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Facile and Rapid Generation of Large-Scale Microcollagen Gel Array for Long-Term Single-Cell 3D Culture and Cell Proliferation Heterogeneity Analysis

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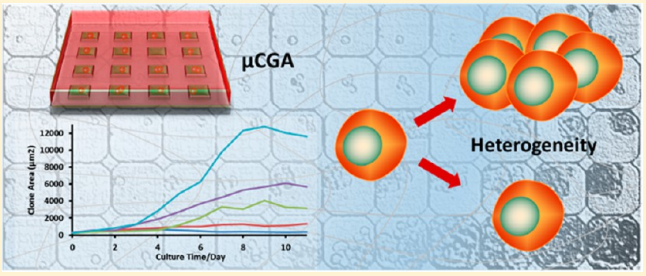
Supporting Information

ABSTRACT: Microfabricated devices are suitable for single-cell analysis due to their high throughput, compatible dimensions and controllable microenvironment. However, existing devices for single-cell culture and analysis encounter some limitations, such as nutrient depletion, random cell migration and complicated fluid shear influence. Moreover, most of the single-cell culture and analysis devices are based on 2D cell culture conditions, even though 3D cell culture methods have been demonstrated to better mimic the real cell microenvironment in vivo. To solve these problems, herein we develop a microcollagen gel array (μCGA) based approach for high-throughput long-term single-cell culture and single-cell analysis under 3D culture conditions. Type-I collagen, a well-established 3D cell culture medium, was used as the scaffold for 3D cell growth. A 2 × 2 cm PDMS chip with 10 000 μCGA units was fabricated to encapsulate thousands of single cells in less than 15 min. Single cells were able to be confined and survive in μCGA units for more than 1 month. The capability of large-scale and long-term single-cell 3D culture under open culture conditions allows us to study cellular proliferation heterogeneity and drug cytotoxicity at the single-cell level. Compared with existing devices for single-cell analysis, μCGA solves the problems of nutrient depletion and random cellular migration, avoids the influence of complicated fluid shear, and mimics the real 3D growth environment in vivo, thereby providing a feasible 3D long-term single-cell culture method for single-cell analysis and drug screening.



It is well established that individual cells, even from the same origin, differ from each other in many aspects due to stochastic biological processes and differences in environmental perturbations.[1−4] Cell heterogeneity has been found to play an important role in many biological processes, including cellular differentiation and immune response, as well as disease development. Traditional ensemble analysis based on averaging a large population of cells, as a result, masks the cellular differences and the presence of rare subpopulations of cells.[5,6] Therefore, over the past decades, much emphasis has been placed on technical advances for single-cell study in order to accurately describe and eventually elucidate the underlying causes of these heterogeneities.[7,8]

Microfabrications, including a variety of integrated microfluidic chips,[9−17] microdroplets based microdevices[18−20] and microwell arrays,[21−24] have been demonstrated as suitable tools to produce massively parallel microcontainers for single-cell trapping and study in a high throughput manner, mainly due to their size compatibility and controllable microenvironments.[25−27] These miniaturized devices have been widely used in cellular heterogeneity studies in recent years. For example, Griffiths et al.[19] used microdroplets to functionally screen individual hybridoma cell clones with a high throughput of about 300 000 cells/day. Lee et al.[9] used a trap-barrier array to capture single cells in a U-shaped hydrodynamic trapping structure for dynamic cellular adhesion and division analysis. Lecault et al.[28] used a microwell array based microfluidic device for hematopoietic stem cell culture and further study of proliferation heterogeneity. Although these methods have shown great potential for single-cell studies, they still encounter some limitations, such as the risk of nutrient depletion and toxin accumulation in enclosed microdroplet environments,[18,27] complex fluidic influences on cell behaviors,[11,26] and inevitable random cell migration in open cell culture conditions by microwell array based devices.[6,21] Furthermore, all of these single-cell analysis approaches are based on a conventional 2D cell culture model, in which cells are suspended freely in culture medium or randomly adhered onto the support surface, even though 3D cell culture has shown great significance in cell study by mimicking the real 3D microenvironment in vivo.[29−32]

To solve the aforementioned problems and demonstrate the feasibility of large-scale single-cell analysis under 3D culture

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conditions, we have developed a microcollagen gel array (μCGA) based approach for high-throughput long-term single-cell culture and single-cell analysis under 3D culture conditions. Type-I collagen, a well-established 3D cell culture medium,[33−35] was used as the scaffold for 3D cell growth. A 2 × 2 cm PDMS chip with 10 000 μCGA units was fabricated to encapsulate thousands of single cells in less than 15 min. Kato III, a human gastric carcinoma cell line,[36] as a model cell, was able to be confined and survive in μCGA units for more than a month. The capability of large-scale and long-term single-cell 3D culture under open culture conditions allows us to study cellular proliferation heterogeneity at the single-cell level. The feasibility for studying cytotoxicity of drugs to cell proliferation at the single-cell level was further investigated. Compared with existing devices for single-cell analysis, μCGA solves the problems of nutrient depletion and random cellular migration, avoids the influence of complicated fluid shear, and mimics the real 3D growth environment in vivo, thereby providing a feasible 3D long-term single-cell culture method for single-cell analysis and drug screening.

EXPERIMENTAL SECTION

Materials and Reagents. SU-8 3050 was purchased from MicroChem (Newton, MA, USA). Polydimethylsiloxane (PDMS, Sylgard 184) and a curing agent were obtained from Dow Corning (Shanghai, China). Film photomask was produced by Qingyi Precision Maskmaking Co. Ltd. (Shenzhen, China). Type-I collagen was purchased from Shengyou Biotechnology Co., Ltd. (Hangzhou, China). Iscove's Modified Dulbecco's Medium (IMDM) was obtained from HyClone (Beijing, China). Fetal bovine serum (FBS) and B-27 were obtained from Gibco through Life Technology (Beijing, China). Penicillin and streptomycin were purchased from Thermo Fisher Scientific Co., Ltd. (Beijing, China). Epidermal growth factor and basic fibroblast growth factor were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Insulin and Propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calcein AM was obtained from Fanbo Biochemicals Co., Ltd. (Beijing, China). Doxorubicin was purchased from Huafeng United Technology Co., Ltd. (Beijing, China).

μCGA Fabrication. The PDMS microwell array used for μCGA fabrication was produced by conventional SU-8 photolithographic and PDMS casting method. Different types of SU-8 photoresist were coated on a silica wafer at different spin rates to produce a photoresist film with certain thickness. SU-8 2015 was used to produce the 15 μm thickness SU-8 template by setting the spin rate at 3250 rpm and SU-8 3050 was used to produce 50 and 100 μm thickness ones at spin rates of 3000 and 1050 rpm, respectively. The array pattern on a film photomask was transferred onto the photoresist via UV exposure. After the patterned silica wafer was developed, washed and dried, the mixture of PDMS elastomer precursor and curing agent (10:1) was poured and spun at 500 rpm for 20s to obtain a thin patterned PDMS layer of about 150 μm in thickness. The resulting PDMS membrane with patterned microwell array was then peeled from the silica wafer and soaked in ultrapure water for sterilization in an autoclave. Before μCGA fabrication, the PMDS membrane was rinsed with culture medium.

To prepare the collagen gel, 200 μL of type-I collagen solution (5 mg/mL) equilibrated in ice water was first added to 17 μL of NaOH (0.1 M) and mixed immediately. The pH of the solution was subsequently adjusted to about 7.0 by adding 283 μL 2× cold PBS buffer (pH 7.2). Finally, 500 μL of cell suspension in culture medium with different cell concentrations (0.16×10^5, 0.8×10^5 and 1.6×10^5 cells/mL for 0.1, 0.5 and 1.0 cell per well, respectively) was added to the collagen solution and mixed well. The ice−water bath inhibited the gelatinization, thus providing enough time for μCGA fabrication. One milliliter of cell suspension in collagen solution was then added to a 35 mm Petri dish to cover the PDMS membrane, which was previously rinsed with culture medium. By shaking the suspension for 3 min, the small amount of pure culture medium in the wells was replaced by the cell suspension. Finally, the membrane with cells trapped in wells was removed and placed on a flat PMMA slice, then pressed by another flat PDMS membrane in a homemade screw pressing device. The pressure was measured by a pressure sensor integrated in the device as 87.5 KPa. The pressure was maintained when the device was incubated at 37 °C for 10 min for gelatinization. The flat PDMS membrane was then removed, and μCGA with cells encapsulated in microgel was obtained.

Long-Term Cell Culture in μCGA. The μCGA with the well size of 100 × 100 × 100 μm (width × length × depth) was used for the long-term single-cell culture. The fabricated μCGA with cells encapsulated was placed on the bottom of a 35 mm Petri dish. Due to the adhesiveness of PDMS, the membrane was well attached and did not float when 2 mL of culture medium was added to supply nutrients for cell growth and proliferation. Culture conditions were set at 37 °C in a humid atmosphere with 5% CO_2. For optimization, the medium conditions of four groups were varied as follows: group A: IMDM, containing 20% FBS and 1% penicillin streptomycin without the addition of growth factor; group B: IMDM, with 1% penicillin streptomycin, 10 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor, 50 μg/mL insulin and 100 units/mL B27; group C: the same culture medium as group B without FBS but with 50% FBS in the collagen gel; group D: Pure IMDM as the culture medium with 50% FBS in the collagen gel. The cells in μCGA were continuously monitored by microscope (Zeiss Axio Observer A1, German) at 24 h intervals.

Cell Proliferation Heterogeneity Analysis. Because of the difficulty in precisely counting cells growing in 3D, a cell clone sectional area was used to estimate cell proliferation ability. The images of single cells/single clones obtained in different culture periods were taken and aligned in time order. ImageJ was then used for image contrast enhancement and cell sectional area measurement. The cell dividing time was collected by manually counting the cell number that divided each day.

Drug Screening. To investigate the influence of doxorubicin on cell proliferation at the single-cell level, different concentrations of the drug ranging from 10 nM to 400 nM were added to the culture medium, and the cells were monitored for 7 days. The first dividing time distribution was collected to estimate the drug influence. As a comparison, the MTT assay was performed according to the standard protocol, where the absorbance at 490 or 580 nm was obtained using a microplate reader (Model 680, Bio-Rad), and the IC_{50} was then calculated.

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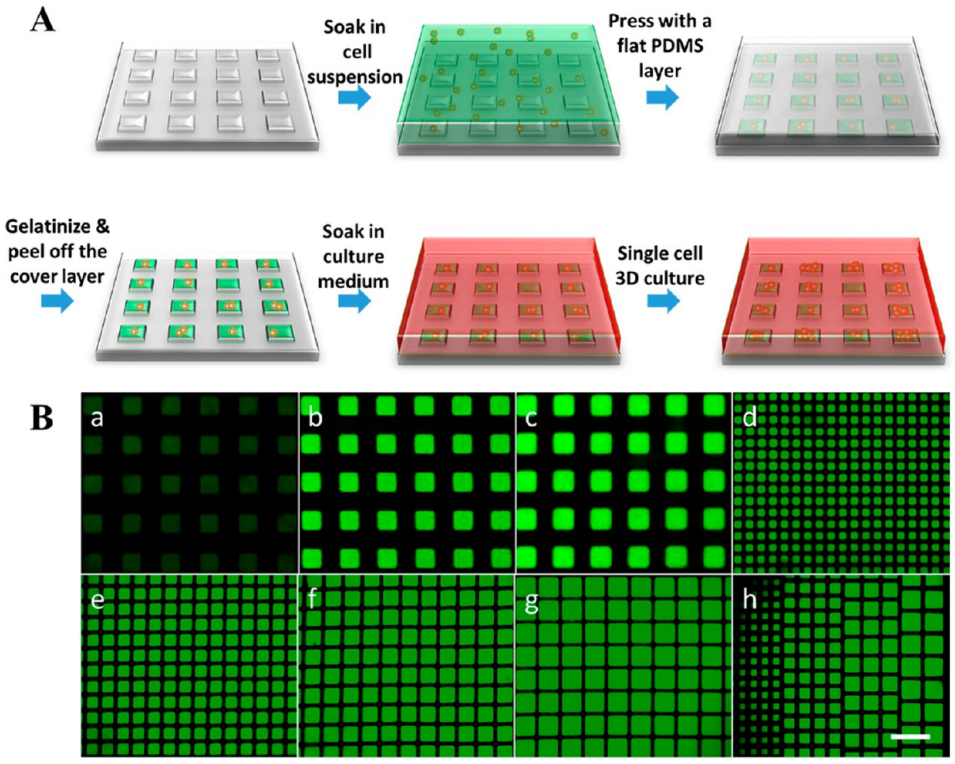

Figure 1. (A) Schematic of μCGA fabrication process for 3D cell culture. (B) Fluorescent images of μCGA with different dimensions. (a)−(c) Fluorescent images of μCGAs with the same size parameter of 114 × 114 μm and different depths of 15, 50 and 100 μm, respectively. (d)−(g) Images of μCGAs with the same depth of 20 μm but different sizes of 40 × 40 μm, 60 × 60 μm, 80 × 80 μm to 100 × 100 μm, respectively. (h) Fluorescent image of a variable-size μCGA containing four different sizes of wells. The scale bar is 200 μm.

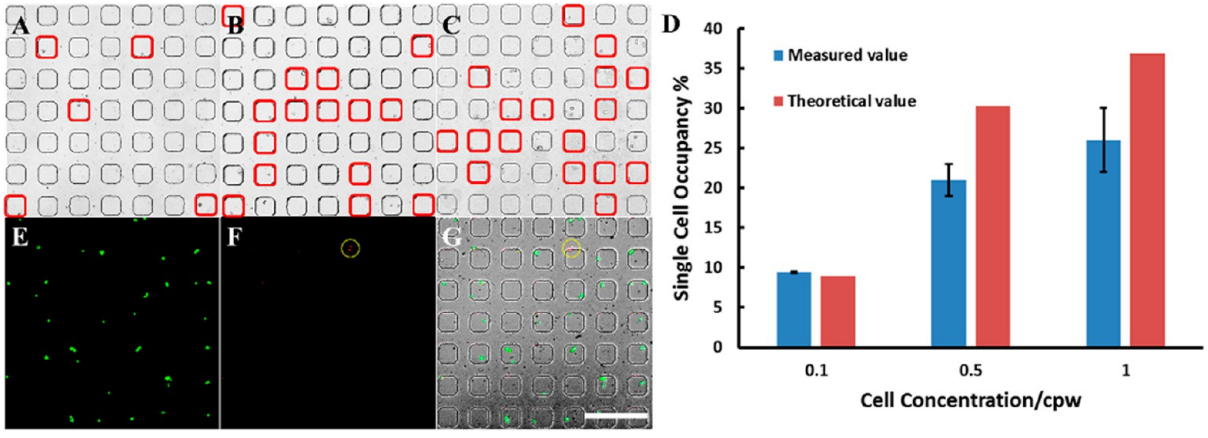

Figure 2. (A−C) Images of cell encapsulated in μCGA with different cell concentrations: 0.1 cpw (A), 0.5 cpw (B), 1.0 cpw (C). μCGA units with single-cell occupancy are highlighted by red squares. (D) Comparison of measured percentage of units with single-cell occupancy and theoretical values according to the Poisson distribution. (E−G) Fluorescent images of a newly produced μCGA costained with Calcein AM and PI: (E) fluorescence image of living cells stained with calcein AM; (F) fluorescence image of dead cells stained with PI; (G) merged image of bright field with (E) and (F). The dead cell in (F) and (G) is highlighted by a yellow cycle. The scale bar is 400 μm.

RESULTS AND DISCUSSION

Fabrication of Large Scale μCGA for Single-Cell Culture and Analysis. The μCGA chip was fabricated as shown in Figure 1A, and the process is described in detail in the Experimental Section. In brief, a PDMS microwell array membrane was first obtained via a conventional SU-8 photolithographic and PDMS casting technique. The membrane was soaked with a cell−collagen suspension in culture medium and then pressed against another flat PDMS membrane in a homemade screw device under a low pressure. The cell−collagen suspension left in the wells was gelatinized by incubation at 37 °C for 10 min. After the flat PDMS layer was peeled, the μCGA with single cells encapsulated in collagen gel was placed in the culture medium for long-term culture and single-cell/single-clone analysis.

To demonstrate the flexibility of producing arrays with different dimensions, μCGAs each containing 10 000 units with different depths (Figure 1Ba−c) and sizes (Figure 1Bd−h) were fabricated. Fluorescein was added to the collagen solution for imaging the resulting array. As shown in the fluorescence images of Figure 1Ba−c, as the gel depth changed from 15 to

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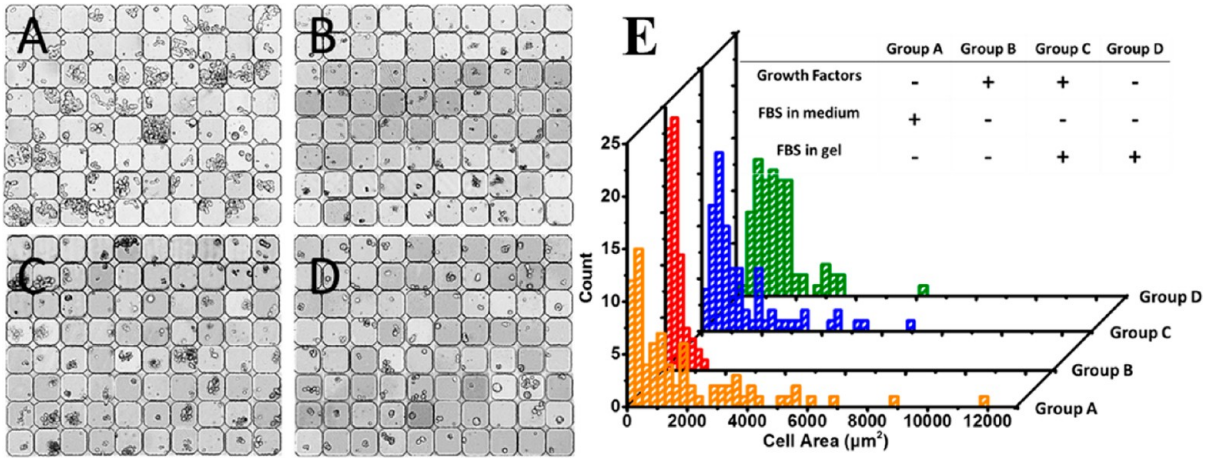

Figure 3. (A–D) Aligned images of the 80 cell/cell clones after 7 day culture with different culture conditions. (A) With 20% FBS in the medium and no growth factor; (B) with growth factors added to the medium, but no serum; (C) with growth factors in the medium and 50% FBS in collagen gel; (D) with 50% FBS in collagen gel but no FBS or growth factors in the medium. (E) Histograms of the cellular sectional area distributions of groups A–D from images (A–D). The table in panel E indicates the existence of serum or growth factors.

50 and 100 μm, the fluorescent intensities were found to increase using the same exposure conditions. Figure 1Bd–g shows images of μCGAs with the same depth of 20 μm but different sizes: 40 × 40 μm, 60 × 60 μm, 80 × 80 to 100 × 100 μm. Using a PDMS template containing different dimensions of units, a variable-size μCGA (Figure 1Bh) was also easily obtained. In all these arrays of different dimensions, the array units were well resolved with clear boundaries, due to the hydrophobic nature of PDMS and mechanical stability of the collagen gel. The ability to rapidly generate large-scale collagen gel bricks with flexible dimensions offers an attractive approach to fabricate single-cell microcompartments for single-cell analysis in complex and diversified microenvironments.

Single-Cell Encapsulation. The cell trapping using μCGA relies on the cell distribution in the collagen solution, which is determined by the cell concentration. Kato III, a human gastric carcinoma cell line, was used as a model cell. Different concentrations of Kato III cells of 0.1 (Figure 2A), 0.5 (Figure 2B) and 1.0 (Figure 2C) cell per well (cpw) were used to investigate the single-cell occupation probability in μCGA. As shown in Figure 2D, the single-cell occupation probability increased as cell concentration increased from 9.4 ± 0.1% (0.1 cpw), 21 ± 2% (0.5 cpw) to 26 ± 4% (1.0 cpw), compared with the theoretical values of 9.04%, 30.3% and 36.8%, respectively, calculated according to the Poisson distribution. The deviation could be explained by cell concentration quantitation deviation and cell aggregation before μCGA fabrication. To obtain the highest throughput for single-cell analysis, the concentration of 1.0 cpw, was chosen for the cellular heterogeneity study at the single-cell level for each assay.

To investigate whether the mechanical press used during the fabrication had any influence on cell viability, the cells in newly produced μCGA were costained with calcein AM (for living cells, Figure 2E) and PI (for dead cells, Figure 2F) and the fluorescent images (Figure 2E,F) and merged image with bright field (Figure 2G) were obtained under fluorescence microscopy. The measured cell viability in a newly fabricated μCGA was found to be about 97.8%, which was comparable to that obtained from the Petri dish culture (97.0%). These results clearly demonstrated the feasibility and reliability of μCGA for single-cell encapsulation with excellent cell viability.

Long-Term 3D Single-cell Culture in μCGA. The variation of some cellular properties and behaviors, such as proliferation, differentiation, cellular response to drug simulation and cellular secretion,[37,38] can hardly be observed during a very short cell cycle or culture period. Thus, a method that allows long-term culture at the single-cell level is of great interest. Compared with recently developed single-cell methods that afford only limited culturing periods,[18,39] μCGA offers the possibility for long-term single-cell culture because of the open culture environment and the stability of the microcollagen gel.

To demonstrate the feasibility of using μCGA for long-term observation, the cell culture conditions were first optimized. Because growth factors (GFs) and FBS are normally used as alternatives for cell culture to supply the nutrients or to stimulate cell growth, we investigated the influence of these two reagents by comparing the sectional area distribution of 80 single-cell clones after 7 day culture at different conditions. The concentrations of FBS and GFs were determined by referring to published works[40] and by the suggestions of the ATCC. Four groups of comparison were conducted as following: 20% FBS in medium without GFs (group A, Figure 3A); GFs in medium without FBS (group B, Figure 3B); GFs in medium and 50% FBS in collagen gel (group C, Figure 3C); 50% FBS in the gel without GFs (group D, Figure 3D). As the results in Figure 3 shown, the presence of FBS (either in medium or in gel) significantly promoted the cell proliferation, while the presence of GFs did not show any positive influence. Moreover, compared with group D, group C with GFs showed a large amount of cellular atrophy in the seventh day, indicating a potentially negative influence of GFs on cells. This may be caused by overly excessive metabolism under the stimulation of GFs, ultimately resulting in cell death. The comparison of groups A and D suggested a better cell proliferation with FBS in medium than that in gel, probably due to insufficient FBS in the gel to supply cell proliferation for long-term cell culture. Therefore the culture condition of group A with 20% FBS in medium was chosen as the optimized culture condition for further experiment.

Under optimized conditions, we prolonged the cell culture time to 11 days (Supporting Information, Figure S1A–C) and further to 1 month (Supporting Information, Figure S1D–F). The cell clones were found continuously growing and

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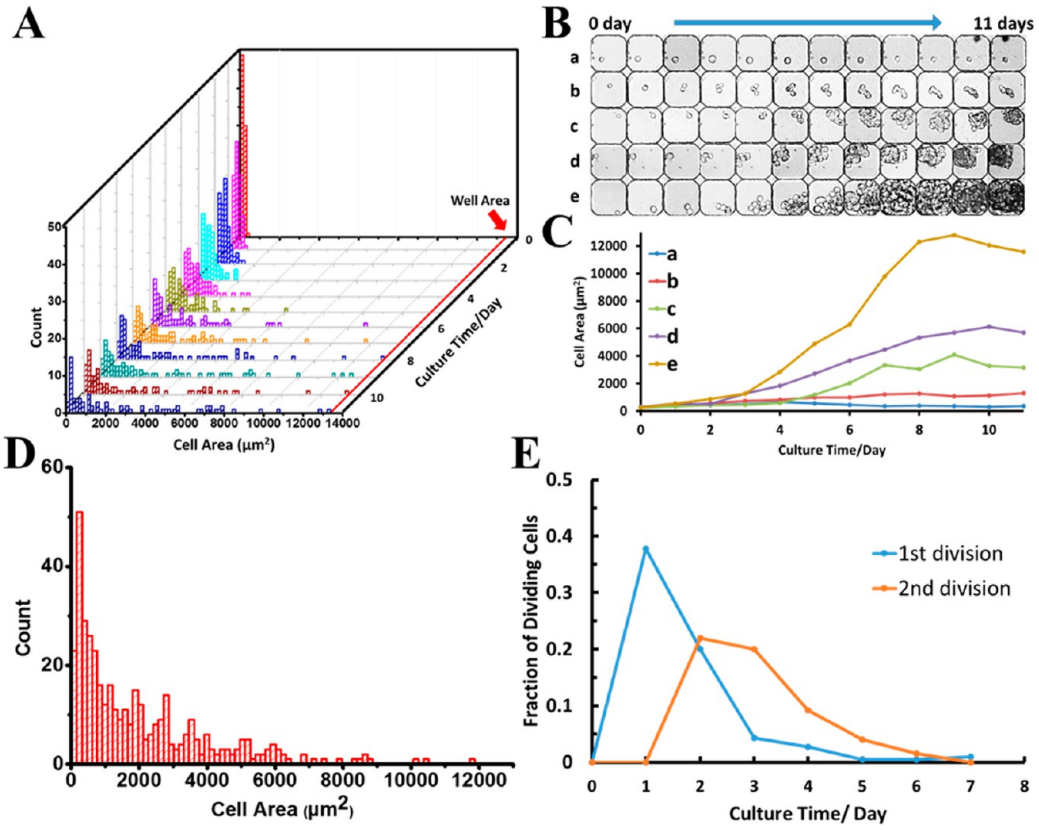

Figure 4. (A) Cell clone sectional area distribution of 80 random continuously recorded single cells during 11 days. The red line in panel A indicates the sectional area of an array unit. (B) a−e are images of five typical single cells with significant cellular proliferation ability heterogeneity during the 11 day culture. The images were taken from the total array and aligned along the time line. (C) The variation of the sectional area of the five single-cell clones during the 11 day culture. (D) The sectional area distribution of single cells/single-cell clones on the seventh day. (E) The histogram distribution of first and second division time of 400 single cells investigated.

proliferating with a low cell death rate. The *z*-axis confocal scanning images of three cell clones after an 11 day culture (Supporting Information, Figure S2) confirm 3D distribution and growth of cells. The experimental results clearly established the feasibility of using μCGA for long-term 3D cell culture.

Analysis of Cell Proliferation Heterogeneity under 3D Culture Conditions. Due to the minimized scale for each microcollagen gel, μCGA allows integration of a large array of microcollagen gels in a very small area for high-throughput single-cell analysis. The μCGA also provides a 3D growth scaffold for single-cell 3D growth with the feasibility for cell proliferation heterogeneity analysis under 3D culture conditions. In our experiment, the μCGA contained 10 000 independent microcollagen gels in a 2 × 2 cm area, in which about 2600 gels were occupied by single cells under 1 cpw cell concentration, providing sufficient throughput for heterogeneity analysis. We continuously recorded 80 random single cells for 11 days. As shown in Figure 4A, the encapsulated single cells exhibited significant proliferation heterogeneity among the individuals, as the sectional area distribution of cell clones became broader when culture time increased. The histogram also revealed that a small group of cells in the 80-cell total had remarkably higher proliferation ability than the others. The growth of five typical cell clones with different proliferation ability during the entire culture period were shown in Figure 4B,C. The cell clones with the highest proliferation ability were found to divide every day and occupy the entire well by the eighth day (Figure 4Be), while the one with lowest proliferation ability did not divide at all during the 11 day culture (Figure 4Ba). The variation of measured sectional area of the five cell clones is shown in Figure 4C. Although the viability of the cell clones was still very high after the 11 day culture in μCGA (Supporting Information, Figure S2A−C), a small decrease of clone area was found after the ninth day, due to the denser cell cluster, formed after a long-term culture with increasing cell−cell interaction in limited well space. Considering that the decrease of clone sectional area after 9 days may lead to more complex difficulties for the heterogeneity analysis, we determined to use the clone area distribution in the seventh day for the detailed cell proliferation heterogeneity analysis.

For proof-of-concept study, we monitored 400 single cells for 7 days at 24 h intervals. The sectional area distribution of these cell clones on the seventh day is shown in Figure 4D. The broad distribution indicates significant variation in proliferation ability among the individual cells, even though they had almost the same initial sectional area and were exposed to identical microenvironments. We recorded the first division time (the division happened for the initial single cell) and second division time (the division took place for either of the two daughter cells divided from the initial single cell) of the 400 cells. As Figure 4E shows, the first division time ranged from the first day till to the seventh day, whereas most took place on the first (38%) and second (20%) days. The second division mainly occurred on the second (22%) and third (20%) days, right after the first division. About 67% of initial single cells were divided and only 85% of these daughter cells were able to further proliferate to

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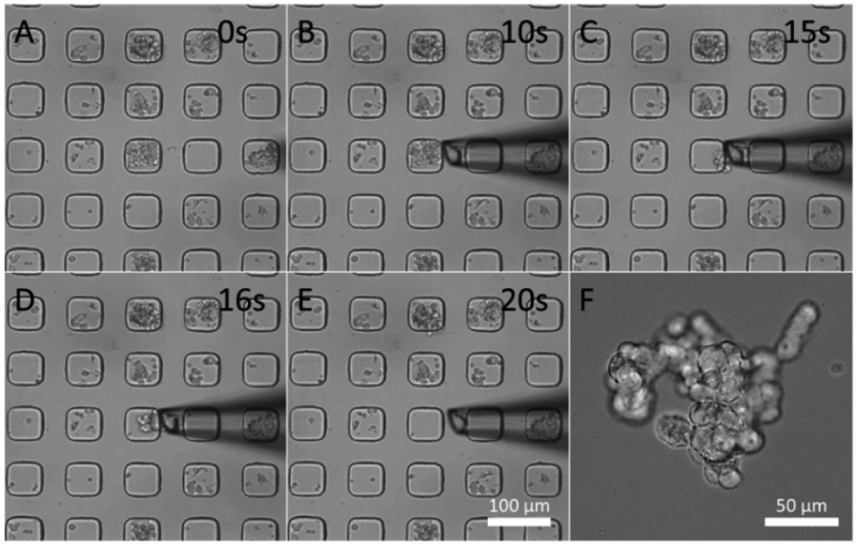

Figure 5. Retrieval process of single-cell clone from μCGA using a capillary tip (A–E). The retrieved single-cell clone (F).

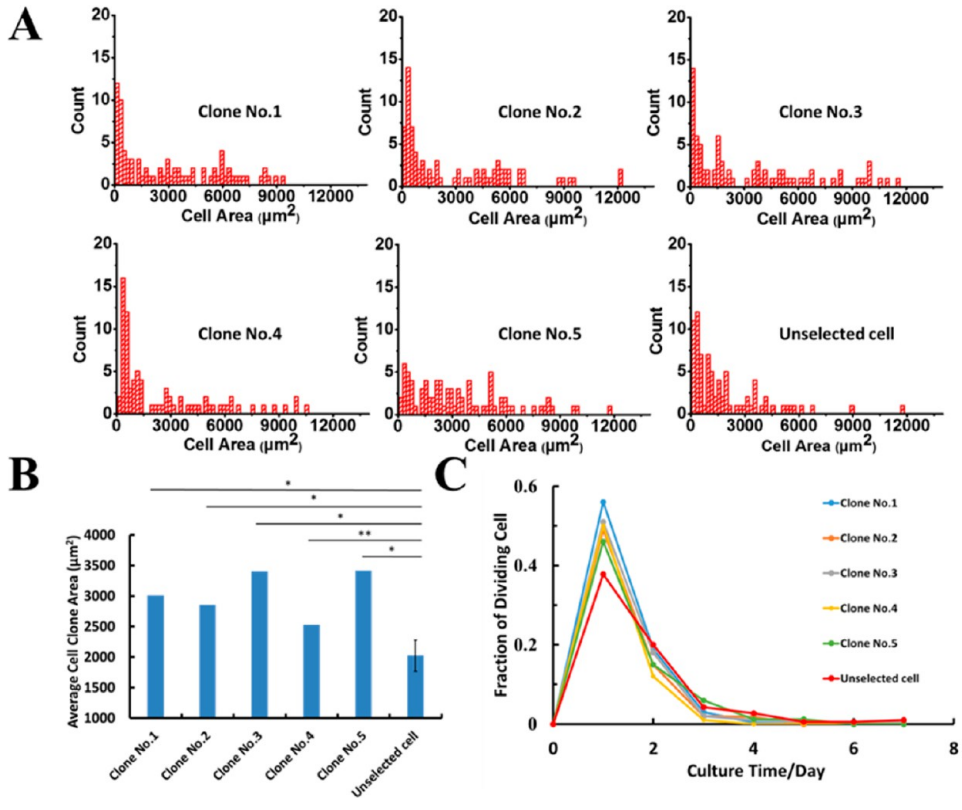

Figure 6. (A) Sectional area distribution and (B) average cell clone area of 80 random single-cell clones in D-μCGA and μCGA. The standard deviation value in B for unselected cell labeled was obtained from the average cell clone area of 5 μCGA groups. * $p < 0.05$, ** $p < 0.15$. (C) The first dividing time distribution of the daughter generation of the five single-cell clones.

generate larger cell clones. Notably, the cell number of the clones with the highest proliferation ability almost doubled each day, while some daughter cells divided asymmetrically and some cells needed much longer time for division. The results clearly established that the proliferation ability of the individual cells differed greatly from each other in terms of the initial single cells as well as the daughter generations of the single cells.

Retrieval of Single-Cell Clone in μCGA. Techniques for cell or cell clone retrieval are highly important for high-throughput single-cell analysis, because in most situations, only a small number of cells or cell clones are desirable for retrieval and further study. The open microenvironment of μCGA is compatible with most existing micromanipulation techniques, thus allowing the easy cell retrieval. A micro-operator (InjectMan NI2, Eppendorf, Germany) with a capillary tip (~100 μm in diameter) was used to retrieve the cell clones of interest microscopically. Figures 5A–E show a retrieval process of a single-cell clone after 7 day culture in μCGA, which was completed easily within 20s. The capillary was moved toward

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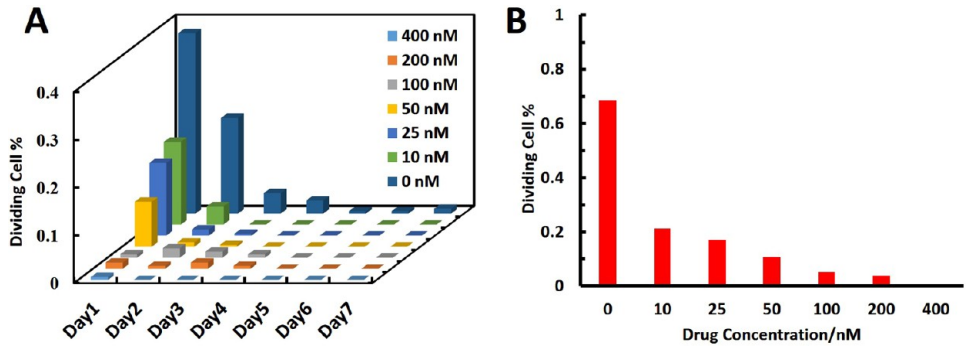

Figure 7. (A) Distribution of cell dividing time under different concentrations of drug stimulation. (B) The total fraction of dividing cell under different concentrations of drug stimulation.

the interested cell clone and a negative pressure was used to suck the clone (with the collagen gel) into the capillary. The retrieved cell clones (Figure 5F) can be collected in 96-well plate for further expansion and analysis.

Investigation of the Inheritance of Proliferation Ability. It is interesting to know whether the descendants of rapidly proliferating cells inherit the rapid proliferation ability after retrieval. On the basis of the aforementioned cell retrieval approach, 5 single-cell clones, which occupied the entire well by the eighth day, were retrieved on the eighth day to investigate the inheritability of cell proliferation ability. The retrieved single-cell clones were then expanded in a 96-well plate and subsequently in a 32-well plate for 1 week of cell culture, until enough descendant cells were obtained. The descendant cells from the five single-cell clones were quantitated and encapsulated separately in five new pieces of μCGAs (D-μCGAs) at 1 cpw concentration. As a comparison, a μCGA with unselected Kato III cells was prepared in parallel. After 7 day culture, the sectional area distributions of 80 random single-cell clones and the first cell dividing time were collected for each group. The broad distributions of clone area were also found in the 5 D-μCGAs (Figure 6A). However, the average area values of the 80 single-cell clones from 5 D-μCGAs were significantly higher than those from unselected cells (factors ranging from 1.32 to 1.78) (Figure 6B). The *t*-test result shown in Figure 6B indicates that the average clone areas of 4 selected cell clones are significantly larger than the area for the unselected cell ($p < 0.05$). The increase in fraction of dividing cells on the first day (from 38% to ~50%) also suggested a more rapid cell division with potentially higher proliferation ability (Figure 6C). The results suggested that the high proliferation ability was obtained by the descendant generations of the rapidly proliferated single-cell clones and indicated a hereditary property for proliferation ability.

Drug Screening under 3D Culture Conditions at the Single-Cell Level. In recent decades, the behavior of cells under 3D culture conditions has been demonstrated to resemble cells in vivo better than dish based 2D culture methods.[25,41,42] Therefore, efforts have been made to enable drug screening in more biomimitic 3D conditions instead of the conventional dish based 2D model. In addtion to the advantages of high throughput and low reagent consumption, μCGA provides a 3D single-cell culture environment. Furthermore, the robust microcollagen gel with cell clones stably localized inside individual compartments effectively avoids cell mixing. Doxorubicin, a widely used drug for cancer chemotherapy that can effectively inhibit the proliferation of cancer cells,[43] was used as the model drug to demonstrate the feasibility of using the system for drug screening under 3D culture conditions at the single-cell level. A series of drug concentrations were used to investigate the inhibition of cell proliferation by doxorubicin at the single-cell level in μCGA. As shown in Figure 7A,B, significant inhibition of cell proliferation was observed under a series of low drug concentrations (about 200 cells were recorded for each concentration). Most of the cell division was found to occur on the first and the second days (Figure 7A) in the presence of different concentrations of drug. The fraction of dividing cells on each day decreased obviously as the drug concentration increased. For example, the fraction of dividing cells was 66.8% without drug in the culture medium, but the fraction sharply decreased to 19.4% with a 10 nM drug and further decreased to 5.2% with a 100 nM drug. When the drug concentration was raised to 400 nM, only 0.6% of the cells were found to proliferate. Moreover, after 7 day culture under the stimulation of a 400 nM drug, the single cells encapsulated in the μCGA showed significant atrophy, indicative of cell death. We did not use the clone sectional area as the estimation of cell proliferation ability, because cell swelling, probably due to cell damage caused by the drug, was found under low drug concentrations (10−100 nM), instead of the cell atrophy under a higher drug concentrations (200−400 nM). The experimental results suggested a remarkably low drug concentration for inhibiting cancer cell proliferation (IC_{50} < 10 nM, Figure 7B). In addition to providing cell proliferation inhibition information, the IC_{50} value for cell viability was obtained by counting the percentage of live cells after exposure to doxorubicin with a value of 3.5 μM (Supporting Information, Figure S3A), which was comparable to that obtained with conventional MTT assay (4.2 μM, Figure S3B). The single-cell assay clearly suggested that lower drug concentration can effectively inhibit cell proliferation, although under such a concentration cancer cell could not be immediately killed. Thus, the single-cell analysis method potentially provides a new strategy for accurately evaluating of drug effects, because excess doses of doxorubicin cause severe side effects inducing a series of diseases.[44,45] Moreover, rather than the average value obtained from conventional MTT assay, μCGA provided much more information at the single-cell level, including the distribution of cell resistance to the drug, the variation of single-cell behavior under drug stimulation, and the change of single-cell morphology.

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CONCLUSIONS

In conclusion, we have developed a facile and rapid fabrication method of μCGA for long-term cell culture and cellular heterogeneity analysis at the single-cell level under 3D culture conditions. Because of the benefit from the mechanical stability of collagen gel, the encapsulated single cells were stably localized, thus allowing for long-term continuous recording of cell proliferation. The collagen gel also provided a 3D scaffold for cell growth and proliferation as a mimic of the real microenvironment in vivo. Four hundred cells out of the total 2600 single cells encapsulated in μCGA were continuously recorded during 7 day culture, and the results showed a significant broad distribution of cell proliferation ability. By integration with a microscale manipulation platform, the cells of interest in μCGA can be easily retrieved, because the open cellular culture conditions enable much more complex and free single-cell/clone manipulation than other methods. We also used the μCGA as a tool for drug screening at the single-cell level to study the heterogeneity of resistance to the drug. A much lower drug concentration was found to cause efficient inhibition to cell proliferation, while not killing the cells. Due to the low cost, low reagent consumption and easy mechanical fabrication method, μCGA offers a new method for long-term single-cell 3D culture and a variety of applications, such as single-cell analysis and drug screening.

ASSOCIATED CONTENT

Supporting Information

Fluorescence images of μCGA after culturing for each 11 days and 1 month, *z*-axis confocal scanning images of three cell clones after 11 day culture, IC_{50} for cell viability obtained by counting percentage of live cells in μCGA (calcein AM and PI co-staining assay) and from conventional MTT assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Supporting Information

Facile and Rapid Generation of Large-scale Micro-collagen-gel Array for Long-term Single Cell 3D Culture and Cell Proliferation Heterogeneity Analysis

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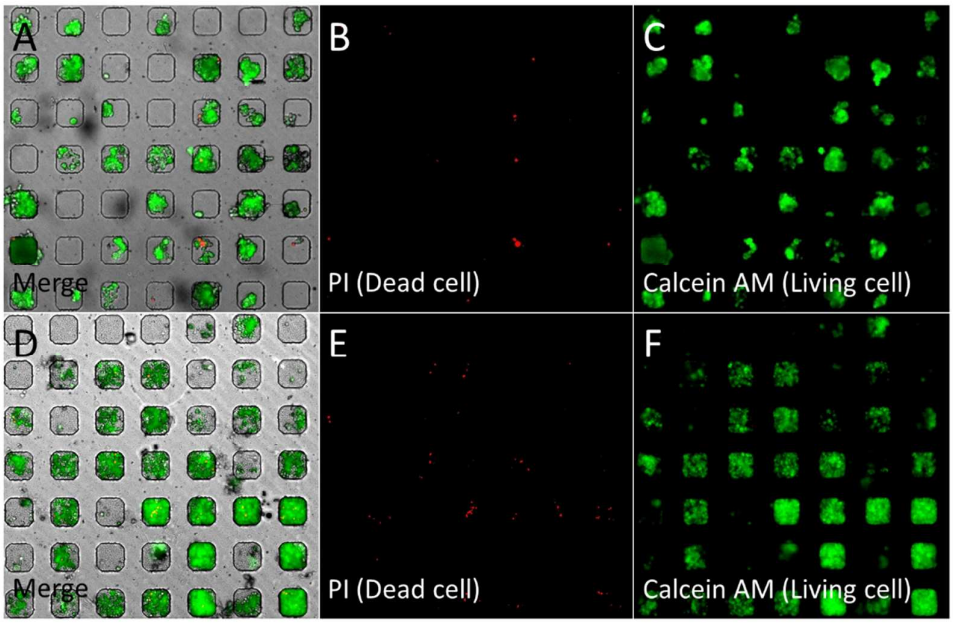

Figure S1. The fluorescence images of μCGA after culturing for 11 days (A-C) and a month (D-F).

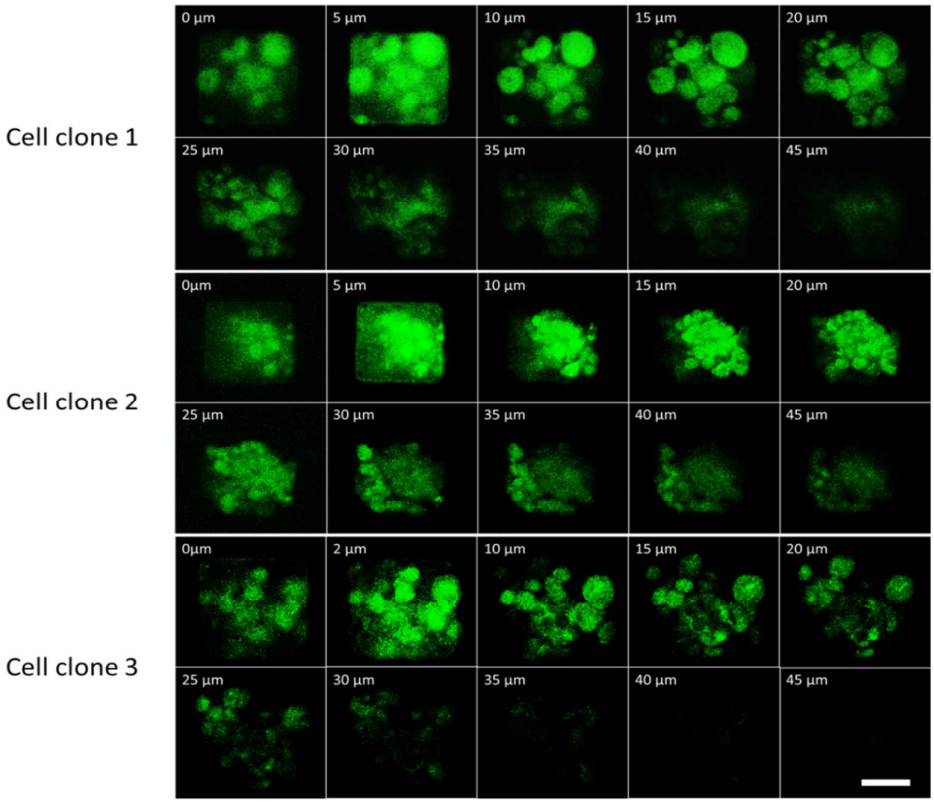

Figure S2. The z-axis confocal scanning images of three cell clones after 11-day culture. The scale bar is 50 μm.

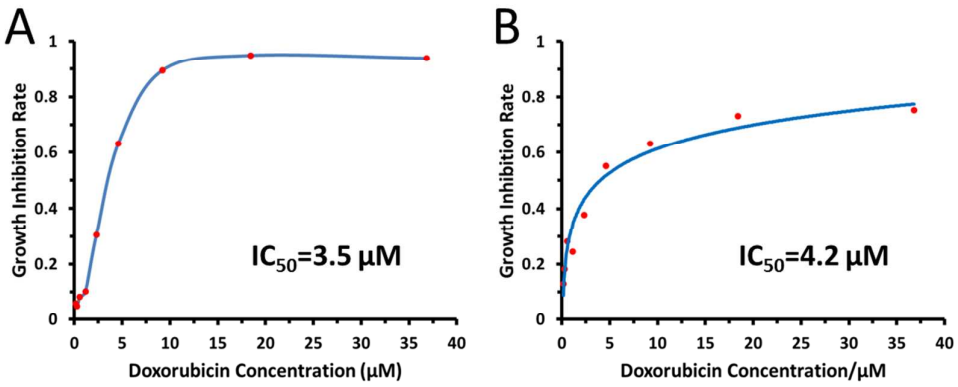

Figure S3. The IC_{50} for cell viability obtained by counting percentage of live cells in μCGA (calcein AM and PI co-staining assay, A) and from conventional MTT assay (B).