



Original

Establishment of a novel method for the production of chimeric mouse embryos using water-in-oil droplets

Hiroyuki IMAI^{1,2)}, Soichiro TSUDA³⁾, Tokuko IWAMORI^{1,4)}, Kiyoshi KANO⁵⁾, Ken Takeshi KUSAKABE²⁾ and Etsuro ONO¹⁾

¹⁾Department of Biomedicine, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

²⁾Laboratory of Veterinary Anatomy, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan

³⁾On-chip Biotechnologies Co., Ltd., 2-24-16 Naka-cho, Koganei, Tokyo 184-0012, Japan

⁴⁾Laboratory of Zoology, Graduate School of Agriculture, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan

⁵⁾Laboratory of Veterinary Developmental Biology, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan

Abstract: Production of chimeric animals is often a necessity for the generation of genetically modified animals and has gained popularity in recent years in regenerative medicine for the reconstruction of xenogeneic organs. Aggregation and injection methods are generally used to produce chimeric mice. In the aggregation method, the chimeras are produced by co-culturing embryos and stem cells, and keeping them physically adhered, although it may not be an assured method for producing chimeric embryos. In the injection method, the chimeras are produced by injecting stem cells into the zona pellucida using microcapillaries; however, this technique requires a high degree of skill. This study aimed to establish a novel method for producing chimeric embryos via water-in-oil droplets that differs from conventional methods. In this study, embryonic stem cells and embryos were successfully isolated in the droplets, and the emergence of chimeric embryos was confirmed by co-culture for 6 h. Using this method, the control and operability of stem cell numbers could be regulated, and reproducibility and quantification were improved during the production of chimeric embryos. In addition to the conventional methods for producing chimeric embryos, the novel method described here could be employed for the efficient production of chimeric animals.

Key words: chimera, droplet, embryonic stem cell, embryos

Introduction

The production of chimeric animals is often necessary for the generation of genetically modified animals and has gained popularity in recent years for the reconstruction of xenogeneic organs [1, 2]. There are two classical methods for producing chimeric animals, especially chimeric mice, using pluripotent stem cells, namely, the injection method [3] and the aggregation method [4]. The culture conditions for stem cells have been improved for the production of chimeric mice, including the use

of chemically defined media or 3i culture systems [5–7]. Previous studies have focused on the number of passages of stem cell lines and the number and ploidy of the host embryos to improve the methods used to induce chimerism [8, 9]. These studies have improved the germline transmission rate in the production of gene knockout mice. However, there are certain issues with the methods currently used for producing chimeric embryos, namely, the injection method and the aggregation method, which are discussed hereafter.

First, in the injection method, chimeric embryos are

(Received 15 May 2020 / Accepted 5 September 2020 / Published online in J-STAGE 1 October 2020)

Corresponding author: H. Imai. e-mail: himai@yamaguchi-u.ac.jp

Supplementary Figures and Table: refer to J-STAGE: <https://www.jstage.jst.go.jp/browse/expanim>



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/4.0/>>.

induced by injecting stem cells into the zona pellucida using a microcapillary. Although specific types of chimeric embryos can be induced at different stages of generation [10, 11], the use of micromanipulators is required, and the operator must be highly skilled. In particular, the success of producing chimeric embryos is influenced by the technique of the experimenter.

In the aggregation method, the embryos and stem cells are placed in small indentations on a petri dish and are allowed to remain adhered to produce chimeric embryos. This is a primitive method that can be used to produce chimeric embryos easily and inexpensively. Although several technical improvements have been proposed [12], problems remain, such as failure to produce chimeric embryos owing to the escape of embryos from the indentations on a petri dish, and difficulties in strictly controlling the number of stem cells used for producing chimeric embryos. In addition to the aforementioned injection and aggregation methods, a microaggregation method has also been developed [13], but no other methods are available for producing chimeric embryos.

To this end, the present study aimed to develop a novel method for chimeric embryo production that differs from conventional methods. Inspired by the combination of the aggregation method and the zona pellucida reconstruction method, we attempted to isolate mouse embryos and mouse embryonic stem cells within water-in-oil (W/O) droplets to keep them attached. Droplet microfluidics has been employed as an ultrahigh-throughput assay technology for a wide range of biological applications, including antibody screening [14] and single-cell RNA sequencing [15]. W/O droplets formed by microfluidic devices are generally monodisperse, which allows the high-throughput creation of millions of tiny ‘test tubes’, which are represented by individual droplets. Typically, the surfactants dissolved in the fluorinated oil maintain stable W/O droplets for over a month. In addition, the surfactants are nontoxic to mammalian cells, and the cells can therefore be cultured for up to 2 weeks [16]. By utilizing these properties of W/O droplets, we attempted to produce chimeric embryos and have described the novel method established in this study.

Materials and Methods

Generation of droplets using microfluidic device

The microfluidic droplet generator used in this study was designed using the CAD software Rhino 6 (McNeel & Associates, Seattle, WA, USA). A polymer mold was used to fabricate the droplet generator, which was prepared using a 3D stereolithography tool (Acculas SI-

C1000, D-MEC, Tokyo, Japan) that constructs three-dimensional structures layer-by-layer with epoxy-based UV curable resin (KC-1257, D-MEC) on a glass substrate. The 3D polymer mold was formed by soaking it in a solvent (EE-4210, Olympus, Tokyo, Japan) for at least 30 min, which dissolved the unreacted remnants. The polymer mold was subsequently rinsed with ethanol and dried thoroughly on a hotplate at approximately 70°C, following which parylene C was vapor-deposited on the polymer mold using a parylene C coating system (SCS Labcoater, Indianapolis, IN, USA). The droplet generator devices were fabricated with polydimethylsiloxane (PDMS, Sylgard 184, Toray Dow Corning, Tokyo, Japan) using a polymer mold by the standard fabrication technique. The cast PDMS was bonded with a glass slide to form closed microfluidic channels by treating with air plasma (PDC-32G, Harrick Plasma, Ithaca, NY, USA) for 30 s. The microfluidic channels in the devices were filled with 2% trichlorosilane (Sigma-Aldrich, St. Louis, MO, USA) in HFE7500 (3M, Saint Paul, MN, USA) to make the surface hydrophobic. The solution was removed, and the devices were baked at 120°C, which increased the hydrophobicity of the microfluidic channels.

To generate W/O emulsions, a sample solution and fluorinated oil were applied to the reservoirs of the PDMS device, and the device was set in a custom-made holder (Fig. 1A). The channels were designed to be wide enough to allow the flow of embryos and mouse embryonic stem cells (ESCs) (Fig. 1B). Air pressure was applied through nylon tubes in the holder to push liquids through the microfluidic channels, which formed droplets (Fig. 1C).

Cells and embryonic culture

EGFP-expressing mouse ESCs, which have been established and described previously [17], were cultured in ESGRO Complete plus serum-free grade medium (Merck, Burlington, NJ, USA) supplemented with 20% Knockout Serum Replacement (ThermoFisher Scientific, Tokyo, Japan). Trypsin solution (1 mmol/l EDTA·4Na, 0.25w/v%, Fujifilm-Wako, Osaka, Japan) was used for cell dissociation.

All protocols for the animal experiments were approved by the Animal Care and Use Committee of Kyushu University (protocol number: A30-304). All mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The embryos were collected from superovulated B6D2F1/Slc female mice at the 2-cell stage, at 1.5 dpc (days post-coitum) and cultured in M16 medium in an atmosphere of 5% CO₂ in air at 37°C.

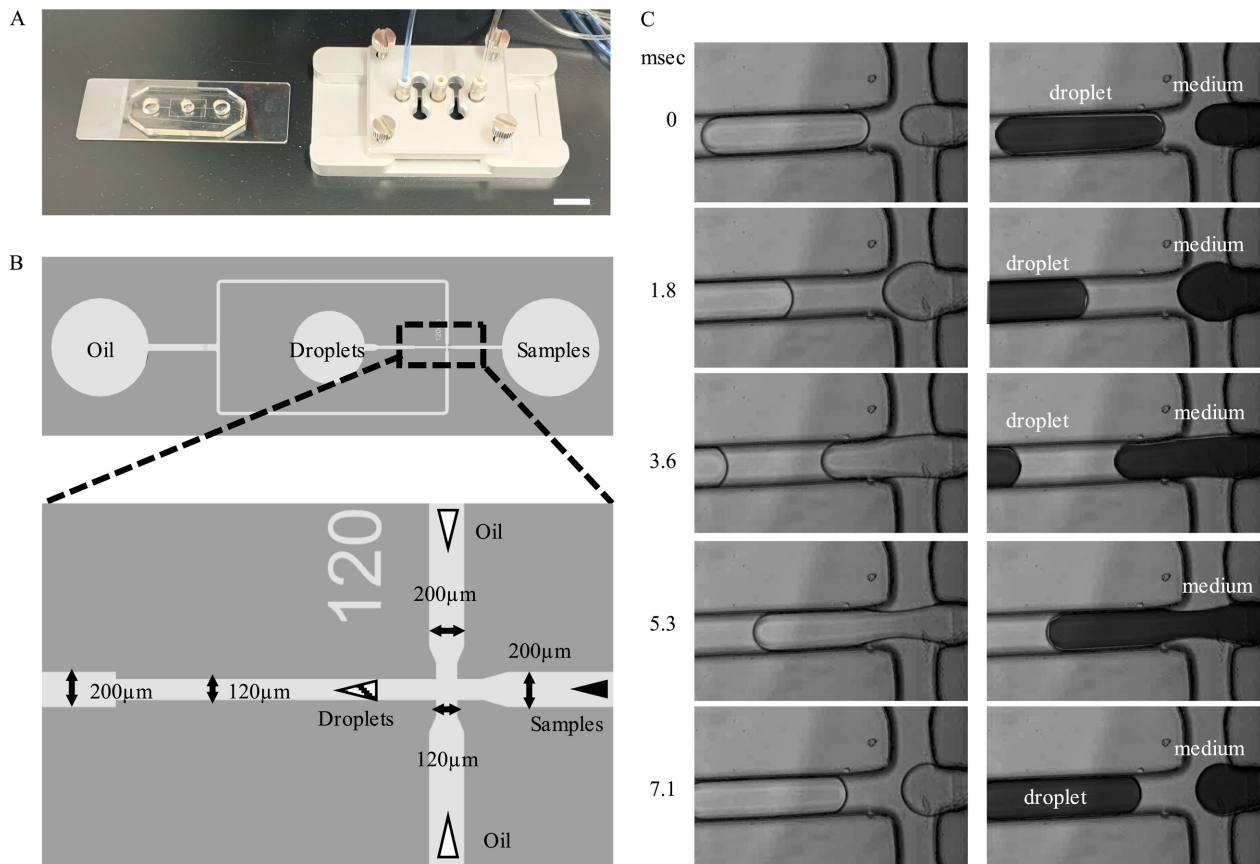


Fig. 1. Custom chip and microfluidic channel. (A) Image of custom device and device holder. See Methods for details; white bar=1 cm. (B) Design of microfluidic channels in the custom chip and a close-up image of the flow focusing junction (dotted box). The white, black, and striped arrowheads indicate the directions of oil, samples, and droplets flow, respectively. (C) Snapshots of droplets generated at the microfluidic junction. The right column emphasizes droplets and medium and shows that the channels are filled with oil.

Formation of chimeric embryos

The embryos at the morula stage were denuded with acidic Tyrode's solution (Sigma-Aldrich) and mixed with EGFP-ESCs suspension, and immediately (within ~3 min) transferred to the custom chip to generate the droplets using an On-chip Droplet Generator (On-chip Biotechnologies Co., Ltd., Koganei, Japan) at the sample pressure and an oil pressure of 8.0 kPa. Fluorinated oil (008-FluoroSurfactant in HFE7500, RAN Biotech., Inc., Beverly, MA, USA) was used in combination with surfactants at a concentration of 2.06%. The resulting emulsion was collected in 0.2 ml PCR tubes, covered with PBS, and cultured in a CO₂ incubator. After mixing the emulsion with an equal volume of 10% PFO (1H,1H,2H,2H-perfluoro-1-octanol, Fujifilm-Wako) in HFE7500, embryos were recovered from the droplets, washed with M2 medium, and subsequently cultured in M16 medium. Cell viability was measured using Propidium iodide (PI) solution (Fujifilm-Wako)

Statistical analysis

Values of $P < 0.05$ were considered statistically sig-

nificant in the Student's t -test for the survival of ESCs and in binominal tests for the survival of chimeric embryos.

Results and Discussion

Encapsulation of mouse ESCs in W/O droplets using microfluidic chips

The custom microfluidic chips were created by the method described in the Materials and Methods section (Fig. 1A). The computer-aided design (CAD) of a microfluidic channel is depicted in Fig. 1B. The width of the channel in the microfluidic chip at the droplet generation point was 120 μm, allowing embryos to pass through the channel. The cell suspension containing embryos and ESCs was broken down into multiple droplets by the fluorinated oil flowing from both sides at the junction of the channel (Fig. 1C). Approximately 150 droplets per second were generated.

EGFP-expressing mouse ESCs, which were established and described in our previous study [17], were used. The ESCs expressed EGFP and showed a naive-

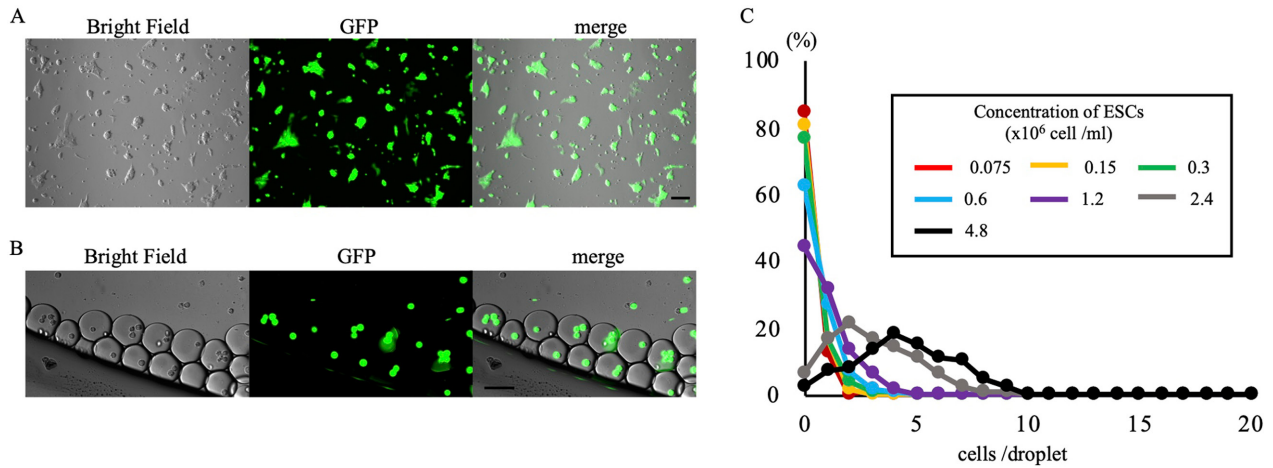


Fig. 2. Isolation of mouse ESCs in the droplets. (A) Morphology and fluorescence imaging of EGFP-expressing mouse ESCs that were subsequently used for the formation of the chimeric embryos; scale bar=100 μ m. (B) Mouse ESCs isolated in the droplets; scale bar=100 μ m. (C) Distribution of the concentrations of mouse ESCs in the suspension and the number of cells encapsulated in the droplets.

type colony morphology (Fig. 2A). After pressurizing the cultured ESCs suspension with the oil in the custom microfluidic chips, the ESCs were successfully encapsulated and isolated into the droplets (Fig. 2B). To control the number of cells that were incorporated into each droplet, droplet generation was performed by altering the concentration of mouse ESCs in the suspension. A two-fold dilution series of the suspension was prepared, ranging from 4.8×10^6 cells/ml to 0.075×10^6 cells/ml. The distribution of the number of cells isolated in the droplets using smear preparation of the generated emulsion is depicted in Fig. 2C and Supplementary Table 1. The results demonstrated that the cells could be isolated in half of the droplets at a concentration of 1.2×10^6 cells/ml, and in 90% of the droplets at concentrations of 2.4×10^6 cells/ml or higher. Subsequent experiments were performed using ESCs at a concentration of 2.4×10^6 cells/ml.

In-droplet culture of ESCs

To determine the period for which the ESCs could be cultured in the droplets, the survival rate following in-droplet culture was measured. The generated droplets were cultured in 0.2 ml PCR tubes overlaid with PBS to avoid evaporation of the volatile fluorinated oil (Supplementary Fig. 1). After culturing the droplet-encapsulated ESCs for 1 and 2 days, it was observed that most of the cells became PI-positive and did not survive (Supplementary Fig. 2A). The cell viability was therefore subsequently measured from 3 h to 12 h, reducing the culture period in the droplets to 1 day or less. It was observed that although the cell viability gradually decreased over the duration of the culture, 75% of the ESCs survived

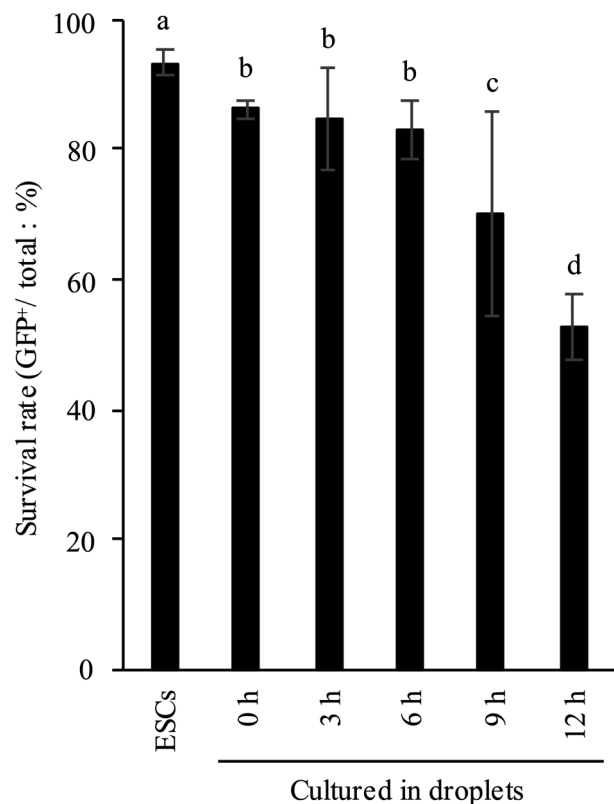


Fig. 3. In-droplet culture of mouse ESCs. Cell viability following in-droplet culture. The number of PI-positive cells was measured, indicating the dead cells, whereas the EGFP-positive cells represented the living cells; a-d, significant differences at $P < 0.05$.

for 9 h and 80% survived for 6 h (Fig. 3, Supplementary Fig. 2B). We therefore considered a culture time of 6 h sufficient to ensure a high survival rate in subsequent experiments for producing chimeric embryos. A previous

report showed that isolation of cells into droplets reduced damage to cells and did not affect subsequent cultivation [18]. Although the cause of cell death in the in-droplet culture after 1 day was unclear, it was assumed that the ESCs did not survive owing to cellular auxotrophy. As the auxotrophy of HEK cells and Jurkat cells is different, the survival rates of these immortalized cell lines in the in-droplet culture may have been different [16]. Mouse ESCs are specifically auxotrophic for substances such as methionine [19], suggesting that cell death could have been induced by the low nutrition environment due to their isolation into microdroplets. However, mechanical damage due to flowing through the microchannel also needs to be examined carefully. By modifying the continued culture of ESCs within the droplets, the method can be applied to the formation of embryoid bodies of uniform sizes [20], which may improve the quality of differentiation.

Production of chimeric embryos via droplets

A schematic diagram of the in-droplet cell culture experiment for the formation of chimeric embryos is depicted in Fig. 4A. The details of the experimental pro-

cedures are described in the Materials and Methods section. By adding the denuded embryos to the ESC suspension and pressurizing with custom chips, we succeeded in isolating the embryos and ESCs into the same droplets (Fig. 4B). Following in-droplet culture, EGFP-positive cells were detected at the morula stage (Fig. 4C). At 3.5 dpc, blastocysts with EGFP-positive cells in the inner cell mass were observed (Fig. 4D), indicating that the formation of chimeric embryos via the droplet method was successful. The production rates of chimeric embryos for the corresponding concentrations of ESC suspensions are listed in Table 1.

There are two methods for reconstructing the embryonic zona pellucida, one using agarose capsules [21] and another using sodium alginate capsules [22]. Both methods have disadvantages, such as the complexity of capsule production or the inhibition of embryonic development by the created capsules [23, 24]. In the present study, it was observed that a 6-h embryonic in-droplet culture did not result in a significant increase in embryo lethality ($P=0.19$), and 87% embryos developed into blastocysts (Table 2). In addition, the embryos could be easily recovered from the droplets by simply adding PFO solution.

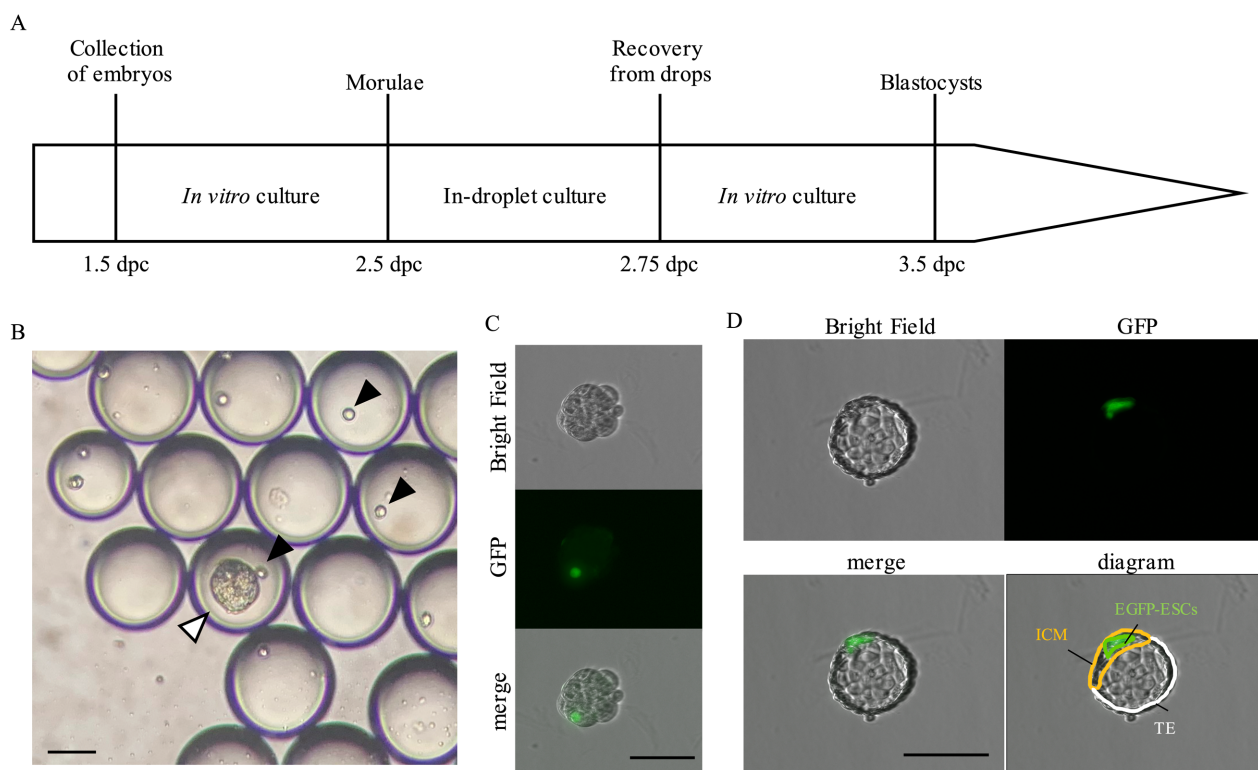


Fig. 4. Formation of mouse chimeric embryos via the water-in-oil (W/O) droplet method. (A) Schematic diagram of the experimental protocol. Morula stage embryos were co-cultured with EGFP-expressing mouse ESCs within the droplets for 6 h. Each experiment was repeated a minimum of five times. (B) The embryos at morula stage and the ESCs can be seen encapsulated in the droplets. The white arrowhead indicates the embryo at the morula stage, while the black arrowhead indicates the ESCs; scale bar=100 μm . (C) A chimeric embryo recovered from the droplets at 2.75 dpc; scale bar=100 μm . (D) Development of the recovered chimeric embryos in (C) blastocyst stage at 3.5 dpc. Diagram of the morphology of the chimeric embryos (ICM, inner cell mass; TE, trophoblast); scale bar=100 μm .

Table 1. Production efficiency of chimeric embryos

Concentration of ESCs (cell/ml)	Total number of embryos	The number of chimeric blastocysts	Percentage of chimeric embryos (%)
1.2×10^6	34	9	26 ^a
2.4×10^6	64	44	69 ^b
4.8×10^6	93	82	88 ^c

a–c, indicates the significant differences at $P < 0.05$.

Table 2. Developmental rate after isolation into droplets

Culture (No. of experiments)	The number of		Rates (%)
	Molura	Blastocyst	
indroplet (n=5)	52	45	87
control (n=10)	121	110	91

In the present study, we established a droplet method for producing chimeric embryos, which was completely different from the conventional methods that are used for the generation of chimeric embryos. The advantages of this droplet method are summarized as follows: 1) The ability to produce chimeric embryos under identical conditions. 2) Experimenter-friendly procedure that does not require prior skills by simplifying and reducing technical intervention. This method, inspired by the combination of the aggregation method and the zona pellucida reconstruction method, will provide a high-throughput platform for the production of chimeric embryos. Embryonic transfer for producing chimeric fetuses was not performed in this study due to microbiological control in the animal-rearing area. If it is possible to sterilize the microfluidic chips and the droplet generator, a series of experiments will be completed within the animal-rearing area. By improving the tolerance for sterilization of the devices, including droplet generators, this study will allow the development of simplified methods for the production of chimeric mammals. In the future, the method established in the present study should be evaluated in more detail by comparing the success rate of chimeric fetuses and chimeric individuals with the conventional methods.

Acknowledgments

This work was supported by the 40th LNest Grant and the Qdai-jump Research (QR) Program.

References

- Wu J, Platero-Luengo A, Sakurai M, Sugawara A, Gil MA, Yamauchi T, et al. Interspecies chimerism with mammalian pluripotent stem cells. *Cell*. 2017; 168: 473–486.e15. [Medline] [CrossRef]
- Yamaguchi T, Sato H, Kato-Itoh M, Goto T, Hara H, Sanbo M, et al. Interspecies organogenesis generates autologous functional islets. *Nature*. 2017; 542: 191–196. [Medline] [CrossRef]
- Gossler A, Doetschman T, Korn R, Serfling E, Kemler R. Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Proc Natl Acad Sci USA*. 1986; 83: 9065–9069. [Medline] [CrossRef]
- Wood SA, Allen ND, Rossant J, Auerbach A, Nagy A. Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature*. 1993; 365: 87–89. [Medline] [CrossRef]
- Cheng J, Dutra A, Takesono A, Garrett-Beal L, Schwartzberg PL. Improved generation of C57BL/6J mouse embryonic stem cells in a defined serum-free media. *Genesis*. 2004; 39: 100–104. [Medline] [CrossRef]
- Kiyonari H, Kaneko M, Abe S, Aizawa S. Three inhibitors of FGF receptor, ERK, and GSK3 establishes germline-competent embryonic stem cells of C57BL/6N mouse strain with high efficiency and stability. *Genesis*. 2010; 48: 317–327. [Medline]
- Yagi M, Kishigami S, Tanaka A, Semi K, Mizutani E, Wakayama S, et al. Derivation of ground-state female ES cells maintaining gamete-derived DNA methylation. *Nature*. 2017; 548: 224–227. [Medline] [CrossRef]
- Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA*. 1993; 90: 8424–8428. [Medline] [CrossRef]
- Ohta H, Sakaide Y, Yamagata K, Wakayama T. Increasing the cell number of host tetraploid embryos can improve the production of mice derived from embryonic stem cells. *Biol Reprod*. 2008; 79: 486–492. [Medline] [CrossRef]
- De Repentigny Y, Kothary R. Production of mouse chimeras by injection of embryonic stem cells into the perivitelline space of one-cell stage embryos. *Transgenic Res*. 2010; 19: 1137–1144. [Medline] [CrossRef]
- Hu M, Wei H, Zhang J, Bai Y, Gao F, Li L, et al. Efficient production of chimeric mice from embryonic stem cells injected into 4- to 8-cell and blastocyst embryos. *J Anim Sci Biotechnol*. 2013; 4: 12. [Medline] [CrossRef]
- Khillan JS, Bao Y. Preparation of animals with a high degree of chimerism by one-step coculture of embryonic stem cells and preimplantation embryos. *Biotechniques*. 1997; 22: 544–549. [Medline] [CrossRef]
- Sumiyama K, Matsumoto N, Garçon-Yoshida J, Ukai H, Ueda HR, Tanaka Y. Easy and efficient production of completely embryonic-stem-cell-derived mice using a micro-aggregation device. *PLoS One*. 2018; 13: e0203056. [Medline] [CrossRef]
- Shembekar N, Hu H, Eustace D, Merten CA. Single-cell droplet microfluidic screening for antibodies specifically binding to target cells. *Cell Rep*. 2018; 22: 2206–2215. [Medline] [CrossRef]
- Macosko EZ, Basu A, Satija R, Nemes J, Shekhar K, Goldman M, et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell*. 2015; 161: 1202–1214. [Medline] [CrossRef]
- Claussell-Tormos J, Lieber D, Baret JC, El-Harrak A, Miller OJ, Frenz L, et al. Droplet-based microfluidic platforms for

- the encapsulation and screening of Mammalian cells and multicellular organisms. *Chem Biol.* 2008; 15: 427–437. [[Medline](#)] [[CrossRef](#)]
17. Imai H, Kano K, Fujii W, Takasawa K, Wakitani S, Hiyama M, et al. Tetraploid embryonic stem cells maintain pluripotency and differentiation potency into three germ layers. *PLoS One.* 2015; 10: e0130585. [[Medline](#)] [[CrossRef](#)]
 18. Takeda K, Fujimura Y, Jimma F. Development of a new compact flow cytometer a using disposable microfluidic chip for contamination – free and biosafety measurement. *Cytometry Res.* 2011; 21: 43–50.
 19. Matsuura K, Kodama F, Sugiyama K, Shimizu T, Hagiwara N, Okano T. Elimination of remaining undifferentiated induced pluripotent stem cells in the process of human cardiac cell sheet fabrication using a methionine-free culture condition. *Tissue Eng Part C Methods.* 2015; 21: 330–338. [[Medline](#)] [[CrossRef](#)]
 20. Miyamoto D, Nakazawa K. Differentiation of mouse iPS cells is dependent on embryoid body size in microwell chip culture. *J Biosci Bioeng.* 2016; 122: 507–512. [[Medline](#)] [[CrossRef](#)]
 21. Nagatomo H, Yao T, Araki Y, Mizutani E, Wakayama T. Agarose capsules as new tools for protecting denuded mouse oocytes/embryos during handling and freezing-thawing and supporting embryonic development in vivo. *Sci Rep.* 2017; 7: 17960. [[Medline](#)] [[CrossRef](#)]
 22. Yániz JL, Santolaria P, López-Gatius F. In vitro development of bovine embryos encapsulated in sodium alginate. *J Vet Med A Physiol Pathol Clin Med.* 2002; 49: 393–395. [[Medline](#)] [[CrossRef](#)]
 23. Westhusin ME, Slapak JR, Fuller DT, Kraemer DC. Culture of agar-embedded one and two cell bovine embryos and embryos produced by nuclear transfer in the sheep and rabbit oviduct. *Theriogenology.* 1989; 31: 271. [[CrossRef](#)]
 24. Willadsen SM. A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. *Nature.* 1979; 277: 298–300. [[Medline](#)] [[CrossRef](#)]