Large-scale high-throughput 3D culture, imaging, and analysis of cell spheroids using microchip-enhanced light-sheet microscopy

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Abstract: Light sheet microscopy combined with a microchip is an emerging tool in biomedical research that notably improves efficiency. However, microchip-enhanced light-sheet microscopy is limited by noticeable aberrations induced by the complex refractive indices in the chip. Herein, we report a droplet microchip that is specifically engineered to be capable of large-scale culture of 3D spheroids (over 600 samples per chip) and has a polymer index matched to water (difference < 1%). When combined with a lab-built open-top light-sheet microscope, this microchip-enhanced microscopy technique allows 3D time-lapse imaging of the cultivated spheroids with \(\sim 2.5\)-\(\mu\)m single-cell resolution and a high throughput of \(\sim 120\) spheroids per minute. This technique was validated by a comparative study on the proliferation and apoptosis rates of hundreds of spheroids with or without treatment with the apoptosis-inducing drug Staurosporine.

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1. Introduction

Three-dimensional (3D) culture preserves numerous significant cell functions that are strongly altered in monolayer cells (2D), leading to growing interest in developing techniques adapted for 3D cultures [1–5]. Among these cutting-edge developments, microchip has become an emerging technique that can generate, culture and manipulate a large number of 3D biological samples, such as embryos, organoids, spheroids and model animals [6–10]. Due to its key advantage of high-throughput culture, typically with hundreds of samples in a chip, microchip has been widely used in applications such as drug screening, cell disease modeling, and tissue engineering [11–13].

While microchip permits high-throughput cell culture, modern microscopy and microscopy-based cellular analysis support the detailed analysis of the structure and function of the cultured biological samples [14,15]. In sharp contrast to the high-throughput nature of microchip, the 3D imaging of the samples is much less efficient. For example, it usually takes several minutes to several hours for a confocal microscope to fully scan a single organoid at single-cell resolution. Therefore, large-scale scanning of hundreds of samples cultured in a microchip would require an impractically long acquisition time. Meanwhile, the low photon efficiency of confocal microscopy...
also leads to significant phototoxicity, which renders long-term live imaging impossible [16]. This challenge has been substantially addressed by the emerging light-sheet fluorescence microscopy (LSFM) technique, in which a thin laser sheet is generated to selectively illuminate a plane of the sample at the vicinity of the focal plane, thereby providing mild 3D imaging of live samples with significantly improved speed and reduced phototoxicity [17–25]. Given these technical advantages, LSFM is especially suited for being combined with microchip to develop various on-chip biomedical assays with superior efficiency compared to traditional methods. However, the major challenge regarding such a combination is that conventional orthogonal LSFM systems require special sample mounting methods to fit their orthogonal dual-objective setups, and are therefore ill-suited for the imaging of samples packed inside a flat microchip. For instance, the early implementations that combined LSFM with microchip scanned the samples while they flowed through the laser sheet and collected the fluorescence signals from the side while the laser sheet illuminated the samples from the top [26–31]. Such hybrid configurations are incompatible with multi-imaging of the same specimens. In contrast, the recent developed open-top LSFM [32–37] and single-objective LSFM [38–44] can overcome such problem. The open-top LSFM has two objectives oriented down at 45° into the sample, thus allowing both oblique excitation and collection. Single-objective LSFM utilizes one high-numerical-aperture (NA) objective for both illumination and detection, which keeps the sample normal to objective at the cost of added complexity. Both imaging modes are compatible with flat microchip on petri dish [45–47]. Unfortunately, in this case, the mismatch in refractive indices (RI) between the sample and the chip architecture becomes the major issue preventing high-resolution high-throughput imaging of 3D cell cultures on a microchip.

To minimize the aberration of RI mismatch, several strategies have been developed to improve the imaging quality, such as adaptive optics [48,49] and tissue optical clearing [50,51]. However, adaptive optics makes the system more complicated and less user-friendly and tissue optical clearing methods are mostly harmful to live cells. Also, custom sample holders can be used to minimize the material thickness or to minimize the RI difference between the material and water by using RI-matching materials, such as Sarstedt Lumox (n = 1.338) [38] and fluorinated ethylene polymer foils (FEP foils, n = 1.344) [52]. However, the RI of such materials is still more or less different to water. More importantly, neither of them is easily to mold thus cannot be used to form microchips with complex tiny structure. Fortunately, Bio 133, a new commercially available UV curable optical polymer (MY Polymers Ltd.), has a RI that exactly matches the water (n = 1.333), has been developed in recent years. More importantly, Bio 133 can be firstly poured onto chip molds and then cured under UV exposure to fabricate many designed microchips theoretically. It has been confirmed to be non-fluorescent, non-toxic and would not introduce additional optical aberrations during the optical imaging process [53], thereby has been preliminarily used in the observation of 2D cells, immobilized Drosophila tissue and C.elegans [53,54]. However, in the previous reports, their synthesized structures of Bio 133 are quite simple, and the compatibility of high-throughput 3D cultivation and imaging based on Bio 133 has not been confirmed as its properties have not been thoroughly understood.

In this study, we integrated Bio 133 into the fabrication of a micro-well-array-based 3D culture chip to realize a uniform refractive index environment (~1.333) suited for high-quality light-sheet imaging from the bottom of the chip. By combining this “aqueous chip” with a custom-made open-top LSFM, we achieved in situ time-lapse imaging of cell spheroids that are three-dimensionally cultivated inside the chip at single-cell resolution and high throughput of 120 spheroids per minute. These superior LSFM results further enable efficient cell counting of hundreds of spheroids, and comparative analysis on cell proliferation rates under normal and apoptosis drug-treated environments.
2. Methods

2.1. Droplet microchip fabrication and non-adherence treatment

A silicon master mold was first prepared using conventional photolithography (Microchem, SU8 2050). Before chip fabrication, the silicon mold was treated with trimethylchlorosilane to render its surface hydrophobic. Next, the Bio 133 polymer was cast on the mold and vacuum-treated for 30 mins to remove the bubbles. The polymer was cured for 10 min by UV light irradiation at 30 W/m², during which a cover glass and a surrounding well were used to isolate the polymer from air (Fig. S1). After that, the cured Bio 133 layer was peeled off and submerged in 100% ethanol for 7 days to completely remove the uncured residues. The cured Bio 133 layer was further washed using 75% ethanol and deionized water for 30 minutes before being attached to the glass bottom of a confocal culture dish (Biosharp, China). The chip was incubated at room temperature for two days until the layer was firmly bonded with the glass bottom.

The chip was first exposed to a UV light for 30 min for sterilization, then submerged in anti-adherence rinsing solution (Stemcell, Canada) for 30 min, during which vacuuming was performed to remove the bubbles. Finally, the chip was left alone overnight for further use.

2.2. Cell culture and seeding

A human MCF-7 colon cancer cell line was maintained on T-25 cm² flasks (Sangon Biotech, China) in a standard CO₂ incubator (Thermo, USA). The culture medium was composed of Dulbecco’s Modified Eagle medium (DMEM) containing high glucose (Gibco, Life Technologies, France) supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1% (v/v) penicillin-streptomycin (Gibco, USA). Culture medium was changed every two days.

Cells were collected with trypsin at 70% confluence. Then, a solution containing 1 × 10⁵ cells in 10 µL medium was mixed with 70 µL Matrigel (Corning, USA), which was added into a chip after removing its remaining anti-adherence solution, followed by 5 min of incubation without movement to allow cell deposition. The above steps were performed at 4 °C to prevent premature gelation of the Matrigel. Then, the Matrigel residues on the surface of the Bio 133 layer were removed and the chip was transferred upside down into a cell incubator for 30 min for Matrigel gelation. Finally, 37 °C cell culture medium was added, followed by 1-2-minutes of vacuum treatment to remove possible bubbles. Culture medium was changed every day.

2.3. 2D image analysis

For 2D image analysis, bright-field images were captured 12 h after cell seeding, and then stitched using a Fiji plugin (Grid/Collection Stitching). The area and shape index of spheroids were analyzed using Fiji’s “Analyze Particle” function.

2.4. Open-top LSFM system

An open-top LSFM system was built for high-throughput 3D imaging of cell spheroids (Fig. S2). The excitation light was transmitted to a collimator (F280APC-A, Thorlabs) by a single-mode fiber with a multi-wavelength DPSS laser (CNI). Then, the injected light passed through a beam expander (GBE02-A) and was reflected to a cylindrical lens (LJ1653RM-A, Thorlabs). Finally, a 45° light sheet was formed by a mirror of 22.5° and an achromatic doublet (AC254-045-A, Thorlabs), which were mounted on the vertical breadboard. The light sheet was used to selectively illuminate the sample. The emission light was detected by a long working distance objective (MY10X-803, Mitutoyo, NA = 0.28, WD = 34 mm) and reflected by a mirror of 22.5°, both of which were mounted on the vertical breadboard. Then, the light passed through a tube lens (TTL200-A, Thorlabs) and was collected by a sCMOS detector (PCO Panda 4.2) for imaging.
The chip was mounted onto a custom-designed holder which was connected to a high-speed motorized XY scanning stage (MLS203, Thorlabs). The spinning stage was used to adjust the orientation to ensure the long side of the chip was aligned with the scanning direction.

2.5. Cell proliferation analysis under normal and apoptosis drug-treated environments

For the analysis of cell proliferation under normal conditions, 12 h after cell seeding, a chip containing 600 spheroids was incubated with 1× SYBR green I (Solarbio, China, for live cell staining) in culture medium for 30 mins. The chip was then washed twice with PBS, followed by imaging using the open-top LSFM system at 12, 18, 24, 36 and 48 h after cell seeding. 3D cell counting was performed in 100 spheroids using Imaris software and the proliferation rate was calculated according to the following formula:

\[
\text{Cell proliferation}_{\text{current}} = \frac{\text{cell number}_{\text{current}} - \text{cell number}_{\text{initial}}}{\text{cell number}_{\text{initial}}} \times 100\%
\]

For the analysis of drug-induced cell apoptosis in spheroids, 5 μM Propidium Iodide (PI, for dead cell staining) and 1 μM Staurosporine (STS, a drug that can effectively inhibit PKC activity in cells) were added into the culture medium 12 h after SYBR green I staining. Then, the spheroids were scanned in both the SYBR Green I and PI channels at 0, 12, 24, 36 and 48 h after STS treatment. Cell counting was performed in 100 spheroids in both channels and the death rate was calculated according to the following formula:

\[
\text{Death rate} = \frac{\text{cell number}_{532\, \text{nm}}}{\text{cell number}_{488\, \text{nm}} + \text{cell number}_{532\, \text{nm}}} \times 100\%
\]

The excitation wavelengths of SYBR Green I and PI were 488 nm and 532 nm, respectively. The excitation laser power is 1.5 mW for 488 nm and 7 mW for 532 nm. The exposure time was set to 20 ms and the speed of the moving stage was set to 0.1 mm/s. The distance between adjacent frames during a scanned acquisition was 2 μm. The camera ROI size was set to 2048 × 512 pixels and the camera pixel sampling within the samples was ~50 million pixels/s.

2.6. Light-sheet image process and analysis

The data was first de-skewed by pixel shift and padding using a MATLAB algorithm (Fig. S3). 3 adjacent strips were stitched laterally using a Fiji plugin (Grid/Collection Stitching) (Fig. S4). The 3D cell counting and visualization was implemented using the Imaris software.

3. Results and discussion

3.1. Micro-well-array platform for the culture of spheroids

We designed a chip consisting of 600 regular hexagonal wells, with a height of 150 μm and inscribed circle radius of 150 μm, for droplet generation and spheroid culture. An interval of 80 μm between any two wells was set for the convenience of additional image stitching after light-sheet imaging. The size of the droplet immobilization region on a single chip was 3 mm × 8 mm.

Since Bio 133 is a non-grid material, we sealed the Bio 133 layer onto a confocal dish for support to implement the sample setup and culture. The bottom glass of the dish is 0.17 mm, with the aberrations minimized. The processes of chip fabrication, droplet generation and immobilization, as well as spheroid formation and cultivation, are shown in Fig. 1(a), and occurred in the device pictured in Fig. 1(b). The whole protocol was designed according to Bio 133’s unique properties (Fig. S5, S6).

Cell seeding was realized by cell deposition into the microwell array, which took only several minutes. During the process, to avoid premature gelation of the Matrigel, the chip was pre-cooled.
and kept at 4 °C. After complete cell deposition, the remaining suspension on the surface of the Bio 133 layer was removed while the Matrigel in the microwells was left to form individual immobilized droplet due to surface tension. The chip was then transferred into a cell incubator for Matrigel gelation. To prevent the cells from sticking to the well walls, low adherence treatment on the chip wells were performed before cell seeding, and the chip was placed upside down during the period of gelation (Fig. 1(c), 1(d) and (S3)). Finally, the chip was covered with culture medium for further culture.

As shown in Fig. 1(e), 600 spheroids could be successfully formed on the chip. Further quantitative analysis indicated that the 2D areas and the shapes of the 600 spheroids inside the chip were generally uniform (Fig. 1(f) and 1(g)).

The above results suggest that the Bio 133-based chip is capable of high-throughput in situ 3D spheroid culture. In the following sections, we demonstrate its capability of aberration-free LSFM imaging for efficient analysis of cell proliferation in hundreds of spheroids under normal conditions or following apoptosis drug treatment.
3.2. Open-top light sheet imaging system

To visualize the 3D spheroids cultivated inside the chip, we custom-built an open-top LSFM system (Fig. 2(a) and Fig. S2a). Both the illumination and detection arms were tilted 45 degrees to the horizontal plane. A chip holder was designed to be contained in the spinning stage (Fig. S4b) to permit fast alignment of the chip to the scanning direction. The thickness of the light sheet was set to 6 µm. The lateral and axial resolutions were measured to be 2.5 µm and 6 µm, respectively (Fig. S7). As shown in Fig. 2(b), when scanning the chip, the light sheet width was set to 1.3 mm, slightly larger than 1/3 the width of the droplet immobilization region. Therefore, the whole chip needed to be scanned 3 times, which only took ~5 min including the on/off time. Figure 2(c) shows a 3D reconstruction of three spheroids with single-cell resolution. Due to the good axial resolving power of this imaging strategy, the cavity structure inside the spheroid could be clearly observed. The above results confirm that the proposed imaging strategy is capable of large-scale high-throughput high-resolution in situ visualization of 3D biological specimens.

Fig. 2. The open-top LSFM system. (a) Schematic showing the open-top LSFM setup. The chip is fixed on a horizontal moving stage. The illumination and detection parts are tilted at an angle of 45° to the chip. I.O: illumination objective; D.O: detection objective; BE: beam expander; CL: cylindrical lens; TL: tube lens. (b) Illustration of light-sheet scanning process (left) and corresponding side view (right). (c) Representative 3D images of the spheroids stained with SYBR Green I, where xy and yz planes are cropped to show inner details. Yellow arrows indicate cavity structure inside the spheroid. Scale bar is 25 µm.

3.3. Large-scale spheroid scanning and quantitative analysis of cell proliferation

Using the open-top LSFM system, 600 spheroids in a chip were scanned in ~5 mins, whereas a confocal microscope would take hours. Figure 3(a) shows the xy-plane projection of the spheroids, where 3 zoomed-in regions are shown in 3D (Fig. 3(b)). The lateral and axial resolutions of LSFM were adequate to permit cell counting in spheroids. Figure 3(c) shows that the cell numbers of the 600 spheroids are normally distributed, with average and median cell numbers of ~50 at 12 h post-seeding. 3D observation provides a more complete picture of cell proliferation than 2D analyses, which can only calculate the 2D area of a spheroid. Time-lapse observation was then performed for 2 days to analyze cell proliferation (Fig. 3(d) and 3(e)). It was calculated that the average growth rates at 18, 24, 36 and 48 h post-cell seeding...
were 8%, 31%, 60% and 94% of the rate at 12 h, respectively. All the spheroids showed steady proliferation, demonstrating the biocompatibility of the cultivation and observation strategy.

3.4. Apoptosis analysis in spheroids with STS treatment

Using the high-throughput culture, imaging, and analysis strategy, cell apoptosis of spheroids treated with STS was studied. We scanned the spheroids 0, 12, 24, 36 and 48 h post-STS treatment and counted live and dead cell numbers to calculate the cell death rate. Figure 4(a) shows a representative 3D view of the spheroids, in which the green channel represents live cells, and the
red channel represents dead cells. It could be observed that low levels of cell apoptosis occurred from 0-24 h, while severe cell apoptosis occurred at 36 h after STS treatment. Figure 4(b), (c) and (d) show the time-lapse analysis of live cell number, dead cell number and death rate in 100 spheroids. The average death rates at 12, 24, 36 and 48 h were 8%, 25%, 61% and 66%, respectively. These results imply that the STS-induced apoptosis is serious but not immediate. Meanwhile, the results also indicate that our proposed strategy is effective and efficient for high-throughput drug evaluation using 3D biological specimens.

![Time-lapse imaging and analysis of spheroid apoptosis induced by STS.](image)

**Fig. 4.** Time-lapse imaging and analysis of spheroid apoptosis induced by STS. (a) Representative 3D reconstruction of spheroids 0, 12, 24, 36 and 48 h after STS treatment. The green channel and the red channel represent live cells stained with SYBR Green I and dead cells stained with PI, respectively. (b-c) Changes in cell number over time for live cells (b) and dead cells (c). (d) Changes in cell death following STS treatment. The histograms in (b-d) indicate distribution of live cell number, dead number and death rate at the end of the experiment, respectively. Scale bars, 100 µm.

### 4. Conclusion

We propose a microchip-enhanced light-sheet microscopy platform enabling large-scale, high-throughput 3D culture, time-lapse imaging and quantitative analysis of cell spheroids on an “aqueous chip”. We fabricated the first Bio 133-based micro-well-array chip with a uniform water-like refractive index, and developed specific droplet generation, immobilization, and spheroid formation methods according to Bio 133’s unique properties. To demonstrate the scale and efficiency of the platform, 600 spheroids were successfully cultured and steadily proliferated on this special chip. A lab-built open-top LSFM system was then combined with this microchip to achieve aberration-free 3D imaging of all 600 cell spheroids at single-cell resolution and within 5 minutes, yielding 1.5 billion-voxel image datasets for a subsequent imaging-based drug testing assay. Using this platform, we successfully tracked the proliferation and apoptosis of the spheroids based on time-lapse 3D imaging results, and analyzed the fate of hundreds of spheroids with or without the drug treatment. In the future work, it is possible to eliminate the glass cover slide, substituting it with a rigid RI-matching material to further improve the imaging quality. Besides, in the Open-top LSFM mode, it is possible to work with high-NA water-immersion objectives to realize even higher resolution [33]. We envision that this platform is also well
suited for the manipulation, cultivation and observation of other biological specimens, such as organoids, tissues and embryos, with notably improved efficiency compared to traditional platforms, thereby functioning as a valuable tool for various biomedical applications.

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**Data availability.** Data relating to the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

**Supplemental document.** See Supplement 1 for supporting content.

**References**


