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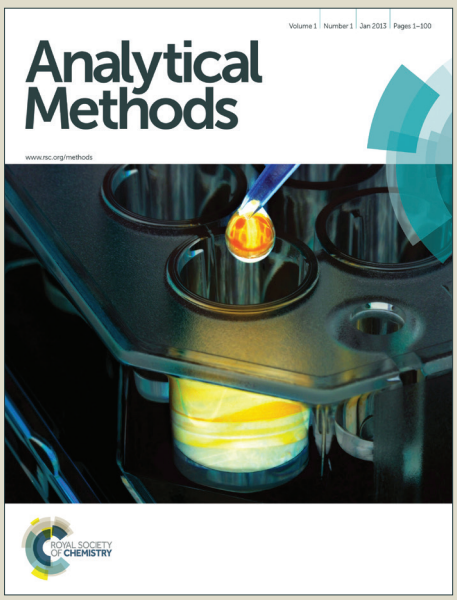

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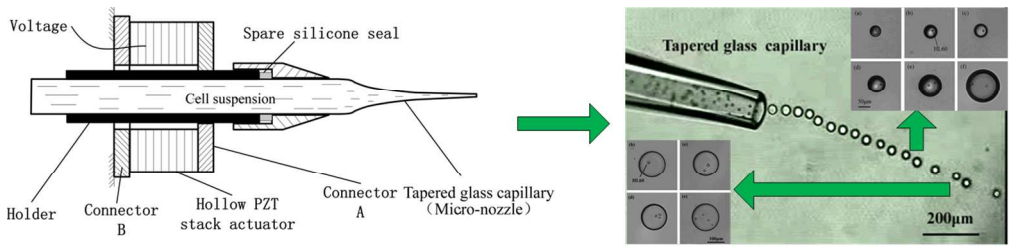

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A novel approach for encapsulating cells into monodisperse picolitre droplets actuated by microfluidic pulse inertia force

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Abstract

Encapsulation of few cells into monodisperse picolitre droplets is an extremely critical step in the process of the droplet based single cell analysis. This paper presents a novel approach, not based on micro-fluidic chip fabricated by standard soft lithography technique, for encapsulating cells into droplets actuated by microfluidic pulse inertia force. The principle of the encapsulating process is to actuate a hollow PZT stack by a signal generator and a voltage amplification to provide an enough pulse inertia force for a tapered glass capillary and the cell suspension inside to eject droplets in mineral oil and a certain number of the cells will be randomly encapsulated in monodisperse picolitre droplets. The tapered glass capillary was fabricated by glass heating process without complicated microfabrication technology. So it has the advantages of good chemical resistance, low friction, easy to manufacture and low cost. The minimum size of the spherical cell droplets can be reached is 20 μm in diameter and about 4 picolitre in volume. The percentage of the droplets with single HL60 cell can reach 42% when the droplet size is 40 μm and the concentration of the cell suspension is 1×10^8 cells per milliliter since the alternate changed pulse inertia force can make the cells well dispersed in the tapered capillary. The percentage of viable cells can be reached is 82% as determined by Trypan Blue staining when the cell droplet size is 120 μm. The experiment results present a novel strategy for droplet-based single cell analysis.

Keywords: microfluidics; droplet; PZT actuator; cell encapsulation; cell analysis;

1 Introduction

In recent years, as a subcategory of microfluidics, droplet-based microfluidics has made great process and its application in single cell analysis has attracted more and more attention [1, 2, 3]. Droplet-based single cell analysis is a method of encapsulating single cell, reagent and reaction product in a small droplet to analyze dynamic behavior [4], directed evolution of enzymes [5], protein crystallization [6], polymerase chain reaction [7] and gene detection [8] and gene expression [9] of single cells. In the droplet-based single cell analysis, each cell can be made to reside within its own picolitre-volume drop, chemically isolated from all the other drops, cell-secreted molecules rapidly achieve detectable concentrations in the confined fluid surrounding the encapsulated cell [10].

The vast majority of reported methods of encapsulating single cells into monodisperse picolitre droplets are based on micro-fluidic chip. In the micro-fluidic chips, T-junction [11, 12] and flow-focusing configuration [13, 14, 15] are frequently adopted, the shearing force of one flowing fluid against another is used and droplets are continuously generated utilizing the oil-aqueous interface instability. In the above two shear-flow-driven droplet generators, microfluidic devices (or microfluidic chips) usually fabricated with polydimethylsiloxane (PDMS) using the standard soft lithography technique are necessary[16, 17, 18]. The fluids are usually driven by syringe pumps and the size of the cell droplets can be changed by altering the fluid flow rates, the channel widths, or the relative viscosity between the two phases [19]. So, the process of loading cells into drops is purely random, the distribution is dictated by Poisson statistics [20]. To control the number of cells in droplets, Edd et al. designed a flow-focusing microfluidic chip with high aspect-ratio microchannels to controllably load cells into drops because the high aspect-ratio microchannels can cause cells to self-organize into two evenly-spaced streams [21].

The other reported encapsulating methods include droplet assembling technique and cell printing technology, which are not based on micro-fluidic chip. Droplet assembling technique [22, 23] uses a capillary sampling probe and array of horizontally

positioned micro-sample vials with a slot fabricated on the bottom of each vial. The cell suspension and the oil are in different vials, which the capillary sampling probe dips into successively. The method has high controllability on the compositions and volumes of droplets in the nanoliter to picolitre range. Cell printing technology uses droplet generators in gaseous environments [24] to place single cells encapsulated in picolitre volume on "drop-on-demand" mode to a desired location. The reported droplet generators mainly include pneumatically actuated droplet generator [25] and acoustically actuated droplet generator [26]. The droplets encapsulated with single cells ejected by cell printing technology are usually prepared for tissue printing and tissue engineering [27, 28].

In our previous work, based on microfluidic pulse inertia force we have developed a drop-on-demand droplet generator for ejecting droplets of low viscosity liquid in gaseous environment [29] and applied it to fabrication of micropellistor [30], paper micro-fluidic device [31] and PDMS microfluidic device [32]. Herein, based on microfluidic pulse inertia force, we developed a novel cell encapsulating approach without micro-fluidic chips. The droplets of cell suspension are ejected from a tapered glass capillary actuated by a hollow PZT stack, without the need of special microchannel networks or external devices.

2 Experiment

2.1 Reagents and materials

Human leukemia cell (HL60) was supplied by Shanghai Meixuan Biological Science and Technology LTD. RPMI1640 culture medium was purchased from Shanghai BioSun Sci&Tech CO., LTD. Mineral oil (M5904) was purchased from American Sigma-Aldrich Company. Span 80 was purchased from Tianjin Hengxing Chemical Preparation Co., Ltd. Trypan Blue was purchased from Beyotime Institute of Biotechnology.

2.2 Experiment apparatus

In Fig.1 (a), the assembly of the picolitre cell droplet generator based on microfluidic pulse inertia force is shown. The structure of the generator mainly

consists of five parts: (1) tapered glass capillary, (2) capillary holder, (3) hollow PZT stack actuator, (4) connector A and (5) connector B. The tapered capillary (usually referred as micro-nozzle) clamped by the capillary holder, which is fixed with the right face of the hollow PZT stack actuator (WTYDCR, CETC) through connector A while the left face is fixed with body frame and kept stationary through connector B. So, the right and left face of the actuator are called movable and stationary face respectively. There are neither micro pumps nor micro valves, which have either micro moving parts or embedded micro electric circuits, therefore significantly simplifying the structure, and decreasing the difficultness in manufacturing and the cost of the generator.

The hollow PZT stack actuator is constructed of several disc-shaped piezoelectric ceramic pieces, the thickness of which is in the range of 0.02~1 mm. There is an approximate linearity between applied voltage amplitude and the right face displacement of the actuator. So, the actuator will cause a larger displacement instantaneously and consequently provide a greater pulse inertia force for the micro-nozzle and cell suspension inside when a higher pulse driving voltage is applied. Fig. 1(b) shows the typical waveform of the driving signal and T is the driving period. The frequency of driving signal is variable from 2~256 Hz.

Glass material was chosen to make the tapered glass capillary because of several advantages, such as good chemical resistance, low friction, easy to manufacture and low cost. The raw material is borosilicate glass capillary (Beijing Zhengtianyi Scientific And Trading Co., Ltd.). The dimensions of glass capillary are 1.6 mm, 1.0 mm and 100 mm in external diameter, internal diameter and length, respectively. Glass heating process was adopted to fabricate the micro-nozzle without complicated microfabrication technology and can be divided into two steps: (1) pulling a capillary to form a micro-nozzle with straight outlet and (2) forging the straight outlet to form a shrinkage one. The detail fabrication process for the micro-nozzle was presented in literature [30]. The micro-nozzles with different outlet diameters can be obtained by varying the control parameters of the voltage amplitude and the balance weights (The outlet diameter in this article means the inner diameter of the nozzle tip). The

minimum outlet diameter of the fabricated micro-nozzle can reach as small as 10 μm. The fabricated micro-nozzle with outlet diameter of 40 μm is shown in Fig.1 (c).

2.3 Process of encapsulating cells into droplets

The driving mechanism of cell droplets formation in the mineral oil actuated by microfluidic pulse inertia force is shown in Fig.2. When being applied with a pulse voltage, the PZT actuator stretches and exerts a driving force F_1 on the solid wall of the glass micro-nozzle through the connector A. In consequence, the glass solid wall and the boundary flow obtains a movement along with the nozzle axis. Then the viscous force V_1 within the cell suspension liquid transfers the movement and the microfluidic body obtains a velocity υ. When the applied pulse voltage decreases rapidly to zero in magnitude, the PZT actuator contracts and the liquid inside the micro-nozzle obtains a pulse inertia force F_2 relative to the micro-nozzle. When the inertia force F_2 is small in magnitude, the viscous forces V_2 within the liquid is greater than the inertia force F_2, the liquid will move along with the nozzle, while, when the inertia force F_2 is larger enough in magnitude, the inertia force F_2 exceeds the viscous force V_2, a droplet of the cell suspension will be thrown out of the micro-nozzle drop by drop in the direction of the inertia force F_2 in aid of the shear force $F\tau$ from the mineral oil. The influence of pulse voltage waveform on the acceleration of micro-channel solid wall, microfluidic pulse inertia force and driving effect of microfluids had been researched in literature [33] and the waveform in Fig.1 (b) can produce a bigger microfluidic pulse inertia force which is more beneficial to droplet formation.

2.4 Encapsulation of cells in picolitre droplets

As is shown in Fig.1 (d), a cell encapsulation experimental apparatus was established, the core component of which is the cell droplet generator actuated by microfluidic pulse inertia force. The pressure regulator is used to produce a small negative pressure in the micro-nozzle and make the micro-nozzle inhaled a certain amount of cell suspension liquid. The droplet generator is fixed on a three-dimension adjusting frame through the connector B. The micro-nozzle orifice is immersed in the mineral liquid in a watch glass, which is placed on the stage of a chatelier-type

microscope (TS-100, Nikon).

In the experiment, Human leukemia cell (HL60) with concentration from 1.0×10^7 to 1.0×10^8 cells per milliliter is used. HL60 is a kind of suspension cell, which is normally used in the research of single cell encapsulation. Fig.3 (b) shows the micrograph of HL60 cells with concentration of 1.0×10^7 cells per milliliter on a glass slide. The diameter of the cells is in the range of 6~12 μm and the average diameter is about 8 μm. Mineral oil was added with 10% span 80, a kind of surfactant, to prevent the generated cell droplets from being fused.

In an additional experiment, we assessed the effect of the applied voltage amplitude and cell droplet size on the cell survival rates which was detected by Trypan Blue Staining. The cell suspension was added with 50% Trypan Blue. So, both of the cells and Trypan Blue would be capsulated in a droplet. The cell survival rates could be counted in the chatelier-type microscope in three minutes.

3 Results and Disscussion

As is shown in Fig.3 (a), the cell droplets were ejected from a tapered capillary with outlet diameter of 80 μm actuated by microfluidic pulse inertia force in mineral oil environment. One cell droplet will be produced in a pulse driving period. In other words, the droplet formation frequency is equal to the frequency of driving signal ($1/T$, shown in Fig.1 (b)), which is 2~256 Hz. So, the maximum rate of droplet generation can be reached is 256 droplets per second. Besides that, the microfluidic pulse inertia force produced by the hollow PZT stack actuator makes the cells uniformly dispersed in the micro-nozzle before being ejected. This is because the direction of pulse inertia force is of alternate change and can make the cell suspension in the micro-nozzle oscillate slightly. The well dispersion of cells is beneficial to the uniformity of cell numbers in droplets. The number of the cells encapsulated in droplets is random and may be zero, one, two or three, as is shown in Fig.3 (c).

The generation of cell droplets is actuated by microfluidic pulse inertia force produced by a piezoelectric actuator, so the size of the droplets is determined mainly by the tapered capillary outlet diameter and frequency, amplitude of the applied pulse

voltage with the invariable of the voltage waveform. Fig.4 (a) shows the variation of cell droplet size with the voltage amplitude for different diameters of capillary outlets. The frequency of the applied voltage is set at a lower value of 2 Hz. Each data point is an average of ten measurements. The hollow PZT stack actuator causes a larger displacement instantaneously and consequently provides greater pulse inertia force for the tapered capillary and cell suspension inside when a higher driving voltage is applied. So, cell suspension will be ejected drop by drop and the droplet size will increase when the voltage amplitude rises. If the voltage is low in magnitude, cell droplets won't be ejected when the micro-nozzle size is relatively small. For instance, the minimum voltage to eject droplets is 20 V for nozzle orifice diameters of 20 μm. On the other hand, when the voltage amplitude is relatively high, the inertia force was big enough to eject more mass of liquid, maybe forming satellite droplets. For instance, the maximum voltage to stably eject droplets without satellite droplets is 50 V when the capillary orifice diameter is 100 μm.

As is shown in Fig.4 (b), the cell droplet size decreased with the increase of the voltage frequency under different voltages. In Fig.4 (b), the diameter of nozzle outlet is set as 60 μm. However, when the voltage frequency is above 30 Hz, the droplets cannot be generated with the voltage amplitudes of 70 V. The reason is that it needs time for PZT stack actuator to have a response to the driving voltage signal. If the voltage amplitude is too high, the actuator will produce a larger displacement and the response time is relatively long, which will cause interference between the neighboring driving voltage periods and the produced pulse inertia force is not big enough to form droplets. The standard deviation of the cells droplets can be obtained and is less than 5%. These HL60 cells were 6~12 μm in diameter in culture whereas the generated droplets were in the range of 20~130 μm in diameter. So, the non uniform size of the cells has little influence on the monodispersity of the fabricated droplets because the cell is much smaller than the droplets. The cell droplets with different sizes are shown in Fig.5.

The number of the cells encapsulated in droplets is random. It is the concentration of the cell suspension and the droplet size that determined the average

number of cells in a droplet. Fig.6 (a) shows the variation of single cell droplet percentage with concentration of the cell suspension for different droplet sizes. Each presented data point represents an analysis of 200 droplets observed by a chatelier-type microscope. If the cell density of the suspension is relatively low (below 6×10^7 cells per milliliter), the fraction of droplets with single cell decreases with the increase of the droplet size and the fraction is relatively low (below 25%), because the number of the cells is too small and a large number of the droplets encapsulating no cells.

If the cell density of the suspension is relatively high (above 6×10^7 cells per milliliter), the fraction of droplets with single cell reaches maximum. For instance, when the cell density is 1×108 cells/mL and if the droplet size is too small (below 40μm) there will be a large number of droplet encapsulating two, three or four cells and the fraction of single cell droplet is not large enough. The droplets capsulating different numbers of cells are shown in Fig.6 (b) ~ (e). When the droplet size is 40 μm with the concentration of the HL60 cell suspension being 1×10^8 cells per milliliter, the fraction of droplets with single cell obtains the maximum value of 0.42.

In the additional experiment of cell viability, the percentage of viable cells decreased slowly with the increase of driving voltage amplitude when the cell droplet size was controlled as 80μm by adjusting the two parameters of the tapered nozzle outlet diameter and voltage frequency, as is shown in Fig.7 (a). Each presented data point represents an analysis of 100 cells observed by a chatelier-type microscope. The outlet diameter of the tapered nozzle (D) and voltage frequency (f) are shown on the top of each data point in Fig.7 (a). The higher applied pulse voltage amplitude causes a larger microfluidic pulse inertia force and a high shear stress which may have an influence on the cell viability. Despite this, the percentage of viable cells is above 75% when the driving voltage amplitude is as high as 70 V.

The percentage of viable cells increased with the increase of the cell droplet size, as is shown in Fig.7 (b). The droplet sizes range from 20 μm to 120 μm were controlled by adjusting the two parameters of the tapered nozzle outlet diameter (D) and voltage amplitude (U) while the voltage frequency (f) was set as 2Hz. The two

parameters are shown on the top of each data point in Fig.7 (b). The experiment result indicates that increasing the amount of the cell nutrient solution in a single droplet can prevent the cells from being damaged by the pulse inertia force or shear stress and is benefit to the cell viability consequently. The percentage of viable cells can be reached is 82% as determined by Trypan Blue staining when the cell droplet size is 120μm. After being stained by Trypan Blue, the living cell has an intact cell membrane while the dead cell has a destroyed membrane and its shape is irregular. The micrographs of the living cell and dead cell are shown in Fig.7 (c) and Fig.7 (d) respectively.

4 Conclusions

In summary, this paper introduced a new approach for encapsulating cells into monodisperse picolitre droplets actuated by microfluidic pulse inertia force. Different from the existent technology, this method does not need micro-fluidic chip fabricated by standard soft lithography technique. A hollow PZT stack is used to provide enough pulse inertia force for a tapered glass capillary and the cell suspension inside to eject droplets in mineral oil and a certain number of the cells will be randomly encapsulated in the droplets. The tapered glass capillary was fabricated by glass heating process without complicated microfabrication technology. So it has the advantages of good chemical resistance, low friction, easy to manufacture and low cost and it won't destroy the cells and other biological reagents during encapsulating process. The cell droplet sizes range from 20μm to 130μm and can be precisely controlled by changing the capillary outlet diameter and the frequency, amplitude of the pulse voltage signal applied on the hollow PZT stack actuator. The percentage of the droplets with single HL60 cell can be reached is 42% when the droplet size is 40 μm and the concentration of the cell suspension is 1×10^8 cells per milliliter since the alternate changed pulse inertia force can make the cells well dispersed in the micro-nozzle. The percentage of viable cells can reach 82% as determined by Trypan Blue staining when the cell droplet size is 120μm.

Acknowledgement

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Figure legend

Fig.1 Experimental apparatus: (a) sketch and photo of the droplet generator actuated by microfluidic pulse inertia force, (b) typical waveform of the driving signal on the hollow PZT stack actuator, (c) micrograph of the tapered glass capillary with outlet diameter of 40 μm fabricated by glass heating process and (d) sketch of the whole experimental system for capsulating single cells in picolitre droplets

Fig.2 Driving mechanism of cell droplets formation in an oil phase liquid actuated by microfluidic pulse inertia force

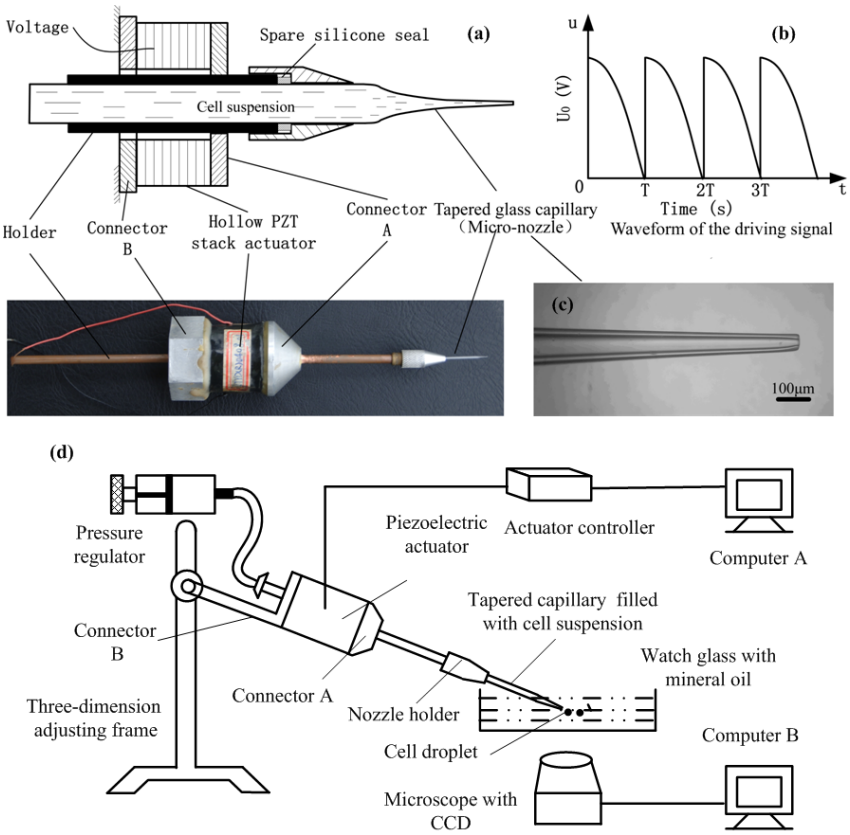
Fig.3 Encapsulating process of HL60 cells in picolitre droplets actuated by microfluidic pulse inertia force: (a) integrated graph of the encapsulating process, (b) micrograph of human leukemia cell (HL60) suspension with concentration of 1.0×10^7 cells per milliliter and (c) detail with enlarged scale of the encapsulating process

Fig.4 (a) Variation of cell droplet size with voltage amplitude for different diameters of tapered capillary outlet when the voltage frequency is 2 Hz and (b) Variation of cell droplet size with voltage frequency for different voltage amplitudes when the outlet diameter of the tapered capillary is 60 μm

Fig.5 Micrograph of picolitre droplets encapsulating single cells with different droplet sizes of: (a) 40 μm, (b) 45 μm, (c) 50 μm, (d) 60 μm, (e) 70 μm and (f) 110 μm

Fig.6 (a) Variation of single cell droplet percentage with density of the cell suspension for different droplet sizes and Micrograph of picolitre droplets encapsulating (b) single cell, (c) two cells, (d) three cells and (e) four cells

Fig.7 Cell Viability of cells encapsulated in picolitre droplets actuated by microfluidic pulse inertia force: (a) the percentage of viable HL60 cells at different driving voltage amplitudes, (b) the percentage of viable HL60 cells at different droplet sizes, (c) micrograph of the living cell after being stained by Trypan Blue and (d) micrograph of the dead cell after being stained by Trypan Blue



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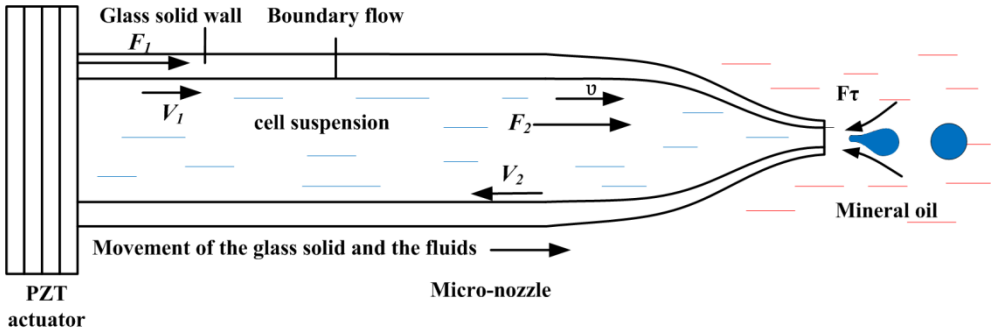

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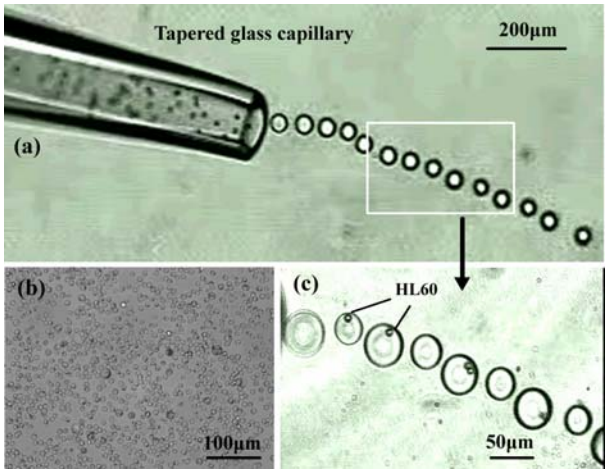

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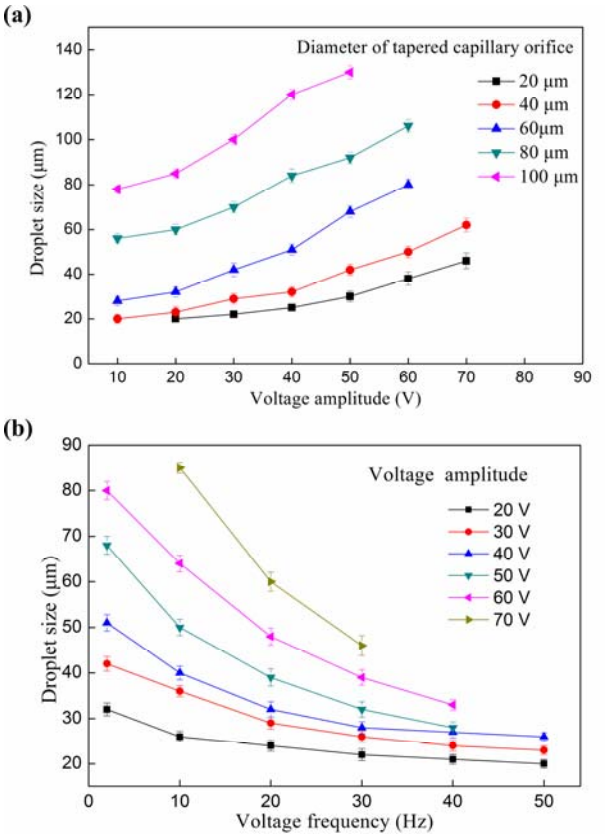

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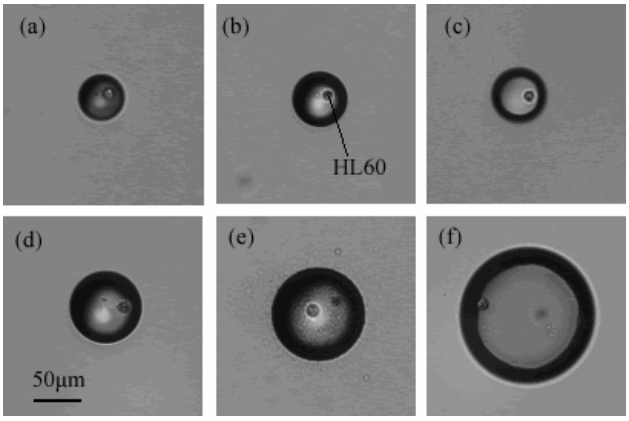

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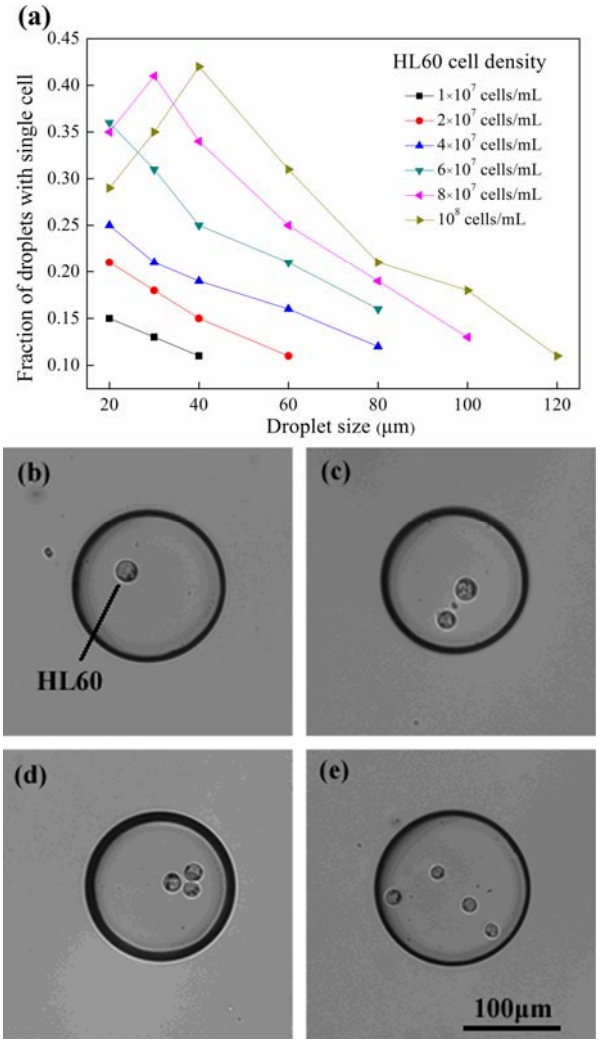

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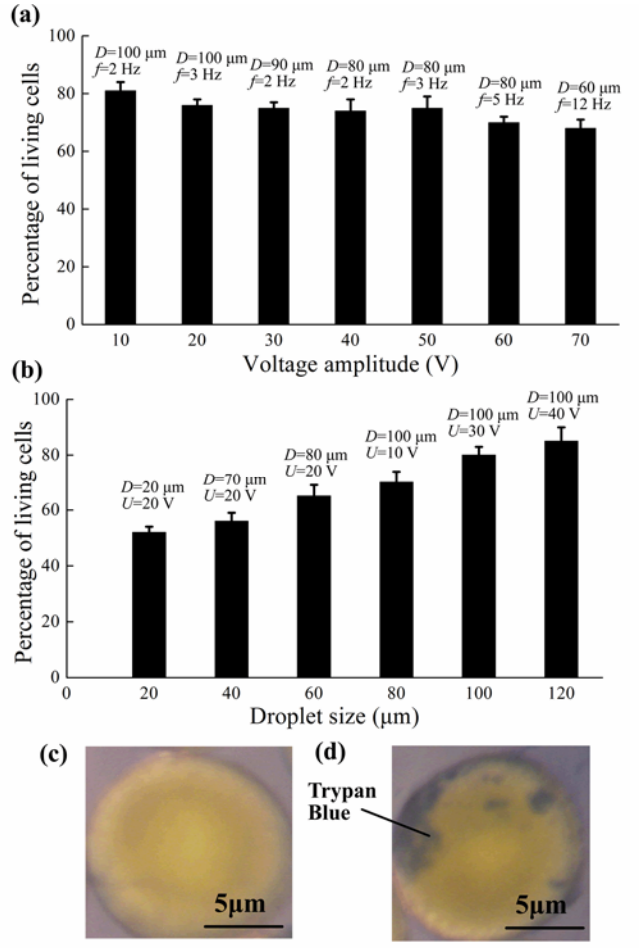

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