sperm sorting such as sperm washing swim-up, density gradient centrifugation have not reached any consensus on the DNA integrity and effect of fertilization rate (Tarozzi et al. 2019). Advanced sperm sorting based on various principles such as surface charge, hyaluronic acid binding, motile sperm organelle morphology examination, magnetic activated cell sorting, and sperm head's birefringence have the limitations in sperm sorting due to either complexity or cost of the method (Henkel 2012). There is an urgent need to develop cost effective, efficient, and portable devices for separation of competent sperm cells for fertilization.

### 1.4.2 Role of Cryopreservation in ART

Cryopreservation is the method of storage of live cells at sub-zero temperatures and retrieval of functional cells after thawing (Pegg 2007). Cryopreservation is an integral part of ART and it is an essential tool for storage and transport of reproductive cells such as sperms, oocytes, embryos, and ovarian tissues (Hiemstra et al. 2006). Slow rate freezing and vitrification are the main methods being used in the ART methods (Gajda and Smorag 2009). Slow freezing method involves the dehydration of cells and conversion of water into ice. Slow freezing is still preferred in ART due to minimal alteration of cellular events. Intra- and extracellular ice crystal formation while slow freezing causes shrinkage in the cell membrane and it leads to the cell damage (Mandawala et al. 2016, Vajta and Kuwayama 2006). Vitrification refers to the rapid freezing and solidification of the cells without ice crystal formation. This method offers preservation of cells in intact state without any mechanical damage to the cell while preservation. High concentration of cryoprotectants is the major limitation of this method (Singh et al. 2017). Miniaturized devices for cryopreservation have been an emerging area of research in cryogenics. These types of devices could help in easy handling and transport of animal gametes, oocytes, and embryos.

### 1.4.3 ART in Animal Production

Livestock production rate depends upon the use of successful ART methods for animal breeding (Velazquez 2008). Several ART methods have been developed, which include in vitro fertilization, embryo transfer, gamete intra-fallopian transfer, zygote intra-fallopian transfer, intracytoplasmic sperm injection, transgenesis, stem cell technology (Verma et al. 2012). Species specific alterations in ART methods result in increased production of livestock. Selection of genetically elite animals, conservation of endangered species, and treatment of reproductive efficiency are the major advantages of ART methods (Lasley et al. 1994). Sperm sexing is an efficient way to increase the productivity of desired sex animals. Specifically in the dairy industry milk producing animals are in high demand to meet the increasing global requirement (Johnson et al. 1999). Cloning is a powerful technique for the multiplication of desired animals without use of conventional breeding techniques. Cloning is the better way to produce highly valuable and endangered species without any genetic variation (Tsunoda and Kato 2002). As the dependency on natural mating in commercial purpose animals has been greatly reduced in recent years, ART methods have gained commercial interests. Development of novel ART methods or improvements in the existing ART methods could lead to the low-cost production of animals.

## 1.5 Conclusions

There has been growing interest in the healthy animal traits with disease resistance and adaptability to extreme climatic conditions. Production of animals with selective breeding results in profitability to the animal farmers. Selection of animals of desired sex and genetic trait is becoming more widespread in food-producing animals. Assisted reproductive technologies offer selective breeding by state-of-the-art methods. Limitations of existing ART methods will be addressed by the use of miniaturized microfluidic devices.

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# Principles, Materials, and Fabrication Methods of Microfluidics

# 2

## Abstract

Microfluidics is an emerging field of research for the manipulation of fluids in microstructures with dimensions of tens to hundreds of micrometers. Microfluidics is characterized by the interplay between physics, material sciences, and engineering. The fluid flow in microscale differs from the macro-scale, and it can be discussed with fundamental principles such as laminar flow, diffusion, surface tension, and electrokinetic forces. Material selection plays a critical role in the design and fabrication of microfluidic devices. Rigidity, softness, compatibility to solvents and chemicals, permeability to oxygen, and optical transparency are some of the parameters of the materials need to be considered for the fabrication of the devices. Micromachining, lithography, 3D printing, and nanofabrication are being employed for the fabrication of microfluidic devices. Droplet-based and digital microfluidics revolutionized the field of microfluidics specifically in DNA, protein, and enzyme-based assays. In this chapter, we summarized the principles of fluid flow and highlighted the characteristics of the materials which are being used for the fabrication of microfluidic devices. This chapter also presents the fabrication methods of microfluidic devices with recent developments.

## Keywords

Microfluidics · Lithography · Laminar flow · 3D printing · Nanofabrication and digital microfluidics

## Abbreviations

DNA      Deoxyribonucleic acid  
GPa      Gigapascals

|      |                           |
|------|---------------------------|
| PDMS | Polydimethylsiloxane      |
| $Pe$ | The Peclet number         |
| PMMA | Poly(methyl methacrylate) |
| $Re$ | Reynolds number           |
| RNA  | Ribonucleic acid          |
| $Sh$ | Sherwood numbers          |
| UV   | Ultraviolet               |

## 2.1 Introduction

Microfluidics is referred to as the science and technology of manipulating small amounts (10<sup>-9</sup> to 10<sup>-18</sup> Liters) of fluids by allowing to pass through channels having dimensions of less than 1 mm (Whitesides 2006). Microfluidic methods exhibit precise control on fluid flows than conventional macroscale methods (Squires and Quake 2005a, 2005b). Microfluidics has attracted significant attention over the past decade, due to the innovative applications in multiple disciplines which include physics, chemistry, and biology. Microfluidic methods offer manipulation of small sample volumes, shorter sample processing times, faster heat and mass transfer rates, and integrating parallel reactions in a single chip (Rohde et al. 2007). Portability, low cost, and high sensitivity are the other important advantages of microfluidics (Gravesen et al. 1993). Applications of microfluidics include chemical synthesis, separation of chemicals, bio-detection, single-cell biology, microdroplets, rheometry, and optofluidics (Ohno et al. 2008). Biological applications of microfluidics include cell sampling, mechanical and electric cell trapping and sorting, cell lysis, electroporation/gene transfer (Beebe et al. 2002a, 2002b). Cell fusion, cell transport, cell cultivation, and electric characterization and single-cell analysis studies have gained the momentum in microfluidic applications (Wheeler et al. 2003). A typical microfluidic device or chip may consist of microchannels, micro nozzles, micropumps, micromixers, and microvalves (Nge et al. 2013; Hardt and Schönfeld 2007). Pressure gradient, capillary effects, electric fields, magnetic field, centrifugal forces, and acoustic streaming are some of the driving forces which can manipulate the fluid flow in microfluidic channels (Squires and Quake 2005a, 2005b). Network connectivity, channel cross section and curvature, surface topography and porosity are the geometric characteristics of microfluidic devices that can influence the fluid flow (Bayraktar and Pidugu 2006). Surface charge, wettability, chemical affinity, PH/ionic strength sensitivity are some of the chemical characteristics need to be considered to design the microfluidic devices (Yang et al. 2010). Piezoelectric actuation electrodes and heaters can be incorporated in the microfluidic devices (Khoshmanesh et al. 2017; Germain et al. 2007). Hot embossing, injection molding, soft lithography, laser photoablation, conventional photolithography/optical lithography, and X-ray lithography are some of the important fabrication methods being used for the fabrication of microfluidic devices.

Silicon/glass, elastomers, thermoset, thermoplastics, hydrogel, and paper-based materials can be used for the fabrication of microfluidic devices (Ren et al. 2013).

## 2.2 Fundamentals of Microfluidics

Manipulation of fluids in microscale channels depends upon the transport mechanisms of fluids within the microchannels. Flow, diffusion, surface effects, and electrokinetics are some of the parameters important for the design and fabrication of microfluidic chips. The transport mechanism for microfluidic applications is summarized in Table 2.1.

**Table 2.1** Summary of transport mechanisms for microfluidics applications

|   | Transport mechanisms |                           | Principles                                                                                                                          | References                               |
|---|----------------------|---------------------------|-------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------|
| 1 | Laminar flow         | Pressure-driven flow      | Hydrostatic pressure gradient results in parabolic velocity profile across the channel                                              | Randall and Doyle (2005)                 |
|   |                      | Electrokinetic fluid flow | Electrical potential with applied voltage across the channel results in plug flow                                                   | Studer et al. (2002)                     |
| 2 | Diffusion            | T-sensor                  | Interface of the two fluids can be controlled by varying the speed of the flows                                                     | Weigl et al. (1999)                      |
|   |                      | H-filter                  | Separate molecules based on differences in size and diffusivity                                                                     | Lewpiriyawong et al. (2008)              |
| 4 | Surface effects      |                           | Movement of fluids by capillary pressure and surface tension                                                                        | Lam et al. (2002), Juncker et al. (2002) |
| 3 | Electrokinetics      | Electrophoresis           | Movement of a particle is towards the electrode of opposite charge in an applied electric field                                     | Wu et al. (2008)                         |
|   |                      | Electroosmosis            | Electrical properties of the fluid and the applied electric field result in electro hydrodynamic forces which act to move the fluid | Wang et al. (2009)                       |
|   |                      | Dielectrophoresis         | Polarization effects of particles in non-uniform electric fields.                                                                   | Çetin and Li (2011)                      |
|   |                      | Electrorotation (ROT)     | Torque is exerted on the induced dipole which causes the particle to rotate                                                         | Wilson et al. (2002)                     |

### 2.2.1 Fluid Flow

The flow of the fluid in microscale is different from a macroscale and it plays an important role in the design and fabrication of microfluidic devices (Ong et al. 2008). The flow of fluids in adjacent solid surfaces called an internal stream, and flow types of internal flows are characterized by Reynolds number ( $Re$ ). It is the ratio of inertial forces to viscous forces, and small Reynolds numbers result in laminar flow for most microfluidic applications.

$$Re = \frac{ud}{\nu}$$

In the above equation,  $u$  is the flow velocity,  $\nu$  is the kinematic viscosity, and  $d$  is the channel diameter.

Laminar flow is the motion of fluid particles in regular and orderly paths in parallel to the walls of the tube or channels. Laminar flow is characterized by  $Re < 2000$ , low viscosity, high momentum diffusion, small momentum convection. Pressure and velocity are almost independent of time in laminar flow. Turbulent flow is the random motion of the fluid particle in a particular one-way direction. Turbulent flow is characterized by  $Re > 4000$ , high momentum diffusion, low momentum convection, a rapid variation of pressure and velocity in space and time. Transition flow is characterized by the transition between the laminar and turbulent flow with  $Re$  between 2000 and 4000. Flow is almost laminar in microfluidic channels due to small dimensions of microfluidic channels (Beebe et al. 2002a, 2002b).

### 2.2.2 Diffusion

Diffusion is the transport of molecules under the influence of the concentration gradient. As diffusion times are short, concentration gradients can be created in miniaturized devices (Dertinger et al. 2001; Jeon et al. 2000). In one dimension, movement due to diffusion can be governed by Einstein–Smoluchowski relation

$$x = \sqrt{2Dt}$$

where  $x$  is the average distance moved by the molecule after time  $t$  in one dimension  $D$  is diffusion constant.

Diffusion coefficient differences lead to the separation of different size molecules in the microfluidic devices (Holmes and Gawad 2010). Stokes–Einstein relation gives the diffusion coefficient for spherical particles

$$D = \frac{kT}{6\pi n r}$$

where  $k$  is Boltzmann's constant,  $T$  is temperature,  $n$  the viscosity of the fluid and  $r$  the hydrodynamic radius of the particle.

Diffusion is more important when Sherwood Numbers ( $Sh$ ) are low (Tian and Finehout 2008). Sherwood Number is the ratio of convective to diffusive mass transfer, and it is small for microfluidic applications

$$Sh = \frac{kd}{D}$$

In the above equation,  $k$  is the mass transfer coefficient,  $d$  is the characteristic length of the.

System, and  $D$  is the diffusion coefficient.

Diffusion is less important in case Peclet number is large (Tabeling 2005). The Peclet number ( $Pe$ ) is defined as the ratio between the advective and diffusive transport

$$Pe = \frac{\text{Advective transport}}{\text{Diffusive transport}} = \frac{lv}{D}$$

$v$  and  $l$  are the characteristic velocities and characteristic length of the system and  $D$  is the diffusion coefficient of the species that is being transported.

### 2.2.3 Surface Effects

Surface area to volume ratio and surface tension forces are important for fluid flow in the microchannels. Large surface area to volume ratio aids in capillary electrophoresis in microchannels by removing excessive heat (Squires and Quake 2005a, 2005b). Surface tension generated from the cohesion between liquid molecules at the liquid and gas interface. Smaller drops exhibit a higher internal pressure than larger drops (Walker and Beebe 2002). Calculations for pressure differences and surface free energy is given by Young–Laplace equation

$$\Delta P = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right)$$

$\Delta P$  Difference between atmospheric pressure and the pressure inside the drop, Radii of curvature  $R_1$  and  $R_2$ ,  $\gamma$  is the surface free energy.

The Bond number enables pumping the fluids via capillary pressure and droplet-based transport systems (Gilet et al. 2009). Bond number ( $Bo$ ) is the ratio of body forces to surface tension forces, and typically  $Bo$  should be small for microfluidics application.

$$Bo = \frac{\Delta \rho \cdot a \cdot L^2}{\gamma_{12}}$$

In this equation,  $\Delta \rho$  is the density difference across the interface,  $a$  is the acceleration associated with the body force, which in most cases is gravity,  $L$  is

the pertinent length scale, and  $\gamma_{12}$  is the surface tension between the two fluid phases.

### 2.2.4 Migration by Electric Force

Coulombic forces result in the movement of electrically charged molecules towards the electrodes in an electric field (Ong et al. 2008). Mobility of the charged particle is governed by

$$\mu = \frac{q}{6\pi\eta r}$$

where  $q$  is the charge on the molecule,  $\eta$  is the viscosity of the liquid,  $r$  is the hydrodynamic radius of the molecule.

## 2.3 Materials for Microfluidic Chip Fabrication

Materials rigidity or softness is measured by Young's (elastic) modulus and the shear modulus (Fallahi et al. 2019). Young's modulus is the ratio of stress and strain in a material and the relation expressed as

$$E = \frac{\sigma}{\epsilon}$$

$E$  is Young's modulus,  $\sigma$  is the stress,  $\epsilon$  is the strain.

The etching is a process of removing selected layers of materials by wet and dry etching methods. Wet etching is a chemical process which involves liquid phase etchants. Wet etchants consist of oxidizing agents such as  $H_2O_2$ ,  $Br_2$ ,  $AgNO_3/CrO_3$ ,  $HNO_3$ , and  $NaOCl$ , which are dissolved in a solvent (Spierings 1993; Grosse et al. 2001). Dry etching involves the removal of the materials by using chemical reactive gases or plasma, sputtering and ion beam-induced etching, and reactive ion etching (Tanzi et al. 2012). Suitable bonding methods are important for sealing the fabricated microfluidic channels. Selection of appropriate bonding method depends upon the materials used for the fabrication of microfluidic devices (Tsao and DeVoe 2009). Adhesive bonding offers high bond strength, low channel clogging, controllable adhesive thickness and this type of bonding can be performed at low temperatures (Saharil et al. 2013). Thermal fusion bonding offers simple, homogeneous channels which are compatible with a wide variety of materials but channel deformation from un-optimized process condition is the major drawback of this method (Sivakumar and Lee 2020). Solvent bonding offers simple, fast, low temperature, high bond strength, low-cost bonding in microfluidic materials (Rahbar et al. 2009). Localized welding is a low temperature, localized bonding and energy director (ultrasonic welding) or metal layer (microwave welding) is required for this type of bonding (Yussuf et al. 2005). Surface treatment and modification offer low-temperature

bonding, low channel deformation but surface chemistry changed after treatment (Zhou et al. 2010a, 2010b).

### 2.3.1 Silicon and Glass

In early studies, glass and silicon are the best available choices for the microfluidic channel fabrication (Iliescu et al. 2012). Glass is preferred over silica due to optical transparency, defined surface chemistry, and high-pressure resistance (Lin et al. 2001; Stjernström and Roeraade 1998a, 1998b). HF/HCl, HF, HF/NH<sub>4</sub> are suitable etchants for wet etching process for glass with a etch rate of 7–8 μm/min. SF<sub>6</sub>, C<sub>4</sub>F<sub>8</sub>, CF<sub>4</sub>, CHF<sub>3</sub> are suitable to dry etchants for dry etching of glass etch rate of 7–8 0.5–0.8 μm/min. Silicon dry etching can be done with an etch rate of 2–30 μm/min, whereas wet etching can be done at a etch rate of 4–90 μm/min depending on the various etchants. Young's modulus for silicon is in the range of 130–180 Gigapascals (GPa), and it is in the range of 50–90 GPa for glass. Silicon and glass based microfluidic devices are fabricated by photolithography with a channel dimension up to less than 100 nm. These materials are highly thermostable, hydrophilic, and resistant to most of the oxidizers. Solvent compatibility is very high for silica and glass materials with stable surface charge. Permeability to oxygen is less than 0.01 barrer for silicon and glass materials (Ren et al. 2013).

### 2.3.2 Elastomers

Elastomers are flexible, stretchable, and weakly cross-linked polymers (Ismagilov et al. 2001). These materials are elongated and compressed when apply and remove the forces. Young's modulus for elastomers is approximately 0.0005 GPa and elastomer microfluidic devices can be fabricated by casting method. Less than 1-micrometer channels are fabricated by using elastomers. Permeability to oxygen is around 500 barrer for elastomers, and optical transparency is high. Most of the elastomers are hydrophobic with low solvent compatibility. Unstable surface charge and moderate resistance to oxidizers are some of the drawbacks of the elastomers (Ren et al. 2013). Polydimethylsiloxane (PDMS) has been preferred material for microfluidic device fabrication for over the decades (Friend and Yeo 2010). The optical detection range of PDMS is from 240 to 1100 nm, and its electric properties allow embedded circuits and intentional breakdown to open connections (Kuswandi et al. 2007). Mechanical properties allow actuation by reversible deformation and facilitate release from molds (Raj and Chakraborty 2020). Thermal properties of PDMS do not allow dissipation of resistive heating from electrophoretic separation. PDMS has low surface free energy and aids in easy removal of replicas from molds. PDMS can be reversibly sealed to materials. PDMS exhibits low permeability to liquid water and good permeability to gases and non-polar organic solvents. PDMS is unreactive towards most of reagents and surface can be etched and modified to be

hydrophilic. PDMS is nontoxic and supports mammalian cell growth (Zhou et al. 2010a, 2010b).

### 2.3.3 Thermosets

Thermosets are rigid, stiff, and strongly cross-linked polymers. Cross-linking in thermosets is irreversible (Kloxin and Bowman 2013). Young's modulus for thermosets is in the range of 2 to 2.7 GPa and these materials can be fabricated by casting, photopolymerization with a channel dimension up to less than 100 nm. Thermosets are highly thermostable and compatible with most of the solvents. These materials are hydrophobic with stable surface charge and optical transparency. Permeability to oxygen is in the range of 0.3–1 barrer (Ren et al. 2013). SU-8 is an epoxy-based, negative near UV photoresist, and it has been used in the form of molds for microfluidic systems (Martinez-Duarte and Madou 2011). It was developed and patented by IBM for the fabrication of microfluidic structures. Poly imide is another used thermoset for microfluidics fabrication, and it offers thermal and chemical stability, low water uptake, and biocompatibility (Abgrall et al. 2007).

### 2.3.4 Thermoplastics

Thermoplastics are highly cross-linked polymers, and these materials can be remolded multiple times by curing and cooling (Liu and Fan 2011). Young's modulus for thermoplastics is in the range of 1.4 to 4.1 GPa, and thermomolding methods fabricate these materials. Permeability to oxygen is in the range of 0.05 to 5 barrer, and these materials are hydrophobic with stable surface charge. These materials have shown medium to high thermostability and solvent compatibility and optical transparency (Ren et al. 2013). Poly(methyl methacrylate) (PMMA) is a commonly used thermoplastic for microfluidic device fabrication. Rigidity, optical transparency, electrophoretic applications, and low price are some of the advantages of the PMMA (Chen et al. 2008). Cyclic olefin copolymer, poly(ethylene glycol) diacrylate, fluorinated ethylene propylene and perfluoro alkoxy, polyfluoro polyether, diol methacrylate, polyurethane, polyetherketone, polyethylene terephthalate, polyvinyl chloride are other thermoplastics that can be used for fabrication of microstructures (Mou and Jiang 2017).

### 2.3.5 Hydrogels

Hydrogels are water containing cross-linked polymers with porous structures which allow the flow of fluids (Erol et al. 2019). Young's modulus for hydrogel is very low in comparison with other materials, and these materials are fabricated by casting, a photopolymerization method with channel dimensions approximately 10  $\mu\text{m}$ . These materials have little thermostability and solvent compatibility resistance in

comparison with other materials. These materials exhibits are greater than one barrer permeability to oxygen and moderate optical transparency (Ren et al. 2013). Agarose, chitosan matrigel, polyethylene glycol diacrylate alginate are some of the important hydrogels being used for the fabrication of microfluidics. Diffusion of small molecules, optical transparency, and low cost are the notable advantages of the hydrogels for the fabrication of microfluidic devices (Verhulsel et al. 2014). Hydrogels can be integrated into microfluidics by use of soft lithography, flow-solidification based integration, gel photopolymerization methods (Burdick et al. 2004; Chung et al. 2012).

### 2.3.6 Paper

Paper is a cellulosic material, and it can allow the flow of fluids by capillary forces. Hydrophilic and hydrophobic microstructures can be fabricated on the paper for chemical, biochemical, and medical applications (Li et al. 2012; Dungchai et al. 2009). Young's modulus for paper materials is in the range of 0.0003 to 0.0025 GPa, and microchannels of approximately 200 $\mu$ m can be synthesized by photolithography and printing methods. Even though, thermal stability, resistance, and solvent compatibility are low for paper materials and permeability to oxygen is greater than one barrer. Amphiphilic materials can be synthesized by paper materials (Ren et al. 2013). Photolithography, plotting, inkjet etching, plasma treatment, wax printing, inkjet printing, flexography printing, screen printing, laser treatment are some of the hydrophilic–hydrophobic patterning techniques on the paper. Colorimetric detection, electrochemical detection, chemiluminescence detection, electrochemiluminescence are some of the detection methods of the paper-based microfluidics (He et al. 2015; Dungchai et al. 2011; Adkins et al. 2015).

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## 2.4 Fabrication methods

### 2.4.1 Typical fabrication techniques

#### 2.4.1.1 Laser photoablation or Laser micromachining

In this method, microchannels are fabricated by applying pulsed laser light on the substrate. Laser energy removes the partial material from the substrates and forms the cavities. Romoli et al. (2011) demonstrated CO<sub>2</sub> laser machining of PMMA for the fabrication of a microfluidic device, and it includes three steps, as shown in Fig. 2.1. The depth and width of channels are controlled by the frequency of the pulsed laser light (Chantal 2006). Ultraviolet (UV) lasers (Roberts et al. 1997; Rossier et al. 1999; Bianchi et al. 2001), Femtosecond or ultra-short-pulse lasers (Jiang et al. 2001; Sugioka et al. 2005; Wolfe et al. 2003; Kim et al. 2005), infrared laser micromachining (Liu et al. 2002; Wang et al. 2005) can be used for micromachining microfluidics. This method is compatible with most of the fabricating materials, and low resolution is the drawback of this method.

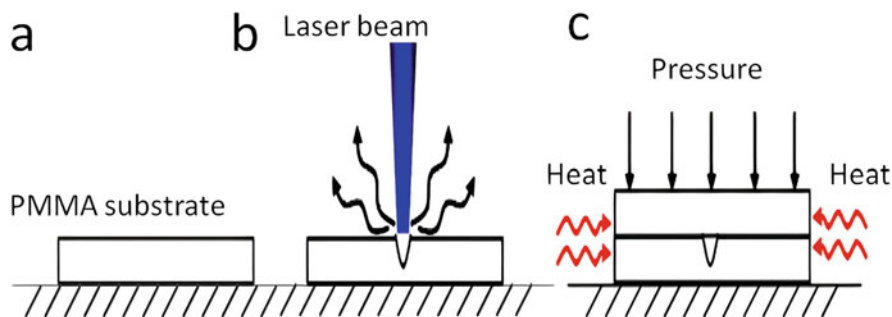


**Fig. 2.1** Main steps: (a) cutting of PMMA substrates, (b) channel fabrication, and (c) thermal bonding. Reproduced with permission from Romoli et al. (2011)

### 2.4.1.2 Lithography

The word lithography comes from the Greek, and it means “writing on stones.” Lithography is a fabrication process of transferring patterns to a reactive polymer using light, laser, or ions (El-Kareh 1995). Based on the source of light, lithography methods can be divided into photolithography, x-ray lithography, electron beam lithography, and ion beam lithography (Thompson 1983). Photolithography is a method that uses the ultraviolet radiation (360–410 nm) to transfer patterns from a mask to the surface of photoresist (photosensitive layer) coated onto a substrate such as a silicon wafer (Vladimirsky 1999). X-ray lithography is similar to photolithography, except it uses much shorter wavelength photons that range in wavelength from 0.4 nm to 1.4 nm (Spille and Feder 1977). Electron beam and ion beam are used as light sources in electron beam lithography and ion beam lithography, respectively (Chérif and Hennequin 1997).

### 2.4.1.3 Soft Lithography

Soft lithography is a set of techniques developed based on conventional lithography methods to fabricate the microstructures on soft matters such as polymers, gels, and organic materials (Qin et al. 2010). Cast molding, embossing, and injection molding are the major types of soft lithography. Xia and Whitesides (1998) explored six other methods, namely, microcontact printing, replica molding, micro-transfer molding, micro-molding in capillaries, solvent-assisted micro-molding, and phase-shift photolithography.

### Casting

The casting process involves creating a negative of desired design with hard material and curing the polymeric materials by using heat or UV light. In a study, PDMS casting against SU 8 master was done by using phosphor bronze and silicon wafers (Schrott et al. 2010). Thian et al. (2008) fabricated the microfluidic channels of 100  $\mu\text{m}$  in width and 50  $\mu\text{m}$  height by vacuum casting method. In this method, silicone rubber was used for the fabrication of the devices. In a cast molding process, PDMS mold was synthesized using a master mold, and it was cut, and holes were

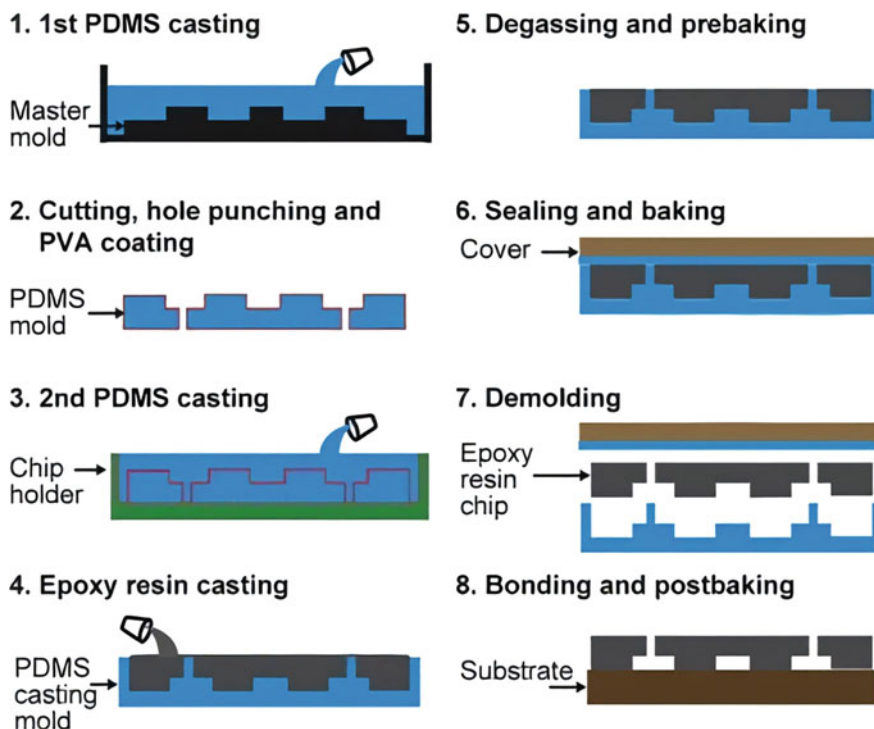


**Fig. 2.2** Steps involved in the PDMS casting and the enclosed epoxy resin casting. Reproduced with permission from Cheng et al. (2017)

punched to fabricate resin chip. The mold was coated with polyvinyl alcohol to prevent the adhesion between two PDMS parts. PDMS casting was performed again using PVA coated PDMS mold to synthesize PDMS casting mold which was used for casting epoxy resin chip (Cheng et al. 2017). Steps involved in the method of PDMS castings and enclosed epoxy resin casting were shown in Fig. 2.2. Russo et al. (2004) developed wet inversion process casting membranes directly on a silicon substrate, and it allows precise control of membrane thickness and pore size. Centrifugal casting and curing of PDMS of microstructures allows concurrent patterning of many sides of component and control the thickness of molds (Mazzeo and Hardt 2013). The lost-wax casting method is advantageous in the fabrication of microstructures with multifaceted geometry without disarrangement of bonding layers (Chung et al. 2015). Sun et al. (2017) demonstrated the iterative casting-shrinkage cycle method to downscale 3D printed microstructures. In a recent study, a transparent ceramic microfluidic module is developed by using aqueous tape casting of  $\text{Al}_2\text{O}_3$  method (Luo and Eitel 2018). A recent study demonstrated the interactions between a single population of leukocytes and two distinctly treated endothelial cell populations by using microfluidic devices cast from 3D-printed molds (Vera et al. 2019).

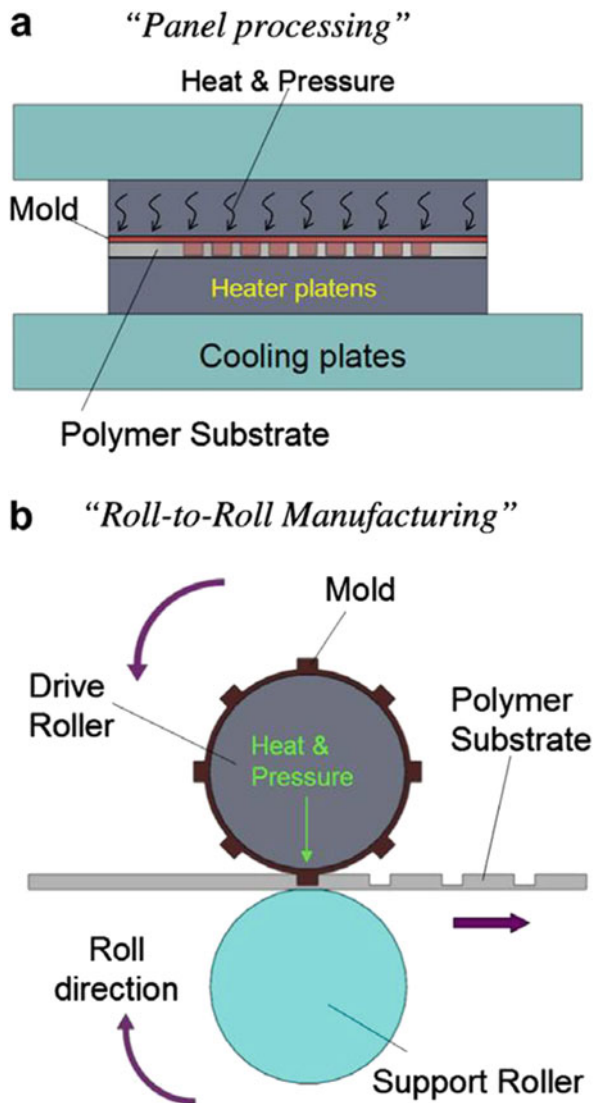
## Embossing

Embossing refers to the transfer of patterns from the molds, which are made up of hard materials, to pliable sheets by applying heat and pressure. Mathur et al. (2009) found that 10 kN applied force and 100 °C embossing temperature as optimum parameters for fabrication of PMMA-based microfluidic channels. Yeo et al. (2009) developed hot roller embossing machine for the fabrication of polymeric microfluidic devices. This machine consists of stainless steel roll, and imprinting mold was attached to the driver roll. Temperature sensing was facilitated inside the core, and roller pressure was supplied from an external source. The substrate samples placed in the preheat conveyor. The difference between panel processing and roll-to-roll manufacturing embossing process is shown in Fig. 2.3. Goral et al. (2010) demonstrated a hot embossing process for fabricating PDMS based microfluidic device by using a standard laboratory oven. In a study, paper-based microfluidics was fabricated by embossing process which offers contact and exchange with environmental gases (Thuvo et al. 2014). Microstructure fabrication on poly(ethylene terephthalate) by specific temperature and pressure profile was demonstrated by Li et al. (2007). In a study, size-based sorting of microparticles and cells was done by using a dispensable microfluidic device by roll-to-roll hot embossing method (Wang et al. 2016). High replication fidelity was observed when hot embossing is combined with milling methods for fabricating thermoplastic microfluidic devices. Altered parameters such as embossing temperature, time, and force on the production of a microfluidic device were investigated. They observed that embossing temperature plays an important role in the fabrication, and other parameters are shown less impact on fabrication process (Çoğun et al. 2017).

## Injection Molding

Injection molding involves the transfer of polymer melts to mold cavities through an injection port. In subsequent steps, polymer solidifies to mold shape upon decreased mold temperatures (Attia et al. 2009). This method offers large-scale production of microfluidic devices with full automation and accurate replication. An injection molding process fabricated disposable, integrated, cycloolefin copolymer microfluidics chips with a cycle time of 2 min (Mair et al. 2006). A typical injection mold consists of a hopper, a heated barrel with a screw, molding tool with injection port, and a mold. The melted polymer in liquid form injected into the mold and molded part will be ejected after a cold cavity process (Becker and Gärtner 2008). Sandström et al. (2015) developed a fabrication method of reaction injection molding in combination with off-stoichiometric thiol–ene epoxy thermosetting polymers. This method offers rapid processing, high replication fidelity, and low residual stress (Sandström et al. 2015). Domansky et al. (2017) fabricated styrene–ethylene–butylene–styrene based microfluidic chip by injection molding method, and it exhibited superior qualities than PDMS based devices for organ on chip allocations (Domansky et al. 2017). Processing parameters such as pre-drying, melt and mold temperatures, injection velocity and pressure, holding pressure and time, and cooling time play an important role in the design of experiments by injection molding methods (Fu et al. 2011). In a study, ultrasonic probes aided in the online quality

**Fig. 2.3** Hot embossing methods (a) panel processing (b) roll-to-roll manufacturing. Reproduced with permission from Yeo et al. (2009)



control by obtaining ultrasonic vibrations during injection molding (Ono et al. 2005). In a recent study, magnetic field-assisted finishing was adopted for injection molding of microfluidic devices to reduce the surface roughness (Guo et al. 2017).

#### 2.4.1.4 3D Printing

3D printing is a technology of fabricating three-dimensional structures in a device by adding the substrate layer by layer. This technology involves the addition of a new layer on the top of the previous layer (Nielsen et al. 2019). Stereolithography is a

three-dimensional printing method which consists of curing layers of liquid photopolymer by exposing them to light. This method offers high resolution, good surface finish, and little topological restriction. Photosensitive materials such as resin and polymers can be used for fabrication of microfluidic devices by stereolithography. High optical absorption, cytotoxicity, low throughput are the possible limitations of this method (Mohamed et al. 2019). Two-photon polymerization is a sister method of stereolithography in which the liquid photopolymer is cured using femtosecond laser energy (Mandt et al. 2018). Selective laser sintering is a 3D printing method that involves laser sintering of a powder bed selectively to fabricate layers of the finished component. It is a fully automated method which offers high resolution. Powders of metal, polymer, and ceramics can be used for the fabrication of microfluidic devices by selective laser sintering (Zhang et al. 2019). Fused deposition modeling is another 3D printing method which involves extruding polymer that is fed as a solid filament to the device. Thermoplastics are used in this method for fabrication of microfluidic devices, and this method exhibits little topological restriction. Slow build time, low accuracy, non-transparency, poor gas permeability are the limitations of this method (Gaal et al. 2017). Electron beam melting, laser engineered net shaping, polyjet, pro metal are the other potential methods for 3D printing of microfluidic devices (He et al. 2016; Chen et al. 2016; Arivarasi and Kumar 2019). Based on the feed material, the 3D technologies are categorized into a solid, liquid, and powder-based methods (Lee et al. 2016) and these methods are illustrated in Fig. 2.4.

### 2.4.2 Nanofabrication

Nanofabrication refers to the creation of structures whose sizes are of less than 100 nm by top-down and bottom-up methods. Self-assembly is a bottom-up method, and it involves atoms and molecules build-up to form nanostructures by chemical or physical forces. The self-assembly may be coupled with external fields such as electric and magnetic fields (Grzelczak et al. 2010). Lithography is a top-down method which involves optical, laser, ions, and electrons to generate nanostructures. Extreme ultraviolet lithography (Bjorkholm 1998; Stulen and Sweeney 1999), electron beam lithography (Altissimo 2010; Kolodziej and Maynard 2012), nanoimprint lithography (Isobe et al. 2012; Cross 2006) are some of the lithographic methods of nanofabrication. Femtosecond laser direct writing system induces irreversible photochemical or photophysical reactions, and it consists of beam direction system, beam steering and motion stage, computer graphic generation and control system, and in situ monitoring system (Zhang et al. 2010). Schematic diagram of a Femtosecond laser direct writing system is shown in Fig. 2.5.

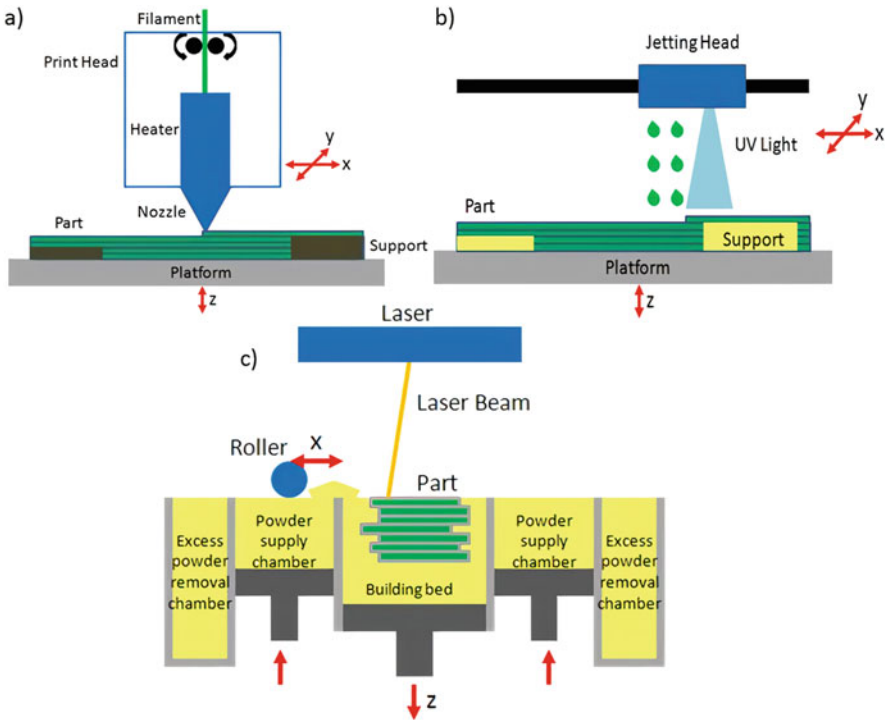


**Fig. 2.4** (a) solid-based (b) liquid-based (c) powder-based 3D printing technologies. Reproduced with permission from Lee et al. (2016)

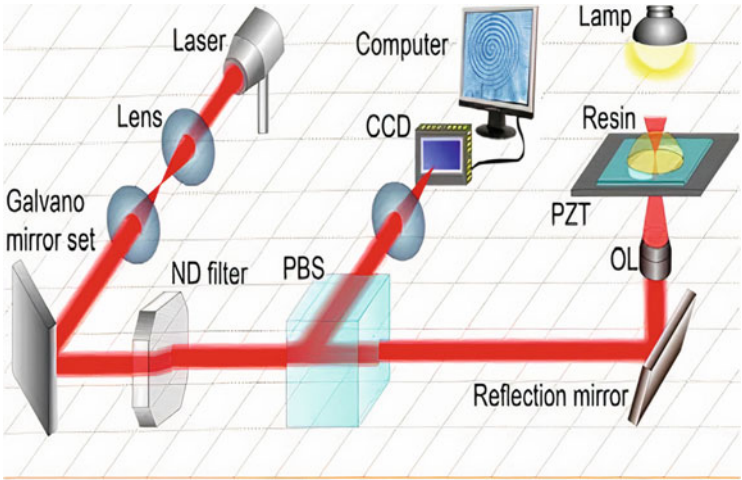


**Fig. 2.5** (a) Schematic diagram of femtosecond laser direct writing system. Reproduced with permission from Zhang et al. (2010)

### 2.4.3 Droplet and Digital Microfluidics

Droplet microfluidics and digital microfluidics are often confused terminology in the field of microfluidics. Droplets can be formed in the microfluidics by balancing the surface tension forces and viscous forces of two immiscible liquids (Shang et al. 2017). Passive droplet generation in microfluidics involves squeezing, dripping, jetting, tip-streaming and tip-multi-breaking methods, and these methods can be tuned to alter the droplet characteristics by applying electrical, magnetic, and centrifugal forces. Viscosity, interfacial tension, channel wettability, and fluid density characteristics of the fluid also play an important role in the droplet formation (Zhu and Wang 2017). Digital microfluidics involves the manipulation of droplets on the electric field (Samiei et al. 2016). Flow actuation is controlled by electrowetting on dielectric or dielectrophoresis in digital microfluidics (Geng et al. 2017). Digital microfluidics has gained the momentum in biological applications such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, enzyme, immunoassays, and cell assays (Wang and Chen 2017). Digital microfluidics offers manipulation of multiple reagents cell-based assays and digital microfluidics-based cytotoxicity assay shown approximately 20 times higher sensitivity than conventional plate assay (Barbulovic-Nad et al. 2008). Magnetic digital microfluidics offers molecular binding on the solid surface, and these devices can be operated manually. Numerous applications of magnetic digital microfluidics have emerged in the field of biosensing and medical diagnostics (Zhang and Nguyen 2017). In a recent study, electro dewetting mechanism was demonstrated for digital microfluidics. In this mechanism, the liquid, solid mechanism is controlled by the attachment and detachment of ionic surfactants to the substrate (Li et al. 2019). Digital microfluidics has been preferred for the nucleic acid amplification methods due to the ease of automation, reduced sample volumes (Coelho et al. 2017). In a recent study, droplet manipulations are effective for conductive and non-conductive fluids by using dielectrowetting.

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## 2.5 Conclusions

The microfluidic research has witnessed tremendous development in the last two decades due to the emergence of technological developments in the manipulation of fluids at the microscale. Microfluidics is implicated in several domains such as biological, pharmaceutical, medical, and physical sciences. Microfluidics has received much attention of biological research because it is being used in various applications such as DNA, RNA, protein and cell analysis, pharmaceutical cellular and enzymatic assays, nucleic acid testing, blood screening with minimum samples. Manipulation of fluids for a potential application can be achieved by the understandings of the governing principles of fluid flow, and this chapter summarized the fundamentals of microfluidics which include the dimensionless numbers, diffusion laws, surface and electric forces. The novel fabrication materials have brought many great changes and new research opportunities in the field of

microfluidics. This chapter explored the various types of materials with pros and cons for the fabrication of microfluidic devices. Design and fabrication of microfluidics chips remain challenging and, therefore, constant and significant efforts have been made to explore the novel methods for fabrication of the microstructures in microfluidic devices. This chapter summarized the conventional and novel fabrication methods of microfluidics with recent developments. Microfluidics is truly interdisciplinary in nature, providing possible synergism in the various fields.

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# Microfluidic and Non-microfluidic Methods of Sperm Sorting and Sperm Analysis

## 3

### Abstract

Sperm sorting and analysis are essential for artificial reproductive methods in animals and humans. Sperm characteristics such as viability, motility, kinetic parameters, and morphology are notable parameters for evaluation of semen samples. Isolation of defective spermatozoa and unwanted cell debris from cell samples was correlated with high fertility rates. Sperm analysis before the use of semen in artificial reproduction is critical and helps to increase the success rate. Conventional methods of sperm sorting are associated with either low efficiency or high cost. Reliability and reproducibility are the major drawbacks of conventional methods which are still in use. Mechanical damage is also one of the problems of conventional and commercial strategies of cell sorting. Computer-assisted sperm analysis (CASA) is the most reliable sperm analysis method, and its use is limited due to its high cost.

Microfluidics offers safe and efficient handling of the semen samples for sorting and analysis. Microfluidics can handle small volumes in biocompatible microchannels which can reduce the mechanical damage of the cells. Portability and reusability of the microfluidic devices attracted the sperm biologists to novel methods of microfluidics-based sperm sorting and analysis. This chapter reviews the microfluidic methods of sperm sorting and analysis. This chapter also covers the conventional and recent methods of sperm processing, and it could help to attract interdisciplinary researchers to develop novel microfluidic strategies for sperm processing.

### Keywords

Sperm sorting · Sperm analysis · Spermatozoa · Microchannels · Sperm damage · Fertility · Semen

## Abbreviations

|       |                                               |
|-------|-----------------------------------------------|
| ALH   | Amplitude of lateral head displacement        |
| ARTs  | Assisted reproductive technologies            |
| BCF   | Beat-cross frequency                          |
| CASA  | Computer-assisted sperm analysis              |
| DNA   | Deoxyribonucleic acid                         |
| IVF   | <i>In vitro</i> fertilization                 |
| LIN   | Linearity                                     |
| LVV   | Average path velocity low cutoff              |
| MACS  | Magnetic activated cell sorting               |
| MAD   | Mean angular displacement                     |
| MSOME | Motile sperm organelle morphology examination |
| MVV   | Average path velocity medium cutoff           |
| STR   | Straightness                                  |
| VAP   | Average path velocity                         |
| VCL   | Curvilinear velocity                          |
| VSL   | Straight-line velocity                        |
| WHO   | World Health Organization                     |
| WOB   | Wobble                                        |

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## 3.1 Introduction

Sperm sorting and analysis methods have made substantial progress in recent years (McDowell et al. 2014, Marzano et al. 2020). Low sperm count, poor viability, low motility damage to deoxyribonucleic acid (DNA), and irregular sperm morphology are some of the problems attributed to a failure of assisted reproductive technologies (ARTs) including *in vitro* fertilization (IVF) and intracytoplasmic sperm injection in animals (Katigbak et al. 2019). Sperm selection is an important step in ARTs which influences both the success rate of artificial insemination and the health of offspring (Said and Land 2011). Sperm selection is also extremely important since current ART methods involve direct insemination by avoiding natural *in vivo* process (Henkel 2012). The main disadvantages of sperm selection methods include the sperm damage that influences the fertilization capability of spermatozoa. The successful sperm sorting method can be developed by sperm behavior in micro-confined environments that mimic the female reproductive tract (Berkovitz et al. 1999).

Semen analysis is the first step in the assisted reproductive technologies, and accurate semen analysis helps to take a decisive decision in the assessment of sperm quality (Rodríguez-Martínez 2006). Conventional semen analysis methods such as chamber counting, viability assays (dye exclusion or hypotonic swelling), and DNA integrity assays (Comet assay or sperm chromatin structure assay) suffer drawbacks that discourage the use of sperm quality assessment (Varum et al. 2007, Franken and

Oehninger 2012, Parekh and Agarwal 2020, Sancho and Vilagran 2013). These methods rely on the skill of the technician and involve a long processing time. Computer-assisted sperm analysis (CASA) is the most reliable automated sperm analysis method over the past four decades (Davis and Katz, 1989). Even though CASA has been proved the most efficient method of sperm quality analysis, the use of CASA has been limited to few laboratories due to its cost.

The advancement of scalable technologies for semen analysis will benefit assisted reproductive technologies in animals. Microfluidic systems have exhibited success in biological applications due to operation with small volumes in microenvironments that mimics the biological systems (Tian and Finehout 2009, Duncombe et al. 2015, Streets and Huang 2014). Recent research on microfluidics has shown promising implications of microfluidic devices on sperm sorting and analysis (Knowlton et al. 2015, Nosrati et al. 2017, Kashaninejad et al. 2018).

## 3.2 Non-microfluidic Sperm sorting methods

In a swim-up method, the semen samples were added to the bottom of the conic tube containing sperm medium and tubes were incubated at 38°C for 1 hr. The upper part of the sperm medium was collected and transferred to a fresh medium (Parrish et al. 1986). Separation of viable sperm and non-viable sperm is shown in Fig. 3.1a. In an in vitro fertilization experiment, more male embryos were observed from sperm collected by swim-up method (Wolf et al. 2018). In a Percoll gradient method, Percoll fractions of various percentages were added to a tube. The sperm sample was placed on top of the gradient and centrifuged. The supernatant was discarded, and the pellet was collected and transferred to a fresh medium (Parrish et al. 1995). In an in vitro fertilization experiment, female embryos have resulted from sperm samples

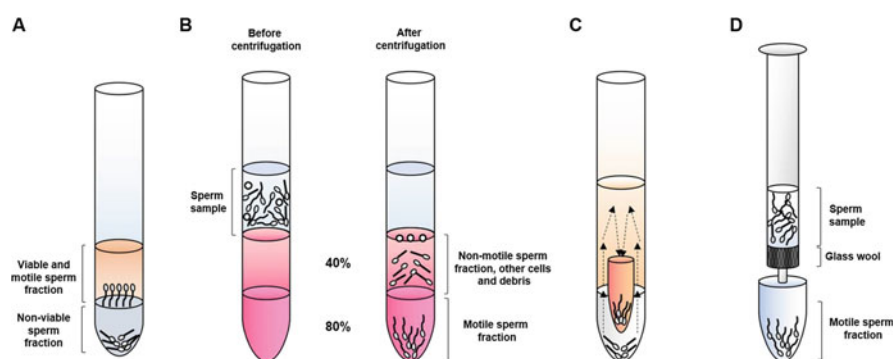


**Fig. 3.1** Diagrams illustrating principles of (a) swim-up (SU) technique, (b) density gradient centrifugation (DGC), (c) migration-sedimentation (MS) procedure: arrows indicate sperm migration direction out of the seminal plasma into the medium and subsequent sperm sedimentation direction towards the central cone. (d) Glass wool filtration (GWF). Reproduced with permission from Marzano et al. (2020)

collected by Percoll gradient method (Wolf et al. 2018). Separation of motile and non-motile sperm by Percoll gradient method is shown in Fig. 3.1b. In a study, Percoll gradient separation showed improved sperm parameters such as intact plasma and acrosomal membranes, motility, and sperm functions. There is no difference in the expression of genes such as leptin, aromatase cytochrome P450, and protamine, after Percoll gradient separation of spermatozoa (Arias et al. 2017). In contrary to this study, Ben et al. 2013, a high percentage of intact plasma membrane and acrosome was observed in yak sperms separated by the swim-up method than Percoll method. This study evaluated the quality of sperms separated by the swim-up and Percoll methods. Similar results were observed upon evaluation of frozen-thawed buffalo semen by swim-up and Percoll gradient methods (Mehmood et al. 2009).

In a modified swim-up method, cell strainer consisting of 70µm pore size nylon mesh was used to collect the spermatozoa. Matured oocytes were placed on the cell strainer to lower the number of polyspermy in boar breeds (Park et al. 2009). In another study, better sperm motility was observed on density gradient sperm separation than dextran swim-up method. This study was conducted in semen samples from Iberian ibex (*Capra pyrenaica*) and European mouflon (*Ovis musimon*) and observed similar results in both the animals (Santiago-Moreno et al. 2014). The effect of incubation time for a swim-up method was studied in dogs. This study indicates that increasing incubation time increased the number of degenerated and damaged cells in the purified samples by the swim-up method (Bukowska et al. 2011). Addition of hyaluronic acid to modified Tyrode's lactate solution for swim-up method isolation sperm cells resulted in an increased percentage of in vitro fertilized oocytes (Shamsuddin and Rodriguez-Martinez 1994).

In a recent study, Kiratli et al. (2018) demonstrated the motile sperm separation method by migration sedimentation method. In this method, sperm cells sediment at the bottom of the tube without centrifugation. Samples were loaded in the upper circular chambers within the migration sedimentation tube and filled the tube with media. Upon incubation of MS tube at 37°C for 30 min, motile sperm cells will move out of the upper circular chambers and settle down at the bottom of the tube (Fig. 3.1c). This method is claimed to have similar efficiency compared to density gradient centrifugation plus a swim-up method of sperm separation. This method greatly reduces sperm damage by avoiding centrifugation (Kiratli et al. 2018). In a typical glass wool filtration method, glass wool was packed in a disposable syringe, and it was attached to the 1.5 ml conical tube. Diluted ejaculates were loaded on the top of the glass wool and enrichment of viable sperm was observed in the first few fractions collected from glass wool column (Fig. 3.1d). This method was claimed to be a simple, inexpensive, and effective method (Jeyendran et al. 1986, Engel et al. 2001, Lee et al. 2009). Glass wool filtration method was found to be successful in removing cryo-damaged canine sperm without affecting DNA integrity of the cells (Kim et al. 2010). Glass wool/Sephadex column sperm separation method improved the fertility in mares (Nie et al. 2003). Magnetic activated cell sorting (MACS) method exhibited the elimination of apoptotic spermatozoa by using paramagnetic annexin V-conjugated microbeads.

Combination of MACS with density gradient centrifugation resulted in effective functional cell separation (Degheidy et al. 2015). Fluorescence-activated cell sorting is an efficient commercial sperm sexing method. This method works based on the difference in the amount of DNA between X and Y chromosome bearing spermatozoa (Garner et al. 2013). Simonik et al. (2015) demonstrated electrophoretic separation of negatively charged spermatozoa from bigger cells such as leukocytes and germ cells. In this study, an electrophoretic apparatus consists of two electrodes, two inner chambers, two outer chambers, two 15 kDa pore-sized polyacrylamide membranes (Simonik et al. 2015). Zeta method is a charge based sperm separation that involves the capturing of negatively charged mature cells to the positively charged tube surface. This method separates the unbound non-mature cells (Chan et al. 2006).

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### 3.3 Microfluidics sperm sorting methods

A microfluidic sperm sorter was demonstrated to isolate spermatozoa to select the spermatozoa with high DNA integrity and lesser reactive oxygen species. This sperm sorter consists of an inlet, two chambers that were separated by a polycarbonate filter of various pore diameters ranging from 3 to 8  $\mu\text{m}$ . The semen samples were loaded via the inlet and motile and functional sperms pass through the filter membrane. The selected spermatozoa were collected at upper retrieval chamber (Asghar et al. 2014). A simple microfluidic device was fabricated with polymethylmethacrylate with a single channel of 7 mm in length and inlet and outlet port sizes of 0.65 mm and mm, respectively. In this study, a lensless charge-coupled device was integrated with a microfluidic chip for a small field of view. This microfluidic device is demonstrated for sorting and tracking the spermatozoa inside the microchannel (Zhang et al. 2011). A two-layered microfluidic device was shown for a selection of spermatozoa with high DNA integrity. The bottom layer contains a network of 500 radial microchannels with a funnel-shaped opening towards the outlet, and size of the radial microchannels was  $100\ \mu\text{m} \times 75\ \mu\text{m}$ . The top layer consists of an injection ring with 1.45 mm depth and 1 ml semen sample can be injected with this injection ring. Viable sperm with high DNA integrity can pass through the radial microchannel and be collected at outlet. The working principle of this microfluidic device is as illustrated in Fig. 3.2. This microfluidic chip offers one step semen purification method with clinical applications (Nosrati et al. 2014). A space-constrained microfluidic sorting was investigated for sperm sorting. In this study, a sorting device was fabricated with microchannels of different lengths with one inlet and one outlet with 0.65 mm and 2mm, respectively. After 30-min incubation of semen samples in the channels, 1-2 fold increase in the motility was observed at outlets (Tasoglu et al. 2013). A microscale integrated sperm sorter was fabricated for isolation of motile sperms from the semen samples. It was designed with two inlets: one inlet is for sample injection, and another inlet is for buffer flow. The sample is allowed to pass through the straight channel and is collected at outlet intended for non-motile sperm collection. Buffer can be passed through another

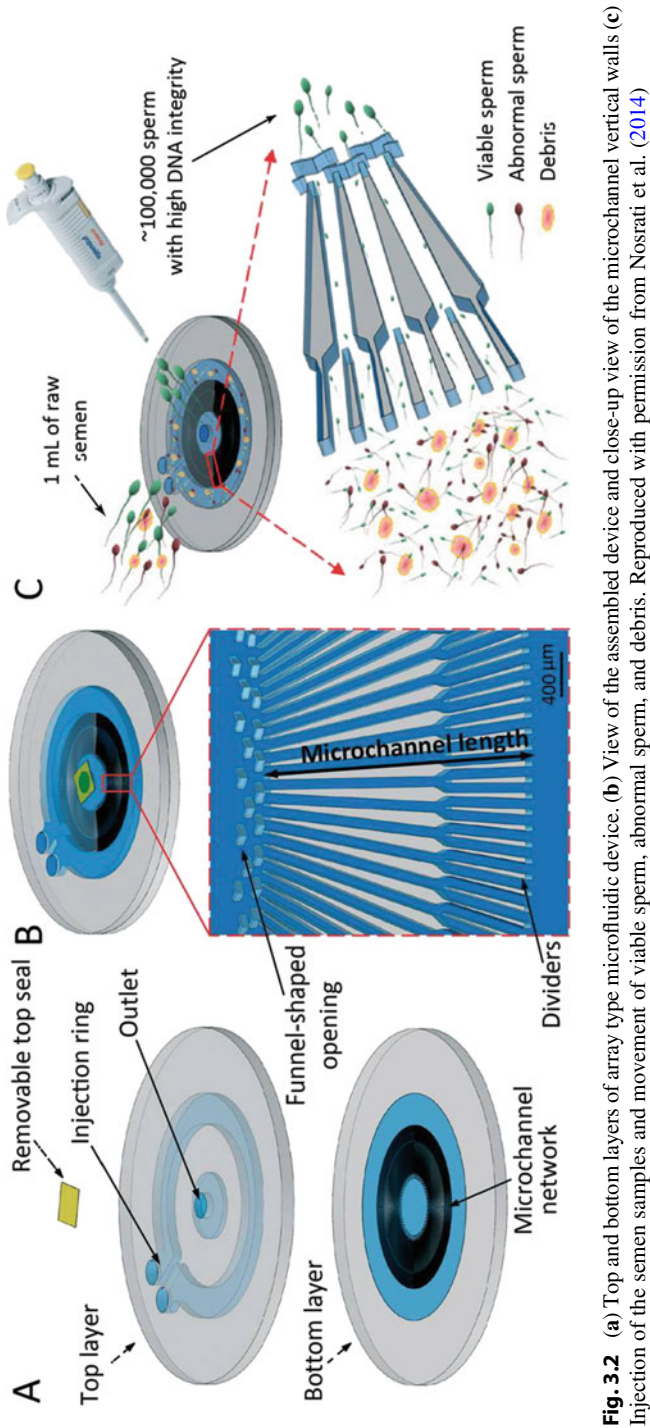


**Fig. 3.2** (a) Top and bottom layers of array type microfluidic device. (b) View of the assembled device and close-up view of the microchannel vertical walls (c) Injection of the semen samples and movement of viable sperm, abnormal sperm, and debris. Reproduced with permission from Nosrati et al. (2014)

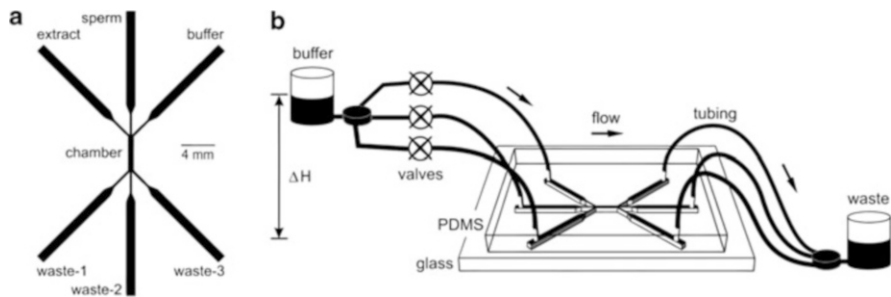


**Fig. 3.3** (a) Diagram of the microfluidic device showing three inlets, three outlets, and a chamber. (b) Diagram of the microfluidic device with fluid control for chemotaxis assays. Reproduced with permission from Koyama et al. (2006)

inlet, and it flows parallel to the sample stream, and motile sperm in sample flow moved into the buffer flow and collected at the separate inlet. This method offers simple, safe, and efficient sperm separation (Cho et al. 2003). A motile sperm sorting microfluidic system was demonstrated, and it consists of 3 reservoirs and three microchannels. Reservoir 1 has two channels and joins at the junction which connects channel two and channel 3. A sample is loaded in channel 2, and the buffer was loaded in channel 1. Due to the height difference between the liquid column between reservoirs 1 and 2 and reservoirs 2 and 3, the reverse flow occurs in reservoir 2. The engineered hydrostatic pressure in microfluidic channels resulted in separating, aligning, and orienting sperm in samples (Seo et al. 2007). A microfluidic device was demonstrated to separate motile spermatozoa from semen samples by using a chemical gradient of acetylcholine. This chip consists of one inlet with eight radial shaped microchannels connected to eight outlets. Eight outlets were used to generate different acetylcholine gradient concentration in a single experiment. Progressive motile sperm were separated by using this method at optimum acetylcholine concentration (Ko et al. 2012). A microfluidic device was fabricated that resembles mammalian female reproductive tract and successfully separated motile spermatozoa by chemoattractant. In this study, the homogeneous cumulus cell suspension was seeded and cultured in the outlet microchannels. These outlets were used as chemoattractant sources and observed chemotactic response in about 10 % of sperm cells (Xie et al. 2010). A microfluidic device was designed for chemotaxis assays, and mouse sperm chemotaxis was demonstrated using mouse ovary extract gradient. This device consists of three inlets, one chemotaxis chamber, and three outlets. Sperm sample was loaded into the middle inlet, and chemical gradient and buffer were loaded from other inlets (Fig. 3.3). This study observed the sperm movement towards the mouse ovary extract than buffer stream (Koyama et al. 2006). An interfacial valve based microfluidic device was demonstrated for thermotaxis response of human sperm. The microfluidic device consists of an air inlet, sperm inlet, and thermal gradient channel and bevel branches. The temperature gradient was created across the microfluidic channel, and thermoresponsive sperm

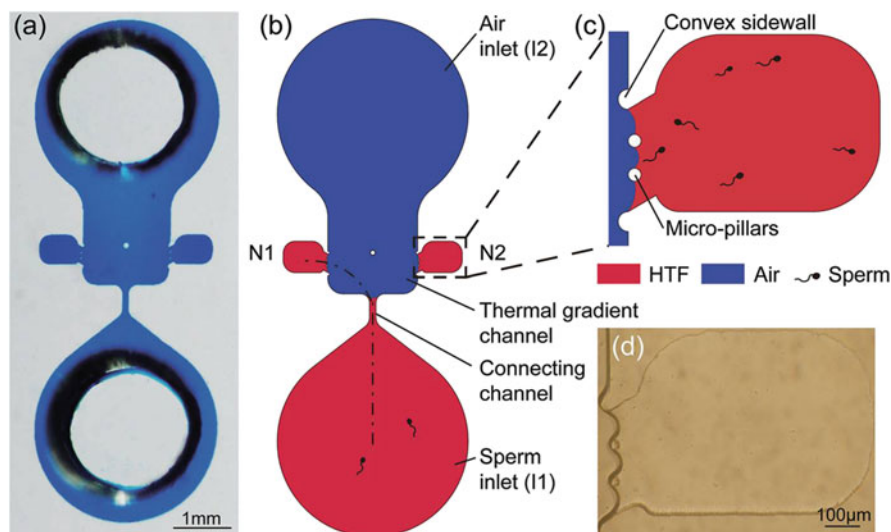


**Fig. 3.4** (a) Diagram of the top view of microfluidic device for sperm thermotaxis assay. (b) Diagram of the assay unit after interfacial valve closure, (c) right branch after interfacial valve closure. (d) Microscopic image of the (c). Reproduced with permission from Li et al. (2014)

were observed at high temperatures (Li et al. 2014). The working principle of this device is shown in Fig. 3.4.

### 3.4 Sperm analysis by non-microfluidic methods

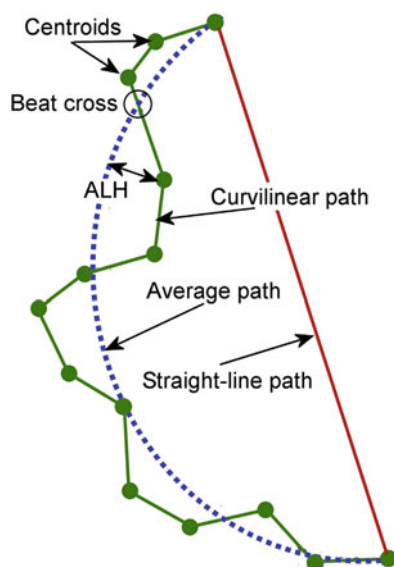
Motile sperm organelle morphology examination (MSOME) is one of the microscopic methods of sperm evaluation using differential interferential contrast microscopy and high magnification (Oliveira et al. 2010). In a study, impaired fertility was reported with a high proportion of sperm head vacuoles which were determined by MSOME (Fekonja et al. 2014). In a transmission electroscopic study, intact acrosome exhibited uniform birefringence, and it was non-uniform in case of acrosomal reaction (Baccetti 2004). The hypoosmotic swelling test was performed to select the viable sperms based on swelling in hypoosmotic solution. Non-viable cells cannot swell and is isolated for selection (Van den Saffele et al. 1992). In a laser-assisted sperm selection, motile sperms are selected based on tail response to a single laser beam (Aitken et al. 1985). Sperm tail flexibility test is based on the tail movement upon mechanically touching sperm cells with an injection needle (Soares et al. 2003). Sperm evaluation and selection also have been reported by hyaluronic acid binding assay and zona pellucida binding assay methods (Prinosilova et al. 2009, Clarke et al. 1989, VandeVoort et al. 1997). These methods rely upon the sperm binding markers with oocytes, and mature and functional sperms are selected by these methods (Sati et al. 2014).

### 3.4.1 Computer-Assisted Semen Analysis (CASA)

The development of CASA was initiated in the year 1974, and complete automated CASA was started in the year 1985 (Amann and Waberski 2014). The CASA system was designed and automated to capture the images from the microscope for digital processing and information analysis by computer software (Lu et al. 2014). CASA provides the evaluation of sperm characteristics like curvilinear velocity (VCL) ( $\mu\text{m/s}$ ), straight-line (rectilinear) velocity (VSL) ( $\mu\text{m/s}$ ), average path velocity ( $\mu\text{m/s}$ ) (VAP), amplitude of lateral head displacement (ALH) ( $\mu\text{m}$ ), (LIN) linearity, wobble (WOB), straightness (STR), beat-cross frequency (BCF) (Hz), mean angular displacement (MAD) (degrees). These parameters were well defined in World Health Organization (WHO) laboratory manual for the examination and processing of human semen (World Health Organization 2010). CASA system consists of a camera attached to a microscope and a computer program that can process the continuous images in a predetermined radius. Sperm trajectories will be constructed with sperm head as a reference point, and sperm parameters will be calculated (Amann and Waberski 2014). Microscope optics and illuminations methods affected the accuracy of image digitalization and targeted tracking in CASA system. Type and depth of the specimen chamber affect the movement of sperm and percent of motility. Temperature, specimen concentration, semen extender, and presence of debris in the sample affect the sperm variables. Video framing rate, number of frames analyzed, number of sperm analyzed, computational algorithms, and statistical methods of a CASA system affect the accuracy of sperm variables (Davis and Katz 1993).

Diagrammatic illustration of CASA terminology is shown in Fig. 3.5. As per the WHO categorization, sperm population is divided into rapid, medium, slow, and

**Fig. 3.5** Diagram illustrating CASA terminology. Reproduced with permission from Amann and Waberski (2014)



static based on the VAP low cutoff (LVV) and VAP medium cutoff (MVV). For example, sperm is fast if VAP is more than MVV and sperm is slow if VAP is less than LVV. Progressive motility includes the cells with VAP more than MVV and straightness with more than a threshold value (World Health Organization 1999). ALH indicates the flagella movement with cell rotation and stronger bending of sperm cells. ALH is important for sperm cells to penetrate the cervical mucus to attach with eggs (Aitken et al. 1985, Versteegen et al. 2002). BCF is proportional to the beating frequency of the sperm tail. In an early study, sperm variables, ALH, BCF, LIN, VAP, and VSL were collectively correlated with high fertility with  $r^2$  value, 0.98 (Farrell et al. 1998, Simonik et al. 2015). In another study, ALH, VCL, and VSL variables of human sperm were correlated with fertilization rates in an in vitro fertilization-embryo transfer method (Hirano et al. 2001).

### 3.4.2 Microfluidics-Based Sperm analysis

In a study, resistive pulse technique, an electrical impedance-based approach was used to characterize the human sperm in a microfluidic device. This device consists of two reservoirs for buffers and another reservoir for a semen sample. An aperture connects all the reservoirs, and it is a long narrow channel with 46 m long, 7 m wide, and 11m height. This study demonstrated the measurement of straight-line velocity (VSL), beat-cross frequency of individual sperm cells by using the microfluidic device. This method is claimed to be economically cheaper than CASA method of sperm characterization (Chen et al. 2013a, b). In another study, sperm concentration was measured on a microfluidic chip that works on the principles of electrical impedance for measurements of spermatozoa. This microfluidic device consists of one inlet, one outlet, and a microchannel with a width of 38 mm at the electrode area. This device was connected to home-made impedance analyzer (Segerink et al. 2010). De Wagenaar et al. (2016) demonstrated the impedance measurement of sperm cells in a microfluidic device. This study observed the changes in impedance upon changes in the orientation and droplet content of the sperm cells. Apart from this, this study sorted the sperm cells using dielectrophoresis based on the impedance of sperm cells. A paper-based microfluidic device was demonstrated to evaluate the live and motile sperm concentrations of human semen samples. This device was fabricated with two paper layers and one laminate layer on the bottom. Concentration spot of the device consists of dried MTT for the generation of colorimetric signals to evaluate the live and dead spermatozoa. Motility spot consists of porous membrane of 8  $\mu\text{m}$ , and it allows passing the motile sperm to reach MTT within the spot to evaluate the concentration of motile cells. This method is a cost-effective method for assessing the semen samples within 10 min (Nosrati et al. 2016). A microfluidic device was fabricated to evaluate the motility of the spermatozoa based on resistive pulse detection. This microfluidic device consists of 3 reservoirs at different heights and interconnected through microchannels. One of the reservoirs loaded with semen samples allowed the sperm to swim against the flow. Motile sperm were detected electronically. A good quality semen sample shows a more

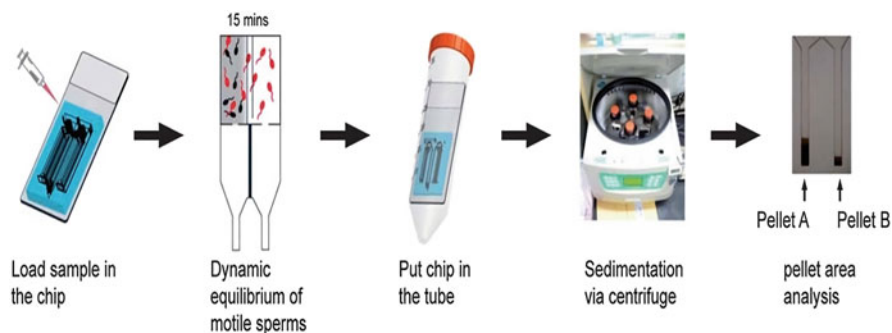


**Fig. 3.6** Steps involved in the sperm quality assessment via separation and sedimentation in a microfluidic device. Reproduced with permission from Chen et al. (2013a, b)

number of pulses than poor-quality samples (Chen et al. 2011). In a study, Chen et al. (2013a, b) demonstrated the sperm quality evaluation by using a microfluidic device that works based on sedimentation upon centrifugation. This microfluidic device consists of two channels separated by a permeable phase guide. One channel was loaded with semen sample, and another channel loaded with buffer solution allowed to pass the motile sperm to the buffer channel until it reaches equilibrium. The cells in both channels were sedimented by centrifugation, and pellet volumes in both the channels indicate the quality of the spermatozoa (Fig. 3.6). Son et al. (2017) demonstrated a spiral microfluidic chip to separate the sperm cells from white blood cells (WBC). This device consists of one inlet, and spiral microchannel ended with one outer outlet and one inner outlet. Samples were injected from the inlet, and sperm cells were collected from outer outlet, and WBC collected at inner outlet. In a study by Eravuchira et al. (2018), integrated quantitative phase microscopy and a microfluidic platform were demonstrated for real-time sperm selection based on morphological characteristics. This microfluidic device consists of one inlet, two outlets, and a T junction. Image analysis was done in the microchannel and based on the sperm quality sperm direction is changed with the help of syringe pumps (Fig. 3.7).

### 3.5 Conclusions

Selection of viable, motile spermatozoa with DNA integrity is an important criterion in assisted reproductive technologies. Sperm evaluation is also equally important for any successful assisted reproductive method. This chapter discussed both microfluidics and non-microfluidics-based sperm sorting and analysis methods. Drawbacks of some of the conventional and commercially available methods can be overcome by developing novel microfluidic methods. As microfluidics are portable and can be fabricated with biocompatible materials, it offers more advantages over non-microfluidic methods. Various cell separation and analysis

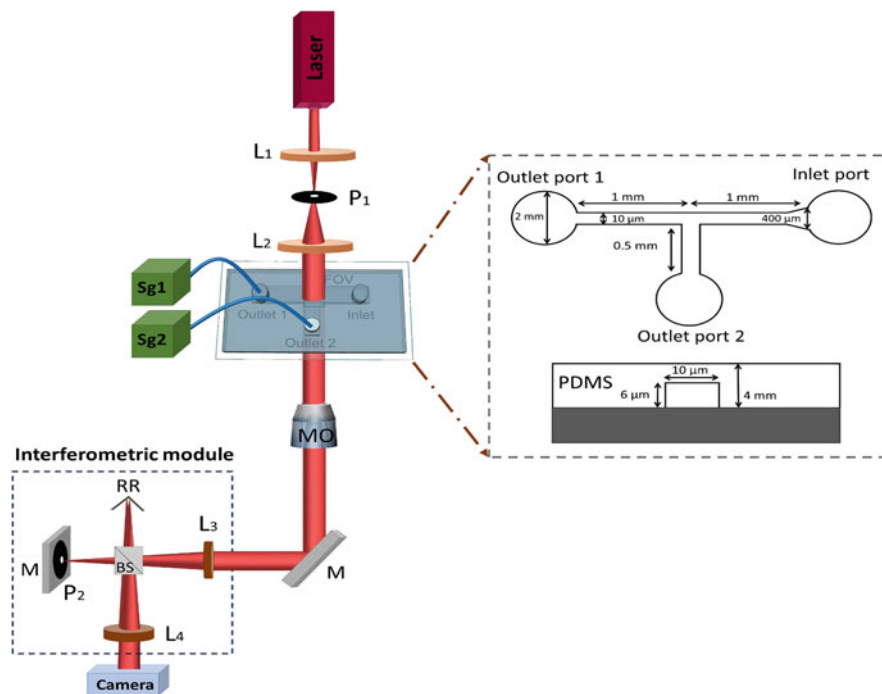


**Fig. 3.7** Diagram of experimental setup containing a disposable microfluidic device integrated with the interferometric phase microscopy setup. *L1-L4* achromatic lenses. *MO* microscope objective, *Sg1*, *Sg2* syringe pumps, *P1*, *P2* pinholes, *M* mirror, *RR* retro-reflector mirror, *BS* beam splitter. Reproduced with permission from Eravuchira et al. (2018)

principles such as velocity, charge, morphology, diffusion, thermotaxis, chemotaxis can be applied by using microfluidic devices. Sperm damage is the major problem in non-microfluidic sperm processing methods, and the use of microfluidics can overcome it. Current sperm research using microfluidics indicates that there will be a lot of scopes to develop microfluidics-based sperm sorting and analysis methods in the near future.

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# Applications of Microfluidics for In Vitro Fertilization and Embryo Culture

## 4

### Abstract

The use of assisted reproductive technology (ART) has remarkable prospective to generate livestock. In vitro fertilization and embryo culture are the key components of ART, which influence the pregnancy rates. Existing methods rely upon the human skills for successful fertilization, embryo selection, and embryo transfer. In vitro fertilization and embryo culture need to be automated to reduce the human intervention and minimize the errors. Microfluidics is an emerging science and technology that offers promising and novel methods for in vitro fertilization and embryo culture.

Microfluidics can integrate several steps of reaction in a single device. The main advantages include the handling of small volumes and single cells. Microfluidic methods are proven to be excellent option for studying live cell dynamics and cell growth. This chapter addresses the constraints in the existing methods and encouraging research results of microfluidics-based in vitro fertilization and embryo culture methods.

### Keywords

In vitro fertilization · Embryo culture · Oocyte · Zygote · Gametes · Livestock · Reproduction

### Abbreviations

|      |                                  |
|------|----------------------------------|
| ART  | Assisted reproductive techniques |
| COC  | Cumulus oocyte complex           |
| DNA  | Deoxyribonucleic acid            |
| EWOD | Electrowetting on a dielectric   |
| ICSI | Intracytoplasmic sperm injection |

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|      |                            |
|------|----------------------------|
| IVF  | In vitro fertilization     |
| IVP  | In vitro embryo production |
| MFSS | Microfluidic sperm sorter  |
| PDMS | Polydimethylsiloxane       |
| ZP   | Zona pellucida             |

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## 4.1 Introduction

The discovery of artificial insemination, advent of cryoprotectants for sperm storage, super ovulation, embryo transfer, in vitro fertilization (IVF), and embryo culture are the sequence of events in between 1930 and 1987, which revolutionized traditional animal breeding methods (Sirard 2018). The collective research efforts in assisted reproductive methods in animals resulted in birth of first somatic cell cloned animal, “Dolly” in 1997 (Edwards et al. 2003). Selective productivity of animals of desired genetic traits has been proven advantageous and profitable for animal farm owners (Velazquez 2008). It also helps in the multiplication of farm animals at faster pace than traditional breeding methods (Evans 1991). Assisted reproductive techniques (ARTs) offer selective breeding of male and female animals (Maxwell et al. 2004). Increasing global animal food demand also necessitated the extensive use of ART in animal reproduction (Hansen 2014).

Microfluidics is an emerging field that attracted the reproductive biologists due to the flexibility in manipulation animal reproduction by using miniaturized devices (Beebe et al. 2002a, 2002b). Microfluidic methods can mimic the animal reproductive tract and experiments can be performed with small volume of samples (Kashaninejad et al. 2018). Continuous supply of nutrients and removal of metabolic wastes are possible with microfluidic designs (Smith and Takayama 2017). Single-cell handling and continuous monitoring of cell growth are some of the other advantages of the microfluidic devices (Wheeler and Rubessa 2017). Analysis and selection of competent embryo is an important step in ART and microfluidics offer multiple steps of ART in a single device (Yanez and Camarillo 2017). Last three decades have witnessed exponential transformation in the field of animal reproduction. Assisted reproductive technologies allow genetic gain of farm animals and it greatly impact the global economy and science. Despite the tremendous success, the efficiency of these ARTs was still low. Microfluidics has become most common in reproductive medicine especially in IVF technology and implications of microfluidic systems greatly enhance the efficiency of ARTs. The microfluidics and non-microfluidic methods of IVF and embryo culture were compared and summarized in Table 4.1.

This chapter discusses the limitations of IVF and embryo culture for animal assisted reproduction with an aim to develop novel microfluidic methods. Recent research on microfluidics for IVF and embryo culture was discussed in detail and it directs the reproductive biologists to implicate the microfluidic methods.

**Table 4.1** Comparison of microfluidic and non-microfluidic methods of IVF and embryo culture

| S. No | Parameter                         | Microfluidics method      | Non-microfluidics methods  | Reference                  |
|-------|-----------------------------------|---------------------------|----------------------------|----------------------------|
| 1     | Culture media                     | Not static                | Static                     | Heo et al. (2010)          |
| 2     | Volume required for culture media | Less( $\mu$ l)            | More ( $\mu$ l-ml)         | Thapa and Heo (2019)       |
| 3     | Disturbance to culture media      | Less                      | More                       | Ma et al. (2011)           |
| 4     | Dynamic fluid environment         | Yes                       | No                         | Smith and Takayama (2017)  |
| 5     | Microenvironment                  | Yes                       | No                         | Smith and Takayama (2017)  |
| 6     | Dynamic chemical environment      | Yes                       | No                         | Smith and Takayama (2017)  |
| 7     | Fluid mechanical stimulation      | Yes                       | No                         | Heo et al. (2010)          |
| 8     | Retention of autocrine factors    | Yes                       | No                         | Heo et al. (2010)          |
| 9     | Manipulation of gametes           | Less laborious/ automatic | Laborious                  | Swain et al. (2013)        |
| 10    | Selection of oocyte               | Possible                  | Difficult (need expertise) | Kashaninejad et al. (2018) |
| 11    | Selection of sperm                | Possible                  | Difficult (need expertise) | Samuel et al. (2018)       |
| 12    | Selection of embryo               | Possible                  | Difficult (need expertise) | Kashaninejad et al. (2018) |
| 13    | Conc. of sperm required           | Low                       | High                       | Suh et al. (2006)          |
| 14    | Flow of oocyte, sperm             | Unidirectional            | Random                     | Smith et al. (2011)        |
| 15    | Fertilization rate                | High                      | Low                        | Chung et al. (2015)        |
| 16    | Cleavage rate                     | High                      | Low                        | Raty et al. (2004)         |
| 17    | Blastocyst development rate       | More                      | Less                       | Kim et al. (2009)          |
| 18    | Pregnancy rate                    | High                      | Low                        | Greco et al. (2017)        |
| 19    | Chances of polyspermy             | Low                       | High                       | Clark et al. (2005)        |
| 20    | Monospermic fertilization         | High                      | Low                        | Sano et al. (2010)         |
| 21    | Handling                          | Easy                      | Hard                       | Wheeler and Rubessa (2017) |
| 22    | Error rate                        | Low                       | High                       | Thapa and Heo (2019)       |
| 23    | Visualization                     | Possible                  | Difficult                  | Horowitz et al. (2020)     |
| 24    | Accuracy                          | High                      | Low                        | Weng (2019)                |
| 25    | Time required                     | Less                      | High                       | Matsuura et al. (2013)     |

## 4.2 In Vitro Fertilization

The in vitro fertilization is one of the revolutionary techniques which got Nobel Prize in 2010 (Johnson 2019). Paradigm shift in the reproductive technologies with the advent of IVF untangles the reproductive problems in humans and farm animals (Casper et al. 2017). IVF is a process in which oocyte and sperm were co-cultured in a microdrop in vitro in order to produce the embryo (Ferré et al. 2020). The whole process is tedious and involves the collection of gametes, selection of oocyte and sperm, maturation, in vitro fertilization then culturing of a embryo (Ventura-Juncá et al. 2015).

IVF has done with different purposes in different species. For example, the main purpose of IVF was to enhance the genetic progress in bovines and ovines, and it was used to demonstrate the genetic modifications in porcine, increasing the reproductive potential in equines, enhancing the basic research in porcine and canine species. Furthermore, IVF is also preferred to increase the beef cattle growth rate, leanness in swine, improvement in milk yield of cattle and improve the reproductive efficiency of farm animals (Sjunnesson 2020). Last decade saw an exponential change in IVF procedures. Microfluidics approach integrates the whole process on one chip and automates the system to reduce the human errors (Le Gac et al. 2020). Acceptable integration is required for any new technology to expand its application wings. Microfluidic platform is a whole new way of approach in handling sperm, oocyte, and embryo which minimizes the human handling, thus decreases human errors. Microfluidics technology has the ability to alter dramatically the fundamental processes of IVF (Swain et al. 2013). As microfluidic systems can mimic the in vivo conditions, microfluidic systems have attracted the attention of reproductive biologists.

### 4.2.1 Microfluidic Platform for Selection and Manipulation of Oocyte

Oocyte collection and processing is a cumbersome process that requires human expertise to handle and time consuming. For the effective assisted reproduction, quality and quantity of oocyte were considered. Successful fertilization in vitro depends on the quality of oocyte which further effect on embryo health. Morphological analysis of oocyte by capturing it and processing the pictures unhide important information about the oocyte quality. Thus isolation and manipulation of oocyte is crucial for fruitful IVF. Microfluidics minimizes the labor intensive, reduces the processing time of oocyte, and allows real-time glimpse at oocyte during IVF. Oocyte is one of the largest cells which make microfluidic systems easy to manipulate oocyte.

Oocyte maturation is a complex process. Successful maturation of oocyte in in vivo conditions requires the coordination of both nuclear and cytoplasmic events. Cytoplasmic maturation of oocyte is necessary for the potential development of embryo after the fertilization. The oocyte cytoplasmic maturation refers to the

gradual increase in the number and migration of mitochondria, cytoplasmic vesicles, golgi complexes, lipid droplets to the periphery which provide oocyte with necessary supplies to perform protein synthesis, post-translational modifications, and reorganization of cytoplasm. These provide machinery for the development of zona pellucida (Fair et al. 1997). Analyzing oocyte morphologically for determination of its quality and selection of oocyte is vital for effective IVF but time consuming. Berenguel-Alonso et al. (2017) fabricated a microfluidic device for culturing of oocyte. Certain limitation of polydimethylsiloxane (PDMS) made them to turn to other materials for fabrication. The cyclic olefin copolymer was used to design microfluidic device which has high-quality optical transparency to study the oocyte maturation. This microfluidic device consists of four ports and a maturation compartment for oocyte which traps oocyte for maturation. Real-time analysis of single cell can be possible using microfluidic system. Earlier studies in microfluidic platform showed successfully removed zona pellucida and cumulus cells.

Angione et al. (2015) engineered a new microfluidic device called simple perfusion apparatus to access the oocyte quality. Control on temperature at various flow rates and high resolution imaging is possible by this device. Zeggari et al. (2007) developed first optical sensor based microfluidic device. Degree of oocyte maturity can be determined by the optical sensors non-invasively. They demonstrated the precise single oocyte trapping and its controlled displacement. This optical sensor can also indicate the fertilization. Morphological parameters were taken into consideration for the selection of quality oocyte (Rienzi et al. 2011). However, there is a lack of accuracy in morphological predictors of oocyte (Wang and Sun 2007) and this initiated the researchers to probe the markers for predicting quality oocyte. Recently, a novel force sensing platform was developed for the mechanical characterization of human oocytes by the using magnetic springs (Gana et al. 2017).

Oocyte is viscoelastic in nature. To measure the solid and liquid mechanical properties of oocyte many methods have been developed. Fertilized and non-viable oocytes show different viscoelastic capacities (Yanez and Camarillo 2017). Relation between the biological properties and oocyte stiffness was demonstrated by the Vidberg et al. (2011). The PDMS based microfluidic device was developed for holding the oocyte by Liu et al. 2010 during micro injection. Defective and healthy oocyte were mechanically characterized ( i.e., stiffness). They also gave hint that mechanical difference in the old and young oocyte is due to the changes in the cytoskeleton and zona pellucida structure. Oocyte deformation during the microinjection was observed in this study (Liu et al. 2010). Softer oocytes with lesser zona pellucida glycoproteins and lesser F-actin amount were also identified in the older oocytes. Significant mechanical input during development or maturation of the oocyte affects its mechanical properties, e.g. rigidity of zona pellucida. During fertilization zona hardening can prevent polyspermy. Cortical granulose plays a critical role in the stiffness of oocyte. Zona hardening is a physical condition and it is initiated by the release of cortical granules during fertilization (Khalilian et al. 2010). Maturation and quality of oocyte are necessary for potential embryo development. (Yanez et al. 2016). There were no specific markers to predict the successful development of embryo during early stages after fertilization. Yanez et al. 2016 in

his paper demonstrated that during early hours after fertilization the formation of blastocysts can be identified by the viscoelastic properties of zygote. They predicted with more than 90% of accuracy. Human, Ovine, Bovine, Porcine have the physically identical embryos during first stages of development. After collecting unfertilized female gamete from the donor, oocyte has to undergo maturation process in media supplemented with hormones as freshly collected ova is not fully equipped for fertilization process. Oocyte is surrounded by a cloud of irregular shape cumulus layer. This spherical cumulus layer is partially responsible for oocyte maturation. Chian et al. (1995) demonstrated that cumulus cells help in the fertilization process even at low sperm concentration. Intact cumulus helps in the prevention of polyspermy condition. Polyspermy is a condition where embryo becomes non-viable after fertilizing with multiple sperm. Hyaluronidase enzymes released from sperm attack the cumulus oophorous and allow sperm to penetrate through zona pellucida to fertilize egg. However cumulus layer dispersed naturally after fertilization in vivo, sometimes it still appears on the oocyte post fertilization which has adverse effect on embryo. In mouse and porcine, cumulus cells removed before IVF, whereas in bovine it was removed after fertilization. The advantageous effects and non-beneficial effects of cumulus cells in different species are still under investigation. The byproduct due to hyaluronidase and cumulus interaction can affect the zona pellucida. The presence of cumulus layer following fertilization significantly decreases the viability of embryos. In addition oocyte should be denuded before ICSI procedure to assess the maturation stages of nucleus and permit the sperm to infuse with oocyte, so cumulus cells are removed. Mechanical stress caused during removal of cumulus cells may have adverse effect like epigenetic modifications and differential expression which results in lesser weight after birth (Craik 2008). There are different approaches adopted so far for the removal of cumulus cells from cumulus oocyte complex (COC)(Lantelme et al. 2001). These approaches involve the flushing of COC into and out through pipetting manually, vortexing at high speed, and using hyaluronidase. Typically combination of both hyaluronidase and manual pipetting is used to denude the oocyte from cumulus cell mass. Pipetting into and out during cumulus removal creates stress on the embryo. Vortexing at high speed to remove cumulus layer alters the location of polar body and chromosome spindles (Li et al. 2006). These ultimately have effects on the fetal development, chance of twin pregnancy or premature delivery (Guo et al. 2016). Cumulus removal using hyaluronidase is advantageous causing little stress but increases number of steps in the removal process.

Removal of zona pellucida is critical for in vitro fertilization. Conventional method involves acidic lysis media (Tyrode's media) to degrade zona pellucida. Embryos are subjected to chemical treatment until degradation of zona pellucida is clearly visible and after degradation it was immediately pipetted out from acidic media to a freshly holding media. This harsh exposure of acidic lysis media partially contributes to low efficiency of embryo. Zeringue et al. (2005) developed a microfluidic device that will minimize the sheer stress on the embryo and precise control of the lysing media during removal of zona pellucida. Moreover lysis of zona pellucida is achieved by gentle flow in less time compared to conventional lysis

methods (static media). Using microfluidic platform chimeric embryos were produced with clear visual access during its development. Spiked magnetically driven micro tool was developed to remove the swine zona pellucida by Yamanishi et al. (2010). This microfluidic system fabricated with PDMS mixed with nano magnetic material guided magnetically to peel off the zona pellucida. Same group successfully demonstrated the removal of ZP on 3D microfluidic system.

### 4.2.2 Microfluidics for Sperm Selection and Isolation

Microfluidics application efficiently improves each step of IVF. Starting from the selection of gametes to development of embryo microfluidics involved in every step of IVF. Recent advances in ART were going against the natural selection. Hence selection step is crucial for successful IVF. Either IVF or ICSI, selection of method is based on the characteristics of sperm (morphology, viability, motility, volume) (Thapa and Heo 2019). Preparation of sperm in farm animals includes washing of sperm, swim-up technique, and density gradient centrifugation (Natali 2011). However centrifugation removes unwanted wastes that interrupt fertilization and used to collect the high motility sperm but it damages the DNA of sperm (Malvezzi et al. 2014). DNA integrity was retained in swim-up method but it is influenced by reactive oxygen species vice versa in case of density gradient centrifugation (Zini et al. 2000). Low pregnancy rates were due to damage in sperm DNA (Loft et al. 2003). Embryologist selects the embryo based on the motility and morphology only during ICSI. DNA integrity of sperm cannot be accessed at that time which further has risk to use it for fertilization with oocyte (Osman et al. 2015). Moreover centrifugation and swim-up processes are time consuming. Therefore paradigm shift is needed in methodology to minimize the DNA fragmentation (Benchaiib et al. 2003). To develop protocol/device to avoid centrifugation step several attempts were made in this field but nothing proves efficient (Lih et al. 1996; Wang 1995; Henkel 2012).

Shirota et al. (2016) compared centrifugation and swim-up procedures and evaluated the efficiency of microfluidic device for achieving minimal DNA damage. They used cycloolefin polymer based microfluidic device, i.e. microfluidic sperm sorter (MFSS) to isolate the sperm. As already mentioned centrifugation and swim-up process were time consuming, microfluidic sperm sorter (MFSS) reduces the semen processing time. Centrifugation step is bypassed in this device. Semen processing using MFSS has reduced the processing time and also bypasses the centrifugation step (Hughes et al. 1998). Removing centrifugation step reduces the chance of exposure to ROS and DNA integrity was protected (Mortimer 1991). Hence they proved that semen processing using MFSS is more reliable and efficient than swim-up and centrifugation procedures. Nagata et al. (2018) designed a diffuser type microfluidic sperm sorter to isolate the high motile bovine sperm which also retains high DNA integrity.

Nosrati et al. (2014) recently developed a one step isolation of quality sperm from raw semen. Microfluidic device they developed consists of 500 parallel microchannels which isolate a sub population of sperms from heterogeneous

semen sample. This device offers a simple and quality sperm isolation procedure with high DNA integrity. Selecting one ml of semen is possible in not more than 20 min based on the motility of sperm. Excessive concentration of sperm in culture media increases chances of polyspermy. Microfluidics allows to overcome this in vitro problem by successful fertilization in vitro at low concentration. Interaction between sperm and oocyte increased in microchannels in comparison with the static systems of IVF. Low concentration of sperm, i.e.  $0.01\text{--}0.08 \times 10^6$  sperm/cell is sufficient for the successful fertilization compared to the traditional method. Microchannel environment mimics the *in vivo* conditions for fertilization. The potential increase in the interaction between oocyte and sperm is due to effective environmental conditions provided in microchannels (Suh et al. 2006). At the time of fertilization only few hundreds of sperms reach ampulla (Settlage et al. 1973). Interaction in the microfluidic channels with decreased sperm concentration resembles more similar to natural conditions for fertilization. Furthermore the use of traditional physical pipetting is minimum in microfluidic systems which minimize turbulence to gamete by allowing fresh media all through the incubation (Beebe et al. 2002a, 2002b). The IVF in porcine commonly results in polyspermy. Lack of optimized conditions for culture results in the low efficiency of IVF in porcine (Dang-Nguyen et al. 2011).

Clark et al. (2005) conducted experiments to optimize the conditions to reduce polyspermy and to increase viable embryos. PDMS based microchannel was designed which moderately resembles the environment conditions of oviduct. Constricted regions were designed to visualize the oocyte and embryo development in this microfluidic system. Microchannels were also used to select sperm in this microfluidic system. Oocytes were covered all sides by microchannel walls which mimic the oviduct. Normally at fertilization site (ampulla-isthmus junction) oocyte retained (parked), isthmus narrowing directs the sperm to the fertilization site. Similar conditions were copied in microchannels where oocyte parks at constriction region of microchannels and sperm guided towards parked oocyte through microchannels. Unlike in microdrop system the sperm concentrations around oocyte were dissimilar in microchannels. The rate of monospermic sperm fertilization increased in microchannels whereas it was low in microdrop system.

Sano et al. (2010) compared the standard static system with microfluidic system and observed that the oocyte developmental competence is double in case of microfluidic system. They also observed the significant increase in the monospermic fertilization in porcine under the microfluidic platform. In female genital tract the sperm is directed by three mechanisms which were known so far, namely rheotaxis, chemotaxis, and thermotaxis (Oseguera-López et al. 2019). Based on the rheotaxis motile sperm was separated. They designed a microfluidic device which separates sperm passively with minimal disturbances from the in vitro environment (Zaferani et al. 2018). In a previous study, Ma et al. (2011) developed a microfluidic system which integrates every step of IVF, i.e. from oocyte manipulation to embryo culture on a single chip. He showed that by associating microfluidics, IVF-on a chip is possible. This device contains four pools for sperm all connected to a octocolumn

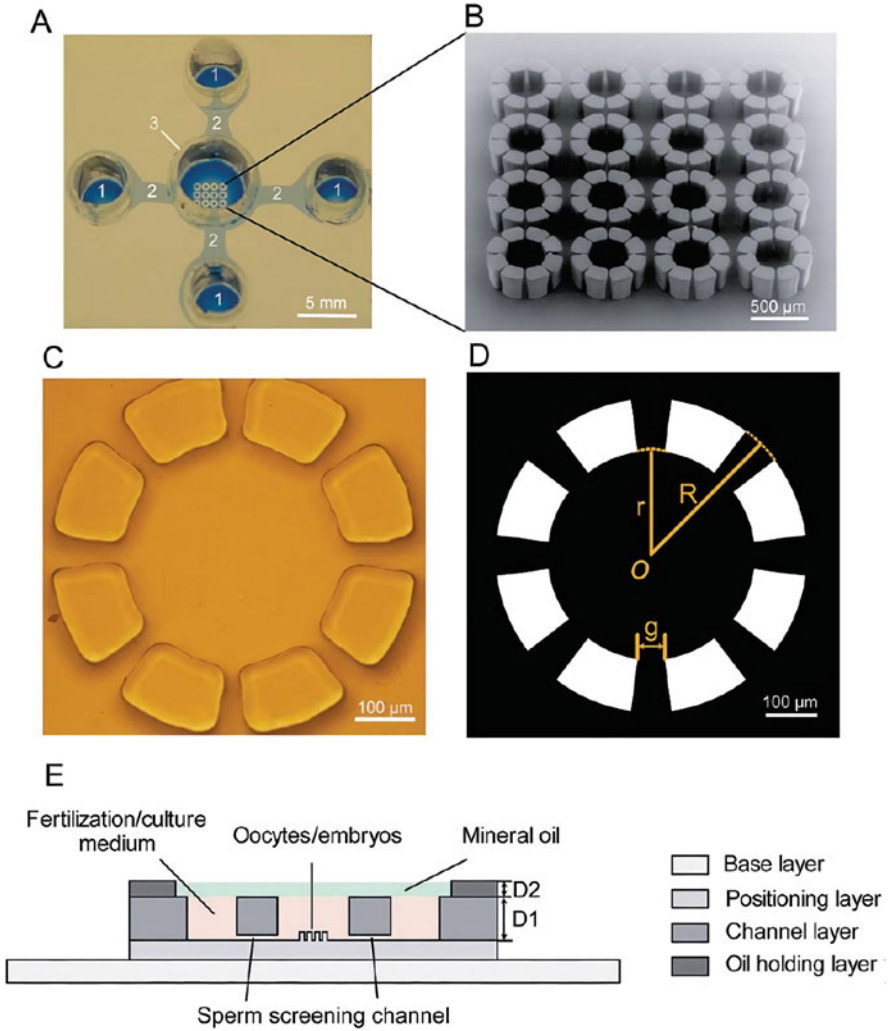


**Fig. 4.1** (a) Microfluidic device containing interconnected three individual sections. 1, 2, 3 represent sperm inlet pool, screening channel, and octacolumn unit, respectively. (b) scanning electron microscopic image of octacolumn (c) Octacolumn under optical microscope (d) structure of octacolumn with representing 200 μm inner radius ( $r$ ), 300 μm outer radius ( $R$ ), and gap ( $g$ ) of 50 μm. (e) Cross section of microfluidic device. This Figure is reproduced with the permission from Ma et al. (2011)

units where oocyte resides. Sperm swims along the centrally screening channel to reach the oocyte placed in the octocolumn (Fig. 4.1).

### 4.2.3 Microfluidics for Intracytoplasmic Sperm Injection (ICSI)

IVF was not successful in all farm animals. Mostly unsuccessful IVF is due to the sperm inability to fertilize oocyte (Bhattacharya et al. 2013). ICSI can be another choice after in vitro fertilization. The IVF is preferred over ICSI in sheep, cattle, etc., due to low rates of blastocyst development and unsuccessful pronuclei formation. However in horse, ICSI is preferred over IVF due to limited availability of ovaries and technical problems in procedures (Galli et al. 2007). Protocols for in vitro embryo production in horse include ovum pick up then ICSI followed by the embryo transfer non-surgically. ICSI was commercialized in horses as it was producing viable embryos consistently (Salamone and Canel 2014). In pigs, more chances of polyspermia in IVF so ICSI can be used as a alternative for fertilization (Coy and Romar 2002). ICSI emerged as an important tool for genetic manipulation after the first transgenic piglet birth (García-Vázquez et al. 2010). Male infertility problems were overcome by ICSI (Van Steirteghem et al. 1993). Pregnancy rates were low (less than 20%) in ICSI but fertilization rate is higher when compared with IVF, and ICSI is a complex process that needs a expertise hand. After maturation a single sperm is injected into the mature oocyte for fertilization in ICSI (Rosen et al. 2006). Risk of oocyte lysis during injecting sperm is quite possible. Hence it is a delicate process, skilled person needed to handle and inject the sperm into oocyte. Oocyte quality (oocyte degeneration) depends up on the operator skills during ICSI procedure (Wilding et al. 2010; Dumoulin et al. 2001). Recent advances in the microfluidic devices are useful in ICSI procedure to some extent. Matsuura et al. (2013) developed a microfluidic device with an objective to reduce the time involved in the ICSI. Comparison between the treatment time for ICSI in conventional microdrop method and microfluidic device clearly shows that microfluidic devices reduce the treatment time. In low quality semen samples, they increased the semen concentration for successful ICSI on microfluidic device. At porcine sperm quantity of  $2 \times 10^6$  cells /mL, ICSI treatment time was significantly reduced on microfluidic device when compared with the conventional microdrop method. Hence ICSI microfluidic method can be a useful approach when the semen sample is of low quality.

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## 4.3 Embryo Culture

Last decade witnessed the exponential increase in the use of assisted reproductive technology for mammalian embryo production. ART's playing a crucial role in solving infertility in humans and domestic animals it is used to produce genetically superior animals. In general, mammalian embryos are grown in vitro in comparatively large amounts of a medium compared to sub-microliters of fluid in vivo in the lumen of the female reproductive tract. In vitro conditions are different from in vivo. Embryo cultured in vitro exposed to physical and chemical stress. A modification in the culture media greatly improves the quality of in vitro produced embryos. However the viability and cleavage rates of in vitro embryo are still low.

Embryos are usually transferred manually, using the pipette, between several separate medium droplets before ultimate transfer to an appropriate recipient, during *in vitro* culture. The *in vitro* embryo production (IVP) procedure embryos undergo through 20 washes and culture drops. Every manual transfer puts the embryos under stress, subjecting them to rapidly changing physical and chemical conditions such as changes in medium composition, volume, pH, osmolarity, and temperature. Microfluidics can overcome the problems involved in the conventional method embryo culture *in vitro*. Decrease in the size of the sample, reaction time, and minimizing the volume of culture media are advantages of microfluidic systems. (Hofmann and Wagner 1997; Beebe et al. 2002a, 2002b). Microfluidic methods decrease the manual handling and creates minimum turbulence to embryo during cumulus cells removal. Furthermore, these methods offer constant influx of fresh media which mimics *in vivo* conditions, and ultimately reduces the physical stress and increases quality of embryo.

Using microfluidic technology, microchannels can help reduce the tension on the embryo. Moreover, the channel walls limit the transport of paracrine and autocrine factors formed by the embryonic cells, leading to a higher aggregation of these factors in the microchannel region around the embryo than in the standard microdrops. Microchannels effectively orchestrate the microenvironment around embryo then standard microdrop technique. Developmental rates of the embryo are significantly improved on the PDMS/borosilicate/silicon microchannels. Embryo degeneration also reduced in the microfluidic system. (Raty et al. 2004). Embryo was observed with solid walls on its all sides during culture in microfluidic system, whereas no walls observed in microdrop except bottom solid glass (Beebe et al. 2002a, 2002b). Microfluidic devices attempt to recreate natural environment *in vitro* for pre-implanted embryo. The rate of blastocyst development was predicted by new efficient biomarker, i.e. embryo mechanical properties. (Yanez et al. 2016). Whether the development of embryo influenced by flowing fluid to answer this question Heo et al. (2010) developed a dynamic micro-funnel culture system which improves the developmental kinetics of embryo. The inactive traditional static surfaces do not provide physical environment conditions like in fallopian tube. So the attempt made to optimize the flow rates to stimulate embryo. The dynamic micro-funnel system mimics the biochemical and fluid mechanics for embryo. It is evident from their work that this microfluidic system enhances the hatching blastocyst percentage. Implantation of embryo in bovines was significantly improvised with this system (Kim et al. 2009). Average number of cells per blastocyst also significantly improved in this system. This microfluidic system has upper hand over static conventional systems. Moreover the number of blastocyst developed *in vitro* on micro-funnel pulsatile system has same number as the *in vivo* developed blastocyst number (Hara et al. 2013). This user friendly microfluidic system achieves genetic gain by overcoming most of the embryo development and pregnancy problems in bovines. Embryo development also influenced by the autocrine factors. Due to autocrine factors the development of embryo improved when it was cultured in group in small volumes.

Toxic material can accumulate in static media (microdrops) during embryo culture and is harmful for the culture. The controlled pulsatile flow of media

periodically can overcome the toxic accumulation problem. This micro-funnel system refreshes the culture with fresh media periodically. Continuous fluid stimulation around embryo allows gaseous exchange, no excessive accumulation of biomolecules in media. Exogenous growth factors addition can cause some aberrations (e.g. large offspring syndrome). Although this system cannot completely mimic the *in vivo* conditions but the embryo development was optimal. Retaining biomolecules was maximum in the micro-funnel system when compared with microdrop system. Mechanically stimulated embryos showed increased development blastocyst. Hara et al. (2013) recreated fallopian tube conditions *in vitro* allowing embryos to experience friction force, compression, and shear stress. Micro vibration increases blastocyst formation, pregnancy rate, and maturation rate in pig (Hur et al. 2013). Membrane based microfluidic system developed by Bae et al. (2011) demonstrated the stimulation time and amplitude of pressure were also crucial for embryo culture. They also observed accurate compressive strain required for embryo development. Membrane based microfluidic system accurately stimulates the embryo *in vitro*.

Initially, Willadsen (1979) reported that microenvironment influences the embryo development. Microfluidics has the capacity to automate the embryo culture procedure which minimizes the human handling and it was first integrated in mice embryo culture by Raty et al. (2004). PDMS and silicon based microchannels were fabricated to culture the embryo and found the embryos cultured in microchannels showed efficient developmental growth rate when compared with conventional culture dish embryo culture. They identified cleavage rates were faster in microfluidic systems. After 48 h the embryos cultured in microchannels acquired blastocyst stage whereas it was still sixteen cell morula stage in conventional microdrop system.

Recent investigations suggest the need for optimizing the *in vitro* environmental conditions for embryo culture (Gruber and Klein 2011). Conventional group embryo culture restricts the probing into single embryo culture. Restricted surface area and lesser media can enhance the embryo development (Thouas et al. 2003) which made researchers to investigate many culture systems. A microwell culture system (PDMS) designed by Chung and co supports the time lapse visualization of developing embryo and selection of embryo for implantation. They observed that blastocyst implantation rate was 70% and all were alive and healthy (Fig. 4.2). Enhancing the viable embryos was challenging in embryo cultures. This can be more critical in case of pig. Optimizing the environmental conditions was necessary for increasing the viable embryos before implantation. Microchannels in microfluidic system were designed to enhance the embryo development. Polyspermy condition is significantly reduced in microchannel than in conventional microdrop system (Clark et al. 2005). Double developmental rates of embryo to blastocyst were observed on microfluidic sperm sorter (MFSS) (Sano et al. 2010). They concluded that concentration of capacitated sperm and denuded oocytes number and its location greatly influence the IVF rates in MFSS system. The rates of embryo and blastocyst development

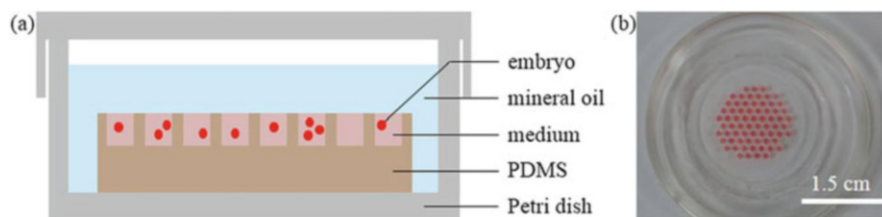


**Fig. 4.2** (a) Cross sectional image of microwell device used to culture single embryo. Sufficient culture media was supplied in each well and mineral oil was added to avoid the evaporation. (b) Top view of the microfluidic device containing 70 microwells. The figure is reproduced with the permission from Chung et al. (2015)

increased in the MFSS chambers produced from monospermic fertilization in porcine after brief co-culture of oocyte with sperm. In a previous study, Huang et al. (2015), developed a novel electrowetting on a dielectric (EWOD) based microfluidic system. In *in vivo* conditions contraction and ciliary actions move the developing embryo from oviduct to uterus. EWOD enhances the movement of embryo present in the droplet and the droplet moves from one electrode to other electrode like it happens *in vivo*. In contrast to the conventional method EWOD offers feasible, accurate regulation of droplet, lowers the contamination chances, fast evaluation. This system is a modified microdrop method where minimum voltage is applied to manipulate the droplet in which the embryo is cultured (Fig. 4.3).

Morphology was the only characteristic marker used to select the embryo pre-implantation. As expected, desirable results were not achieved with this probe into other criteria was investigated widely. Substantial changes occur in the embryo metabolism during developmental stages *in vivo*. Glucose plays a critical role in embryo development suggesting that it can be used to select the efficient embryo for implantation (Renard et al. 1980 ;Gardner et al. 2001) Studies found that embryo viability affected by increased glycolytic pathway (Lane and Gardner 1996). Alteration in the metabolisms from normal is detrimental to embryo development. Hence the quantification of nutrients was required to select the best grade embryo. Removing all the challenges for quantifying embryonic metabolism, Urbanski et al. (2008) designed a microfluidic device which acts as a non-invasive, standalone device to address the metabolism of a single embryo culture. This microfluidic system works automatically to aliquot the enzymes and mixe the reagents. Furthermore, these devices collect and analyze the data with less human interruptions. Metabolism of an embryo can be addressed by analyzing the media used for its culture. This system measures the energetic substrates from the pre-implantation embryo (Fig. 4.4). Determining the developmental stages and quality of embryo during its growth is tough for an embryologist. Microfluidic methods have attracted the researchers to stretch its application in the field of metabolic measurements. Consumption of oxygen by embryo is a early parameter to check the embryo quality (Houghton et al. 1996). Correlation was established between consumption rates of oxygen and

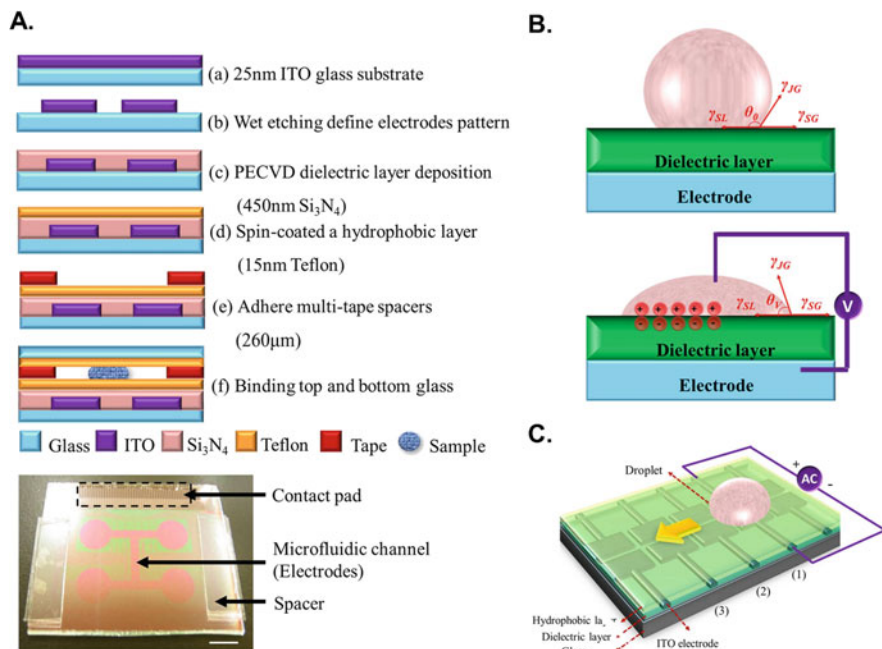


**Fig. 4.3** (a) Fabrication of microfluidic device consisting of a ground and 5 electrodes mounted on same plane with a gap of 20  $\mu\text{m}$  between each electrode. (b) Contact angle changes on application of voltage. (c) Movement of the droplet when EWOD was ON. This figure is reproduced with the permission from Huang et al. (2015)

embryo development by using scanning electrochemical microscope (Shiku et al. 2004). Microfluidic devices were employed to measure the embryo oxygen consumption during the developmental stages. Wu et al. (2007) constructed a microfluidic chip with an automated detector to measure the oxygen consumption rate of a single embryo during its development (Fig. 4.5). They used 10  $\mu\text{L}/\text{min}$  flow rates with an amperometric detector to access the embryo oxygen consumption of single embryo.

## 4.4 Conclusions

Success of traditional ART methods relies upon the skill of the personnel involved in the in vitro fertilization and embryo culture steps. Automation of these steps in miniaturized devices greatly reduces the failures in ART. The field of microfluidics-based ART needs an interdisciplinary approach and development of microfluidic methods requires involvement of at least an engineer and a biologist. In vitro fertilization and embryo culture will be more successful if these steps are mimicked in vivo like conditions such as continuous nutrient supplements and waste removal.

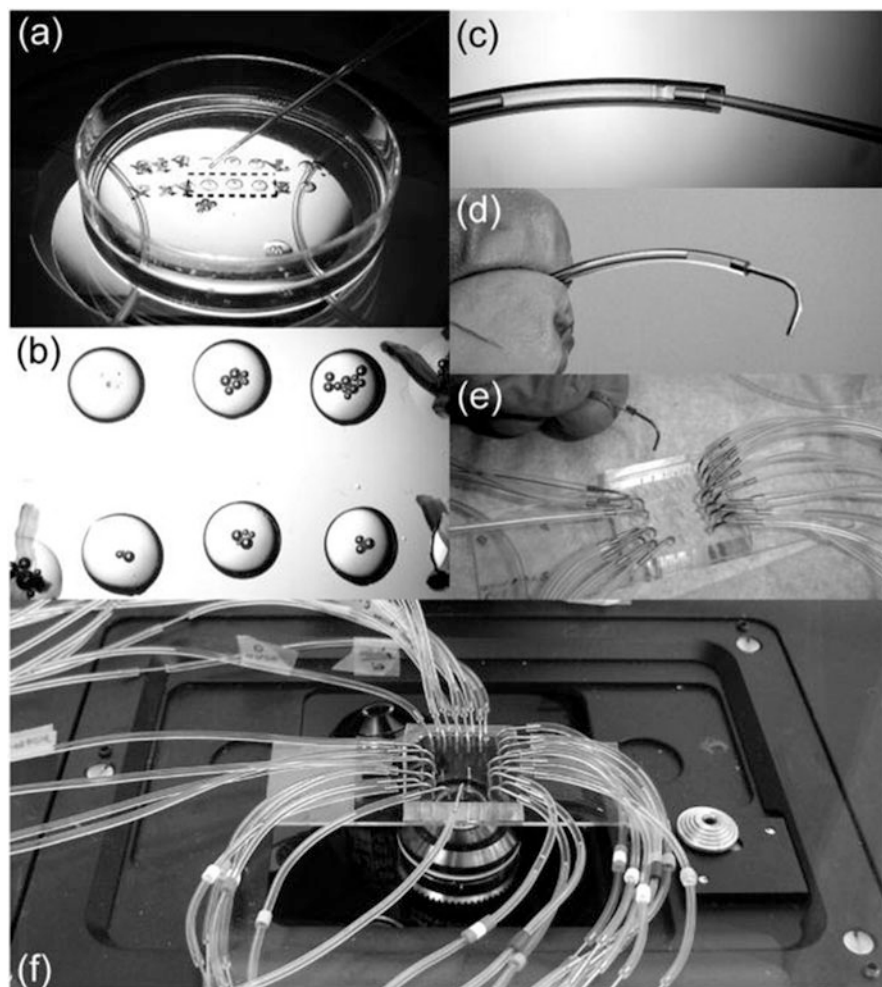


**Fig. 4.4** (a) and (b) showing the oil droplets used to cover the collected embryo cultured media samples on a Petridish (c) Narrow gel loading tips to avoid the flow of mineral oil into the circuit. (d) Before adding a sterilized pin a cut was made at the end to prevent the mineral oil flow. (e) Preparation of the interface (f) Non-invasive measurement of embryo metabolism under epifluorescent microscope. This figure is reproduced with the permission from Urbanski et al. (2008)

Microfluidics offers monitoring the growth of reproductive cells and selection of competent reproductive cells such as spermatozoa and embryos. Microfluidic methods have potential to mold as per the ART requirements. Substantial research in this field would help to realize the dream of in vitro production of embryos in a single microfluidic device.

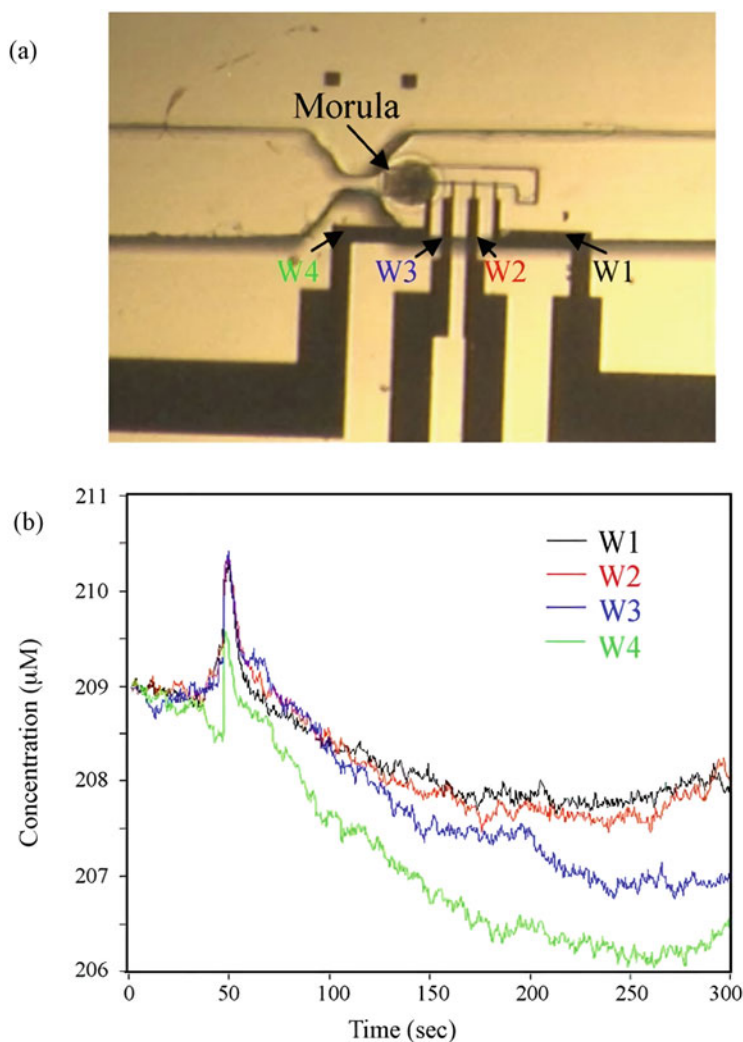


**Fig. 4.5** (a) Microfluidic device to immobilize embryo safely with limited gate size and W1, W2, W3, W4 were amperometric detector array electrodes to access the oxygen consumption of morula stage embryo. (b) Plot determining the oxygen consumption rates of 6th day morula stage embryo at different electrodes. This figure is reproduced with the permission from Wu et al. (2007)

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# Microfluidics in Cryopreservation of Animal Gametes and Embryos

# 5

## Abstract

Cryopreservation technology has revolutionized the field of reproduction and reproductive technologies. Cryopreservation enables to preserve gametes for long periods and allow carrying gametes over long distances. Gametes can be cryopreserved and used when a suitable donor or recipient is present. Preservation of fertility is now feasible with embryo preservation. High-quality embryos can be cryopreserved at  $-196^{\circ}\text{C}$  in liquid nitrogen and can be transferred at the required time in female uterus. The in vitro fertilization (IVF) technique provides an opportunity to transfer high-quality embryos to female uterus without extra IVF cycle which reduces the cost of IVF at infertility centers.

Cryopreservation is now seen as a treatment for infertility in male and female animals. This review provides the history and recent developments in the field of cryopreservation with focus on microfluidics-based cryopreservation of animal gametes and embryos. This chapter also includes the discussion on available cryopreservation techniques and external factors affecting the efficiency of cryopreservation. This review would help to design and develop novel microfluidic devices for cryopreservation.

## Keywords

Cryopreservation · Cryoprotectants · Slow freezing · Vitrification · Thawing · Oocyte · Embryo

## Abbreviations

|      |                        |
|------|------------------------|
| CPAs | Cryoprotectants        |
| DMSO | Dimethylsulphoxide     |
| IVF  | In vitro fertilization |

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|         |                                |
|---------|--------------------------------|
| NP-CPAs | Non permeating cryoprotectants |
| P-CPAs  | Permeating cryoprotectants     |
| PDMS    | Polydimethylsiloxane           |
| PROH    | 1,2 propanediol                |

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## 5.1 Introduction

Cryopreservation is a milestone in reproductive technology, and cryopreservation techniques have been employed for long-term storage of spermatozoa embryos and oocytes over three decades (Sherman 1973). Cryopreservation enables the later use of gametes and embryos in assisted reproductive technology. Nowadays, donor sperm/egg bank establishments are minimizing medical risks related to fertility and patient's synchronization increasing live birth rate (Macaldowie et al. 2012). Long-term preservation of reproductive cells by maintaining the viability after long-term storage has tremendous animal and human reproductive applications (Woods et al. 2004; Gosden 2005). The cells and tissues can be cryopreserved by cooling to extremely low temperatures, such as  $-196^{\circ}\text{C}$  which halts all biological activities in a section including cell death. Cryopreservation of embryos has greatly improved in vitro fertilization (IVF). Some theories on cryopreservation indicated that there could be high success rate in embryo transfer with cryopreserved embryos. The activation of repair mechanisms may be initiated after freezing and thawing of embryos in pre-implantation (Vladimirov et al. 2017). Thawing process imposes low levels of stress, which leads to hormesis. This low controlled level of stress could allow mitochondrial damage to be repaired and protein to be properly folded.

In cryopreservation, allowing the cells and tissues to sub-zero temperatures is performed to prevent biological activities that help in long-term storage. The cells, gametes, and embryos lack established internal defense mechanisms to cope chilling, ice formation. The main problem is that the scientists are still tackling to find a perfect recipe which ensures cell survival and also maintains physiological functions necessary for cells to be alive.

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## 5.2 Cryoprotectants

Cryoprotectants are the chemicals employed in cryopreservation to shun ice formation and to provide strength to cells from freeze shock. The use of cryoprotectants (CPAs) in cryopreservation helps to shield the cells from cryoinjuries such as ice crystal formation and concentrated chemicals (Mazur 1963; Kleinhans and Mazur 2007). The CPAs also stabilize the intracellular structures and membranes. CPAs are categorized as permeating (P-CPAs) or nonpermeating (NP-CPAs) based on ability to enter inside the cells. The glycerol, ethylene glycol, dimethylsulphoxide (DMSO), 1,2 propanediol (PROH) are some of the important CPAs being used as P-CPAs.

Sugars such as sucrose and glucose are commonly used NP-CPAs in cryopreservation. The determination of optimum levels of CPAs is an important step to reduce the side effects such as cytotoxicity (Jain and Paulson 2006). Trehalose has been used as NP-CPA due to its high glass transition temperature (Eroglu et al. 2003). Polymers such as polyethylene glycol, polyvinylpyrrolidone, ficoll, and polyvinyl alcohol are being used in cryopreservation to save the zona pellucida against mechanical injuries (Lane et al. 1999; Zech et al. 2005). Antioxidants should be added in the cryopreservation to prevent the oxidative damage of reproductive cells during cryopreservation (Pangestu 1996). In a previous study, it is evident that the use of antioxidants in culture improves life of blastocysts after cryopreservation (Nedambale et al. 2006).

### 5.3 Microfluidic Methods of Cryopreservation

In early studies, an individual cell-based cryochip was demonstrated for cryopreservation and functional activity of cryopreserved cells. The device consists of a cell chamber with pico wells, the plastic body, the coverslip, open conduit, and waste accumulation area (Fig. 5.1). In this study, freezing and thawing effects of individual cells observed in a microfluidic device and 97% of cells were found in pico wells after freezing and thawing experiments (Deutsch et al. 2010; Afrimzon et al. 2010).

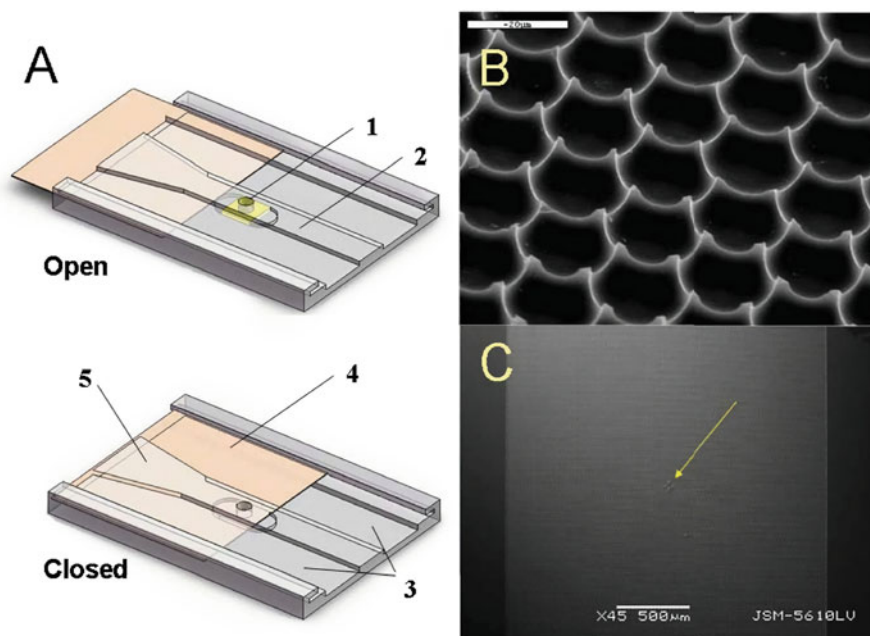


**Fig. 5.1** (a) Cell cryo-chip components. (1) Sample chamber, (2) flow conduit, (3) two engraved longitudinal grooves, (4) coverslip, (5) waste accumulation area. (b) and (c) are SEM images of the sample chamber. Reproduced with permission from Deutsch et al. (2010)

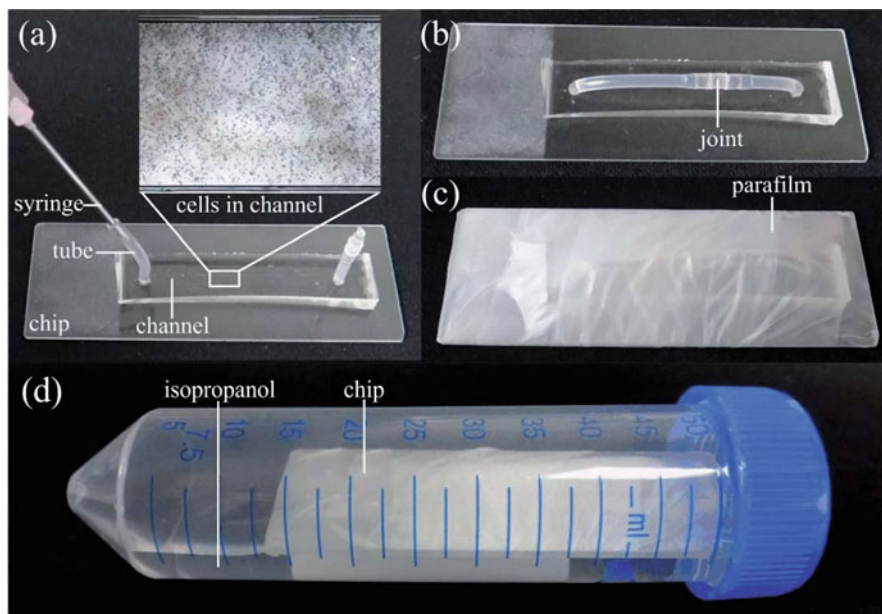


**Fig. 5.2** Images of the microfluidic chip and steps of packing (a) injection of cells into the microchannel, (b) plugging the inlet and outlet tubes, (c) packed chip by parafilm, (d) the packed chip in a 50 mL centrifuge tube. Reproduced with permission from Li et al. (2014)

In a study, direct freezing and thawing of mammalian cells were demonstrated on PDMS based microfluidic chip, and it offers the storage of cells up to months. The microfluidic chip consists of a microchannel with an inlet and an outlet. The tubes of inlet and outlet can be connected to close the channel while storage (Fig. 5.2). In a typical protocol, the cell suspension was injected into a channel through the inlet and tubes of the inlet and outlet were connected. The chip was packed in parafilm and placed in a 50 mL centrifuge tube that is filled with 25 mL of isopropyl alcohol. The centrifuge tube with a microfluidic chip is placed in -80°C refrigerator. The thawing of frozen microfluidic chip involves the centrifugal removal tube from refrigerator and replacement of isopropyl alcohol with 37 °C sterile water. Chip was removed from the centrifugal tube and parafilm was also removed from the chip. The chip was incubated at 37 °C, 5% CO<sub>2</sub> incubator after unplugging the inlet and outlet joint (Li et al. 2014). In a previous study, a microfluidic device was demonstrated for biological cell cryopreservation. This device consists of a microchamber, fluidic channels, inlet and outlet ports, and microheater with an insulation layer. Results showed 74% of yeast cell survival in this method, and it was suggested for cryopreservation of sperm and embryo applications (Li et al. 2010). In a study, a microfluidic device was fabricated with polydimethylsiloxane (PDMS) for CPA loading and unloading during cell cryopreservation. This device consists of a flow focus region, a serpentine channel, and a parallel channel. The focus region consists of two inlets, one is for loading the cells, and another is for loading CPA. The serpentine channel

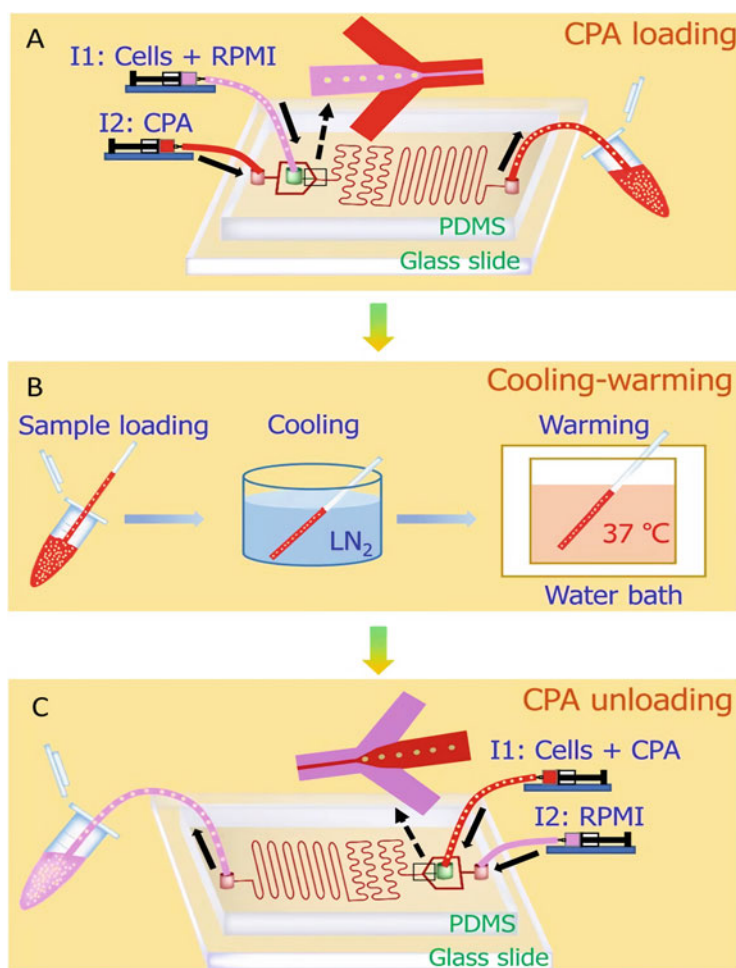


**Fig. 5.3** Schematic diagram of (a) CPA loading, (b) ultra-rapid cooling-warming, (c) CPA unloading. Reproduced with permission from Zheng et al. (2018)

was intended for mixing, and the parallel channel was designed for equilibration (Fig. 5.3). This method improved cell viability and less mechanical damage after CPA loading and unloading (Zheng et al. 2018). In a previous study by Song et al. (2009), similar results were obtained by using a microfluidic device that consists of a long microfluidic microchannel with three inputs and one outlet. Cryopreservation of cells in this method consists of CPA loading step, freezing and thawing step, and CPA unloading step (Fig. 5.4).

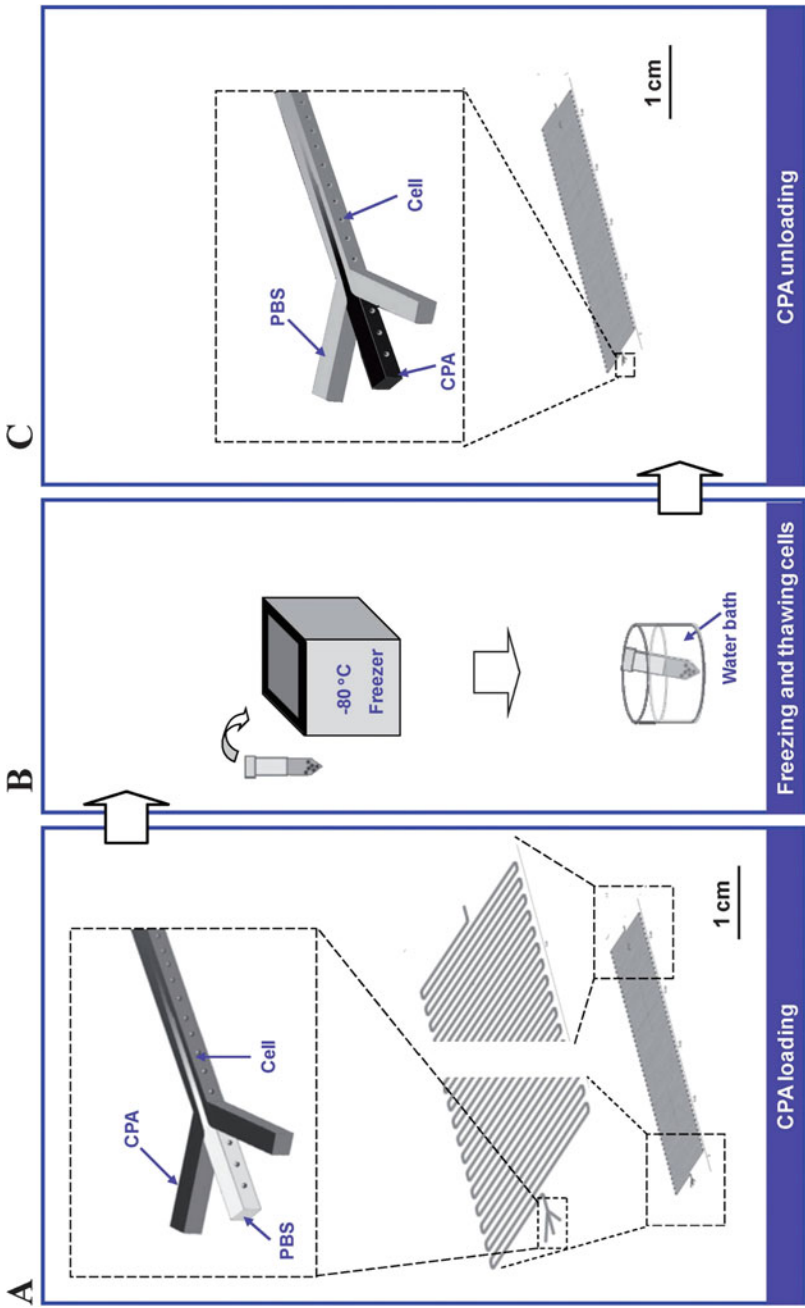


**Fig. 5.4** Schematic diagram depicting the steps involved in cryopreservation of cells by using microfluidic device. (a) CPA loading; (b) freezing and thawing; (c) CPA unloading. Reproduced with permission from Song et al. (2009)

## 5.4 Animal Oocyte, Embryo, and Sperm Cryopreservation

The oocytes can be preserved for long term by cryopreservation and the cryopreserved oocytes can be utilized in case of female death or future infertility (Ledda et al. 2001; Checura and Seidel Jr 2007). Embryo banks help to conserve the endangered species and superior genetic traits by the cryopreservation of embryos (Gama 2006; Woelders et al. 2006; Andrabi and Maxwell 2007). Freezing temperatures and external agents added in the cryopreservation exhibited the harmful effects to oocytes and embryo in several studies (Rojas et al. 2004; Succu et al. 2007). The unsystematic microtubules, disorganized chromosomes, polyspermy, alteration in the zona pellucida are some of the side effects observed in cryopreservation of oocytes and embryo (Mavrides and Morroll 2005; Morató et al. 2008). Meiotic tubule disorganization was observed after the cryopreservation of the cells by vitrification method (Pereira et al. 2008). Cytoskeletal relaxants such as cytochalasin B have been employed to reduce the stiffness of the cells (Silvestre et al. 2006). Lipid content is another factor that affects the cryopreservation of cells. Lipid content in the oocytes and embryo is directly related to the cryotolerances. Higher lipid content resulted in more cell damage in the oocyte and embryo cryopreservation (Zeron et al. 2001; McEvoy et al. 2001). The bovine oocytes are reported to be more tolerant than porcine oocytes due to low lipid content (Niemann and Rath 2001; Genicot et al. 2005). The previous studies on delipidation of the cells resulted in more cryotolerance and the exact mechanism needs to be studied to develop novel method of cryopreservation (Nagashima et al. 1999).

Cryopreservation has emerged as an effective way to treat male infertility since the first human sperm cryopreservation (Sherman 1973). Sperm cryopreserved by use of nitrogen vapors and successful artificial insemination and birth of healthy offspring has led to the use of nitrogen vapors in sperm cryobanks (Sherman 1990). Permeating CPAs such as glycerol, DMSO, ethylene glycol, PROH are able to alter the spermatozoa acrosomal membrane. These CPAs also cause cytotoxic effects on cryopreserved cells. The NP-CPAs such as polymers and sugars act as osmotic buffers and these can reduce the side effects of CPAs (Sieme et al. 2016). Addition of semen extenders such as egg yolk in semen samples improves the viability of spermatozoa and preserves the fertilization competency of cryopreserved samples (Jeyendran et al. 1995; Varisli et al. 2013). Semen sample preparations before the cryopreservation such as washing, density gradient centrifugation, and swim-up methods help the isolation of dead cells cell debris from the semen samples. These steps help in the long-term preservation of cells in cryopreservation (Henkel and Schill 2003; Opuwari and Henkel 2016). Glass ampoules, cryovials, and plastic vials have been used as physical carriers for sperm freezing (Mortimer 2004).

## 5.5 Cryopreservation Protocols

The slow freezing, vitrification, and rapid freezing are the three methods that have been used to cryopreserve oocytes and embryos at various developmental stages. Cryoprotectants concentrations vary in these methods. These methods are used according to the type of specimen and conditions suitable for preserving the specimen. The existing cryopreservation protocols are summarized in Table 5.1.

### 5.5.1 Slow Freezing

Slow freezing in cryopreservation refers to the programmable method of freezing and gradual cell dehydration, with small concentrations of CPAs and slow cooling rates (Edgar and Gook 2012). Slow freezing offers reduced cytotoxicity over other methods due to low CPA concentrations. Slow freezing process involves the equilibration of cells with CPAs, slow cooling at the rate of  $-2^{\circ}\text{C}/\text{min}$  until it reaches  $-6^{\circ}\text{C}/-8^{\circ}\text{C}$ , rapid cooling at the rate of  $50^{\circ}\text{C}/\text{min}$  until it reaches to  $-150^{\circ}\text{C}$  and storage in liquid nitrogen. The DMSO and PROH are the commonly used CPAs in the slow freezing process and the use of a low sodium medium was also preferred in the slow freezing process. But optimum protocol is still not developed, especially in case of slow oocyte freezing, which has low survival rate, developmental potential, and clinical outcomes (Rienzi et al. 2017). Membrane damage, low motility, reduction in the membrane fluidity are some of the limitations in slow freezing of spermatozoa by slow freezing process (Centola et al. 1992; Critser et al. 1988; Holt 2000; Giraud et al. 2000).

### 5.5.2 Vitrification

Vitrification has been preferred over the slow freezing for cryopreservation of cells to overcome the problem of ice crystal formation. Slow freezing method is associated with the ice crystal formation which is the possible reason for more cell deaths. Vitrification involves the ultra-rapid cooling at the rate of  $>10,000^{\circ}\text{C}/\text{min}$  until it reaches  $-196^{\circ}\text{C}$ , and this method requires high concentrations of CPAs. Even though this method is preferred over the slow freezing method, the use of high concentrations of CPAs leads to cytotoxicity of the gametes and embryos. Replacement of CPAs in vitrification was demonstrated by Nawroth et al. (2002), and they observed improved motility after cryopreservation.

### 5.5.3 Rapid Freezing

This method is an ultra-rapid cooling procedure. In this approach, ultra-cooling temperature strategy is applied to cryopreserve the samples. In this approach, very low or no cryoprotectant is used as used is slow freezing or vitrification. Due to very

**Table 5.1** Summary and comparison of cryopreservation techniques

| S. No | Property                     | Slow freezing                                                                                                                                                                     | Vitrification                                                                                                                                    | Ultra-Rapid freezing                                                                                                                                                               |
|-------|------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1     | Principle                    | Cells are cooled slowly in steps under computer control to $-196$ degrees Celsius                                                                                                 | Vitrification is a method in which not only cells but also the whole solution is solidified without the crystallization of ice by rapid freezing | Cells/embryos/oocytes are directly submerged into liquid nitrogen after brief exposure to cryoprotectant                                                                           |
| 2     | Process                      | Specimen is allowed to freeze slowly at a slow cooling rate of $0.5-5^{\circ}\text{C}$ from $0^{\circ}\text{C}$ to $-100^{\circ}\text{C}$ and then transferred to liquid nitrogen | Specimen is rapidly cooled down from $37$ to $-196^{\circ}\text{C}$ in $<1$ s, resulting in extremely fast rates of cooling                      | Specimen is ultra-rapidly cooled by dipping directly into liquid nitrogen in a single step process                                                                                 |
| 3     | Cryoprotectant concentration | Low concentration of cryoprotectant is used to protect the cells/oocytes/embryos/sperm from damage caused due to slow freezing                                                    | High concentration of cryoprotectant is used to protect the cells/oocytes/embryos/sperm from damage caused due to rapid drop in temperature      | Relatively low concentration of cryoprotectant as compared to vitrification is used to protect the cells/oocytes/embryos/sperm from damage caused due to rapid drop in temperature |
| 4     | Sample volume                | Sample volume used is high, i.e. $100-250\ \mu\text{L}$                                                                                                                           | Sample volume used is low, i.e. $1-2\ \mu\text{L}$ due to vitrification devices used                                                             | Sample volume used is low, i.e. $1-2\ \mu\text{L}$                                                                                                                                 |
| 5     | Cost                         | Expensive as controlled slow freezing requires use of freezing machines in a controlled manner                                                                                    | Inexpensive as no control freezing is required                                                                                                   | Inexpensive as no control freezing is required                                                                                                                                     |
| 6     | Ice crystal formation        | Ice crystal formation is observed due to slow freezing and low concentration of cryoprotectants                                                                                   | Due to rapid cooling and with high concentration of cryoprotectants, amorphous glass like structure is formed which avoids ice crystal formation | Due to rapid cooling and with high concentration of cryoprotectants, ice crystal formation is minimized                                                                            |
| 7     | Injuries                     | Slow freezing could not effectively avoid ice crystal formation which leads to cell membrane damage and low survival rate                                                         | Rapid freezing effectively avoids ice crystal formation during freezing and increases the survival rate                                          | Less injuries due to less ice crystal formation and increased survival rate. Survival rate is less than vitrification and high than slow freezing                                  |

low or no use of cryoprotectants, cytotoxic effects of cryoprotectants can be eliminated in this approach. Despite its benefits, this approach is considered to be uncontrollable and not consistent (Vajta and Nagy 2006). Sherman (1990) proposed the rapid freezing method. In this procedure cryoprotectant solution is added to sperm sample in a straw, and then straw containing sperm and cryoprotectant solution is kept at 15–20 cm beyond the liquid nitrogen height for 15 min. Finally the straws containing cells were submerged in the liquid nitrogen at  $-196^{\circ}\text{C}$  for long-term storage (Sherman 1990).

### 5.5.4 Thawing

Thawing is also an important step affecting the success of cryopreservation. Nonpermeating cryoprotectants play the main role in thawing. They protect the cell against cell damage due to their osmotic buffer ability which protects the cell from induced swelling when CPAs are removed from the cell. The thawing temperature is usually  $37^{\circ}\text{C}$  and it may be at room temperature (Di Santo et al. 2011).

## 5.6 Microfluidic Methods of Oocyte, Embryo, and Sperm Cryopreservation

In a study, Heo et al. (2011) fabricated a microfluidic system consists of two inlets, mixing channel, oocyte analysis chamber, and an outlet channel. Oocyte analysis chamber was fabricated with oocyte loading channel and control valve. The oocyte trapping was managed by two shallow channels located on both sides of the oocyte analysis chamber with a height of  $30\text{ }\mu\text{m}$ , and it prevents the movement of the oocyte from the chamber (Fig. 5.5). This study demonstrated the loading of propanediol, a cryoprotectant, to oocyte by minimizing the volume changes in less

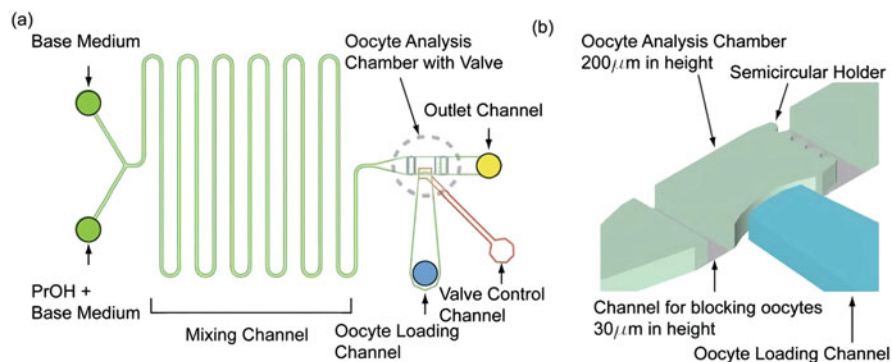


**Fig. 5.5** Microfluidic design for loading of cryoprotectants (CPAs) to the oocyte. Reproduced with permission from Heo et al. (2011)

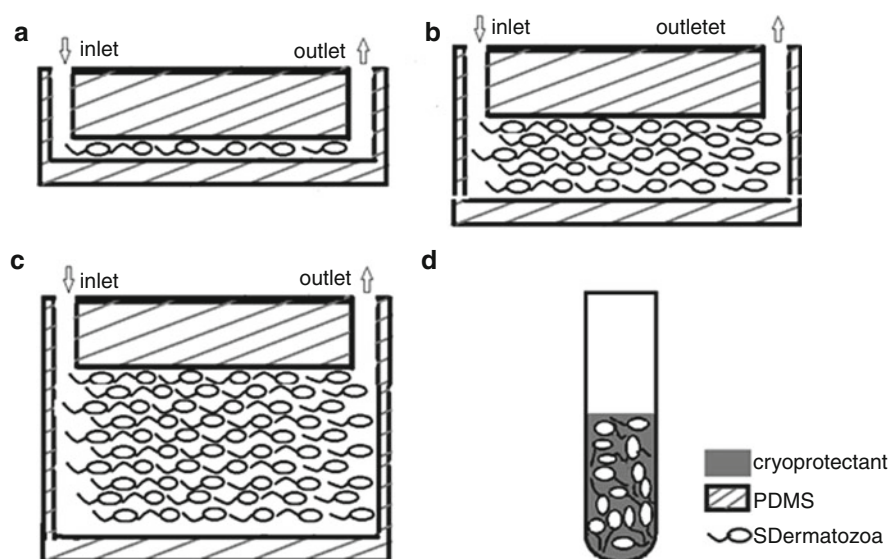


**Fig. 5.6** Diagram of spermatozoa cryopreserved in microchannel with height of (a) 10 mm; (b) 50 mm; (c) 100 mm. (d) Spermatozoa cryopreserved disorderly in the freezing tube. Reproduced with permission from Zou et al. (2013)

than 15 min. Minimization of cryoprotectant volume and loading time greatly reduces the toxicity to the cells. In another study, a similar type of microfluidic system was fabricated with some changes. It consists of two inlets, one serpentine mixer and an exposure chamber with loading or withdrawal port. Loading and release of cells from the microfluidic device were done with the help of suction port. This study demonstrated, cryoprotectant exposure through this microfluidic device resulted in lower shrinkage rate, smoother cell surface, higher cytoplasmic lipid retention, less cytoplasmic leakage, and improved developmental competence (Lai et al. 2015). Embryo vitrification reduces intracellular ice formation, and it leads to higher cell survival. A digital microfluidic device was demonstrated for processing mouse embryos for vitrification. This microfluidic device consists of an array of electrodes, embryo inlet/outlet, dispensing reservoir, waste reservoir, mixing/splitting channel. Digital microfluidic device has shown an automated generation of cryoprotectant gradient on the droplet containing the embryo. This device also demonstrated convenient loading and retrieval of embryos (Pyne et al. 2014). In a study, a microfluidic chip was developed for the cryopreservation of small volume of human spermatozoa without any cryoprotectant. This chip consists of spermatozoa inlet, spermatozoa store channel, and spermatozoa outlet (Fig. 5.6). Three chips were fabricated with a channel height of 10, 50, and 100  $\mu\text{m}$  and polydimethylsiloxane (PDMS) was used as a fabrication material. Microfluidic chips with 10  $\mu\text{m}$  showed higher viability than other two chips after direct plunging into liquid nitrogen. In this study, PDMS was also found to be compatible with liquid nitrogen storage (Zou et al. 2013).

## 5.7 Hurdles in Cryopreservation

Though cryopreservation technology has advanced considerably in the past years, there are still hurdles which hinder the success rate of sperm, oocyte, and embryo cryopreservation. The cryopreservation protocol causes harmful effects on sperm morphology and kinematics (Di Santo et al. 2011; Kopeika et al. 2015). Further studies are required to establish the effect of sperm cryopreservation and thawing on DNA integrity (Kopeika et al. 2015). Different freezing protocols have a different range of effects on DNA integrity. Spermatozoa of infertile men can have more chances of chromatin disorganization in cryopreservation and low resistance to denaturation (Donnelly et al. 2001). Studies performed on animal models showed that the use of spermatozoa with damaged DNA in fertilization could result in defects that may appear later in life (Fernández-Gonzalez et al. 2008). Longer-term studies should be performed on parameters that are not usually studied like tumor susceptibility, physiologic aging, and psychosocial health in adulthood (Di Santo et al. 2011). Stress related disorders in pregnancy and long gestational age risks are suspected in embryo freezing (Alviggi et al. 2018; Maheshwari et al. 2018). The vitrification method of cryopreservation of cells may also be associated with aberrant expression of some stress related genes (Bartolac et al. 2018). There are many cryopreservation and thawing protocols available these days both lab-specific and commercial but proper composition of CPAs/NP-CPAs, cryo-solutions is still unknown, which can be adopted universally. Cryopreservation protocols are not standardized universally which leads to varying results. There should be development of universal solution composition and protocol which can be adopted everywhere required with slight modifications (Parmegiani et al. 2018; Serdarogullari et al. 2019).

## 5.8 Conclusion

Cryopreservation is proved to be a big achievement in the field of reproductive technologies. Various cryopreservation techniques made it possible to achieve long-term preservation of gametes in a viable form. Multiple cryopreservation techniques can preserve genetic material preservation in endangered species. Various fertility clinics are using cryopreservation protocols for preserving gametes, embryos up to seventh day with significant positive results and getting better outcomes in IVF procedures. Despite development in cryopreservation, there is still a lack of proper standardization protocol, including thawing as it also affects the potential of gametes and embryo developmental capacity. Various laboratories have their protocols with less reproducibility in other labs. The efficiency with the same protocol varies when performed by different persons. There is a need for development of standardized cryopreservation protocol which can be adopted universally in all labs and fertility clinics for gametes and embryos. Gametes and embryos display significant viability during cryopreservation and fertilization and embryo development rates. But the optimum concentration of cryoprotectants is still to be elucidated for each gamete

type, and embryo as cytotoxicity of cryoprotectants also reduces the viability of gametes which affect fertilization potential and development of the transferred embryo in female after thawing. Microfluidics offers fast cryopreservation methods with less volume of animal gametes and embryo samples. Individual cell cryopreservation would be a reality with the development of a novel microfluidic device.

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# Sperm Sexing: Methods, Applications, and the Possible Role of Microfluidics

## 6

### Abstract

Controlling the sex of livestock offspring is a great interest of animal farmers for economic reasons. Sexed semen of desired animals has been in great demand over the years and commercially available FACS based method has some limitations such as high cost and sperm damage. Production of desired sex animals is the prime application of sperm sexing, and sexed semen has some other potential applications such as skewing sex ratio and conservation of endangered species.

Microfluidics is an emerging field of handling the fluids at the microscale, and its cell separation applications attracted sperm biologists to separate sperm cells. Successful attempts have been made to separate the sperm cells based on sperm characteristics such as motility, swimming behavior, and external sperm stimulants. This chapter discusses the differences in X and Y chromosome bearing spermatozoa with previous failed attempts to sperm sexing. Possible novel strategies for sperm sexing by using microfluidic methods are also discussed in this chapter.

### Keywords

Sperm sexing · Livestock · Microfluidics · Spermatozoa · Sexed semen

### Abbreviations

|      |                                        |
|------|----------------------------------------|
| AI   | Artificial insemination                |
| ALH  | Amplitude of lateral head displacement |
| BCF  | Beat-cross frequency                   |
| CASA | Computer-assisted sperm analysis       |
| DEP  | Dielectrophoresis                      |
| DNA  | Deoxyribonucleic acid                  |

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|      |                                     |
|------|-------------------------------------|
| FACS | Fluorescence-activated cell sorting |
| FAO  | Food and Agriculture Organization   |
| FISH | Fluorescence in situ hybridization  |
| LIN  | Linearity and are                   |
| MAD  | Mean angular displacement           |
| PCR  | Polymerase chain reaction           |
| STR  | Straightness of the path            |
| TTCD | 2,3,7,8-Tetrachlorodibenzo-p-dioxin |
| VAP  | Average path velocity               |
| VCL  | Curvilinear velocity                |
| VSL  | Straight-line velocity              |

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## 6.1 Introduction

Similar to humans, animals have two sex chromosomes, the X and the Y. Female diploidic somatic cells contain two X chromosomes, whereas male diploid somatic cells contain one X and one Y chromosome. Animal gametes are haploid, and egg contains X chromosome, whereas sperm contains either X or Y chromosome. Sex of the progeny is determined by the chromosome present in the sperm. Fertilization of egg with X chromosome bearing spermatozoa results in female progeny, whereas fertilization of egg with Y chromosome bearing spermatozoa occurs in male progeny (Hafez and Hafez 2000). Animal semen samples contain 50% of X chromosome bearing spermatozoa, and another 50% is Y chromosome bearing spermatozoa (Parati et al. 2006). Artificial insemination (AI) is the process of inseminating semen samples into the reproductive tract of female animals. AI is a potential alternative to the natural mating of animals for conception (Foote 2010). As there is a rising demand for the desired sex animals for various reasons such as female animals are required for milk production, and male animals are advantageous for the meat industry. Sperm sexing is a better way to produce the desired sex animals (Prakash et al. 2014). Sperm sexing refers to the method of separating either X chromosome bearing spermatozoa or Y chromosome bearing spermatozoa from semen samples. Successful sperm sexing method provides the live and dynamic spermatozoa with an ability to fertilize the egg (Vishwanath and Moreno 2018).

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## 6.2 Methods of Sperm Sexing

Conventional sperm sexing methods such as Percoll density gradient method (Upreti et al. 1988; Mothé et al. 2018a, 2018b), albumin gradient centrifugation (Claassens et al. 1995; Flaherty et al. 1997), swim-up or swim down (Wolf et al. 2018; Lucio et al. 2012), free-flow electrophoresis (Manger et al. 1997a, 1997b; Blottner et al. 1994a, 1994b; Engelmann et al. 1988a, 1988b), Sephadex columns (Check et al.

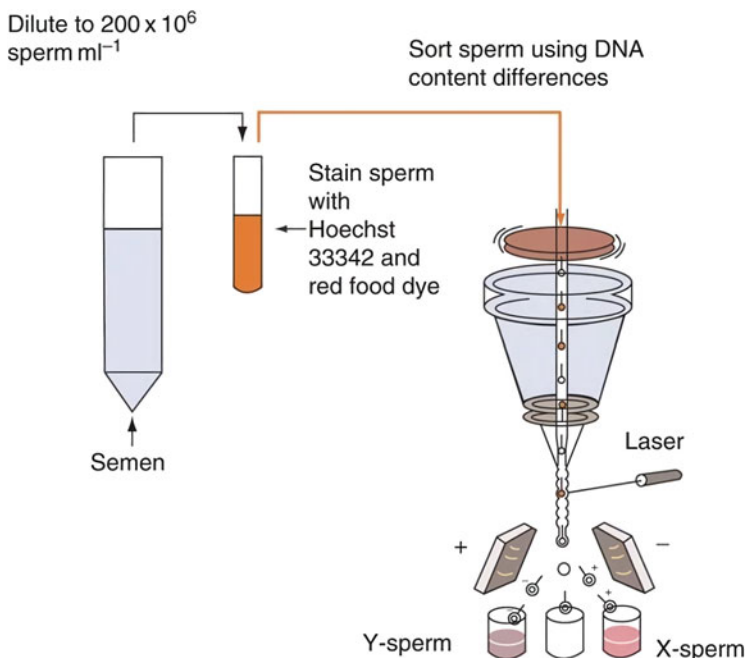


**Fig. 6.1** Principle of fluorescence-activated cell sorting based sperm sexing. Reproduced with permission from Hasler and Garner (2011)

1992; Quinlivan et al. 1982), H-Y antigen-based method (Wachtel 1984; Bennett and Boyse 1973), sperm sorting based on volumetric differences (van Munster et al. 1999a, 1999b), centrifugal countercurrent distribution (Ollero et al. 2000) failed to reproduce the results. As of now, fluorescence-activated cell sorting (FACS) based method is the only commercially successful sperm sexing method across the globe (Rens et al. 1999). The basic principle of sperm sexing by FACS is shown in Fig. 6.1. This method is based on staining sperms cells with the nucleic acid fluorophore and subsequent laser excitement of fluorophore and measurement of fluorescence by a photomultiplier tube. The deoxyribonucleic acid (DNA) content difference in the X and Y chromosome bearing spermatozoa results in fluorescence difference. FACS allows charging the microdroplets containing the single spermatozoa to positive, negative, and no charge based on the fluorescence information obtained from each cell. For example, FACS allows choosing and assigning positive charge to sperm droplet with high fluorescence, negative charge to sperm droplet with low fluorescence, and other microdroplets with no charge. Finally, charged microdroplets are separated by positive and negative electrodes (Garner 2006; Johnson 1995; Hasler and Garner 2011; Garner et al. 2013). Sperm damage, low conception rates, lower sorting speed and efficiency, and high cost are some of the constraints in FACS based sperm sexing method (Carvalho et al. 2010). Raman spectroscopy-based method (De Luca et al. 2014; Managò et al. 2014), CRISPR/Cas

system for genome editing (Kurtz and Petersen 2019), activation of toll-like receptor 7/8 (Umehara et al. 2019, 2020) are some of the new strategies being applied for the sperm sexing. The Engender, a New Zealand based start-up company developed a new microfluidics-based sex sorting technology. Recently, this technology has been transferred to another company for commercialization (Simpson and Rohde 2019).

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## 6.3 Applications of the Sperm Sexing

### 6.3.1 Production of Female Animals

As per the Food and Agriculture Organization (FAO) statistics, more than 6 billion people consume milk and milk products (FAO 2020a). Global milk production depends on female animals such as cattle, buffaloes, goats, sheep, camels, yaks, horses, reindeers, and donkeys. Cattle have been the highest global milk producers, with 81 percent of global milk production. Buffaloes, goats, sheep, and camels produce 15, 2, 1, 0.5 percent of global milk production, respectively. Milk production from horses, reindeers, donkeys, and yaks are confined to some regions of the world. For example, milk production from yaks prevalent in high mountainous areas (FAO 2020b). World milk production was expected to be like 859 million tons in the year (2020 FAO 2020). Sperm sexing is the better possible way to meet the global milk demand by preselection of female animals.

### 6.3.2 Production of Male Animals

World meat production was 342 million tonnes in 2018, and it is projected to double by 2050 FAO (2020b). Cattle, pigs, poultry, buffaloes, sheep, and goats are the main sources of animal meat and meat products. Commercial meat producers prefer male animals for various reasons such as high muscle mass and high meat yield in comparison with female animals and males are bigger and grow resourcefully (Seideman et al. 1982). Production of male animals is beneficial for the meat industry and the use of sexed semen helps to produce male animals with high meta yield.

### 6.3.3 Production of Genetically Superior Animals

“Genetically superior” refers to the superior in terms of a particular set of characteristics such as productivity in the environmental circumstances, fertility, disease resistance, or longevity (Harris and Newman 1994). Production and conservation of genetically superior dams are possible by the use and preservation of sexed semen (Seidel Jr 2007). Even though indigenous breeds have low productivity of animals source foods, these are well adapted to local conditions such as the thermal environment, available feed and water resources, endemic diseases, and parasites.

Management cost is very less for indigenous animal breeds, and these contribute greatly to the rural economy sustainable development (Mendelsohn 2003). Some specialized dairy breeds (Friesian and Jersey) have high milk yields but are not as much adaptable to harsh environments as indigenous breeds such as Sahiwal, Red Sindhi, Tharparkar, Kankrej, Gir, Kenana, and Butana (Mote and Gaikwad 2018). The commercial animal farming industry is looking forward to the crossbreeds, which can be adaptable to harsh environmental conditions with high milk yield (Wakchaure et al. 2015). Genetic selection of low-methane emitting animals and animals with a less feed requirement is a viable option by sexed semen applications (Weller et al. 2017).

### **6.3.4 Farm Management and Reducing the Number of Unproductive Animals**

Artificial insemination by sexed semen is a flexible option for herd replacement in animal farms (Holden and Butler 2018). Dairy animal framers prefer more female animals, whereas meat animal farmers prefer more male animals on the farm (Butler et al. 2014). Dystocia is referred to as abnormal fetal size or position, which can lead to the death of animals from birth to weaning (Mee 2008). Dystocia is reported to be more in the bull calves than heifer calves (Meijering 1984; Purohit et al. 2011). This problem can be overcome by a selection of well-grown heifers plus sexed semen to produce heifer calves. Animal farmers also prefer the animal breeds with less feed and water requirement with a high yield of animal source food. Sexed semen allows choosing the desired breed of animals for better farm management (Norman et al. 2010).

### **6.3.5 Sex Ratio Control of Animals**

Sperm sexing is expected to play a key role in controlling the sex ratio of animals being used for work, recreation, or sports and companion animals (Gledhill 1983). Draught animals are still being used for agriculture work in the low income and developing countries (Ramaswamy 1998). Maintaining proper sex ratio is necessary for the draught animals to empower the rural economy. Recreation or sports animals gained a lot of interest to the animal breeders as there is a high demand for animals of specific qualities of the desired sex. In recent years, there is a huge commercial demand for companion animals of desired sex and elite breeds (Flint and Woolliams 2008; Kaushik 1999).

### **6.3.6 Conservation of Endangered Animals**

Small population animal propagation is important for biodiversity, and sperm sexing is crucial, especially, for the single-sex dominant small population animals.

Production of female offspring is advantageous in the management of endangered mammalian species (Bainbridge and Jabbour 1998). In practice, available sperm sexing technologies are less for endangered species, and there is an urgent need to develop species-specific sperm sexing technologies to conserve endangered animals (Andrabi and Maxwell 2007).

## **6.4 Clues for Sperm Sexing in Animals and Possible Microfluidics Approaches**

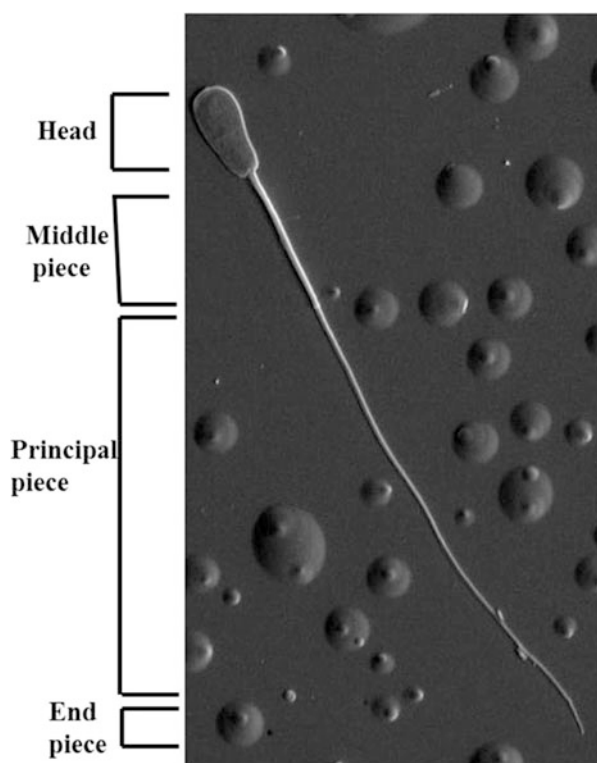
### **6.4.1 Characteristic Features of Animal Semen and Sperm Morphometry**

Ejaculated semen of animals consists of spermatozoa and seminal plasma. The average concentration of spermatozoa in each ejaculate differs extensively among the different species (Garner and Hafez 2000). Seminal plasma contains proteins, organic and inorganic compounds. Seminal plasma helps in the transport of spermatozoa in the female reproductive tract (Druart and de Graaf 2018). The average range of bull, ram, stallion, boar ejaculate volume is 2–10, 0.5–2, 30–100, and 30–300 ml, respectively. The concentration of spermatozoa in these species ranges from 100 to 6000X  $10^6$  /ml (Vajta et al. 2009). Rheological study of bull semen revealed that it follows the pseudoplastic flow and viscosity of semen decreased with shear rate. The sperm morphology image was captured by scanning electron microscopy and depicted in Fig. 6.2. The head of the Karan Fries spermatozoa is flat and paddle-shaped with smooth plasma, and approximate length/width of the Karan Fries spermatozoa head was found to be  $9.05 \pm 0.62\mu\text{m}/4.09 \pm 0.21\mu\text{m}$ . The average values of mid-piece length, principal piece length, end piece length were found to be  $13.6 \pm 1.8\mu\text{m}$ ,  $43.95 \pm 2.95\mu\text{m}$ , and  $2.28 \pm 0.54\mu\text{m}$ , respectively (Yata et al. 2020).

### **6.4.2 Size and Shape Differences between and X and Y Chromosome Bearing Spermatozoa**

Size and shape of X and Y chromosome bearing spermatozoa are of great interest for developing sperm sexing methods (Gledhill 1988). In an atomic force microscopy study, nanoscale differences of sexually mature Nellore bull X and Y chromosome bearing spermatozoa were studied. A high-speed cell sorter (MoFlo SX, Beckman Counter, CA, USA) was used for sperm sexing at ABS Pecplan, Uberaba, Brazil. Size and shape differences sexed for X-sperm, sexed for Y-sperm analyzed by using SPM-9600 equipment (Shimadzu, Japan) and cell measurements were performed on the sperm head. In his study, sperm head perimeter was observed to be  $29.9 \pm 2.2.5$  and  $28.9 \pm 1.59\mu\text{m}$  for sexed for X-sperm and sexed for Y-sperm, respectively. There is no significant difference found in the surface area and three-dimensional volumes of the sperm head. This study indicates that there is no significant difference

**Fig. 6.2** Scanning electronic microscopy image of bull spermatozoa showing the head, middle piece, principal piece, and end piece



between the X and Y chromosome bearing spermatozoa (Carvalho et al. 2013). This lack of differentiation could be the reason for previous methods to fail to sex the sperm based on the size and shape.

### 6.4.3 Motility and Swimming Patterns in X and Y Chromosome Bearing Spermatozoa

Penfold et al. (1998) evaluated the motility of X and Y chromosome bearing spermatozoa of bull semen. Sperm cells were stained with 5 $\mu$ g/ml of Hoechst 33342 fluorochrome, and sexing was done by using modified Epics V flow cytometer/cell sorter (Coulter Corporation, Miami, FL). Sexed sperm samples were evaluated by computer-assisted sperm analysis (CASA), curvilinear velocity (VCL), straight-line velocity (VSL, average path velocity (VAP), mean angular displacement (MAD), beat-cross frequency (BCF), amplitude of lateral head displacement (ALH), linearity (LIN) (ratio of VCL and VAP), and straightness of the path (STR) (ratio of VSL and VAP). This study confirms that there is no significant difference between the kinetic parameters VSL, VCL, and VAP of X and Y chromosome bearing spermatozoa. These results were not supporting the previous

claims of Y chromosome which moves faster than the X chromosome. Still, significant differences were observed on the LIN and STR of X and Y chromosome bearing spermatozoa. Linearity was found to be 31 and 27  $\mu\text{m}/\text{sec}$  and STR is found to be 65 and 58  $\mu\text{m}/\text{sec}$  for X and Y chromosome bearing spermatozoa, respectively. This finding forms the basis for design and fabrication of velocity-based design and fabrication of microfluidic devices.

In a study, highly motile sperm has been separated inside the microfluidic cell by providing rheotaxis zone in front of the corral of the microfluidic system (Zaferani et al. 2018; Schuster et al. 2003). A passively driven microfluidic system was fabricated to separate highly motile sperm by deviating the path of motile sperm from an initial straight path of the microfluidic system (Cho et al. 2003). In a recent study, motile sperm are separated by applying hydrostatic pressure, and this microfluidic system allows the motile sperm moment against flow direction (Seo et al. 2007). Motile spermatozoa were separated by centrifugation of a microfluidic chip that contains two channels separated by a permeative phase-guide structure (Chen et al. 2013a, 2013b). In another study, motile sperms were separated by inducing chemotaxis with a chemical gradient of 0.625 (mg/ml)/mm of acetylcholine in the microfluidic system (Ko et al. 2012).

#### **6.4.4 DNA Content Difference in the X Chromosome Bearing Sperm and Y Chromosome Spermatozoa**

In early research, Garner et al. 1983 identified the difference in the relative DNA content of presumed X and Y chromosome bearing spermatozoa of Holstein, Jersey, Angus, Hereford, Brahman breeds. Based on the difference in the DNA content of the X and Y chromosome bearing spermatozoa, FACS based sperm sexing has been developed. Still, sperm sexing methods depend on this small difference of the DNA content in both the spermatozoa. So far, there is no concrete evidence reported except DNA content difference for developing novel sperm sexing methods. Amplification of DNA content signals provides the scope to separate X and Y chromosome bearing spermatozoa (Johnson et al. 1987; Kawarasaki et al. 1998a, 1998b). Fluorescent and non-fluorescent particle or cells were isolated in microfluidic systems by using pulsed laser microbeams. Chen et al. (2013a, 2013b) demonstrated a 3D microfluidic device to separate fluorescent cells and particles. In this device, fluorescent signals are detected by a photomultiplier tube, and this information triggers a laser pulse to generate a cavitation bubble with optimal delay time. Cavitation bubble generation leads to the deflection of detected fluorescent cells or particles, whereas non-fluorescent cells or particles pass through the regular path without deflection (Fig. 6.3).

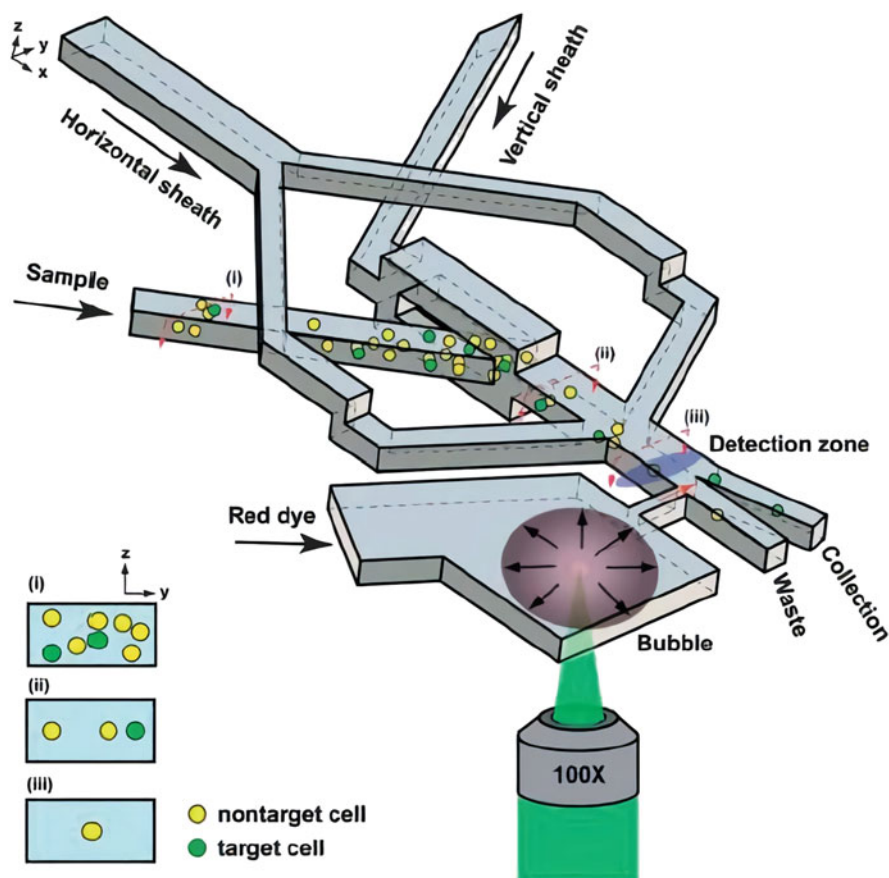


**Fig. 6.3** Schematic of a 3D pulsed laser activated cell sorter. Reproduced with permission from Chen et al. (2013a, 2013b)

#### 6.4.5 Differentially Expressed Proteins or Sperm-Specific Proteins in X and Y Chromosome Bearing Spermatozoa

So far, 30 up-regulated proteins were identified in X chromosome bearing spermatozoa, and nine up-regulated proteins were identified in Y chromosome bearing spermatozoa (De Canio et al. 2014; Scott et al. 2018; Chen et al. 2012). In a preliminary study, bovine sex-specific protein, 30 kDa, was identified that could present X chromosome bearing spermatozoa. In this method, rabbit antisera were generated against sex-sorted sperm (Sang et al. 2011). Identification of such kind of sex-specific proteins on the surface of sperm cell could help to develop membrane-based microfluidic devices for continuous-flow separation by capturing the specific sex spermatozoa through a specific antibody.

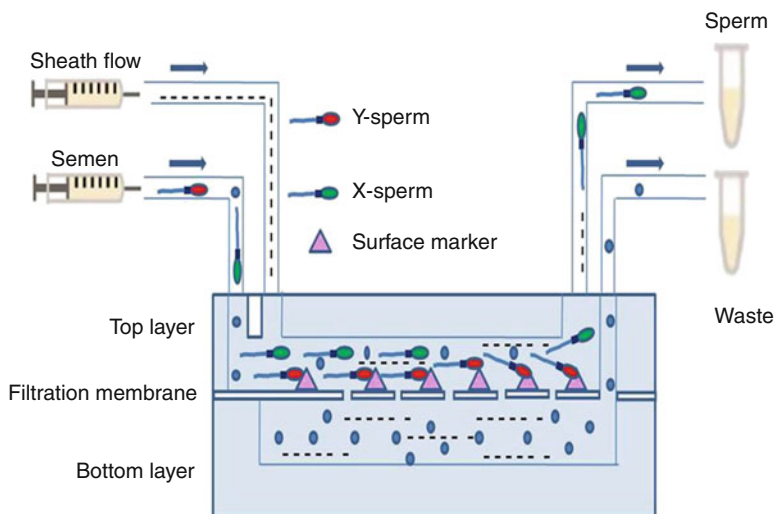


**Fig. 6.4** Hypothesis and design of a microfluidic device with sex-specific biomarker on the membrane for capturing either male or female sperm

A radial flow microfluidic device was reported for the separation of circulating epithelial tumor cells. In this study, anti-epithelial cell adhesion molecule was coated on bean-shaped microposts within the microfluidic chip, and this antibody captures epithelial cell adhesion molecule antigen expressed on epithelial tumor cells (Murlidhar et al. 2014). In a study, importance of inlet designs and advantageous of parallel inlets was studied in affinity microfluidics (Li et al. 2011a, 2011b). In another study, the advantages of multiple vertical channels were demonstrated. In a recent study, antibody functionalized herringbone-grooved microfluidic device was shown to capture cancer exosomes from serous ovarian cancer serum. Cancer exosome membrane biomarkers (CD9 and EpCAM) were targeted to bind with antibody within the microfluidic system (Hissey et al. 2018). Identification of high-affinity sex-specific surface marker on spermatozoa may lead to the development of membrane-based microfluidic devices. Hypothetical sperm sexing method is illustrated in Fig. 6.4, and this design consists of immobilization of sex-specific surface marker on the porous membrane, which can capture either male or female sperm. In contrast, another type of spermatozoa will be collected at the outlet.

#### 6.4.6 Surface Charge on X and Y Chromosome Bearing Spermatozoa

Previous literature on the surface charge on X and Y chromosome bearing spermatozoa of human and bovine sperm samples provided contradictory results. Some of the studies reported electrophoretic mobility of X and Y chromosome bearing spermatozoa towards cathode and anode, respectively. In contrast, some other studies had shown the results vice versa (Kaneko et al. 1984; Manger et al.

1997a, 1997b; Blottner et al. 1994a, 1994b; Engelmann et al. 1988a, 1988b). Ishijima et al. (1991) determined the zeta potential of human spermatozoa by using electrophoretic light scattering spectrophotometer and laser-rotating prism methods. In this study, net negative charge on Y-bearing sperm and X-bearing sperm was found to be approximately  $-16$  mV and  $-20$  mV, respectively. This study indicates that X chromosome bearing spermatozoa has more negative charge than Y chromosome bearing sperm.

In a recent study, a microfluidic device was fabricated for cell enumeration based on impedance change, and it was capable of separating circulating tumor cells based on electrophoresis and dielectrophoresis forces within an electrical field (Dabighi and Toghraie 2020). Moon et al. (2011) developed a microfluidic device for the separation of human breast cancer cells from blood cell sample by combining multi-orifice flow fractionation and dielectrophoretic field. Interdigitized electrode-based dielectrophoresis (DEP) microfluidic devices have been used for the separation of fetal nucleated red blood cells, MDA-MB-231 cells, HL-60 cells with 80–85% purity (Xu et al. 2006; Becker et al. 1994, 1995). Field-flow fractionation in DEP based microfluidic devices is capable of isolating HL-60 cells (Huang et al. 1997), human breast cancer cells (Gascoyne et al. 2009), hematopoietic CD34 + stem cells (Huang et al. 1999), putative stem cells (Vykoukal et al. 2008), human ovarian carcinoma cells (Gupta et al. 2012). Gravitational field-flow fractionation in DEP based microfluidic device was demonstrated to isolate MDA-MB-435 cells from normal blood cells (Yang et al. 1999). Microscope slides coated with electrode arrays in a DEP based microfluidic device were able to separate MDA-MB-435 cells and HL-60 cells from blood cells (Das et al. 2005). DEP microfluidic devices with ring traps (Thomas et al. 2009), interdigitated comb-like electrodes (Alazzam et al. 2011), 3D asymmetric electrodes (An et al. 2009), silicon chip containing a five-by-five array of micro-locations (Cheng et al. 1998), electrosmeared slide patterned with gold microelectrodes (Cristofanilli et al. 2008), castellated (Tai et al. 2007), and angled electrodes (Kim et al. 2007) exhibited electrophoretic isolation of various types of cells. Köhler et al. (2012) designed and fabricated micro free-flow electrophoresis by an injection molding process, and it has been demonstrated for the separation of proteins (Fig. 6.5). This device could be applied for sperm sexing when X and Y chromosome bearing spermatozoa differ significantly in charge.

### 6.4.7 Sex-Specific Response to External Stimulants

In a recent report, the effect of pH and temperature on X chromosome bearing spermatozoa and Y chromosome bearing spermatozoa was studied. The study was conducted on human X and Y chromosome bearing spermatozoa at three temperatures (4, 22, and 37 °C) and three pH conditions (6.5, 7.5, and 8.5). The study revealed that Y chromosome bearing spermatozoa are more susceptible and less viable to temperature stress (You et al. 2017). In another study, the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on X chromosome bearing spermatozoa and Y chromosome bearing spermatozoa was studied. This study

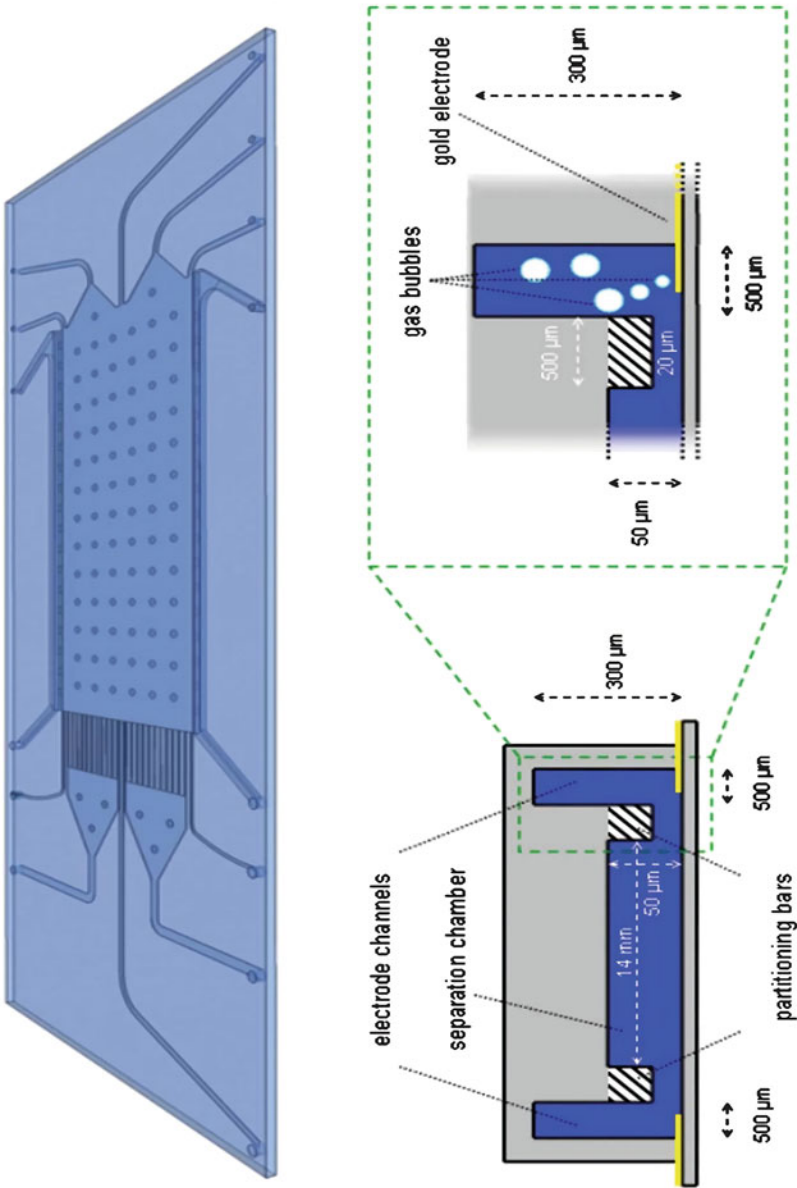


**Fig. 6.5** Micro free-flow electrophoresis designed and fabricated for separation of biomolecules. Reproduced with permission from Köhler et al. (2012)

revealed that Y chromosome viability is lesser than X spermatozoa upon exposure to TTCD at high concentration (You et al. 2018).

It is evident from previous reports that sperms can navigate towards the eggs due to chemotaxis (Friedrich and Jülicher 2007). The sperms are attracted to natural chemoattractants such as hormones, the oocyte microenvironment, and follicular and oviductal fluids (Kaupp et al. 2008). Koyama et al. (2006) demonstrated mouse sperm chemotoxicity towards the mouse ovary extract in poly(dimethylsiloxane) based microfluidic device. In another study, chemotaxis was evaluated using acetylcholine as a chemoattractant in a microfluidic system (Bhagwat et al. 2018). An interfacial valve-based microfluidic chip microfluidic device was demonstrated to study the thermotaxis of human sperm within a temperature range of 34 to 38.3 °C (Li et al. 2014). In a study, sperm chemotaxis and thermotaxis were demonstrated in a microfluidic system that consists of one inlet, one outlet, sperm migration channel, and microheater glass substrate. Chemotactic attractant concentration and temperature gradients are generated within the microfluidic system to separate capacitated sperm and non-capacitated sperm (Ko et al. 2018). Sperm response to the reversed flow direction in a microfluidic device was demonstrated and provided the mechanism of bull sperm movement in the upstream direction (El-Sherry et al. 2014). Rheotaxis study on mammalian cells confirmed the similar mechanism of upstream navigation of sperm cells in a microfluidic system (Kantsler et al. 2014).

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## 6.5 Recent Research in Sperm Sexing by Using Microfluidics

Engender Technologies Ltd., New Zealand based start-up recently obtained a US patent for a microfluidic method for sexing the sperm. In this method, a microfluidic device consists of a focusing stage, orientation stage, detection stage, switching stage, and two outputs. In brief, samples (fluorescent particles or cells) are placed in the focusing stage and they are oriented in the proper direction at the orientation stage, and fluorescence measurement takes place at the detection stage. Fluorescent measurement information of each particle or cell is passed to switching stage where the controller is connected to turn the radiation energy to change the direction of desired particles or cells. Separated particles are collected at outlets based on the fluorescence intensity of particles/cells (Simpson and Rohde 2019).

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## 6.6 Validation Methods of Sperm Sexing

Validation of sperm sexing methods is critical for the development of novel microfluidic sperm sexing techniques. Commercially available FACS based sperm sexing method provides the validation with more than 100% accuracy, but this method is not available in most of the research laboratories. The best alternative to FACS based validation of sperm sexing is the quantitative real-time polymerase chain reaction (PCR) (Maleki et al. 2013; Awan et al. 2017; Mothé et al. 2018a, 2018b). In this method, X and Y chromosome-specific genes are amplified using primers, as shown in Table 6.1. Fluorescence in situ hybridization (FISH) is also

**Table 6.1** Sex-specific primers for the real-time PCR analysis

|         | Gene/location                               | Forward primer          | Reverse primer          | References                  |
|---------|---------------------------------------------|-------------------------|-------------------------|-----------------------------|
| Bovine  | SRY gene/Y chromosome                       | GAACGAAGACGAAAGGTGGCT   | GCGGGTAAGAAACTCCTCC     | Maleki et al. (2013)        |
|         | Proteolipid protein gene (PLP)/X chromosome | TAAAGGTGGGTAGGGTCAAGG   | GCTGTGAATACGACGGGTGTAG  |                             |
| Buffalo | SRY gene/Y chromosome                       | GTTGTGCCAAGACCACATATTC  | ATAGGGTAACA TTGGCTACACG | Awan et al. (2017)          |
|         | Proteolipid protein gene (PLP)/X chromosome | GTTC TTCAGGTCACAGGGTAAG | CTCTTTGTGACCCCTATGGACTG |                             |
| Dog     | SRY gene/Y chromosome                       | CTCGCGATCAAAAGGCGCAAGAT | TTCGGCTTCTGTAAGCATTTTC  | Mothé et al. (2018a, 2018b) |
|         | Coagulation factor IX (F9)/X chromosome     | CCCAAGAGGCCACTGGAAAT    | TGTGCAAGGAGCAAGGTTGA    |                             |

another method of validation sperm sexing methods, and it involves the hybridization by use of fluorescent probes to detect sex-specific DNA. FISH is more complicated than quantitative real-time PCR (Rens et al. 2001; Habermann et al. 2005; Kawarasaki et al. 1998a, 1998b; Di Berardino et al. 2004). Quinacrine mustard staining for Y chromosome bearing sperm (Ogawa et al. 1988) and semi-quantitative PCR methods (Reynolds and Varlaro 1996) are not preferred for the sperm sexing validation due to less accuracy and reproducibility.

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## 6.7 Conclusions

Owing to the high demand for sexed semen in the animal industry and limitations of commercially available sperm sexing methods, it is an urgent need to develop novel sperm sexing methods. Small differences between X and Y chromosome bearing spermatozoa have been posing a great challenge to sperm biologists. The recent success of sperm sexing by microfluidic method generated hope for the development of novel microfluidics-based sperm sexing methods. Microfluidic method may offer some advantages over current methods such as the speed of sorting, cost, and minimal sperm damage.

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