Microwells support high-resolution time-lapse imaging and development of preimplanted mouse embryos

Yu-Hsiang Chung,¹ Yi-Hsing Hsiao,¹ Wei-Lun Kao,¹ Chia-Hsien Hsu,² Da-Jeng Yao,¹ and Chihchen Chen^{1,a)}

¹*Institute of Nanoengineering and Microsystems, National Tsing Hua University, 30013 Hsinchu, Taiwan*

²*Institute of Biomedical Engineering and Nanomedicine, National Health Research Institutes, 35053 Zhunan, Miaoli County, Taiwan*

(Received 31 December 2014; accepted 8 April 2015; published online 28 April 2015)

A vital aspect affecting the success rate of *in vitro* fertilization is the culture environment of the embryo. However, what is not yet comprehensively understood is the affect the biochemical, physical, and genetic requirements have over the dynamic development of human or mouse preimplantation embryos. The conventional microdrop technique often cultures embryos in groups, which limits the investigation of the microenvironment of embryos. We report an open microwell platform, which enables micropipette manipulation and culture of embryos in defined sub-microliter volumes without valves. The fluidic environment of each microwell is secluded from others by layering oil on top, allowing for non-invasive, high-resolution time-lapse microscopy, and data collection from each individual embryo without confounding factors. We have successfully cultured mouse embryos from the two-cell stage to completely hatched blastocysts inside microwells with an 89% success rate ($n=64$), which is comparable to the success rate of the contemporary practice. Development timings of mouse embryos that developed into blastocysts are statistically different to those of embryos that failed to form blastocysts (p -value $< 10^{-10}$, two-tailed Student's *t*-test) and are robust indicators of the competence of the embryo to form a blastocyst *in vitro* with 94% sensitivity and 100% specificity. Embryos at the cleavage- or blastocyst-stage following the normal development timings were selected and transferred to the uteri of surrogate female mice. Fifteen of twenty-two (68%) blastocysts and four of ten (40%) embryos successfully developed into normal baby mice following embryo transfer. This microwell platform, which supports the development of preimplanted embryos and is low-cost, easy to fabricate and operate, we believe, opens opportunities for a wide range of applications in reproductive medicine and cell biology.

© 2015 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4918642>]

I. INTRODUCTION

The use of assisted reproductive technology (ART) is rapidly expanding globally as the number of couples resorting to ART for the delivery of their baby increases. In *in vitro* fertilization (IVF), embryos developed in the laboratory are graded, selected, and transferred back to the uterus; unfortunately, the life birth rate is still below 30% after over 30 yr of marked advances in ART.¹ As a result, multiple cycles and/or multiple embryos per cycle are often required, increasing the rate of multiple gestations and imposing great economical and psychological burden to patients. Selection of the most promising embryos to implant is one of the important steps to enhance cycle outcomes. However, contemporary embryo culture and selection

^{a)} Author to whom correspondence should be addressed. Electronic mail: chihchen@mx.nthu.edu.tw.

methodologies are limited in providing the most suitable embryo for a successful pregnancy.² In addition, the optimal practice of embryo culture, duration, and grading has not yet been reached.³ It is generally considered that transferring blastocyst-stage embryo(s) offers a higher implantation rate than transferring cleavage-stage one(s).⁴ However, the occurrence of preimplantation genetic disorders also increases with the duration of extended culture of embryos.⁵ Incorporating noninvasive observation of embryo development by capturing the images with a time-lapse device may allow embryologists to be more objective in scoring embryos, and hence a better selection of embryos at earlier stages for transfer or cryopreservation.⁶

Many culture systems have been developed to support early embryo development *in vitro*. Embryos are often cultured in groups inside a microdrop, that is, several tens of microliters in volume.⁷ It is found that the rate of forming blastocysts decreases with an increased size of the microdrop.⁸ Although microdrops have long been the common practice to confine embryos to a small area, they are commonly created in a serial, pipette-based fashion, a process that is laborious and suffers from evaporation when more and/or smaller microdrops are desired. In addition, microdrops can coalesce and/or split, which alters the culture conditions and makes the tracking difficult. Hence, several culture systems, such as specialized microdrop dishes,⁹ glass oviduct,¹⁰ well-of-well (WOW),¹¹ and microfluidic devices,¹² have been developed to reduce the volume of culture medium and yielded viable blastocysts with a better consistency. However, the imaging quality of cultured embryos is often compromised due to the curved culture surface found in the microdrop, glass oviduct, and WOW techniques. The WOW and variations of microwell approaches, including microwell inserts,¹³ permit culture of single or group embryos in confined space while grant access to a larger reservoir of media and have yielded successful culture of embryos from different species, including human, mouse, pig, and cow.¹¹ The impact of the size and spacing of microwells on the autocrine/paracrine compounds and embryo development are investigated in a continuous effort. Microfluidics can offer various structures and functionalities for different applications.¹⁴ For instance, a microfluidic device mimicking the *in vivo* fertilization and reducing polyspermy has been developed and commercialized.¹⁵ Dynamic culture systems have been developed to recapitulate the *in vivo* embryo growth environment, to activate potential beneficial signaling pathways, and to disrupt gradients of molecules, such as factors released from degenerating embryos,¹⁶ ions,¹⁷ oxygen,¹⁸ nutrients,¹⁹ and waste products,²⁰ built up around embryos.²¹ Here, we report the use of microwells for culturing early mouse embryos as schematically shown in Figure 1. Embryos in suspension are seeded onto the device and allowed to sediment into microwells, which eliminates expensive costs associated with manual or robotics-based operation. Embryos remain in microwells after aspirating the excess solution from the top surface. Mineral oil is then layered on top to prevent the medium from evaporation and to spontaneously isolate culture microwells from each other. The passive confinement of embryos in an array of secluded microwells allows for time-lapse tracking as well as culturing embryos in solitary. Compared with WOW, the microwell platform reduces the manual labor involved in loading embryos sequentially using a pipette and minimizes adverse impacts from degenerating embryos if present in a separate microwell. In contrast to many microfluidic embryo culture platforms embracing closed fluidic

FIG. 1. The schematic diagram of the microwell device for the *in vitro* culture of mouse embryos. (a) Schematic cross-sectional view of the microwell device. Embryos in suspension are applied onto the device and allowed to sediment into microwells. Mineral oil is then applied on top to prevent the medium from evaporation and to isolate culture microwells from each other. (b) A picture of an actual device, in which microwells are filled with the dye solution for visualization. There are 70 microwells and each microwell is 500 μm in height, 1000 μm in diameter, and 393 nl in volume.

channels, the microwell platform is simple, easy to fabricate, and operate, allows for micropipette access, complete fidelity of embryo recovery, and time-lapse imaging on multiple embryos cultured in individual defined volumes. It also permits increased cell-surface contact, a potential beneficial factor in embryo development. We envision that the proposed microwell device can be made of other biocompatible plastic materials easily using high-throughput fabrication techniques, such as hot embossing and injection molding. These microwell devices, we believe, are user-friendly and open opportunities for a wide range of applications in reproductive medicine, cell biology, tissue engineering, stem cell biotechnology, and high-throughput pharmaceutical testing.

II. MATERIAL AND METHODS

A. Materials

For microwell fabrication, SU-8 photoresist and developer were obtained from MicroChem (Newton, MA); polydimethylsiloxane (PDMS) and curing agent were obtained from Dow Corning (Midland, MI). Phosphate buffered saline (PBS) was obtained from Mediatech (Herndon, VA). Lyophilized bovine serum albumin (BSA) and ingredients in embryo culture medium were obtained from Aldrich Chemical Co. (Milwaukee, WI). M2 medium and 2,2,2-tribromoethanol, or Avertin, were obtained from Sigma-Aldrich (St. Louis, MO). Mineral oil, OVOIL™-100, was obtained from Vitrolife AB (Goteborg, Sweden). Live/Dead viability kit was obtained from Life Technologies (Grand Island, NY).

B. Device design and fabrication

Microwells were fabricated by replica molding PDMS from the photolithographically defined SU8 photoresist master mold consisting an array of posts that were 1000 μm in diameter and 500 μm in thickness. A base and a curing agent of PDMS were mixed in 10:1 w/w ratio, poured onto the mold, and cured at 65 °C for at least 4 h to make the microwell replica. There were 70 microwells in one device and the volume of each microwell was calculated to be 393 nl as shown in Figure 1(b). The PDMS microwell replica was treated with oxygen plasma to facilitate the filling and sterilized by exposure to UV light for 20 min and 75% ethanol. Fluorescent rhodamine dye solutions were used to assess the loading procedure to confirm that no solution was remained outside the microwells (see Ref. 22 for supplementary material, Figure S1).

C. Embryo preparation, seeding, and culture

Embryos at the two-cell stage were retrieved from six-week old imprinting control region (ICR) mice provided by Animal Technology Institute Taiwan under Institutional Animal Care and Use Committee (IACUC) approved protocols (IACUC approval No. 103089). Mice were exposed to a daily photoperiod of 14-h light and 10-h dark. Female mice were injected with 5 IU pregnant mare's serum gonadotropin (PMSG) and 5 IU human chorionic gonadotropin (hCG) followed 42–48 h later. Female and male mice were placed together for mating. The appearance of vaginal plug was checked to confirm mating in the following morning. After 42–44 h from the hCG injection, female mice were sacrificed with cervical dislocation and the oviducts were flushed with M2 medium to obtain embryos at two-cell stage. Embryos from individual mice were suspended in 150 μl pre-equilibrated potassium simplex optimized medium (KSOM), applied onto separate microwell devices, and allowed to sediment into microwells. After the excess medium was aspirated carefully with a pipette, 2.5 ml mineral oil was layered on the top of microwell device. Embryos were maintained at 37 °C under humidified atmosphere with 5% CO₂ and 95% air without medium exchanges in an incubator or in a custom-made microscope stage incubator. Briefly, a Plexiglas enclosure around the body of the microscope was heated to 37 °C using an air blower. The cell culture dish was placed on a heating insert and covered with an on-stage Plexiglas box with a heater on the top to prevent condensation. The on-stage box was perfused with 5% CO₂ and 95% air heated and humidified

through a humidifier placed inside the enclosure. The viability was assessed using the acetomethoxy derivate of calcein (calcein AM) and ethidium homodimer staining.

D. Time-lapse microscopic imaging and development staging

Early mouse embryos from two-cell stage were seeded and maintained inside microwells, permitting time-lapse imaging to track the development of each embryo with good temporal resolution. Optical images were captured automatically at pre-programmed locations every 30 min for the duration of the experiment (~90 h) on a Leica DMI 6000 B microscope using a Leica DFC 310 FX digital camera. The blastocyst rate, development timing, and the morphology change of every embryos were observed and recorded. Receiver operating characteristics (ROC) curves were used to assess the prediction power of parameters for selecting embryos. The cleavage timings were used to distinguish blastocyst-competent and blastocyst-incompetent embryos. A greater cleavage-time threshold increased the sensitivity as fewer blastocyst-competent embryos were excluded; however, the specificity would decrease as blastocyst-incompetent embryos might be included as well. Embryos were also cultured in microdrops of 10 μ l in volume for comparison and were examined every 24 h.

E. Embryo transfer (ET)

Implantation was conducted using both cleavage-stage and blastocyst-stage embryos. Embryos that have developed into eight-cell stage or blastocysts after 2 days or 3.5 days in culture were selected, respectively. Pseudopregnancy was induced by placing six-week old ICR female mice with vasectomized ICR males and the vaginal plug was checked the next day. Embryos in microwells were aspirated using a glass micropipette of 350 μ m in diameter and implanted to the uterine horn of the 2.5-day pseudo-pregnant ICR mice anesthetized by the injection of 250 mg/kg avertin solution of 20 mg/ml in concentration.²³ Successful implantation was confirmed by observing obvious physical changes in the surrogate mice after 14 days and the delivery of mouse pups after 19 days of surgery.

III. RESULTS AND DISCUSSION

A. Seeding and culture of embryos in microwells

Embryos were seeded at a number of 12–28 embryos per device. The majority of the microwells were empty due to the larger number of microwell per device available than the number of embryos used. The percentages of microwells occupied by 0, 1, 2, or >2 embryos were $84 \pm 5\%$, $11 \pm 4\%$, $3 \pm 2\%$, or $2 \pm 2\%$, respectively (mean $\pm$ std, $n = 5$ independent experiments). We did not notice apparent difference in the blastocyst rate or development timing of embryos in single-embryo or multiple-embryo occupancy. We investigated and compared the development of two-cell embryos cultured in microwells and with the conventional microdrop method. Representative time-lapse micrographs of mouse embryos cultured from two-cell to hatching blastocysts stage in microwells were shown in Figure 2. The hatching rate of embryos cultured in microwells was 89% ($n = 64$), averaged from five independent experiments, where n was the total number of two-cell embryos used. Embryos were also cultured using the conventional microdrop method. Five two-cell embryos were cultured in each microdrop of 10 μ l KSOM. The blastocyst rate was 91% ($n = 115$). The blastocyst rates were summarized in Table I and shown in Figure 3. Although the blastocyst rates were similar for two culture techniques (p -value = 0.66, two-tailed Student's t -test), the results showed significant differences in blastocyst rates among embryos cultured in different microdrops, varying from 20% to 100%. In comparison, a better consistency of blastocyst rate was demonstrated using the microwell platform. It might be beneficial in confining factors released from degenerating embryos to their own isolated culture space using the microwell platform, in which an array of culture space of sub-microliter in volume could be achieved and created in parallel without pipette-based manual labor or automated equipment. Embryos successfully developed into blastocysts even when they were cultured inside microwells that were

FIG. 2. Representative time-lapse micrographs of a mouse embryo cultured from two-cell stage to hatching blastocysts stage in the microwell. Pictures of the embryo at (a) two-cell stage, (b) four-cell stage (10 h in culture), (c) eight-cell stage (21 h in culture), (d) morula stage (37 h in culture), (e) blastocyst stage (61 h in culture), and (f) completely hatched (94 h in culture). The blastocyst has come out of its zona pellucida. White arrows point to a spot near the microwell, suggesting that time-lapse images are in good registration.

TABLE I. Summary of blastocyst rates of microwell- and microdrop-based cultures of mouse embryos.

Embryo culture technique	Microwell ^a	Microdrop ^b
Blastocyst rate (blastocysts/two-cell embryos used)	15/18	5/5
	8/8	5/5
	13/15	5/5
	9/9	5/5
	12/14	4/5
		4/5
		5/5
		5/5
		5/5
		5/5
		5/5
		4/5
		2/5
		5/5
		2/5
		4/5
		5/5
		5/5
Total	57/64	105/115

^aEach data was based on the development of embryos from a single mouse.
^bEach data was based on the development of embryos inside a single microdrop.

FIG. 3. Blastocyst rates of embryos cultured in microwells and microdrops are comparable. The blastocyst rate is $89 \pm 7\%$ (mean $\pm$ std, $n = 64$ embryos obtained from five ICR mice) for microwell-based cultures, while it is $91 \pm 18\%$ (mean $\pm$ std, $n = 115$ embryos cultured in twenty-three drops of $10 \mu\text{l}$ in size) for microdrop-based cultures. The p -value is 0.66, suggesting insignificant difference in blastocyst rates between methods (two-tailed Student's t -test).

$200 \mu\text{m}$ in diameter and 16 nl in volume (unpublished results). Embryos were characterized by high viability ($>95\%$ by calcein AM, see Ref. 22 for supplementary material, Figure S2).

B. Time-lapse imaging of embryos developed in microwells

Time-lapse imaging of embryos cultured in microwells allows us to track the development of each embryo with good temporal resolution (see Ref. 22 for supplementary material, Movie S1) to provide information of improved quality and the quantity. In addition, these noninvasive observation on embryos can be conducted in less-disturbed incubation environment created by integrating the microscope and incubator into one system.²⁴ The blastocyst rate, development timing, and the morphology change of every individual embryos were observed and recorded. When zygotes developed to two-cell stage, following cleavages took place about every 12-h intervals. Embryos that successfully developed into blastocyst matched the interval time of typical cleavage time.²⁵ We went on to sort the embryos into two groups based on whether they reached the blastocyst stage at the end of culture and the results were summarized in Table II. Embryos at the early two-cell stage were used in order to increase the percentage of less

TABLE II. Differential development timing of mouse embryos.

Embryos hours in culture ^a	Development timing of mouse embryos cultured in microwells			
	4-cell stage	8-cell stage	Morula	Blastocyst
Well-developed ^b ($n = 49$)	15.4 ± 5.1^c	30.5 ± 7.8^d	46.5 ± 8.1	67.2 ± 10.4
Abnormal ^c ($n = 16$)	27.8 ± 5.5^c	58.3 ± 13.6^d	...	... (hours)

^a*In vitro* culture time from two-cell staged embryos.

^bEmbryos developed into blastocysts.

^c p -value = 10^{-11} , two-tailed Student's t -test.

^d p -value = 10^{-10} , two-tailed Student's t -test.

^eEmbryos that did not develop into blastocysts.

blastocyst-competent embryos. Note that the blastocyst rate decreased to 75% when embryos were retrieved ~ 6 h earlier than usual. The decrease of the blastocyst rate was likely due to the increased sensitivity of the embryo at earlier stage to its environment, although the impact of the imaging process could not be ruled out. Two-cell mouse embryos of inferior quality on average took ~ 12 h and ~ 28 h longer and exhibited a greater variance than the well-developed ones to develop to four-cell and eight-cell stages, respectively. Blastocyst formation *in vitro* was used as an endpoint and was counted as a positive event. The sensitivities and specificities based on the time the embryo took to develop into the four-cell and eight-cell stage were calculated. The threshold values were adjusted from 5 to 40 h and 17 to 81 h for generating the 4-cell and 8-cell ROC curves shown in Figures 4(a) and 4(b), respectively. Both ROC curves indicated that the cleavage time of the embryo could be potentially exploited as an indicator of the competence of an embryo to form blastocyst *in vitro*, allowing for the early prediction of the fate of each embryo. Embryos that reached the blastocyst stage could be predicted, with a 92% sensitivity and 94% specificity, by having a 21-h or shorter time in culture to reach the four-cell stage as the cut-off value. Similarly, embryos that reached the blastocyst stage could be predicted, with a 94% sensitivity and 100% specificity, by having a 41-h or shorter time in culture to reach the eight-cell stage as the cut-off value. Early prediction of the fate of embryos may contribute greatly to the assisted reproductive technologies by reducing the needed amount of oocytes, cycle of implantation, and duration of *in vitro* embryo culture and the associated epigenetic disruptions.

C. Implantation of embryos developed in microwells

Embryos that were cultured and developed with a typical development timing in microwells were chosen and aspirated with glass micropipettes (Figure 5(a)). Ten embryos at eight-cell stage were implanted into a surrogate mouse, while six and sixteen embryos at blastocyst stage were implanted into two surrogate mice, respectively. All three implantation surgeries resulted in successful pregnancy and the delivery of live-born mouse pups 19 days after the surgery as shown representatively in Figure 5(b), demonstrating the microwell platform support the development of preimplantation mouse embryos. The live-birth rates were 40% and 68% for eight-cell and blastocyst implantation, respectively. The results were summarized in Table III.

FIG. 4. The ROC curves based on the development timing of 2-cell embryos into (a) four-cell stage and (b) eight-cell stage, respectively. Some threshold values are indicated. The sensitivity and specificity are 92% and 94%, respectively, when the threshold value of 4-cell cleavage time is 21 h, while the sensitivity and specificity are 94% and 100%, respectively, when the threshold value of 8-cell cleavage time is 41 h. The areas under both ROC curves were close to 1 and much greater than that of a random selected case in which the ROC curve was shown as a straight line at a 45° angle.

FIG. 5. Microwells support the development of preimplanted mouse embryos and live-born pups have been delivered. (a) Well-developed embryos cultured inside microwells are selected and aspirated with a glass micropipette. (b) A representative image shows five of six implanted embryos at the blastocyst stage that have turned into healthy live-born pups.

TABLE III. Summary of live-birth rates of mouse embryos implanted at eight-cell and blastocyst stage cultured in microwells.

Stage of embryos implanted	Eight-cell	Blastocyst
Live-birth rate (live-born mouse pups/ embryos implanted)	4/10	5/6 10/16
Total	4/10	15/22

We can collect experiment data from every single embryo cultured inside the isolated microwell and observe the different morphology changes or the relationships from each other. Mineral oil applied onto the top of the open microwell platform serves as a virtual ceiling enclosing microwells while grants the convenient access of pipettes. Isolated embryo culture space allows the study of the concentrating effect of growth-promoting factors as well as metabolic and waste products. However, due to the small volumes, particular attention is required to the shift of media properties, such as pH and osmolality. Counterintuitively, it was reported that manual renewal of media at regular intervals was not beneficial in comparison to no renewal of media to embryos cultured in microdrops for 72 to 186 h.²⁶ Incorporation of time-lapse imaging in the field of IVF has provided much information about embryo development.²⁷ The combination of the embryo appearance (morphology) and the importance of when and how the cellular processes that lead to this appearance occur (kinetics) are now integrated into the unique concept of morphokinetics. At present, efforts are focused on using this information to improve embryo selection and existing success rates without losing sight of the ever-present objective of implementing a single ET strategy to avoid multiple gestations. We have identified the development timing of the embryo into four-cell and eight-cell stage as predictive morphokinetic variables for successful development to the blastocyst stage and implantation potential. Promising ET results support the embryo selection based on time-dependent markers and further results are pending to confirm the clinical validity of these morphokinetic variables.

IV. CONCLUSIONS

The open PDMS-based microwell platform developed in this study enables easy and low-cost fabrication, micropipette operation, and time-lapse microscopy. When the scale of the microwell is down to the single cell level, it is especially suitable for the study of embryo development. We have successfully long-term cultured mouse embryos from the two-cell stage to completely hatched blastocysts in microwells, which allows us to address cells in each

microwell individually. Time-lapse microscopy was used to conduct longitudinal studies on the same embryo and to correlate the fate of the embryo and its morphology and development timing, which could provide early predictions on the quality of the embryos. Selected embryos successfully developed into normal offsprings after implantation.

ACKNOWLEDGMENTS

We thank Kuei-Jung Chang for valuable assistance with animal care and surgery. This work was supported in part by the Taiwan National Science Council Grant No. NSC 101-2221-E-007-101-MY3 (CC).

- ¹C. Moutou, V. Goossens, E. Coonen, M. De Rycke, G. Kokkali, P. Renwick, S. B. SenGupta, K. Vesela, and J. Traeger-Synodinos, *Hum. Reprod.* **29**(5), 880 (2014); S. Sunderam, D. M. Kissin, S. Crawford, J. E. Anderson, S. G. Folger, D. J. Jamieson, and W. D. Barfield, *MMWR Surveill. Summ.* **62**(9), 1 (2013).
- ²T. C. Cheng, C. C. Huang, L. S. Huang, C. I. Chen, M. S. Lee, and J. Y. Liu, *Chin. J. Physiol.* **47**(1), 43 (2004).
- ³B. D. Bavister, *Hum. Reprod. Update* **1**(2), 91 (1995).
- ⁴Y. Goto, Y. Noda, M. Shiotani, J. Kishi, T. Nonogaki, and T. Mori, *J. Assisted Reprod. Genet.* **10**(3), 197 (1993).
- ⁵S. K. Kalra, S. J. Ratcliffe, K. T. Barnhart, and C. Coutifaris, *Obstet. Gynecol.* **120**(1), 69 (2012).
- ⁶S. Niimura, *J. Reprod. Dev.* **49**(6), 413 (2003).
- ⁷S. Salahuddin, S. Ookutsu, K. Goto, Y. Nakanishi, and Y. Nagata, *Hum. Reprod.* **10**(9), 2382 (1995); M. Lane and D. K. Gardner, *ibid.* **7**(4), 558 (1992); J. Moessner and W. C. Dodson, *Fertil. Steril.* **64**(5), 1034 (1995); T. Fujita, H. Umeki, H. Shimura, K. Kugumiya, and K. Shiga, *J. Reprod. Dev.* **52**(1), 137 (2006).
- ⁸S. Kito, A. Iritani, and B. D. Bavister, *Theriogenology* **47**(2), 541 (1997); R. M. Rivera and P. Rinaudo, *Mol. Reprod. Dev.* **80**(3), 184 (2013).
- ⁹D. Rieger, T. Schimmel, J. Cohen, and M. Cecchi, in *Proceedings of the 14th World Congress on In Vitro Fertilization* (2007), p. 141.
- ¹⁰G. A. Thouas, G. M. Jones, and A. O. Trounson, *Reproduction* **126**(2), 161 (2003).
- ¹¹G. Vajta, T. T. Peura, P. Holm, K. Paldi, T. Greve, A. O. Trounson, and H. Callesen, *Mol. Reprod. Dev.* **55**(3), 256 (2000); G. Vajta, T. Korosi, Y. Du, K. Nakata, S. Ieda, M. Kuwayama, and Z. P. Nagy, *Reprod. BioMed. Online* **17**(1), 73 (2008).
- ¹²T. C. Esteves, F. van Rossem, V. Nordhoff, S. Schlatt, M. Boiani, and S. Le Gac, *RSC Adv.* **3**(48), 26451 (2013); S. Raty, E. M. Walters, J. Davis, H. Zeringue, D. J. Beebe, S. L. Rodriguez-Zas, and M. B. Wheeler, *Lab Chip* **4**(3), 186 (2004); J. E. Swain, D. Lai, S. Takayama, and G. D. Smith, *ibid.* **13**(7), 1213 (2013); S. Le Gac, T. C. Esteves, F. van Rossem, A. van den Berg, and M. Boiani, *Hum. Reprod.* **26**, 1172 (2011); G. D. Smith, S. Takayama, and J. E. Swain, *Biol. Reprod.* **86**(3), 62 (2012); J. Melin, A. Lee, K. Foygel, D. E. Leong, S. R. Quake, and M. W. M. Yao, *Dev. Dyn.* **238**(4), 950 (2009); S. Raty, J. A. Davis, D. J. Beebe, S. L. Rodriguez-Zas, and M. B. Wheeler, *Theriogenology* **55**(1), 241 (2001).
- ¹³R. L. Krisher and M. B. Wheeler, *Reprod., Fertil. Dev.* **22**(1), 32 (2010); T. Somfai, Y. Inaba, Y. Aikawa, M. Ohtake, S. Kobayashi, T. Akai, H. Hattori, K. Konishi, and K. Imai, *Reprod. Domest. Anim.* **45**(6), 1104 (2010); P. J. Booth, T. J. Watson, and H. J. Leese, *Biol. Reprod.* **77**(5), 765 (2007).
- ¹⁴J. Mizuno, S. Ostrovidov, H. Nakamura, K. Akaishi, H. Inui, Y. Sakai, T. Fujii, K. Anzai, and A. Watanabe, *Hum. Reprod.* **22**, 1169 (2007); J. Mizuno, S. Ostrovidov, Y. Sakai, T. Fujii, H. Nakamura, and H. Inui, *Fertil. Steril.* **88**, S101 (2007); C. Han, Q. Zhang, R. Ma, L. Xie, T. Qiu, L. Wang, K. Mitchelson, J. Wang, G. Huang, J. Qiao, and J. Cheng, *Lab Chip* **10**(21), 2848 (2010); R. Ma, L. Xie, C. Han, K. Su, T. Qiu, L. Wang, G. Huang, W. Xing, J. Qao, J. Wang, and J. Cheng, *Anal. Chem.* **83**(8), 2964 (2011).
- ¹⁵S. G. Clark, K. Haubert, D. J. Beebe, C. E. Ferguson, and M. B. Wheeler, *Lab Chip* **5**(11), 1229 (2005).
- ¹⁶R. E. Spindler and D. E. Wildt, *Biol. Reprod.* **66**(1), 167 (2002); R. E. Spindler, E. G. Crichton, Y. Agca, N. Loskutoff, J. Critser, D. K. Gardner, and D. E. Wildt, *Theriogenology* **66**(1), 82 (2006).
- ¹⁷J. R. Trimarchi, L. Liu, P. J. S. Smith, and D. L. Keefe, *Biol. Reprod.* **63**(3), 851 (2000).
- ¹⁸J. R. Trimarchi, L. Liu, D. M. Porterfield, P. J. S. Smith, and D. L. Keefe, *Biol. Reprod.* **62**(6), 1866 (2000).
- ¹⁹M. Lane, K. Hooper, and D. K. Gardner, *J. Assisted Reprod. Genet.* **18**(9), 519 (2001); D. K. Gardner and M. Lane, *Biol. Reprod.* **48**(2), 377 (1993); M. Lane and D. K. Gardner, *J. Reprod. Fertil.* **102**(2), 305 (1994).
- ²⁰M. Lane and D. K. Gardner, *Biol. Reprod.* **69**(4), 1109 (2003); D. L. Zander, J. G. Thompson, and M. Lane, *ibid.* **74**(2), 288 (2006).
- ²¹Y. S. Heo, L. M. Cabrera, C. L. Bormann, G. D. Smith, and S. Takayama, *Lab Chip* **12**(12), 2240 (2012); Y. S. Heo, L. M. Cabrera, C. L. Bormann, C. T. Shah, S. Takayama, and G. D. Smith, *Hum. Reprod.* **25**(3), 613 (2010); E. Isachenko, R. Maettner, V. Isachenko, S. Roth, R. Kreienberg, and K. Sterzik, *Clin. Lab.* **56**(11-12), 569 (2010); V. Isachenko, R. Maettner, K. Sterzik, E. Strehler, R. Kreienberg, K. Hancke, S. Roth, and E. Isachenko, *Reprod. BioMed. Online* **22**(6), 536 (2011); Y. Mizobe, M. Yoshida, and K. Miyoshi, *J. Reprod. Dev.* **56**(2), 285 (2010); C. Y. Bae, M. S. Kim, and J. K. Park, *Biochip J.* **5**(2), 106 (2011); T. Koike, K. Matsuura, K. Naruse, and H. Funahashi, *J. Reprod. Dev.* **56**(5), 552 (2010); K. Matsuura, N. Hayashi, Y. Kuroda, C. Takiue, R. Hirata, M. Takenami, Y. Aoi, N. Yoshioka, T. Habara, T. Mukaida, and K. Naruse, *Reprod. BioMed. Online* **20**(3), 358 (2010).
- ²²See supplementary material at <http://dx.doi.org/10.1063/1.4918642> for assessing the formation of isolated embryo culture space using fluorescence-based readouts, the viability, and the time-lapse movie of cultured embryos.
- ²³T. Rulicke, A. Haenggli, K. Rappold, U. Moehrlen, and T. Stallmach, *Reprod., Fertil. Dev.* **18**(8), 885 (2006).
- ²⁴M. Meseguer, I. Rubio, M. Cruz, N. Basile, J. Marcos, and A. Requena, *Fertil. Steril.* **98**(6), 1481 (2012); I. Rubio, A. Galan, Z. Larreategui, F. Ayerdi, J. Bellver, J. Herrero, and M. Meseguer, *ibid.* **102**(5), 1287 (2014).
- ²⁵N. Saiz and B. Plusa, *Reproduction* **145**(3), R65 (2013).

²⁶J. Ali, Clin. Embryol. **7**, 1 (2004); Y. Fukui, E. S. Lee, and N. Araki, J. Animal Sci. **74**(11), 2752 (1996).

²⁷J. Conaghan, A. A. Chen, S. P. Willman, K. Ivani, P. E. Chenette, R. Boostanfar, V. L. Baker, G. D. Adamson, M. E. Abusief, M. Gvakharia, K. E. Loewke, and S. Shen, *Fertil. Steril.* **100**(2), 412 (2013); K. Kirkegaard, U. S. Kesmodel, J. J. Hindkjaer, and H. J. Ingerslev, *Hum. Reprod.* **28**(10), 2643 (2013); C. C. Wong, K. E. Loewke, N. L. Bossert, B. Behr, C. J. De Jonge, T. M. Baer, and R. A. Reijo Pera, *Nat. Biotechnol.* **28**(10), 1115 (2010); Y. H. Liu, V. Chapple, P. Roberts, and P. Matson, *Fertil. Steril.* **102**(5), 1295 (2014).