Lab on a Chip

TECHNICAL INNOVATION

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Spinning micropipette liquid emulsion generator for single cell whole genome amplification†

Zitian Chen,[ab] Yusi Fu,[ab] Fangli Zhang,[ab] Lu Liu,[ab] Naiqing Zhang,[a] Dong Zhou,[a] Junrui Yang,[c] Yuhong Pang[ab] and Yanyi Huang*[abd]

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Many on-chip approaches that use flow-focusing to pinch the continuous aqueous phase into droplets have become the most popular methods that provide monodisperse emulsion droplets. However, not every lab can easily adapt a microfluidic workflow into their familiar protocols. We develop an off-chip approach, spinning micro-pipette liquid emulsion (SiMPLE) generator, to produce highly stable monodisperse water-in-oil emulsions using a moving micropipette to disperse the aqueous phase in an oil-filled microcentrifuge tube. This method provides a simple way to produce picoliter-size droplets *in situ* with no dead volume during emulsification. With SiMPLE, single-cell emulsion whole genome amplification was performed to demonstrate that this novel method can seamlessly be integrated with experimental operations and supplies that most researchers are familiar with. The SiMPLE generator has effectively lowered the technical difficulties in applications relying on emulsion droplets.

Recently, single cell analysis has experienced a paradigm shift owing to the rapid progress of 'omics' technologies and high-throughput sequencing, revealing the heterogeneity and dynamics among complex biological systems.[1–10] Single cell genomic DNA (gDNA) sequencing, which has become the most promising technological breakthrough for studying single cells, needs a specific DNA amplification process to produce a large amount of replicated DNA fragments from limited copies of gDNA in a cell.[11–19] Typically, each single diploid human cell contains about 6 pg of DNA, while most sequencing library preparation protocols require input samples on the order of ng to μg. To amplify the whole genome of a single cell, the amplification method needs to be accurate to avoid generating many false positives. In addition, the amplification needs to be highly efficient to cover the major part of the whole genome. Besides, an ideal amplification should also have small amplification bias and generate uniform gain across the whole genome.

Most available methods relied on one of three amplification strategies: degenerate oligonucleotide-primed PCR (DOP-PCR),[20] multiple displacement amplification (MDA),[21] and multiple annealing and looping-based amplification cycles (MALBAC).[22] We reported a method, eWGA (emulsion Whole Genome Amplification),[23] wherein whole-genome MDA reaction separates a single cell into a large amount of water-in-oil droplets to improve the amplification performance, thus enabling identification of both small-scale copy number variations (CNVs) and high confidence single nucleotide variations (SNVs) at the same time, with significantly improved accuracy and precision. CNV and SNV are two major genomic variations that are the main research aspects in single cell genomic studies. Evenness of amplification is essential for the amplification of single cell genomic DNA for CNV identification. Through emulsions, the amplification bias between fragments has been greatly suppressed and hence enables uniform amplification across the whole genome. In the eWGA approach, a high-quality emulsion is prominent and a prerequisite. In our previous work, we applied the most widely used flow-focusing microfluidic devices[24–28] to produce an emulsion. Similar approaches have also been reported by other research groups for amplifying the whole genome of single bacteria.[29,30]

However, although microfluidic approaches are straightforward, chip fabrication and operation can be a skill barrier for inexperienced researchers. An alternative approach, which may be integrated with conventional supplies and operation skills in the majority of biology labs, is desired. It has been tested that uniformity of the emulsion droplets is critical for eWGA,[23] hence vortexing is not an ideal approach. In this communication, we report an off-chip approach to produce

[a] *Biodynamic Optical Imaging Center (BIOPIC), School of Life Sciences, and College of Engineering, Peking University, Beijing, China.*
E-mail: yanyi@pku.edu.cn
[b] *Beijing Advanced Innovation Center for Genomics (ICG), Peking University, Beijing, China*
[c] *School of Electronics Engineering and Computer Science, Peking University, Beijing, China*
[d] *Peking-Tsinghua Center for Life Sciences, Peking University, Beijing, China*
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highly uniform emulsions in conventional micro-centrifuge tubes to better popularize emulsion-based reactions. This off-chip droplet generation set-up, the spinning micro-pipette liquid emulsion (SiMPLE) generator, uses a micropipette to deliver and break aqueous solutions into monodisperse picoliter droplets in oil. The SiMPLE generator is easy to operate and can be readily adapted by researchers in the fields of chemistry and biology. It utilizes a low-cost instrument and supplies and greatly minimizes the experimental difficulties of emulsion generation, providing a universal and scalable solution to produce high-quality droplets with high throughput.

In our experimental set-up (Fig. 1), a hydrophobic-coated glass micropipette (the inner diameter of the tip is in the region of 10 μm) was attached to a load platform. The spinning was generated by a speed-controlled servo motor and an eccentric wheel, and the motion is delivered to a micropipette through a load platform, which may potentially be used for the multiplexing operation. A syringe, controlled by a syringe pump, is connected to the end of the micropipette through a flexible micro-tubing to generate a constant flow rate in the aqueous phase. The micropipette tip is immersed in the oil phase which has been preloaded in a micro-centrifuge tube. The aqueous solution is pushed out of the micropipette while it is spinning, and then the continuous phase is broken into uniform droplets. Although some satisfactory results may be achieved through a much simpler and inexpensive fashion (see the ESI†), a precisely controlled set-up as shown in Fig. 1 can produce much more robust results to better assess the capability of this approach. Once the operational parameters for an application are fixed, a simple motor driven device will work well.

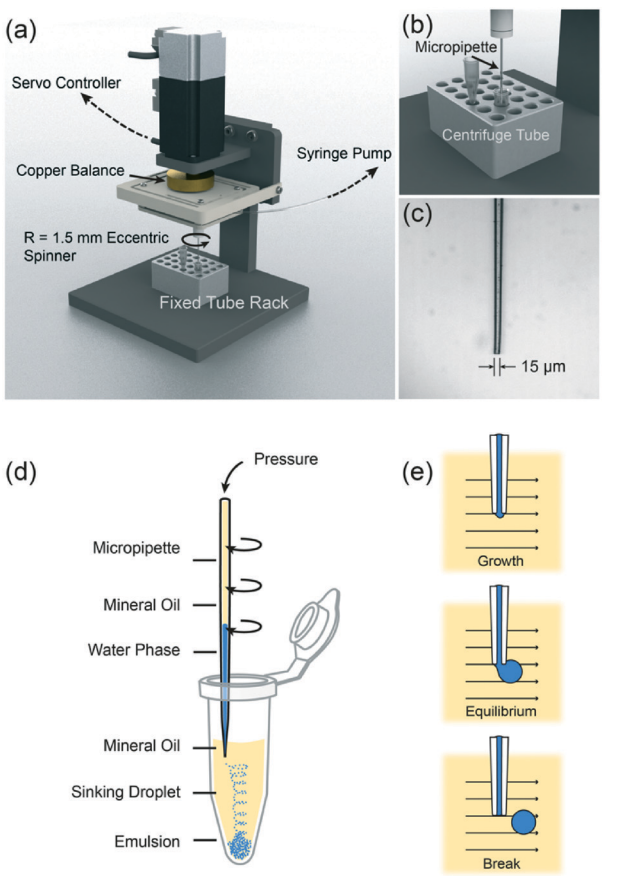

Fig. 1 Schematic illustration of a SiMPLE generator. (a) The major components of the set-up. (b) A glass micropipette with its tip in oil to produce w/o emulsion droplets. (c) A microphotograph of the glass micropipette tip. (d) SiMPLE generated aqueous droplets, with densities higher than that of oil, sinking to the bottom of the centrifuge tube. (e) Process of droplet formation.

In a previously reported method,[31] a stationary capillary with moving oil phase has been demonstrated to generate high-quality emulsion droplets. However, for eWGA, this approach would have created challenges in completely recovering all droplets in the subsequent reactions. Instead of such a configuration that combines moving oil phase with a stationary aqueous input, which is much easier to realize using a chip-based T-junction or similar formats,[32–36] the off-chip operation with microcentrifuge tubes seems to favor the SiMPLE configuration. The droplets will be generated and reacted within a single tube without transferring. This advantage greatly eliminates cross-sample contamination or the loss of materials, and is critical for single cell analysis.

To better understand the details of the droplet break-off process, we used a simple model,[31] which has been proved adequate enough in similar jetting approaches, to analyze our system (see the ESI† for details). With the aqueous phase injection, the growing drop is initially spherical, as confirmed under high-speed microscopic observation (Fig. 2), and then the drop is distorted under the force coming from the viscous flow of oil. A neck is formed on the tip of the micropipette and then the drop breaks when the interfacial tension cannot balance the other forces acting upon it.

Through the spinning motion in oil with a density lower than that of the aqueous solution, the net force on the drop growing on the tip of the micropipette is the combination of interfacial tension, viscous drag force from the relative motion of oil, buoyancy, gravity, pressure-difference induced lift force, and centrifugal force. Under our experimental conditions, only interfacial tension and drag force play the major roles in the net force and therefore are considered in the model (ESI†). The balance between these two major forces leads to a simple inverse proportionality between the droplet diameter and the relative motion velocity of the micropipette.

At a modest dispensing rate and spinning speed, all the droplets generated at the tip of the micropipette were monodispersed, and sank down toward the bottom of the micro-centrifuge tube due to their higher density compared with oil (Fig. 2). It is worth pointing out that although in principle any oil with a suitable surfactant can be used in the SiMPLE set-up, in practical application this method does not work if oil with a density higher than that of the aqueous dispersed phase is used because, in such a case, the droplets leaving the micropipette tip will be elevated and float on the top surface of the oil and consequently be smashed by the spinning

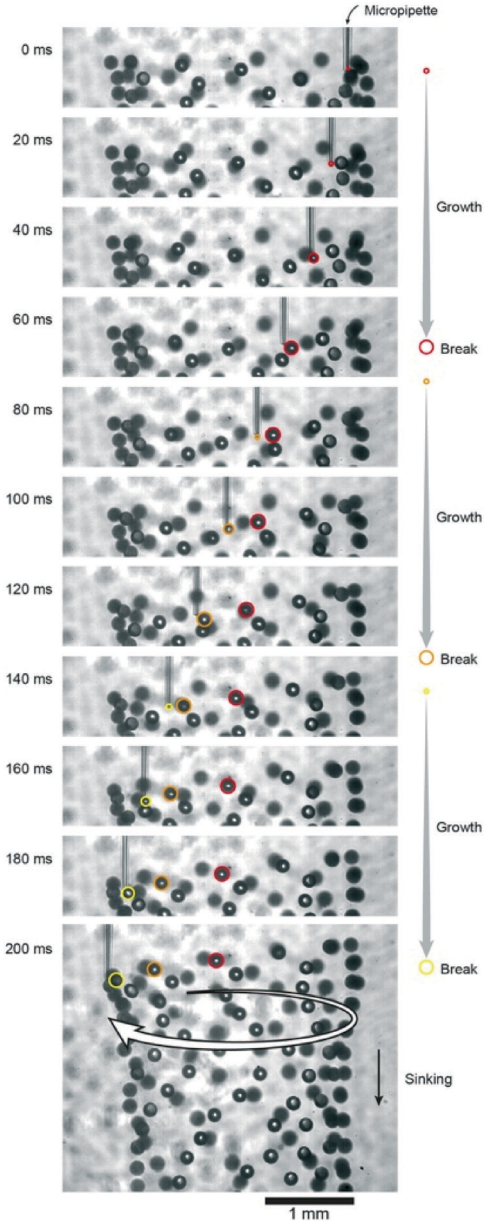

Fig. 2 Time-lapse microphotographs of droplet generation. The pictures were obtained by using a high-speed camera. A few specific droplets generated are indicated in frames.

micropipettes. When using mineral oil, we found that the dispersity of droplet size would be affected by two major experimental factors. First, the high dispensing rate of the aqueous solution ($q > 4$ μL min^{-1}) caused high polydispersity of emulsions through producing satellite droplets. Second, the high motion velocity of the micropipette (>10 cm s^{-1}) resulted in low pressure behind the micropipette tip and trapped the generated droplets near the rotation region of the tip, causing these trapped droplets to be smashed by the fast moving micropipette.

The SiMPLE generator can efficiently produce monodisperse (dispersity not more than 5%, minimum 2% achieved) emulsion droplets with diameters ranging from 25 to 230 μm (standard deviation 2–8 μm, Fig. 3). The droplet size is dependent on both the dispensing rate of the aqueous phase and the linear motion velocity of the micropipette. At a constant aqueous dispensing rate, the size of a droplet is inversely proportional to the micropipette motion velocity, as indicated by the simple model (Fig. 3c). However, we have found that the size of aqueous droplets will increase upon elevation of the aqueous dispensing flow rate, which has not been shown in the simple model. We suspect that this dependence is probably due to the post-equilibrium fluid transfer through the neck to the droplet during separation. With an empirical fitting formula, we can control the droplet size with high precision by tuning both the spinning speed (1–9 cm s^{-1}) and the dispensing rate (0.1–2 μl min^{-1}) of the micropipette.

The SiMPLE generator holds a few competitive advantages in performing biological or clinical experiments, which typically have very limited tolerance to transferring samples between reaction vials as a way of preventing cross-contamination especially in the DNAs after amplification. In addition, each micro-pipette costs only $0.2 ($49 for 225/pack), which is an insignificant amount compared to the cost of single cell WGA and sequencing; thus, it is disposable after one-time usage. To most researchers, the in-tube operation of the SiMPLE approach is convenient, and the micropipette is a common tool for experimentalists in the field of single cell analysis. More importantly, when the dispersed phase, the solution that contains the biological analytes,

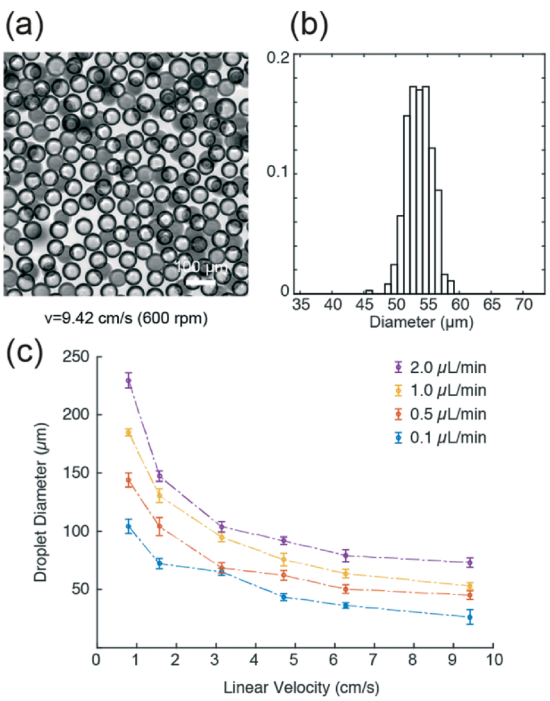

Fig. 3 The size control and dispersity of w/o emulsion droplets. (a) Monodisperse emulsion droplets generated when the micropipette motion velocity was at 9.42 cm s^{-1} (600 rpm). (b) Histogram of the diameters of droplets. (c) The relationship between droplet diameter and linear velocity of the moving micropipette.

forms droplets *in situ*, the aqueous dispersed phase will be isolated from the external environment, without the need to worry about material loss during liquid handling. The typical jetting rate of a droplet is about 200 Hz, enabling hundreds of thousands of droplets to be produced within 10 min, which is suitable for reactions that require a large number of compartments, such as digital PCR or eWGA. At room temperature, the emulsion generated by SiMPLE is very stable, without any noticeable fission or fusion for at least 10 h (see the ESI†). eWGA usually takes 8 h and the segregated reactions need to remain separated during this period.

We performed eWGA of single mouse embryonic stem cells (mESCs) using SiMPLE-generated emulsions. We manually picked single mESCs from a suspension under a microscope using a glass micropipette and lysed the single cells with 2 μl of lysis buffer. After adding 8 μl of MDA reaction buffer containing Phi-29 polymerase, random primers, and dNTP, this 10 μl mix was dispersed into an emulsion with a droplet number of about 1.5×10^5 and a diameter of 50 μm. The above operations were carried out at 4 °C to prevent the reaction from initiating during emulsion generation, which was completed within 10 min. Based on statistical averaging, each droplet contains only a few DNA fragments of the lysed single cell, significantly reducing the amplification bias of the MDA reaction.

The number of droplets was determined by keeping a balance between two considerations. One is that the number needs to be large enough, otherwise each droplet may contain too many fragments and the amplification bias within a droplet will impair the evenness of amplification. The other is that the number should not be too large, otherwise there will be many droplets that do not contain a template and produce a considerable amount of non-specifically amplified 'junk' products, wasting the sequencing cost. When all the reaction mix had been dispersed into droplets, the temperature was elevated to 30 °C and kept isothermal for 8 h. The reaction was terminated by heat inactivation of the polymerase, and the amplified product was collected by demulsification followed by purification.

Each single-cell eWGA reaction yielded a total amount of DNA of around 600 ng, indicating 10^5-fold amplification of genomic DNA in a single cell. This amplification gain is reproducible, as validated by qPCR (quantitative PCR) (see the ESI†), and is also comparable to conventional MDA reactions in a tube.[21] We amplified 10 single mESCs using eWGA with SiMPLE-generated emulsions, as well as two additional single mESCs using conventional MDA for comparison. After constructing sequencing libraries, we sequenced about 0.3G bases (1.5 M sequencing reads, 2 × 100 bp each) for each sample. Unlike conventional MDA reactions, the SiMPLE-eWGA approach shows extremely even distribution of the read coverage across the whole genome for every single cell (Fig. 4). This improvement in amplification evenness is significant, as we have described in our previous work.[23] One of the major improvements of this work is the elimination of

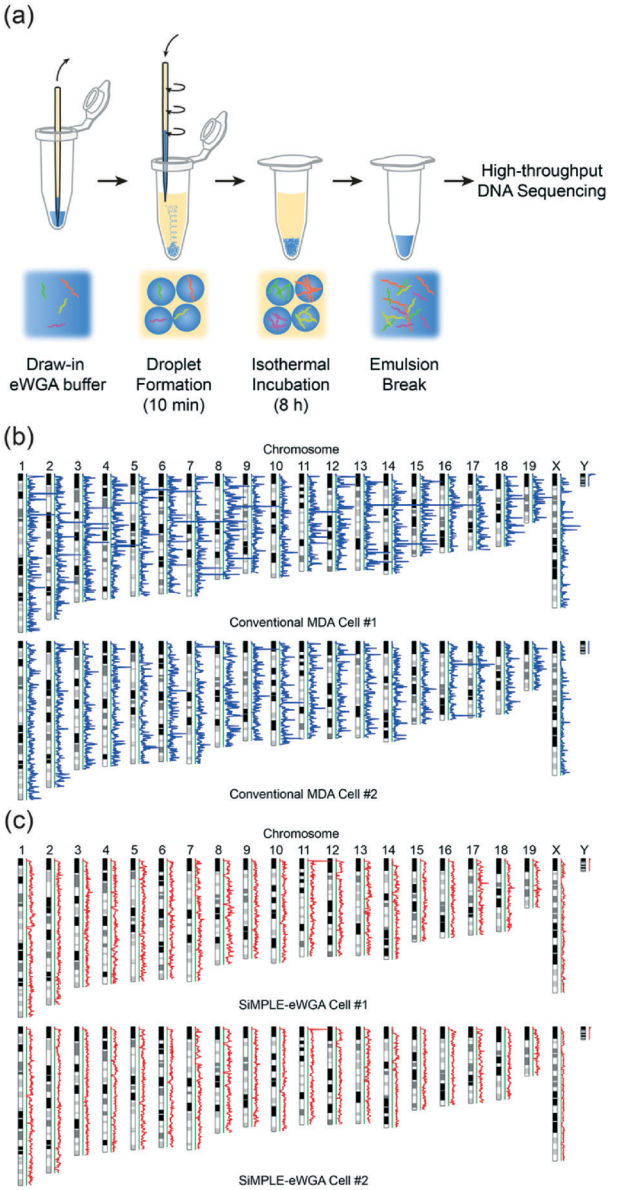

Fig. 4 Emulsion whole genome amplification (eWGA) and sequencing results of single mouse embryo stem cells. (a) Schematic illustration of the eWGA experimental process. (b and c) Comparison of the coverage profiles of mESC genome sequencing using different amplification methods (conventional MDA (b) and SiMPLE-eWGA (c)) with 1.5 M reads per sample. The distribution of the read coverage shows greatly improved evenness for the SiMPLE-eWGA approach.

microfluidic devices and the non-ideal interface between 'world' and the chip.[37]

In summary, we have developed a novel monodisperse emulsion generation technique, SiMPLE, which uses a moving micropipette in oil to dispense the continuous aqueous phase into uniform and stable emulsion droplets. Compared to other emulsion generation methods typically based on microfluidic approaches, this SiMPLE generator greatly simplifies the experimental set-up and operation procedure, and rules out sample loss or contamination during liquid transfer. In addition, the size of monodisperse picoliter droplets

can be precisely regulated by the flow rate of the continuous phase and the motion velocity of the micropipette tip. We demonstrate single cell eWGA with SiMPLE-generated emulsions, which has greatly lowered the experimental difficulties for single cell genomic studies. With further improvements in the engineering of multiple micropipette platforms and integration with other biological assay chemistries, we believe this technique will become achievable in more emulsion-based reactions in biological and chemical research studies.

Acknowledgements

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Electronic Supplementary Material (ESI) for Lab on a Chip.

Supporting Information

Spinning micro-pipette liquid emulsion generator for single cell whole genome amplification

Zitian Chen,[a,b] Yusi Fu,[a,b] Fangli Zhang,[a,b] Lu Liu,[a,b] Naiqing Zhang,[a] Dong Zhou,[a] Junrui Yang,[c] Yuhong Pang,[a,b] and Yanyi Huang*[,a,b,d]

[a] Biodynamic Optical Imaging Center (BIOPIC), School of Life Sciences, and College of Engineering, Peking University, Beijing, China

[b] Beijing Advanced Innovation Center for Genomics (ICG), Peking University, Beijing, China

[c] School of Electronics Engineering and Computer Science, Peking University, Beijing, China

[d] Peking-Tsinghua Center for Life Sciences, Peking University, Beijing, China

* Corresponding author. E-mail: yanyi@pku.edu.cn

Part I. Materials and methods

1. Experimental setup of SiMPLE generator and protocol of w/o emulsions generation.

A glass micropipette is attached to a load platform (Figure S1). The platform, made of polyoxymethylene (POM), is connected to a speed-controlled servo motor (YZ-ACSD608) through an eccentric wheel, made of copper. The eccentric distance, i.e. the rotation radius of the glass micropipette tube, is 1.5 mm. Glass micropipettes are fabricated by a micropipette puller (Sutter P-1000). The inner diameter of micropipette tip is around 10 μm. The surface of the glass micropipettes is cleaned by a plasma cleaner, and then modified by 1H,1H,2H,2H-perfluorooctyl trichlorosilane (TCPFO) vapor in a vacuum desiccator for 40 min to become hydrophobic.

The tip of micropipette was immersed into oil, and the other end of the tube is connected to a 1 ml syringe held on a syringe pump (Longer Pump TJ-2A, China) via FEP microbore tubing to generate constant flow rate. The buffer used in dispensed phase was filtered by a 0.22μm filter to prevent clogging at the micropipette tip.

For generating w/o emulsions, we use mineral oil (SIGMA M3516) supplemented with 4.5% Span80 (SIGMA S6760) , 0.4% Tween80 (SIGMA P8074) and 0.05% Triton X-100 (Beyotime ST795) in volume as continuous oil phase and MiliQ water supplemented with 1x Phi29 buffer(NEB) as dispersed phase. Density of dispersed phase and continuous phase are 1.002 kg/l and 0.784 kg/l, respectively. Interfacial tension between dispersed and continuous phase is 6.27 dyn/cm, obtained using the pendant drop measurement. Viscocity of continuous phase is 48.65 cP. All the physical properties above are measured under temperature of 25 ℃.

2. Scale analysis of forces on a drop with the specific experimental parameters.

We assume the drop as a sphere and simplify the forces acting upon it in our analysis. The force balance of the drop can be described by the following equation.

$$(F_b + F_l)^2 + {F_n}^2 + {F_d}^2 = {F_\gamma}^2 \qquad (1)$$

where F_b is the difference between buoyancy force and drop gravity, F_l is lift force, F_n is

centrifugal force, F_d is drag force, and F_γ is interfacial tension.

The interfacial tension, which holds the drop on the tube, is $\mathrm{F_\gamma = \pi d_n \gamma}$, where γ is the interfacial tension between the continuous phase and disperse phase, d_n is the diameter of the neck during droplet generation. We find that d_n is in the same level with d_i ($d_n \approx d_i$), where d_i is the inner diameter of the micropipette tip. The drag force is a modification of the Stokes formula $\mathrm{F_d = 3\pi\eta_c d(v - v_d - v_c)}$ in the situation with low Reynolds number ($\mathrm{Re} = \frac{\rho_c v d}{\eta_c} \leq 1$), where d is the diameter of the drop, and η_c and ρ_c are the dynamic viscosity and density of the continuous phase respectively, and v is the relative velocity between the micropipette tip and the centrifuge tube, and v_c is the relative velocity between the continuous phase and the centrifuge tube near the tip, and $v_d \approx \frac{\mathrm{q}}{\pi d^2}$ is the velocity of the expanding drop relative to the tip. The buoyancy force, considering the gravity of the drop, is $F_b = \frac{1}{6}\pi d^3 g \Delta\rho$, where $\Delta\rho = \rho_c - \rho_d$ is the density difference of the continuous and dispersed phase, and g is gravitational acceleration. The centrifugal force is $F_n = \frac{1}{6}\pi d^3 \Delta\rho \omega^2 l$, where ω is the angular velocity of the tip, and l is the rotation radius of the tip. A lift force F_l will act on the drop because of the low pressure behind the micropipette tip.

With our specific experimental parameters, γ = 6.27 dyn/cm, η_c = 48.65 cP, $\Delta\rho = 218\ \mathrm{kg/m^3}$, $\mathrm{l} = 1.5\ \mathrm{mm}$, d_i = 15 μm, we analyze the scale of forces on a drop during its formation, assuming $\omega = 400\ \mathrm{rpm}$ (assume $\omega \leq 600\ \mathrm{rpm}$), q = 0.5 μL/min, d = 50 μm.

$$\mathrm{F_\gamma = \pi d_i \gamma} \sim 3\times10^{-7}\mathrm{N}$$

$$\mathrm{F_d = 3\pi\eta_c d(v - v_d - v_c)} \sim \mathrm{3\pi\eta_c dv} \sim 1\times10^{-6}\mathrm{N}$$

where $\mathrm{v_c}$ and $\mathrm{v_d} \approx \frac{\mathrm{q}}{\pi \mathrm{d}^2} \sim 1\ \mathrm{mm/s}$ are much smaller than $\mathrm{v} = \omega\times\mathrm{r} \sim 63\ \mathrm{mm/s}$ and are neglected.

$$\mathrm{F_b} = \frac{1}{6}\pi \mathrm{d}^3 \mathrm{g}\Delta\rho \sim 1\times10^{-10}\mathrm{N}$$

$$\mathrm{F_n} = \frac{1}{6}\pi \mathrm{d}^3 \Delta\rho\omega^2 \mathrm{l} \sim 1\times10^{-12}\mathrm{N}$$

So the buoyancy force F_b, the lift force F_l and the centrifugal force F_n are all small in comparison to the viscous drag force F_d and interfacial tension $\mathrm{F_\gamma}$ and are neglected in Equation (1).

When F_b, F_l and F_n are all neglectable comparing to the viscous drag force F_d, thus

Equation (1) can be simplified as balance between the interfacial tension and the stokes drag, which leads to

$$\frac{d}{d_i} \sim \frac{\gamma}{3\eta_c v} = (\frac{v}{v_0})^{-1} \qquad (2)$$

where d is drop diameter, d_i is inner diameter of the micropipette tip, γ is interfacial tension between the continuous phase and dispersed phase, η_c is dynamic viscosity of the continuous phase, v is the relative velocity between the micropipette tip and the centrifuge tube, and we set variable $v_0 = \gamma/3\eta_c$.

3. Droplet size control, dispersity, and curve fitting.

We took bright field microscopic images using an inverted microscope (Nikon Ti-E) with a CCD camera (Qimaging 2000R). We analyzed the pictures and calculate the size of each droplets using MATLAB. The results are shown in Figure S2.

We used an empirical formula for predicting droplet diameters:

$$\frac{\mathrm{d}}{\mathrm{d_i}} = \mathrm{A_1}(\frac{\mathrm{q}}{\mathrm{q_0}})^{\frac{1}{3}}(\frac{\mathrm{v}}{\mathrm{v_0}})^{-1} + \mathrm{A_2}(\frac{\mathrm{q}}{\mathrm{q_0}})^{\frac{1}{3}} + \mathrm{A_3}(\frac{\mathrm{v}}{\mathrm{v_0}})^{-1} + \mathrm{A_4}$$

with $\mathrm{A_1} = 1.76, \mathrm{A_2} = 3.14, \mathrm{A_3} = 0.24, \mathrm{A_4} = 0.79$.

4. Experimental procedure and protocol of eWGA

We lysed single mouse ES cells in tube with volume of 2 μl, releasing genomic DNA (gDNA) fragments. Then we dehybridize the double-strand gDNA into single strands by heat (95 ℃ for 5 min). Prior mixed MDA reaction buffer (8 μl, containing 0.8 μl of Phi-29 polymerase (NEB), 1 μl of 50 μM random hexamer primers (Invitrogen), and 1 μl of 1 mM dNTP (NEB)) was added to each tube at 4 ℃. 10 μl reaction solution was immediately tranferred into the glass micropipette and dispersed into droplets in oil, at 4 ℃, within 10 min by SiMPLE generator. We controlled the diameter of droplet (about 50 μm) by tuning the spinning speed of the micropipette and the delivering rate of reaction buffer. As a result, 10 μl reaction solution was separated into $\sim 1.5\times10^5$ droplets. Isothermal amplification reaction started when we placed the microcentrifuge tubes in thermomixer at 30 ℃. The whole amplification time is about 8 h. The eWGA reaction was terminated by heat inactivation of the polymerase at 60 ℃ for 10 min and demulsification by votexing with 700 μl isopropanol.

The amplification was reproducible and validated by quantitative PCR (Figure S3). We chose 10 single-cell eWGA products to construct libraries for next-generation sequencing. Meanwhile, two mouse ES single cell were selected to perform the MDA reaction in tube, and then sequenced as well, for comparison. We sequenced about 0.3G bases for each library using Illumina Hiseq platform. The coverage distribution across the whole genome of each sample was listed in Figure S4.

5. A simpler SiMPLE generator combining pipette with electrical toothbrush (For Fun!)

In a very beginning of this project, we have decided to perform a 'quick and dirty' experiment to test the idea of generation of emulsion via spinning a glass micropipette in oil. We purchased a specific electro toothbrush (Panasonic) and replaced its brush head with a glass micropipette. We just simply taped the micropipette to the toothbrush, and used a 20 μl conventional manual pipette (Eppendorf) to slowly push the aqueous liquid out of the glass pipette. Although with no precise control at all, we found this simple combination could produce a large amount of w/o droplets within very short period of time. We noticed that the distribution of the droplets was not monodisperse, but majority of droplets are about 50 - 100 μm in diameter, which is actually the best size for eWGA. We also immediately realized that the motion speed of the glass micropipette is critical since the droplet size would also be affect by the motion speed. Interestingly this finding was verified by testing other electrical toothbrushes. Panasonic electrical toothbrush uses circular motion to drive the brushhead, hence the linear motion speed of micropipette is constant. While another popular brand, Philips, uses reciprocating motion which does not provide constant linear motion speed of the brushhead, and cannot be used in our application.

Part II. Supporting Figures

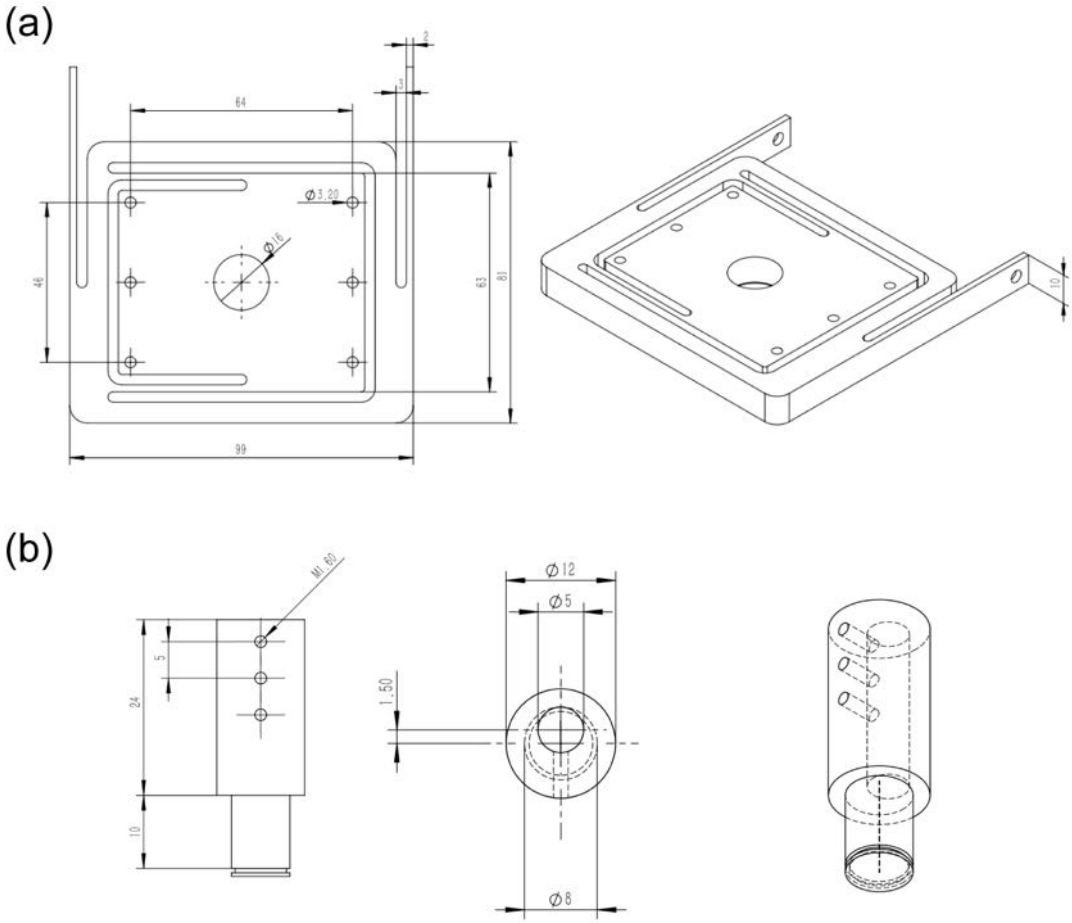
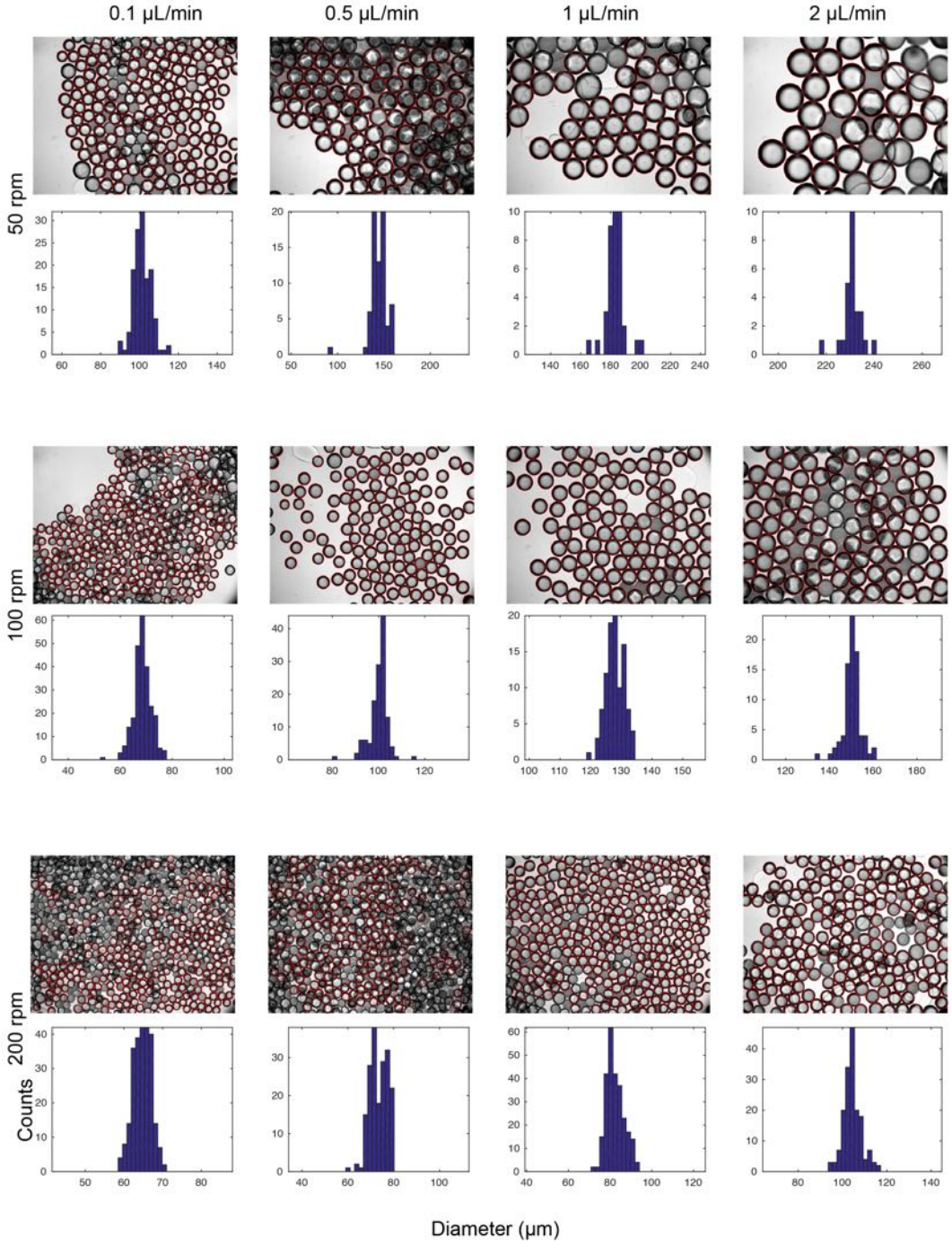


Figure S1. The design details of the loading platform (a) and the 1.5 mm off-axis eccentric shank (b).


0.1 µL/min
0.5 µL/min
1 µL/min
2 µL/min
50 rpm
100 rpm
200 rpm
Counts
Diameter (µm)

Cont.

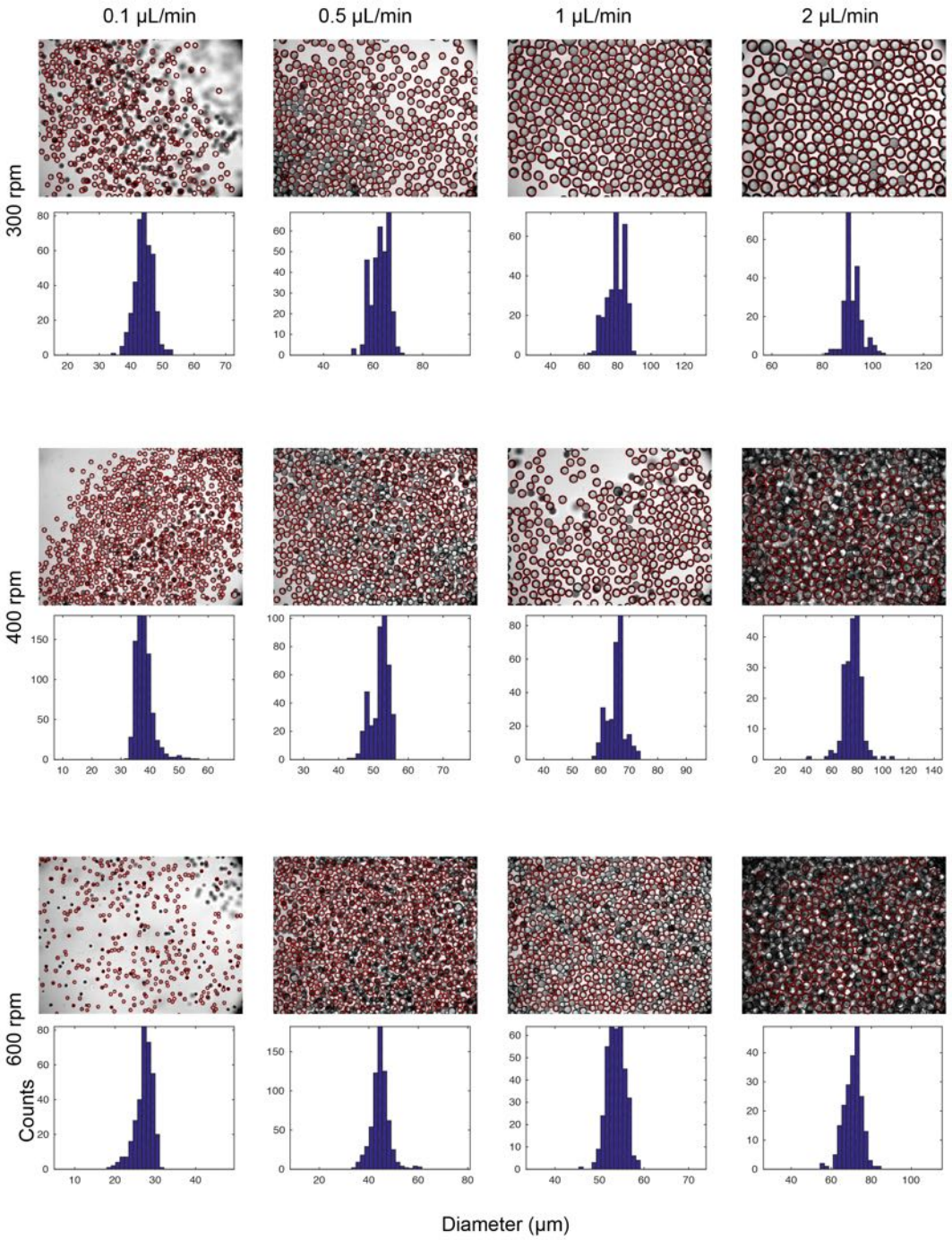


Figure S2. Microscopic observation and size distribution of the w/o emulsion droplet generated by SiMPLE generator.

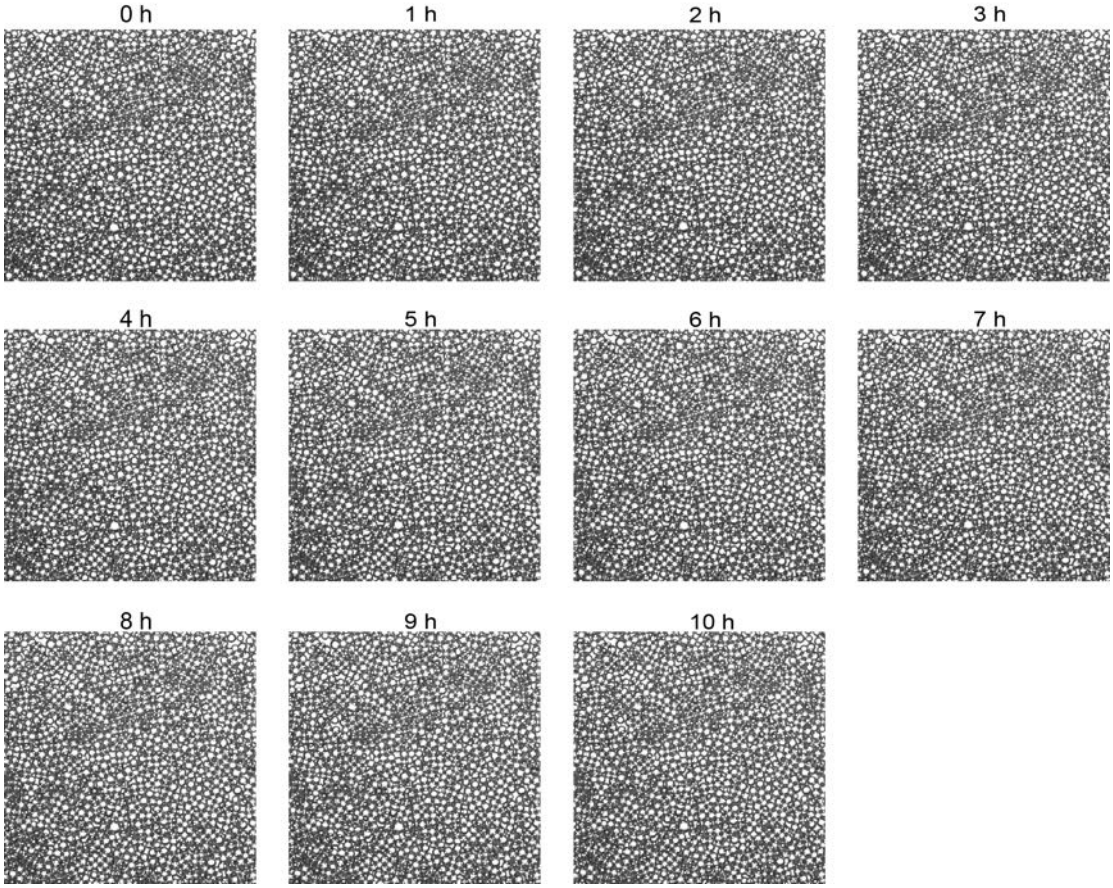


Figure S3. Stability of w/o emulsion. The generated emulsion are placed in Nunc TopYield strips for microscopic imaging. Microphotographs (field of view 1.5 mm x 1.5 mm) are taken at the 1 h interval for 10 h. No noticeable fusion of fission of the droplets has been observed during this period of time.

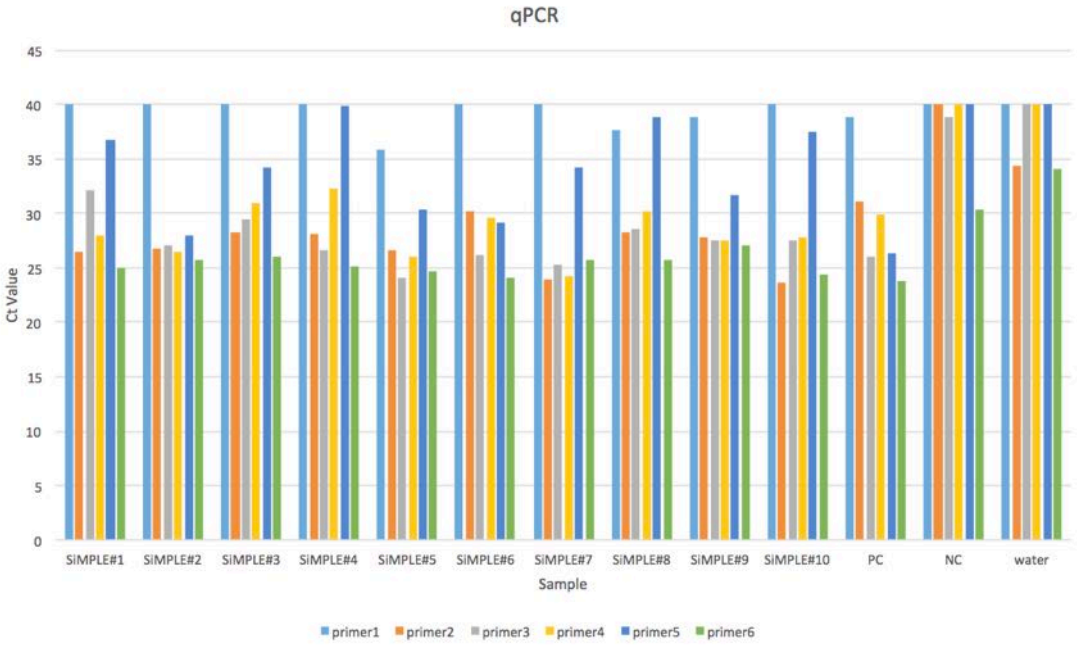


Figure S4. The quantitate PCR result of the amplified products of single cells.

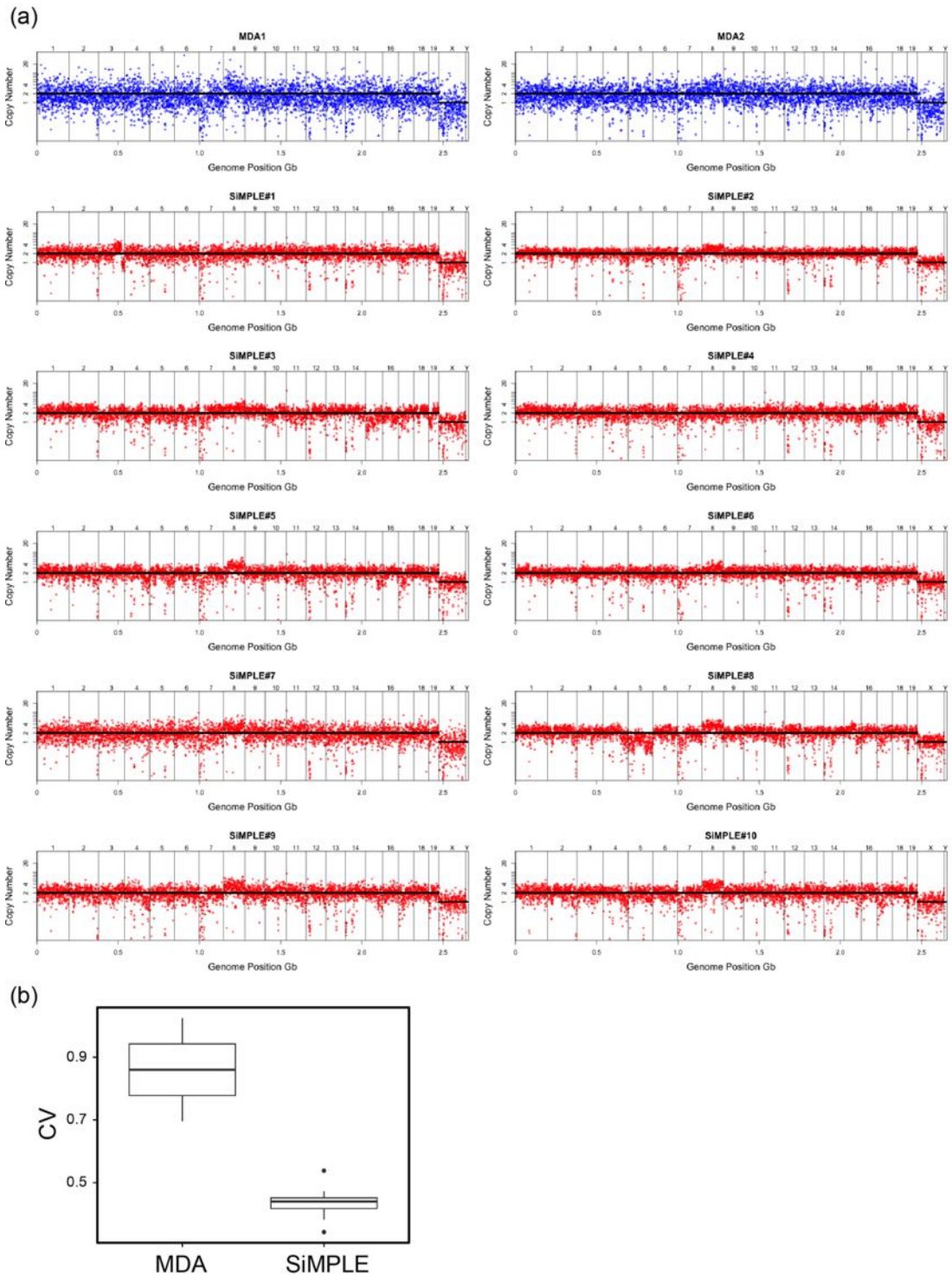


Figure S5. (a) The coverage distribution across the whole genome of the sequencing results of single cells, with two single-cell MDA samples for comparison. (b) The coefficient of variation (CV) of the coverage flutuation using different amplificaiton methods.