Microfluidics for Cryopreservation

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1 **Abstract**

2 Cryopreservation has utility in clinical and scientific research but implementation is
3 highly complex and includes labor-intensive cell-specific protocols for the
4 addition/removal of cryoprotective agents and freeze-thaw cycles. Microfluidic
5 platforms can revolutionize cryopreservation by providing new tools to manipulate
6 and screen cells at micro/nano scales, which are presently difficult or impossible with
7 conventional bulk approaches. This review describes applications of microfluidic
8 tools in cell manipulation, cryoprotective agent exposure, programmed
9 freezing/thawing, vitrification, and *in situ* assessment in cryopreservation, and
10 discusses achievements and challenges, providing perspectives for future
11 development.

12

13 **Keywords:** microfluidics, cryopreservation, freezing/thawing, cryoprotective agent
14 loading/unloading, vitrification

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1. Introduction

Cryopreservation is a technique using very low temperatures to preserve structurally intact living cells and tissues. Cryopreservation is a science/technology with the goal of long-term storage of cells, tissues, and organs at cryogenic temperatures (usually in liquid nitrogen or liquid nitrogen vapors), allowing resumption of normal functions after retrieval from a cryobank (Kuleshova and Huttmacher, 2008). To date, cryopreservation has permitted breakthroughs in biomedical applications including assisted reproductive medicine, stem cell technologies, cell therapies, tissue engineering, development and *in vitro* screening of anticancer drugs, pharmacology, and basic scientific research (Benelli et al., 2013; Feng et al., 2011; Julca et al., 2012; Palasz and Mapletoft, 1996; Popova et al., 2016; Smorag and Gajda, 1994; Teixeira da Silva, 2003; Wang, B. et al., 2014; Wang et al., 2012). Trillions of cells are biopreserved globally for daily clinical use. For example, cryopreservation of human oocytes preserves future fertility of young females who may experience infertility due to exposure to environmental/occupational hazards or aggressive medical treatments and such cryopreservation avoids moral, ethical, and religious issues associated with human embryo preservation (Choi et al., 2015a; Nagashima et al., 1995; Palasz and Mapletoft, 1996; Rall and Fahy, 1985; Redmond et al., 1988; Smorag and Gajda, 1994; Steponkus et al., 1990; Trounson and Mohr, 1983). Similarly, due to the significant decline in sperm quality in global males (Merzenich et al., 2010), increased incidence of azoospermia and oligospermia, and rapid increases in testicular cancer in young males, spermatozoa and spermatogonia stem cell preservation can be undertaken (Zou et al., 2013) and cryopreservation of sperm, ova, and fertilized eggs is currently in clinical practice worldwide.

In addition, stem cell cryopreservation is indispensable to ensure that manufacturing, distribution and unpredictable demands of stem cells can be addressed, and efficient cryopreservation can enable marketing of stem cell products (Xu, 2011). Cryopreservation of immune cells with high efficiency and quality is an important prerequisite for meeting bio-immunotherapy demands, which is an emerging cancer treatment with reportedly fewer side effects and more promising outcomes. In general, efficient and reliable storage of large quantities of diagnostically and therapeutically relevant cells, including blood cells, gametes, embryos, stem and immune cells, is crucial to guarantee their permanent availability (Ihmig et al., 2013).

Current cryopreservation techniques fall into three categories (He, 2011): programmable slow freezing, vitrification, and low-CPA vitrification (ultra-rapid freezing). Among the three categories, programmable slow freezing has been the most common method, allowing freezing of low-CPA cell solutions but with ice crystallization in both extra- and intracellular solutions. In programmable slow freezing, most mammalian cells are frozen at 1 °C / min with 1.5 M CPA (Zhang et al., 2011a). With this optimal cooling rate, both the solution and intracellular ice injuries are minimized to support cell survival (Leibo et al., 1970; Mazur, 1984). Cryo-injuries introduced by solution effects (elevated concentration caused by ice formation and growth), extra- and intracellular ice formation (EIF and IIF) and osmotic pressure-driven cell dehydration, are inevitable even after adequate optimization of

freeze-thaw cycles. Vitrification is an alternative well-established technique offering the benefits of cryopreservation without ice crystal formation damage (Fahy, 1981; Rall and Fahy, 1985). Vitrification normally uses a high concentration of CPAs to prevent ice formation. However, low-CPA vitrification uses ultra-rapid freezing to suppress ice formation under a low CPA concentration condition. Of note, high-CPA and rapid cooling can promote vitrification but compared with programmable slow freezing, vitrification and low-CPA vitrification usually require greater CPA (4–8 M) and more rapid cooling (up to $\sim 10^5$ °C/min). Both the loading/unloading processes of such high concentrations of CPAs, and the ultra-rapid cooling rates required for vitrification, complicate this method within the scope of traditional freeze-thaw techniques for mass volume cell suspensions within routinely used containers. Slow freezing techniques are commonly used for manipulating larger samples, where cell-specific CPA loading/unloading, cell scale optimal freezing/thawing, and ultra-rapid and uniform freeze/thawing are hard to achieve. However, microfluidic applications may address these challenges.

Recently, rapid development of micro- and nanotechnologies has allowed implementing entire cryopreservation procedures on-chip. Owing to numerous intrinsic features, such as ease of mass production and specific designs, ease of component integration, disposability, low cost, and requirement of less reagents or analyte volumes, as well as ease of rapid implementation, microfluidics techniques emerged in early 1980 have been introduced for cryopreservation studies. These studies include controlled loading/unloading of CPAs into cells with stepwise, linear and complex CPA profiles (Heo et al., 2011; Park et al., 2011; Song et al., 2009), programmable freeze-thawing cycles (Afrimzon et al., 2010; Deutsch et al., 2010; Li et al., 2010), and low-CPA vitrification by ultra-rapid freezing (Choi et al., 2015a; He et al., 2008; Zou et al., 2013) using microfluidics. Such innovative microfluidic applications offer a tremendous potential to revolutionize cryopreservation.

This review highlights applications of contemporary microfluidic techniques in cryopreservation, discussing new insights into highly cell-type specific cryopreservation approaches and describing new tools to manipulate cells and their interactions with the extracellular environment.

2. Fundamentals of Cryopreservation

Cryopreservation deals with long-term maintenance of cellular or tissue biological function at low temperatures, suppressing metabolism and biochemical reactions, effectively ceasing “biological time” (Mullen and Critser, 2007). Although ultra-low temperatures offer long-term preservation, cryo-injuries such as ice formation-induced mechanical stress and freeze concentration-induced physiochemical deviation from physiological states are inevitable (Gao and Crister, 2000; Mazur, 2004; Mazur et al., 1972). To prevent cell death by freezing/thawing, CPAs have been introduced and are widely used; although some are toxic (Meryman, 1971a).

The underlying theory of cryopreservation is shown in Figure 1. The inverted ‘U’ shaped relationship between cell survival and cooling (Figure 1A) has been

1 summarized in the literatures prior to understanding the mechanism behind it (Acker,
2 2007; Mazur, 1984, 2004; Mazur et al., 1972; Mullen and Critser, 2007). During slow
3 freezing cryopreservation, cells may suffer injuries caused by deviations of the extra-
4 and intracellular solutions from the physiological environment and extra- and
5 intracellular ice formation (Figure 1B), as summarized by the “two-factor hypothesis”
6 offered by Mazur (Mazur, 1970, 1984). Briefly, both prolonged exposure to
7 extracellular freezing (“solution injury”) and intracellular freezing (“IIF injury”) are
8 determining factors for cell survival (Mazur, 1970, 1984, 2004). Both solution injury
9 caused by over-slow cooling and IIF injury caused by over-rapid cooling are fatal to
10 cells. Thus, a cryopreservation protocol minimizing both solution and IIF injuries
11 offers the best cell survival and corresponds to optimal cooling (He, 2011; Mazur,
12 1970, 1984, 2004; Shimada, 1978; Toner et al., 1990; Zhao et al., 2006; Zhao, G. et al.,
13 2014) (Figure 1A).

14 Temperatures and corresponding cell volume excursions during cryopreservation
15 (Acker, 2008; Mazur, 1984, 2004) appear in Figure 1C (a, b) and 1 D (a, b). Typical
16 cryopreservation procedures involved in programmable slow freezing can be
17 summarized this way (Gao and Crister, 2000; Mazur, 1984, 2004; Zhao et al., 2016):
18 addition of CPAs to cells before cooling; cooling cells below the solution freezing
19 point; triggering ice formation by seeding to eliminate overcooling; sample freezing
20 towards a low temperature ($-196\text{ }^{\circ}\text{C}$) at a controlled rate; storage; thawing and
21 re-warming; and CPA removal. Cell volume excursion during cryopreservation
22 appears in Figure 1C (b) and it can be used as an intuitive indicator of osmotic injury
23 (Benson et al., 2005a; Benson et al., 2012; Davidson et al., 2014; Lusianti et al., 2013):
24 the cell may be damaged once the volume change is beyond upper/lower limits. Of
25 note, storage at a very low temperature ($< -180\text{ }^{\circ}\text{C}$) is not as important as freeze-thaw
26 cycles across the intermediate temperature zone ($-15\text{ }^{\circ}\text{C}$ to $-60\text{ }^{\circ}\text{C}$) which are most
27 damaging (Gao and Crister, 2000; Mazur, 1984). Thus, optimization of CPA
28 loading/unloading and freezing/thawing is needed for successful slow-freezing
29 cryopreservation.

30 For vitrification or low-CPA vitrification, the cooling process is relatively simple
31 compared to programmable slow freezing because the sample is directly immersed in
32 liquid nitrogen to achieve the fastest cooling possible (Fahy, 1986b; Fahy et al., 1984;
33 Huang et al., 2015; Wang et al., 2016) (Figure 1D (a)). Because no ice forms in either
34 extra- or intracellular solution, no water or CPA transport across the cell membrane
35 during freezing/thawing occurs (Fahy and Wowk, 2015) (Figure 1D (b)). However,
36 because vitrification or even low-CPA vitrification requires a high (which are toxic)
37 concentration of CPA (4-8 M) compared to programmable slow freezing, severe
38 osmotic injury during addition and removal of CPA may occur (Fahy and Wowk,
39 2015). Thus, protocols for adding/removing CPAs during vitreous cryopreservation
40 must be carefully designed, and multi-step addition and removal is often needed to
41 minimize osmotic injuries (Benson et al., 2012; Davidson et al., 2014; Lusianti et al.,
42 2013). Of note, for simplicity, three-step addition and removal of CPAs as a
43 representative case, is shown in Figure 1D (b). Ultra-rapid cooling is key to low-CPA
44 vitrification; however, it is technically difficult for large volume samples to cool

1 rapidly (Fahy and Wowk, 2015; Fahy et al., 2009; Wowk, 2010). Furthermore,
2 Mazur's group recently suggested that a high warming rate is considerably more
3 important to attaining high cell survivals than is a high cooling rate (Jin and Mazur,
4 2015; Kleinhans et al., 2010; Seki et al., 2014; Seki and Mazur, 2008, 2009). Ideal
5 rewarming should be rapid enough to avoid devitrification and recrystallization,
6 which may fatally injure cells (Fahy and Wowk, 2015). However, it is also hard to
7 achieve a sufficiently rapid warming rate for large volume samples. Cell survival and
8 cooling for vitrification, including low-CPA vitrification (He, 2011), is depicted as the
9 dash line in Figure 1A.

11 **2.1 CPA loading and unloading**

12 Although cryopreservation offers a powerful tool for long-term storage and
13 off-shelf availability of biological materials, CPAs are required. CPA received great
14 attention since its discovery in 1948 (Meryman, 1974; Polge et al., 1949; Smith and
15 Polge, 1950); they prevent stress of freeze/thawing while causing cytotoxicity and
16 osmotic shock (Fahy, 1994; Luyet and Rapatz, 1971; Meryman, 1971b). CPA loading
17 prior to freezing and unloading after thawing expose cells to a series of anisotonic
18 solutions, and as a result, cells undergo volume excursions (Acker, 2008; Gao and
19 Crister, 2000; McGrath, 1997) (Figures 1C (b), 1D (b)). During loading of a
20 permeable CPA, the chemical potential of intracellular water exceeds that of
21 extracellular water, and cells first dehydrate with the outflow of cell water, and then
22 re-swell due to backflow of water plus the CPA (Gao and Crister, 2000). In contrast,
23 during CPA unloading, the opposite occurs (Gao and Crister, 2000). Transport of
24 water across a cell membrane is always more rapid than diffusion of larger CPA
25 molecules (Gao and Crister, 2000). Complete cell volume excursion and how this
26 occurs appears in Figures 1C (b) and 1D (b). As shown, minimal and maximal values
27 for cell volume occur during CPA addition and removal, respectively, and these
28 volumes during osmotic responses may cause osmotic injuries that reduce cell
29 recovery and survival (Song et al., 2009). Accordingly, optimization of CPA
30 loading/unloading requires confining cell volumes in a minimal/maximal range
31 (Benson et al., 2011; Benson et al., 2012; Davidson et al., 2014; Lusianti et al., 2013).

32 Except for osmotic injuries, CPA toxicity is also a dominant factor for successful
33 cryopreservation of living systems by both freezing and vitrification (Benson et al.,
34 2012; Cordeiro et al., 2015; Davidson et al., 2015; Fahy, 1981, 1986a, 2010; Fahy and
35 Karow, 1977; Fahy et al., 1990; Fahy et al., 1984; Fahy et al., 2004a, b; Fahy et al.,
36 2004c). Significant effort has been devoted to the study on CPA toxicity neutralization,
37 especially for organ vitrification (Fahy, 1981, 1986b, 1987, 1994; Fahy et al., 1985;
38 Fahy et al., 2004c; Khirabadi et al., 1988). Benson et al. pioneered the development of
39 a toxicity cost function (it reflects the cumulative damage caused by toxicity) based
40 on mathematical optimization of procedures for CPA equilibration (addition and
41 removal). Predictions on human oocytes using this function yield significantly less
42 toxicity than conventional stepwise procedures (Benson et al., 2012; Davidson et al.,
43 2014). Some extension of the toxicity cost function was further successfully used for
44 the design of CPA equilibration for adherent endothelial cells (Davidson et al., 2015).

1 Compared to programmed slow freezing, vitrification/low-CPA vitrification requires
2 relatively more CPA; therefore CPA addition and removal processes are more time
3 consuming and costly (Choi et al., 2015a; Choi et al., 2015b; Huang et al., 2015;
4 Wang et al., 2016).

5 A routine method for CPA loading/unloading involves repeated centrifugation,
6 supernatant removal, addition of new solutions, and cell resuspension (Lusianti et al.,
7 2013; Lusianti and Higgins, 2014; Zhurova et al., 2014a; Zhurova et al., 2014b).
8 Although the manpower and material resources involved are expensive and the
9 samples are exposed to contamination risk, this process is difficult to automate within
10 the framework of traditional processing (Lusianti et al., 2013). More recently,
11 however, microfluidic platforms have been adopted to avoid potential osmotic and
12 toxic injuries to cells using programmed, controlled, complex CPA loading and
13 unloading profiles (Heo et al., 2011; Liu et al., 2015; Lusianti and Higgins, 2014).
14 This approach has been more efficient, robust, and safe, while still enabling high
15 throughput.

16 17 **2.2 Cooling and warming**

18 Contrary to intuition and common sense, cell fates are determined by the
19 lethality of the temperature “danger zone” ($\sim -15\text{ }^{\circ}\text{C}$ to $-60\text{ }^{\circ}\text{C}$) that a cell must
20 traverse twice — once during cooling and once during warming, instead of the storage
21 at very low temperatures (Mazur, 1984, 2004). The typical physical events that occur
22 during slow-freezing cryopreservation process (Acker, 2007) are depicted in Figure
23 1C, and more detailed cooling rate-dependent cell freezing responses (Mazur, 1984;
24 Toner et al., 1990), including low-CPA vitrification by ultra-rapid freezing, are shown
25 in Figure 1B. After exposure to subzero temperatures below the CPA solution freezing
26 point, cells and their surrounding medium may experience different physical events
27 that are cooling rate dependent (Acker, 2007; Mazur, 1984). In Figure 1B, Case I
28 depicts slow freezing that allows sufficient cell dehydration to minimize supercooling
29 of intracellular solutions, resulting in severe cell shrinkage-induced osmotic injuries
30 and high CPA concentration-induced solution injuries (Zhao, G. et al., 2014). In
31 contrast, preventing freezing of intracellular water, as seen in Case II, allows optimal
32 freezing and partial cell dehydration plus innocuous IIF, resulting in comparatively
33 high cell survival corresponding to tolerable compound injuries (Zhao, G. et al., 2014).
34 For Case III, rapid freezing causes insufficient cell dehydration and increased super
35 cooling of the intracellular solution, which may attain re-equilibrium by intracellular
36 freezing, resulting in serious IIF injuries (Zhao, G. et al., 2014). For Case IV,
37 ultra-rapid freezing induces both extra- and intracellular solutions to form a glass
38 instead of crystallizing, resulting in most cell survival corresponding to complete
39 prevention of cryo-injuries by vitrification (namely, low-CPA vitrification) (Zhao, G.
40 et al., 2014). The schematic of cooling rate-dependent cell survival (He, 2011) is
41 depicted in Figure 1A. As shown for conventional programmable slow freezing,
42 significant IIF and freeze concentration-induced excessive dehydration are the two
43 dominant cryo-injuries that determine cell survival (Gao and Crister, 2000; Mazur,
44 1984). The freeze concentration-induced cell damage effect is apparent for

slow-cooling rates, which is decreased with an increased cooling rate (Mazur, 1984). However, IIF becomes dominant only when the cooling rate is sufficiently high (Mazur, 1984, 2004). How these damage factors are related to cooling rate is depicted by a classical inverted U curve (Figure 1A, solid line) (He, 2011; Mazur, 1984). For conventional high-CPA vitrification, the cooling rate is not as important; the CPA concentration is high enough to suppress ice formation (Fahy and Wowk, 2015). Instead, osmotic stress and toxicity introduced by high CPA concentrations are more damaging (Fahy and Wowk, 2015). For low-CPA vitrification by ultra-rapid cooling, the classical inverted U curve can be extended to the ultra-rapid cooling rate domain, in which cell survival increases with the cooling rate between 10^3 – 10^6 °C/min (dashed line in Figure 1A) (He, 2011) (Fahy and Wowk, 2015; Karlsson et al., 1994; Zhao et al., 2006; Zhao, G. et al., 2014; Zhao et al., 2013).

With respect to conventional cryopreservation platforms, difficulty arises in ensuring that all cells in a large volume are cooled at a prescribed optimal cooling rate for the slow freezing program (Allen et al., 1975; Kilbride et al., 2016; Slabbert et al., 2015). Furthermore, preventing cytotoxicity from high CPA concentrations required to decrease critical cooling rates necessary for vitreous cryopreservation is of importance (Fahy and Wowk, 2015). Unprecedented successes in cell cooling and warming have been accomplished with emerging novel microfluidic platforms which have intrinsic advantages, especially for cell-specific manipulation and controllability for rapid thermal responses (Huang et al., 2015; Lai et al., 2015a; Pyne et al., 2014; Vom et al., 2013; Zhou et al., 2013; Zhou et al., 2015).

3. Characterization of Cell Membrane Transport Properties

To minimize negative effects caused by CPA addition prior to freezing and removal after thawing, carefully designed protocols based on fundamental biophysical principles are needed (Liu et al., 2015; McGrath, 1997). Cell membrane transport properties must be determined using osmotic shift experiments to optimize the addition and removal of CPAs. Cell membrane transport property (or permeability)-related parameters include (Mcgrath and Krings, 1986; Woods et al., 1999) hydraulic conductivity (L_p), CPA permeability (P_s), and activation energies of both parameters (E_{Lp} , E_{Ps}).

Microfluidics provide an ideal platform for microscale cell manipulation and permit precise description of cell osmotic behaviors with controlled CPA loading/unloading. Various microfluidic devices have been applied for characterization of cell membrane transport properties by studying the cell osmotic response.

Microfluidic approaches have also been adopted for quantification of cell membrane permeability for more than 10 years and these are classified in two categories according to the materials from which they are made: polydimethylsiloxane (PDMS) and non-PDMS materials. To study osmotic responses, Chen et al. (Chen et al., 2007; Chen et al., 2008) reported the development of a PDMS microperfusion chamber and Takamatsu and coworkers (Takamatsu et al., 2004) developed a non-PDMS sandwich structured microperfusion chamber, which was improved by Liu

1 and coworkers (Liu et al., 2015). Both chamber types have been implemented for
2 accurate depiction of cell volume responses after osmotic shifts. Although there also
3 have been interesting studies using microfluidics for characterization of cell
4 membrane transport properties of adherent cells (Fry and Higgins, 2012; Verkman,
5 2000), this review focuses on microfluidic devices and methods for suspended cells.
6 Hereafter, we summarize microfluidic-based perfusion chambers for measuring cell
7 membrane biophysical properties and minimization of osmotic injury by accurately
8 controlled biotransport across the cell membrane.

9 10 ***3.1 Sandwich-structured microfluidic perfusion chamber***

11 Takamatsu's group used a 50- μ m-thick silicone rubber sheet (40 mm x 4 mm)
12 sandwiched between two silicone glass slides to form a single straight microfluidic
13 channel (Takamatsu et al., 2004). Before perfusion, the upper silicone glass was
14 removed and the cell suspension was pipetted into the channel prior to replacing the
15 top silicone glass. After about 10 min, cells had settled on the upper surface of the
16 bottom glass. When the perfusion solution was controlled at low velocity, most cells
17 did not move against the fluid. Unlike conventional microdiffusion designs (McGrath,
18 1985) or the microfluidic perfusion chamber method (Gao et al., 1996), the
19 extracellular solution was directly shifted from low to high concentration (or inversely)
20 without the aid of a dialysis/porous membrane. To improve data accuracy, the
21 concentration change of the extracellular solution was simultaneously measured using
22 a laser interferometer during perfusion experiments.

23 Compared to traditional microscale devices or the more recent PDMS
24 microfluidic perfusion chambers, the sandwich-structured microfluidic perfusion
25 chamber is low cost, easy to make, and can be accurately configured for controlling
26 extracellular solution concentrations. However, inconvenient features of this design
27 include a limited observation area (especially after the aluminum
28 temperature-controlled stage is mounted, the view field is confined by a
29 10-mm-diameter hole at the center of the aluminum block, making it hard to move the
30 chamber independent of the stage). Also, there is an inconvenient sample loading
31 process (the upper silicon glass has to be removed to load cell suspension for each
32 assay). Temperature control of the extracellular solution can also be inaccurate due to
33 the thermocouple configuration outside of the microchannel, and the laser
34 interferometer is relatively expensive with complicated optic setup required.

35 36 ***3.2 PDMS microfluidic perfusion chamber***

37 More recently, Chen and colleagues (Chen et al., 2007; Chen et al., 2008)
38 fabricated a PDMS-based microfluidic perfusion system using soft lithography
39 (Figure 2A). The PDMS layer containing a microchannel was inverted and sealed on a
40 glass slide to form the microfluidic chamber, and the entire construct was placed into
41 the temperature-control chamber. Advantages of this microfluidic perfusion chamber
42 include cost effectiveness, disposability, reduced labor requirements and the ability to
43 wash and reassemble for multiple use. Also, customized microchannels allow cell
44 confinement in a monolayer state to prevent cell overlapping. Disadvantages of the

1 microfluidic perfusion chamber are that masks have to be re-designed and
2 re-manufactured to generate microchannels with different sizes and geometries, which
3 can be time consuming. Moreover, cells can be trapped and enriched by the block
4 during perfusion and shear force from the upper stream and the counter force from the
5 block tend to distort cells, inevitably introducing cell volume measurement errors that
6 can affect theoretical calculations. A T-type thermocouple temperature sensor is
7 directly embedded in the PDMS near the microchannel instead of being in the
8 extracellular solution, to ensure accurate temperature monitoring.

9 Later, Tseng and coworkers (Tseng et al., 2011) described another design for the
10 PDMS microfluidic perfusion chamber (Figure 2B), whereby the block of the PDMS
11 layer (Figure 2A) was removed, and a PDMS layer with a rectangular microchannel
12 ($200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m} \times 2\text{ cm}$) was bonded onto a glass slide (Figure 2B). The upper
13 surface of the glass was treated with $50\text{ }\mu\text{g/mL}$ poly-D-lysine hydrobromide to
14 immobilize cells. During perfusion, cell volume responses were observed and
15 recorded *in situ*. To minimize inconsistency between experiments and theoretical
16 assumption (step-wise change) for extracellular solution concentration profiles, the
17 perfusion flow velocity was increased to $36\text{ }\mu\text{L/min}$ to minimize the solution
18 replacement time to $< 0.4\text{ s}$ (Tseng et al., 2011). Of note, commercially available
19 cryostage (HSC 601; INSTEC Inc., Boulder, CO) was used for temperature regulation
20 in the perfusion system for ease of measurement of cell membrane transport
21 properties at very low temperatures (-5 , -10 , and $-20\text{ }^{\circ}\text{C}$). Temperature dependence
22 in both L_p and P_s of megakaryocytes was discontinuous between $37\text{ }^{\circ}\text{C}$ and $-20\text{ }^{\circ}\text{C}$
23 (Tseng et al., 2011). Compared to the PDMS microfluidic perfusion chamber
24 developed by Chen (Chen et al., 2007; Chen et al., 2008), cell volume responses were
25 more accurately measured in the device developed by Tseng et al. (Tseng et al., 2011),
26 since the aforementioned cell distortion caused by the upper stream and the counter
27 force from the block was avoided.

28 Heo et al. (Heo et al., 2011) developed a two-layer, PDMS oocyte-specific
29 microfluidic device for quantification of cell volume responses during CPA loading
30 (Figure 3A). In their design, a series of semicircular holders were introduced to hold
31 single oocytes for perfusion. Cell volume excursions during various CPA loading
32 protocols, including step-wise, linear, and complex, were compared. Using this
33 microfluidic device, CPA loading processes of oocytes were optimized by minimizing
34 exposure time and cell volume excursion. Lai and colleagues (Lai et al., 2015a)
35 developed a novel microfluidic CPA exchange device (Figure 3B) and reported that
36 minimizing cell shrinkage at a given minimum cell volume and CPA exposure time
37 improves cell survival. Furthermore, this approach allows a new CPA exchange
38 protocol using automated microfluidics to improve oocyte and zygote vitrification.
39 Except for the above mentioned microfluidic devices that have been directly used for
40 investigation of cell membrane transport properties of oocytes, there are also several
41 reports on microfluidic platforms for assisted reproduction, where microfluidic
42 perfusion chambers or channels may be adopted for CPA perfusion. Clark et al. and
43 Wheeler et al. (Clark et al., 2005; Wheeler et al., 2007) designed a microchannel
44 device (Figure 3C) that mimics the function of the oviduct and creates a flow pattern

1 of spermatozoa past the oocytes similar to the pattern in the oviduct, and successfully
2 performed fertilization of pig oocytes. Suh et al. (Suh et al., 2006) proved that *in vitro*
3 fertilization of murine oocytes can be conducted within microfluidic channels (Figure
4 3D). Later, Han et al. reported a novel microwell-structured microfluidic device
5 (Figure 3E) that integrates single oocyte trapping, fertilization and subsequent embryo
6 culture, this device can create a well-controlled microenvironment for individual
7 zygotes. All these microfluidic devices are well-designed with controlled sizes of the
8 microstructures, and may be potentially adopted for investigation of osmotic
9 behaviors of oocytes, zygotes or embryos.

10 More recently, Lyu et al. (Lyu et al., 2014) developed a PDMS microfluidic chip
11 with hydrodynamic switching to measure cell membrane hydraulic conductivity
12 (Figure 4). A specially designed block structure in the PDMS layer was used for cell
13 trapping, similar to the block design used by the Chen' laboratory (Chen et al., 2007;
14 Chen et al., 2008) (Figure 2A). Cells were first trapped by the block using controlling
15 syringes (s2) and (s3) at a constant flow while keeping the syringe (s1) inactive
16 (Figure 4A). The syringe pump (s1) was then triggered to aspirate the isotonic
17 solution containing cells to induce hydrodynamic switching and thereby flush the
18 hypertonic solution over trapped cells (Lyu et al., 2014) (Figure 4B). By changing the
19 aspiration rate of the syringe pump, the exchange rate of extracellular solution can be
20 controlled for cells trapped at locations in which the solution switching dynamics are
21 characterized (Figure 4B), and cell responses are simultaneously recorded (Figure 4C)
22 (Lyu et al., 2014). Using this PDMS microfluidic chip with hydrodynamic switching,
23 hydraulic conductivities of articular cartilage chondrocytes were measured with two
24 different switching time constants (Figure 4D), with results suggesting that L_p is
25 independent of extracellular solution change rates. Although L_p value is independent
26 of shear force caused by perfusion flow, obvious cell reshaping was observed (Figure
27 4C), which may introduce error into subsequent data analysis because non-spherical
28 cells are not used by cryobiologists for calculating permeabilites (Takamatsu et al.,
29 2004; Zawlodzka and Takamatsu, 2005). It should be noted that the main idea for
30 controllable mixing of extracellular solutions was firstly accomplished by
31 Takamatsu's group in 2004 (Takamatsu et al., 2004) using a much simpler device,
32 whereby quantitative analysis of extracellular solution concentration profiles during
33 osmotic shift experiments was performed. The PDMS microperfusion chamber has
34 similar advantages and disadvantages to those mentioned previously (Chen et al.,
35 2007; Chen et al., 2008).

36 37 **3.3 Improved sandwich structured microfluidic perfusion chamber**

38 Based on the sandwich-structured microfluidic perfusion chamber originally
39 developed by the Takamatsu's group (Takamatsu et al., 2004), Zhao's group (Liu et al.,
40 2015; Wang et al., 2013; Wang, J.Y. et al., 2014; Yue et al., 2014; Zhao, 2012)
41 proposed an improved design (Figure 5) by avoiding most of the disadvantages. First,
42 a transparent plexiglass temperature control unit was introduced to provide an
43 expanded vision compared with metal units (Figure 5A). Then, a tiny thermocouple
44 was embedded into the microchannel during manufacturing to ensure accurate

1 monitoring of temperature in the local extracellular solution. Next, a dedicated
2 capillary for cell loading was introduced and the profile for extracellular solution
3 concentration changes during CPA loading/unloading was pre-determined with
4 experimentally validated finite element analysis (Figures 5B and C). A Teflon (PTFE)
5 tube with a thicker wall (1.2 mm) was used to connect the system to minimize the
6 pressure fluctuation upon various flow controls, and instead of assembling with bolts
7 or glue, the components shown in Figure 5A were tightly clamped with a file clip,
8 providing tight adherence but sufficient protection by stress relaxation.

9 The features of this improved perfusion system make it robust, low cost, easy
10 and fast to assemble, highly precise and reusable and durable. Due to the fact that the
11 sealing process of PDMS on glass with oxygen plasma is irreversible (Chen et al.,
12 2007; Chen et al., 2008), the PDMS microfluidic perfusion chamber cannot be
13 disassembled for washing and reuse. The sandwich structured microfluidic perfusion
14 chamber, however, is able to address this issue. Till now, this sandwich structured
15 microfluidic perfusion chamber has been successfully used for optimization of CPA
16 loading/unloading for human umbilical vein endothelial cells (Niu et al., 2016),
17 investigation of dual dependence of transport properties of *Spodoptera frugiperda*
18 (Sf21) insect cells on temperature and CPA concentration (Wang et al., 2013),
19 measurement of transport properties of porcine adipose-derived stem cells (Wang, J.Y.
20 et al., 2014), and to explore the effect of nanoparticles on osmotic responses of pig
21 iliac endothelial cells and Sf21 cells (Yue et al., 2014).

22 23 **3.4 MEMS based microfluidic coulter counter**

24 The aforementioned microfluidic microperfusion chambers are all originally
25 designed to work under a microscope (Gao et al., 1996; Liu et al., 2015). Cell volume
26 changes are usually determined through imaging using a CCD or digital camera
27 mounted on the microscope, with the assumption that the projected area of cell can be
28 converted into its volume (McGrath, 1985; Takamatsu et al., 2004; Yoshimori and
29 Takamatsu, 2009; Zhao et al., 2012). However, these microfluidic devices are not
30 applicable for cell volume measurements for non-spherical or irregularly shaped cells,
31 *i.e.*, human erythrocytes, platelets and sperm.

32 The Coulter counter, an electrical impedance measurement tool for quantitative
33 measurements of the size and concentration of biological cells or particles
34 independent of their shapes, has been well-developed and widely used in the medical
35 and industrial fields (Nieuwenhuis et al., 2004; Zhe et al., 2007) since its invention by
36 Wallace H. Coulter in 1949 (Wallace H., 1953). The Coulter principle demonstrates
37 that a change in impedance being proportional to the volume of a particle traversing
38 an orifice will be induced by pulling the particle through the orifice concurrent with
39 an electrolyte (Wallace H., 1953; Walter R. and Wallace H., 1971). The Coulter
40 counter has become an utmost important method for characterization of cell
41 membrane transport properties in the field of cryopreservation (Agca et al., 2005;
42 Benson et al., 2005b; Ebertz and McGann, 2002, 2004; Elmoazzen et al., 2002;
43 Glazar et al., 2009; Liu et al., 1995; Liu et al., 1997; Si et al., 2006; Toupin et al.,
44 1989a, b; Woods et al., 1999). Commercially available Coulter counters are usually

costly, relatively large in size, require large sample volumes, and are unsuitable for rapid processing of samples (Wu, Y.F. et al., 2010). In addition, they do not support successive volume measurements for single cells (Sun et al., 2010). Consequently, diverse microelectromechanical system (MEMS) based microfluidic Coulter counters have been successfully developed to overcome all the disadvantages mentioned above, while retaining the features of being cost effective, low volume requirement for both cells and reagents, being compact and portable, and possible integration with other systems (Gawad et al., 2001; Jasim et al., 2015; Koch et al., 1999; McPherson and Walker, 2010; Murali et al., 2009; Nieuwenhuis et al., 2004; Rodriguez-Trujillo et al., 2008; Scott et al., 2008; Sun et al., 2010; Wood et al., 2007; Zhang, M. et al., 2012; Zhe et al., 2007; Zheng et al., 2008). Most of the microfluidic Coulter counters can only be used for cell counting and static cell sizing, and do not support successive volume measurements for single cells, since they have only a limited number of electrode pairs (Wu, Y. et al., 2010; Wu et al., 2012; Wu, Y.F. et al., 2010).

Wu et al. developed a novel MEMS Coulter counter that support continuously detecting and monitoring dynamic cell impedance changes for single cells (Wu, Y. et al., 2010; Wu et al., 2012; Wu, Y.F. et al., 2010) (Figure 6). In their design, the mixing, focusing and sensing regions are integrated into a single microdevice (Figures 6A and B), which are constituted by a 'S' shaped microchannel (Figure 6C), a ramp down vertical electrode pair (Figure 6D), and multi-electrodes with vertical sidewalls (Figures 6A and D). Theoretical analysis indicates that sufficient passive mixing with high efficiency (Figure 6E), adequate flow focusing (Figure 6F), and measurement of transient impedance changes with significantly enhanced sensitivity (benefited from the uniform electrical field over the entire height of the microchannel produced by a vertical electrode pair, Figure 6G) are all achieved by this microfluidic Coulter counter (Wu, Y. et al., 2010; Wu et al., 2012; Wu, Y.F. et al., 2010). The MEMS Coulter counter was then successfully applied for measurements of the impedance changes of yeast cells after mixing with dimethyl sulfoxide (Me₂SO) at four different temperatures (Figure 6H). This MEMS-based Coulter counter has greatly enriched the scope of microfluidic devices for characterization of cell membrane transport properties, by rapid measurements of impedance changes of cells regardless of cell shapes, while maintaining high precision and low consumption of samples and reagents.

4. Controllable addition and removal of CPAs

Optimization of CPA loading/unloading protocols based on precisely determined cell membrane transport properties, including water and CPA permeability and their activation energies, is a prerequisite for pre- and post-processing of cell suspensions prior to and after freeze-thaw cycles. Exploiting flow characteristics in microchannels, considerable efforts have focused on using microfluidics to accomplish CPA loading/unloading on a chip. Chandran and colleagues (Bala Chandran et al., 2012) investigated the influence of buoyancy-driven flow on mass transfer in a two-stream microfluidic channel, and successfully introduced the typical CPA (10% v/v DMSO) into a cell suspension (Jurkat cells) using this device (Figure 7A). Scherr and

co-workers (Scherr et al., 2013) conducted a numerical study of flow field and the concentration distribution during CPA loading into cells in a three inlet T-junction microchannel, and found that each cell has a unique path in the flow field, and thus a distinctive extracellular concentration profile (Figure 7B). For practical use with more realistic and more complicated mixtures of cryoprotectants, they then simulated loading of cryoprotectant cocktails-on-a-chip (Scherr et al., 2014a, b). Data show that the chip provided both controlled loading of CPA to minimize osmotic shock.

Lusianti and colleagues (Lusianti and Higgins, 2014) developed a microfluidic membrane device, consisting of two laser-patterned Kapton sheets, an AN69 hemodialysis membrane, and a clear acrylic housing, which could be used for continuous removal of glycerol from freeze-thawed erythrocytes (Figure 7C). Fleming et al. (Fleming et al., 2007) further developed a diffusion-based model to investigate DMSO extraction in a fully developed channel containing a washing flow parallel to a DMSO-laden cell suspension, with theoretical predictions consistent with experimental observations. Song et al. (Song et al., 2009) designed and fabricated a microfluidic device with a three-input channel ($100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m} \times 1.5\text{ m}$), and reported that the long channel offered continuous changes in CPA concentration along the length by diffusion (Figure 7D). The device allows control of loading and unloading of CPAs in the microchannel using diffusion and laminar flow. Although CPA loading/unloading of large sample volumes is challenging, microfluidic devices provide a feasible, robust, and easy-to-implement method for effectively controlling osmotic shock, and can be exploited for future uses.

Additionally, sperm cryopreservation is a critical part of assisted reproductive technology (ART) (Araki et al., 2015; Tomita et al., 2016; Zou et al., 2013). While most of the applications of microfluidics in sperm processing were focused on sorting or isolation of sperm from semen sample, there are a few studies conducted on CPA addition and removal (Cho et al., 2003; Huang et al., 2014; Li et al., 2016; Samuel et al., 2016; Sano et al., 2010; Schuster et al., 2003). Fortunately, both the designs of the staggered herringbone microfluidic mixer (Park et al., 2012) and the passive, planar micromixer based on logarithmic spirals (Scherr et al., 2015; Scherr et al., 2012) (Figure 7E), which are initially developed for sperm activation, can be potentially adopted for CPA processing, since controllable extracellular mixing is applicable in these devices.

5. Cooling and warming: Programmable freezing and vitrification

Cell-based measurement and diagnosis with microfluidic devices are increasingly being used with the implementation of freeze-thaw cycles on a chip to offer potentially seamless cell preparation and use. Cryopreservation of limited cells such as oocytes, sperm (azoospermia; severe oligozoospermia), and spermatogenic stem cells remains a challenge for traditional methods but cooling and warming on a chip may address these challenges and offer new opportunities. Also, microfluidics can be used for microscale encapsulation of cells, which allows cell vitrification with low CPA concentration (Huang et al., 2015; Zhang et al., 2013; Zhao, S.T. et al., 2014).

5.1 Controlled slow freezing and programmable freezing

Li et al. (Li et al., 2014) proposed a novel cryopreservation method for directly freezing and thawing mammalian cells on a PDMS-based microfluidic chip with a straight microchannel into which the cell suspension was injected. The chip was then packed with parafilm or placed in a small Ziploc bag and put into a centrifuge tube filled with isopropyl alcohol, and maintained in a $-80\text{ }^{\circ}\text{C}$ cryogenic refrigerator for further use (Figure 8A). With this technique, several mammalian cells were successfully cryopreserved for several days/months, including cancer cells (SKBR3 and HepG2), endothelial cells (human umbilical vein endothelial cells), and fibroblasts (3T3 cells). By supporting direct freezing and thawing cells on chip, instead of thawing and culturing cells in flasks before transferring them into chips, this approach offers the advantages of requiring minimal cell numbers and reagent volume and preparation time for on-chip cell-based experiments, and provides ready-to-use kits for on-chip cell-based experiments (Li et al., 2014).

The Toner's group (Roach et al., 2009) creatively developed a microwell array for studying cryo-responses in an array of single cells, where thousands of individual cells are seeded in a high density grid of cell-sized microwells, allowing high-throughput tracking and imaging of cells during cryopreservation. Later, Deutsch et al. and Afrimzon et al. (Afrimzon et al., 2010; Deutsch et al., 2010) introduced an individual cell-based cryo-chip (i3C) with an array of picowells (Figure 8B), enabling individual cell cryopreservation, observation, and retrieval for the first time. Furthermore, i3C is compatible with commonly used, commercially available cryostage (MDBCS 600; Linkam Instruments, Tadworth, UK), which extends its application to include programmed freezing and thawing on a chip.

Another microfluidic device, consisting of a PDMS microchamber with fluidic channels and a microheater/temperature sensor covered with an electrical insulation layer on top of the silicon substrate (Figure 8C), has been successfully used for two-step, temperature-controlled, on-chip cryopreservation of yeast cells (Li et al., 2010). Controlled freezing on the chip offers better survival (74% and 38% at temperatures of $-25\text{ }^{\circ}\text{C}$ and $-40\text{ }^{\circ}\text{C}$, respectively) compared to direct freezing with liquid nitrogen vapor (27%). Likely a more accurate temperature sensor, such as the recently reported submicrometer and nanoscale sensors (Huo et al., 2014), and feedback control on the microheaters, can further improve cell survival (Li et al., 2010).

5.2 Ultra-rapid cooling and vitrification

As an important alternative to programmed freezing, microfluidics is widely tested and has been adopted for vitreous cryopreservation. With conventional vitrification, the greatest challenge is the addition/removal of high concentration CPAs that are toxic to cells. Due to the fact that ultra-rapid cooling is technically difficult to achieve, high concentration CPAs are routinely needed to decrease the critical cooling rates for vitrification, and therefore various microfluidics have been applied for addition/removal of high concentration CPAs (Bala Chandran et al., 2012; Fleming et al., 2007; Heo et al., 2011; Park et al., 2011; Pyne et al., 2014; Song et al.,

2009). Exposure time and osmotic stress could be well controlled by slow cell shrinkage on a microfluidic platform, and current achievements with mammalian oocytes, zygotes, and embryos are promising (Lai et al., 2015a; Pyne et al., 2014).

For ultra-rapid cooling and warming, microfluidics can be used to address the major challenge to cell vitrification: devitrification and/or recrystallization during rewarming of vitrified cells (Fahy et al., 1984; Fahy and Wowk, 2015). Till now, only limited sample volumes (up to 2.5 μ L) and/or high CPA concentrations (up to 8 M) allow vitrification (Huang et al., 2015). Most recently, a nonplanar microfluidic flow-focusing device was developed to encapsulate cells in alginate hydrogel at a micrometer scale (Huang et al., 2015) (Figure 9(A)), and then cells encapsulated could be loaded into plastic straws (PS) for vitrification with only 2 M of penetrating CPAs in up to 100 times greater sample volumes (up to 250 μ L) (Figure 9B). Experiments with mouse embryonic stem cells (mESCs) and human adipose-derived stem cells (hADSCs) indicate that stress-sensitive stem cells can be vitrified using this approach with survival exceeding 80% (Figure 9C). Further studies indicate that devitrification could be effectively inhibited by alginate hydrogel microencapsulation and no significant IIF could occur in encapsulated cells during warming (Huang et al., 2015).

Because cell suspensions can be confined to a microscale, at least in one dimension such as a thin liquid film, the surface-area-to-volume ratio can be greatly increased and accordingly heat transfer can be easily and significantly enhanced. Typically, if the cell suspension is dispersed into microscale droplets, and then the cells encapsulated in the CPA droplets with appropriate sizes are ejected directly into liquid nitrogen, vitrification at low cryoprotectant concentration is possible (Demirci and Montesano, 2007; Samot et al., 2011; Song et al., 2010; Zhang, X.H. et al., 2012). For all such studies using ultra-rapid cooling, the CPA concentration involved is similar to that used in slow freezing protocols. Thus, compared to traditional freezing methods, ultra-rapid and uniform cooling is potentially available with a microfluidic platform. In cryopreservation, low-CPA or CPA-free vitrification is the ultimate objective and microfluidics platforms are promising solutions to achieve this objective (Huang et al., 2015; Zou et al., 2013). So far, microfluidics for vitrification based cryopreservation is still being explored.

6. Conclusions and Future Perspectives

Significant advances in the application of microfluidics for cryopreservation of cells while maintaining their viability and biological functionality have been achieved. However, the full potential of microfluidics for cell cryopreservation remains to be explored. Precise addition and removal of CPAs is routinely required for cell cryopreservation, and this is especially a challenge for vitrification because of the need for high concentration CPAs. Although multi-step CPA introduction and washing is currently used in vitrification, manual manipulation makes these processes labor intensive, time consuming, and lacking quality controls. Microfluidics can provide efficient and reliable methods for examining different CPAs, their concentrations and exposures as well as optimizing CPA exchange protocols by controlling exchange

1 rates and minimal and maximal cell volume values. Similarly, cooling and warming
2 on a chip is also an emerging important application for cryopreservation by controlled
3 or programmable freezing, since microfluidics can offer cell-specific temperature
4 control with high precision. Furthermore, the high surface-area-to-volume ratio of cell
5 suspension on a chip intrinsically enables rapid cooling and warming needed for
6 vitrification. More importantly, microfluidics provides an urgently needed method for
7 preserving very limited volumes of precious biological samples, as often occurs with
8 assisted reproductive technologies and stem cell research (Araki et al., 2015; Cohen
9 and Garrisi, 1997; Cohen et al., 1997; Montag et al., 1998; Rao et al., 2015; Wang et
10 al., 2016). Microfluidics can further be used to encapsulate biological agents and cells
11 in micro-scale volumes, enabling low-CPA droplet or encapsulation vitrification (Choi
12 et al., 2015a; He et al., 2008; Huang et al., 2015; Risco et al., 2007; Tasoglu et al.,
13 2013).

14 As an important future direction, microfluidics can be used for all-in-one and
15 ready-to-use experimental platforms for cell-based assays (Kondo et al., 2016).
16 Interestingly, procedures including cell culture, CPA addition, freeze-thaw cycle, CPA
17 removal, and subsequent cell viability evaluation, including cell diagnosis,
18 cytotoxicity testing, and culture and investigation, could be integrated on a single chip.
19 Thus, cryopreservation and cell-based diagnostic chips could be potentially integrated,
20 allowing a powerful tool for both diagnostic and therapeutic applications (Li et al.,
21 2014).

22 Microfluidics is also an enabling technology for fertility preservation, including
23 gamete and embryo cryopreservation (Heo et al., 2011; Pyne et al., 2014). Significant
24 progress has been made in recent years for applications of microfluidics in ART, e.g.,
25 sperm activation, sorting and isolation, oocyte processing, fertilization, and embryo
26 culture (Cho et al., 2003; de Wagenaar et al., 2016; Huang et al., 2014; Li et al., 2016;
27 Ohta et al., 2010; Samuel et al., 2016; Sano et al., 2010; Scherr et al., 2015; Schuster
28 et al., 2003; Suh et al., 2003; Tsai et al., 2010; Zhang et al., 2011b). As an important
29 and indispensable part of fertility preservation, cryopreservation on-chip has achieved
30 considerable progress, e.g., on-chip CPA-free cryopreservation of small amounts of
31 human spermatozoa (Zou et al., 2013). Single or a small number of human sperms
32 have also been successfully cryopreserved using microcapsules (Araki et al., 2015;
33 Cohen and Garrisi, 1997; Cohen et al., 1997; Montag et al., 1998). Given that
34 microfluidics is a well established method for cell encapsulation (Huang et al., 2015;
35 Koster et al., 2008; Zimmermann et al., 2007), we believe that cryopreservation of
36 gamete and embryo on-chip is an emerging area which will receive extensive
37 attention in the future.

38 Regarding the need for rapid screening of optimal cryopreservation protocols in
39 ART, such as *in situ* sperm and oocyte preservation, a microfluidic device-based
40 automatic screening system for cryopreservation is urgently needed. As mentioned
41 above, most of the individual procedures involved in ART, including sperm activation,
42 sorting and isolation, oocyte processing, fertilization, embryo culture, and gamete and
43 embryo cryopreservation are now possible to be performed on-chip (Heo et al., 2011;
44 Lai et al., 2015b; Pyne et al., 2014; Swain et al., 2013), thus the time is ripe to

1 develop a micro-total-ART system for fertility preservation, with all these functions
2 integrated on a single chip.

3 In spite of the aforementioned advantages of microfluidics for cryopreservation,
4 low production rate of cell encapsulation (by droplets, alginate hydrogel, or some
5 other biocompatible materials) and very limited handling sample volume have
6 become major roadblocks for their widespread applications at the laboratorial and
7 clinical scale (Jeong et al., 2016). Fortunately, several reports have recently shown
8 promising implementations of parallelizing a large number of microfluidic cell
9 encapsulation units and networks of microchannels onto a single chip or a chipset
10 (Korczyk et al., 2015; Mulligan and Rothstein, 2012; Tendulkar et al., 2012). In this
11 way, widespread utilization of microfluidics for cryopreservation is entirely feasible.

12 Last but certainly not least, automation is also a very important aspect in the
13 design and application of microfluidic chips for cryopreservation applications.
14 Automation of complex procedures of cryopreservation in microfluidic chips could
15 eliminate run-to-run and operator-to-operator variability in the laboratory and in
16 clinical applications, and promote process standardization by increasing consistency
17 and reliability. Integrated microfluidics for cryopreservation with a high level of
18 automation is promising, as it can lower manufacturing cost, reagent and sample
19 consumption, decrease labor intensity, increase production rate, improve ease of use,
20 offer high throughput, and may promote point-of-care cryopreservation. On-chip
21 cryopreservation is an emerging technique that may fulfill novel needs for
22 functionality and clinical applications. Highly integrated, automatic and standardized
23 microfluidic platforms will optimize cryopreservation to support technologies in
24 engineering and life sciences.

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Figure Legends

Figure 1. Basic theory for cryopreservation. (A) Cell survival corresponds to cooling rate. (B) Cooling-rate dependent cell fates during freezing. Typical thermal profiles and cell volume responses for slow programmable freezing (C) and vitrification (D). (a) Temperature profiles; (b) Cell volume excursions. (A) adapted from (Mazur, 1984) and (He, 2011) with permission; (B) Reproduced from (Zhao, G. et al., 2014) with permission; (C) and (D) adapted from (Acker, 2008) and (Zhao et al., 2016) with permission.

Figure 2. PDMS microfluidics for quantitative examination of cell osmotic responses. Mechanical block- (A) and biological settling- (B) based microfluidic chips for cell perfusion. (a–d) are schematics of the microfluidic perfusion chamber, the microperfusion system, and the typical micrograph of cells perfused in the microchannel of the two microfluidic chips, respectively. (A) Reproduced from (Chen et al., 2007; Chen et al., 2008) with permission and courtesy of Prof. Chen; (B) Reproduced from (Tseng et al., 2011) with permission and courtesy of Dr. Shu.

Figure 3. Schematics of the representative oocyte-specific microfluidic perfusion devices. (A) A two-layer PDMS device on glass, including a microfluidic network layer (lower layer) and a control layer (upper layer) to handle single oocyte for perfusion. Reproduced from (Heo et al., 2011; Lai et al., 2015) with permission. (B) A two-layer PDMS device, including a microfluidic channel layer that houses the oocytes and delivers CPA solutions, and a holding pipette layer for suction of the oocytes. Reproduced from (Lai et al., 2015a) with permission. (C) A first microfluidic device used for *in vitro* fertilization of pig oocytes. (D) A modified microfluidic device used for *in vitro* fertilization of mouse oocytes. (C) and (D): Reproduced from (Swain et al., 2013) with permission. (E) A novel microwell-structured microfluidic device that integrates single oocyte trapping, fertilization and subsequent embryo culture. Reproduced from (Han et al., 2010) with permission.

Figure 4. Measurement of cell membrane transport properties using hydrodynamic switching. (A) Cell trapping by two laminar flows with the same velocity. (B) Changing the extracellular solution by adjustment of the velocity ratio of the two flows. (C) Cell volume responses to the change in extracellular osmolality (switching time constant $\tau = 0.23$ s). (D) Relative cell volume corresponds to time during hydrodynamic switching with two different time constants. Reproduced from (Lyu et al., 2014) with permission.

Figure 5. Improved sandwich-structured microperfusion chamber for quantitative characterization of cell membrane permeability. (A) Sectional view of microchamber. (B) Transient concentration distributions in the microchannel. (C) Concentration profile of point ‘P’ during osmotic shift. (D) Micrographs for typical cell volume responses. Reproduced from (Liu et al., 2015; Niu et al., 2015; Wang et

al., 2016) with permission.

Figure 6. A MEMS-based Coulter counter for cell counting and sizing using multiple electrodes. (A) Three-dimensional schematic of the MEMS Coulter counter. (B) A complete fabricated and packaged Coulter counter with PDMS cover. (C) SEM image of the microfluidic channel for mixing and sensing. (D) SEM image of gold electroplated focusing and detection electrodes. (E) Simulation of the mixing of two dyes. (F) Simulation of electric field and its gradient distribution in the focusing electrode pair. (G) Simulated electric field in a vertical electrode pair. (H) Measurement of the impedance changes of yeast cells after mixing with dimethylsulfoxide (Me₂SO) at four different temperatures. Reproduced from (Wu, Y. et al., 2010; Wu et al., 2012) with permission.

Figure 7. Microfluidics enabled CPA loading/unloading. (A) Two-stream microfluidic device. (B) three-inlet T-junction microchannel. (C) membrane-based microfluidic device. (D) three-inlet Y-junction microchannel. (E) A sequential logarithmic microfluidic mixer for zebrafish sperm activation. Reproduced from (Bala Chandran et al., 2012; Lusianti and Higgins, 2014; Scherr et al., 2013; Song et al., 2009)(Scherr et al., 2015) with permission.

Figure 8. Microfluidics for controllable freezing and thawing. (A) On-chip direct freezing/thawing of mammalian cells. (B) Individual-cell-based microfluidic chip mounted onto a cryostage for programmed freezing/thawing of cells. (C) A microfabricated chip with an incubation microchamber, microfluidic channels, and microheaters for on-chip cell cryopreservation. Reproduced from (Afrimzon et al., 2010; Deutsch et al., 2010; Li et al., 2014; Li et al., 2010) with permission.

Figure 9. Microfluidics facilitate microencapsulation of cells for low-CPA vitreous cryopreservation (Huang et al., 2015). (A) A nonplanar microfluidic flow-focusing device for microencapsulation of cells. (B) Vitrification of VSP but not VSQ during cooling with PS, and devitrification of the vitrified VSP during warming with the PS. (C) Viability of mESCs and hADSCs with (W/) or without (W/O) alginate hydrogel encapsulation (Enap) before and after vitrification in VSP. **: $p < 0.01$. VSP: cell culture medium containing 2M PROH and 1.3M trehalose. VSQ: cell culture medium containing 1.5M PROH and 0.5M trehalose. PS: conventional plastic straw. mESCs: mouse embryonic stem cells. hADSCs: human adipose-derived stem cells.

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