1 Generation of Monodisperse Cell-Sized Microdroplets using a Centrifuge-Based
2 Axisymmetric Co-Flowing Microfluidic Device

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20

21 **Abstract:**

22 We report an easy-to-use generation method of biologically compatible monodisperse
23 water-in-oil microdroplets using a glass-capillary-based microfluidic device in a tabletop
24 mini-centrifuge. This device does not require complicated microfabrication; furthermore, only a
25 small sample volume is required in experiments. Therefore, we believe that this method will assist
26 biochemical and cell-biological experiments.

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28 **Main text:**

29 In recent years, cell-sized water-in-oil (W/O) microdroplets have attracted much attention
30 as powerful methods (1-5) that enable miniaturized chemical experiments, saving of chemical
31 samples, saving of experimental time, parallel and high-throughput analyses, quantitative chemical
32 experiments, and so on. These microdroplets have been applied to a wide range of biological studies
33 such as protein synthesis (6); single-molecular DNA PCR (7,8); cell encapsulation (9); and
34 simplified models of living cells called artificial cells (5,6). In general, the powerful advantages of
35 W/O microdroplets are derived by producing them monodispersely using microfluidic technologies.

36 Three methods have usually been employed for producing monodisperse W/O
37 microdroplets (10): the co-flowing method (11,12), flow-focusing method (13,14), and T-junction
38 method (15). In all these methods, microdroplets are formed in precisely fabricated microfluidic
39 channels, in which immiscible liquid flows composed of the oil phase and aqueous phase are
40 generated and spontaneous breakups of the aqueous flow generate monodisperse W/O
41 microdroplets. However, some difficulties hamper the use of microfluidic channels, such as
42 time-consuming photolithography-based fabrication of microfluidic channels in clean rooms,
43 precise control of the liquid flow in the microfluidic channels using microsyringe pumps, and
44 requirement of a large amount of biological- or chemical-reaction samples because of dead volumes

in syringe pumps and injection tubes. To be able to take advantage of W/O microdroplets, the generation process of monodisperse microdroplets needs to be simplified. Here, we report an easy method for generating monodisperse cell-sized microdroplets. This method requires just a tabletop mini-centrifuge and a capillary-based axisymmetric co-flowing microfluidic device fixed in a sampling microtube, named a centrifuge-based axisymmetric co-flowing microfluidic device. The proposed method does not need complicated fabrication, precise control of liquid, or a large sample volume when it is employed. We demonstrate the generation of monodisperse W/O microdroplets in a size-controlled manner by using the above-mentioned device. We also present cell-free protein synthesis and cell encapsulation in microdroplets and discuss the potential applicability of the proposed method to biological studies.

The centrifuge-based axisymmetric co-flowing microfluidic device (Fig. 1) is composed of two kinds of round capillaries (inner and outer capillaries), a capillary holder, and a sampling microtube. This device was developed on the basis of a centrifuge-based droplet shooting device (16). The inner capillary was inserted into the outer capillary, and both were fixed with the holder in the microtube. The holder consisted of an upper part to hold the inner capillary and a lower part to hold the outer one. The distance L between the upper and lower parts of the holder can be adjusted by screwing one of these parts (Fig. 1B). Thus, distance w (Fig. 1A) can be controlled precisely. The detailed design of the holder is shown in Fig. 1B.

The holders were fabricated by cutting a 2-mm-thick polyacetal plastic plate with a milling machine (MDX-40A, Roland DG). The glass capillaries were fabricated using a glass capillary puller (PC-10, Narishige) and a microforge (MF-900, Narishige), and the diameters of the inner and outer capillary orifices were adjusted to d_i and d_o , respectively (Fig. 1A).

For synthesis of the microdroplets, 10 μ L of hexadecane (Wako Pure Chemical Industries) containing 2% (w/w) Span 80 (Tokyo Chemical Industry) was introduced into an outer glass

capillary (1B200-6, World Precision Instruments), and the outer capillary was set in the lower part of the holder. Then, about 0.1 μL of an aqueous solution was introduced into an inner glass capillary (G-1, Narishige) by the capillary phenomenon, and the inner capillary was set in the upper part of the holder (Fig. 1C). After confirmation of insertion of the inner capillary into the outer capillary, the holder was installed in a sampling microtube, the bottom of which was filled with hexadecane containing 2% (w/w) Span 80. Then, the microtube was centrifuged using a tabletop swinging-out-type mini-centrifuge (ATT101, HITECH Co., Ltd.) at a gravity of 1600 G (rotation speed: 4200 rpm) for 2–3 s to generate microdroplets. All experiments were carried out at a room temperature.

Fig. 2A shows a microscope image of cell-sized W/O microdroplets generated by the centrifuge-based axisymmetric co-flowing microfluidic device ($d_i = 5\ \mu\text{m}$, $d_o = 80\ \mu\text{m}$, $w = 115\ \mu\text{m}$), as captured by a digital microscope (VHX-2000, Keyence). This figure shows that many W/O microdroplets were generated in a single centrifuge experiment. Fig. 2B shows digital microscopy images and size distribution histograms of the W/O microdroplets generated with this device when the diameter d_i was varied while keeping d_o and w constant at 60 μm and 115 μm , respectively. For $d_i = 5\ \mu\text{m}$, microdroplets with an average diameter of 6.6 μm (standard deviation (SD): 1.1 μm , coefficient of variation (C.V.): 17%) were generated, indicating that monodisperse W/O microdroplets were successfully obtained by the proposed method. For $d_i = 10$ and 20 μm , monodisperse W/O microdroplets with an average diameter of 8.6 μm (SD: 1.3 μm , C.V.: 15%) and 13.8 μm (SD: 1.9 μm , C.V.: 14%) were obtained respectively. Consequently, the diameters of generated droplets were comparable with the diameter of the inner capillary orifice, and their SDs were small. Fig. 2C shows a microscope image and a size distribution of W/O microdroplets generated by a tapping method; the method is a well-used generation method for microdroplets. The microdroplets were generated by 50 times hand-tapping of 50 μL of hexadecane with 2% (w/w)

Span 80 including 2 μL of water. The size distribution of microdroplets by the tapping method (average diameter: 10.7 μm , SD: 5.3 μm , C.V.: 50%) was very wider than that by our device. These results show that the diameters of the W/O microdroplets were controlled by changing d_i .

We next investigated the relationship between the diameter of the inner capillary orifice (d_i) and the diameter of the generated microdroplets under varying diameters of the outer capillary orifice (d_o) while keeping w constant at 115 μm (Fig. 2D). We found that the diameters of the generated W/O microdroplets were proportional to d_i and did not depend on d_o . In general, it is known that the diameter of a microdroplet (r) generated by droplet breaking via the Plateau–Rayleigh instability of a jetting flow of the water phase is proportional to the capillary orifice diameter (d_i) (17), that is,

$$r \sim d_i. \quad (1)$$

On the other hand, the diameter of a microdroplet generated by dripping of the water phase is proportional to the third root of the capillary orifice diameter (d_i) (16,17), that is,

$$r \sim \sqrt[3]{d_i / G}. \quad (2)$$

Thus, from the experimental results (Fig. 2D), we consider that the droplet generation mechanism by this method is droplet breaking by the Plateau–Rayleigh instability of a jetting flow.

Fig. 2E shows probabilities of generation of monodisperse microdroplets for various values of d_i and d_o , with w fixed at 115 μm . These results show that monodisperse W/O microdroplets were stably generated when d_i was about 5–20 μm and d_o was about 60–80 μm .

Finally, we demonstrated the possibility of biological application of this method (Fig. 3). Fig. 3A shows differential interference contrast microscopy (IX71, Olympus) images (upper) and fluorescence microscopy (IX71, Olympus) images of a W/O microdroplet encapsulating a cell-free protein synthesis reaction system. The W/O microdroplets were generated under the condition of $d_i = 10 \mu\text{m}$, $d_o = 60 \mu\text{m}$, and $w = 115 \mu\text{m}$. We used a gene encoding green fluorescent protein (GFP)

117 (18) and a cell-free protein synthesis reaction kit PUREfrex (GeneFrontier). A reaction solution
118 containing 1 μ L of dsDNA-encoding GFP (conc. 330 ng/ μ L), 5 μ L of feeding solution (instruction
119 manual of PUREfrex; containing main nutrients, ribonucleotides, and amino acids), 0.5 μ L of
120 various proteins in 30% glycerol (such as T7 RNA polymerase), 0.5 μ L of ribosome (20 μ M), and 3
121 μ L of MilliQ water was introduced into the inner capillary for the generation of the W/O
122 microdroplets. After 120 min incubation of the W/O microdroplets, the fluorescence in the droplets
123 was observed by laser scanning microscopy (IX-81, Olympus) (Fig. 3A). As a result, it was
124 observed that the GFP was expressed in the microdroplet and exhibited its fluorescence. This result
125 suggests that our proposed method can be applied to various types of biochemical reaction systems.

126 Fig. 3B shows a digital microscopy (VHX-2000, Keyence) image of W/O microdroplets
127 encapsulating yeast (*S. cerevisiae*) cells. In this experiment, a SD medium (D-Glucose (Wako) 20
128 g/L, Yeast Nitrogen Base w/o amino acid (Difco) 6.7 g/L) containing 23 mg/mL of dry yeast (Super
129 Camellia, Nissin Foods) was introduced into the inner capillary for the generation of W/O
130 microdroplets. As shown in Fig. 3B, 0–2 yeast cells were encapsulated in a single microdroplet. Fig.
131 3C shows superimposed images of differential interference contrast images and fluorescence
132 images of yeast cells before encapsulation and retrieved after encapsulation. By staining the yeast
133 cells with 43 μ M of propidium iodide (PI) for 15 min, dead cells fluoresced by 559 nm of excitation
134 laser light. From the experiments, we investigated cell viability (Fig. 3D). Even after the
135 encapsulation, 87% of yeast cells were alive compared to the cells before the encapsulation. These
136 results demonstrate that the proposed method can encapsulate living cells into monodisperse
137 microdroplets.

138 In this paper, we demonstrated the generation of monodisperse W/O microdroplets using
139 the centrifuge-based axisymmetric co-flowing microfluidic device. The device was constructed with
140 thin capillaries, and the centrifuge force was used for solution sending in the capillaries. Thus,

141 compared to usual microfluidic devices (1-5,10), it was easy to fabricate the device because of no
142 requirement of any photolithography equipment. In addition, since the oil and water flows were
143 produced by the centrifuge force, there were no dead volume; the dead volume is inevitable in
144 syringe pumps and tubing for solution sending in the usual devices (several to several tens μL)
145 (1-5,10). As well as them, size controllability of generated microdroplets were comparable to
146 previous microfluidic devices because SDs of generated droplet diameters in our device were
147 several or 10% of the average diameters, which were similar to those in previous devices (14). In
148 summary, the advantages of our proposed method for microdroplet synthesis are as follows: (i) easy
149 fabrication, (ii) requirement of only a small volume ($\sim 0.1 \mu\text{L}$) of the encapsulated sample solutions,
150 (iii) and size controllability in the generation of monodisperse microdroplets through a change in
151 the inner capillary orifice. These advantages widen the applicability of these microdroplets to
152 biological studies such as single-molecule analysis (7,8), single-cell enzymatic assays (9), chemical
153 microreactors (19), development of artificial biochemical reaction networks (20).

154

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215 **Figure Legends**

216 FIG. 1. Centrifuge-based axisymmetric co-flowing microfluidic device. A) Schematic illustration of
217 the device and droplet formation in it. The inner capillary is inserted into the outer capillary. The
218 aqueous phase flows in the inner capillary, and the immiscible oil phase flows around the aqueous
219 phase in the outer capillary. These flows are produced by a centrifugal gravity force. d_i is the
220 diameter of the inner capillary orifice; d_o is that of the outer capillary orifice; and w is the internal
221 diameter of the outer capillary, in which the inner capillary orifice is placed. B) Design of the
222 capillary holder made of polyacetal plastic. The holder consists of an upper part and a lower part. L
223 is the length between the upper part and lower parts. The unit of diameters in the holder is mm. C)
224 Photograph of fabricated device.

225

226 FIG. 2. A) Digital microscopy image of W/O microdroplets generated from about 0.1 μL of
227 ultrapure water using capillaries with $d_i = 5 \mu\text{m}$, $d_o = 80 \mu\text{m}$, and $w = 115 \mu\text{m}$ under a centrifugal
228 force $G = 1.6 \times 10^3 \text{ G}$. B) Microscopy images of droplets generated using capillaries of various
229 diameters, $d_i = 5$ (blue), 10 (orange), and 20 μm (green), and size distribution histograms of
230 generated microdroplets with $d_o = 60 \mu\text{m}$ and $w = 115 \mu\text{m}$ (n=50). C) Digital microscopy image and
231 size distribution of W/O microdroplets obtained by hand tapping (n=180). D) Relationship between
232 inner capillary diameters d_i and droplet diameters for varying d_o and fixed w of 115 μm . E)
233 Probabilities of generation of monodisperse microdroplets. The probability was calculated by
234 dividing the number of centrifuge experiments in which monodisperse droplets were obtained by
235 the total number of centrifuge experiments.

236

237 FIG. 3. A) Differential interference contrast microscopy images (upper) and fluorescence
238 microscope images (lower) of W/O microdroplets encapsulating a cell-free protein synthesis
239 reaction system, in which dsDNA strands encoding GFP and the PURE system were included. The
240 fluorescence of GFP increased over time ($t = 0$ and 120 min), where the microdroplets were
241 incubated at 37°C. The conditions for droplet synthesis were $d_i = 10\ \mu\text{m}$, $d_o = 60\ \mu\text{m}$, and $w = 115$
242 μm . B) Digital microscopy image of W/O microdroplets encapsulating yeast cells with SD medium.
243 The conditions of microdroplet synthesis were $d_i = 10\ \mu\text{m}$, $d_o = 60\ \mu\text{m}$, and $w = 115\ \mu\text{m}$. C)
244 Superimposed images of differential interference contrast images and fluorescence images of yeast
245 cells before encapsulation and retrieved after encapsulation into W/O microdroplets. PI only stained
246 the dead yeast cells by binding to DNA in nucleus. D) Cell viability of yeast cells before
247 encapsulation and retrieved after encapsulation into W/O microdroplets.

Figure 1

Figure 2

Figure 3