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# Quantitative Monitoring of the Response of Tumor Spheroids Cultured in 3D Environment by Optical Coherence Tomography

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**ABSTRACT** In order to have greater clinical prediction during the early stage of drug development process, tumor spheroid culture model is generally used to investigate the drug response. Since the tumor spheroids are cultured in three-dimensional environment, quantification under optical microscope may not be objective and the process is labor intensive. As such, we developed an inverted swept-source optical coherence tomography (OCT) system to quantitatively monitor the response of tumor spheroids under tested condition. The tumor spheroids were cultured in standard 96-well microplates mounted on a special-designed platform of the system. A $3 \times 3 \times 3$ mm$^3$ OCT reconstructed image could be captured within 5 seconds to enable high throughput assay. The tumor spheroids exposed to anti-cancer drug and modified by gene silencing were respectively monitored by the OCT system. Dynamic response of the tumor spheroids could be quantitatively analyzed to study the efficiency of anti-cancer drug and gene therapy during tumor development. The proposed OCT system has the compatibility of conventional cell culture consumables and practicability of drug development process. Non-invasive and quantitative assessment could be realized for the routine analysis in biological laboratories.

**INDEX TERMS** 3D cell culture, drug development, gene therapy, tumor spheroids, optical coherence tomography.

## I. INTRODUCTION

In the development of cancer therapy, living cell culture, animal study, and clinical trial are the three major steps to be sequentially conducted before getting the listing approval of a new anti-cancer drug. Although animal study can acquire good clinical prediction and obtain important information of the drug response, but the running cost is high and the ethical regulation becomes tight nowadays. In the earliest phase of the drug development, the technique of living cell culture provides a fast and less expensive method to investigate cellular response under tested conditions. It is desired that greater clinical prediction is generated from the cell culture experiments, and shorter period of animal study is achieved for accelerating the drug development process.

Living cell culture is a conventional bio-technology that biological cells are seeded on culture vessels, e.g., Petri dish and multi-well microplate. The cells spread on solid glass or plastic surface of the vessel as a monolayer format and are cultured for studying cellular response under tested condition. This culture model has been adopted for decades and most of the research studies are based on this monolayer culture model. However, in the research of cancer chemotherapy, low clinical prediction was found to correlate to the monolayer cell culture results [1]. That might be because the monolayer culture model cannot physiologically mimic the native microenvironment. The difference between the behavior of solid tumor existing in tissue and the response of tumor cells cultured as monolayer is large. Recently, cancer biologists move to culture tumor cells in three-dimensional (3D) environment [2], [3]. The tumor cells are encapsulated and suspended in polymeric scaffold material. They are cultured and proliferate to multi-cellular spheroids, or called tumor spheroids, for mimicking *in vivo* early tumors [4], [5]. There are several significant differences between monolayer cells and tumor spheroids. For example, higher drug resistance

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and genomic change were found in tumor spheroids compared with monolayer cells [6]–[8]. As such tumor spheroid culture model is believed to bridge the gap between cell culture and live tissue [9]. It obtains greater clinical prediction in drug development and has attracted extensive attention recently [10].

In biological laboratory, analysis of the response of tumor spheroids is generally based on counting the number and size of the spheroids under optical microscope [11]. The optical imaging method is a non-invasive and non-label approach. The spheroids can be counted at preset schedule during the culture course. It is widely used currently; but the measurement result may vary among different operators. Alternatively, optical coherence tomography (OCT) has been reported to image two/three-dimensional microstructure without labelling or adding contrast agent [12], [13]. It mainly consists of an optical interferometer receiving backscattered or reflected signal from target object. By analyzing the signal, reconstructed images of the target object can be obtained. A number of clinical applications have been reported for determining blood-flow velocity [14], [15], angiography [16], [17], birefringence [18], [19], and elasticity [20], [21]. Moreover, some research groups demonstrated on applying OCT to the analysis of tumor spheroids [22]–[25]. For example, time-domain OCT was reported for imaging the growth dynamics of the tumor spheroids [22]. The spheroid volume (V) was calculated by the formula, $V = 4\pi a^2 b/3$, where $a$ and $b$ are the radius of the short and long axes of spheroid, respectively. Since the spheroid shape varies with the growth time and the cultured environment, the calculation may cause a large estimation error. Y. Jung *et al*. utilized OCT for quantitative tracking of cell death after chemotherapy and photodynamic therapy (PDT) [23]. The normalized surface-area-to-volume was calculated for the investigation of the relationship between the treatment outcome and PDT light dose. However, since the OCT system with an A-scan rate of 5 kHz took 100 seconds for each volumetric imaging, it may not suitable for high throughput screening of multi-well microplates. Additionally, because the OCT system in 800-nm regime was used for cell imaging, the imaging depth may not be enough to penetrate the entire spheroid at the late stages of cell growth. Y. Huang *et al*. developed a spectral-domain (SD) OCT system at 1310 nm with a spectral range of 110 nm for the detection of necrotic regions of the tumor spheroids [24]. The interference spectrum was detected by an InGaAs linescan camera composed of 1024 pixels with an A-scan rate of 20 kHz. However, the depth range was inversely proportional to the spectral resolution which is defined as the spectral range divided by the camera pixels. Moreover, for cell imaging with OCT, the OCT beam should penetrate through the bottom layer of microplate which ranges from 1 to 2 mm. The thickness caused the degradation of system sensitivity and limited the imaging depth range.

In this study, we developed a swept-source (SS) OCT system in the 1310-nm regime with an A-scan rate of 100 kHz with an inverted probe for cell imaging. Instead of using an InGaAs camera, the sampling points of each A-scan is twice of the SD OCT system as shown in the previous report [24]. Additionally, the sensitivity roll-off of the proposed SS OCT system was also investigated. Furthermore, in order to show the practicability of the proposed system, two experiments that are usually conducted in the drug development process were demonstrated including anti-cancer drug screening and gene therapeutic screening. In summary, the proposed OCT system has fast scan rate and high penetration depth for imaging cells/spheroids cultured in a standard culture microplate. It is compatible to the conventional cell culture equipment and consumables. It was shown to be practically used for the routine analysis of the response of tumor spheroids under tested conditions.

## II. MATERIALS AND METHODS

### A. CELLS AND FORMATION OF TUMOR SPHEROIDS

A hepatocyte-derived carcinoma cell line of Huh7 was used in this study and is a widely used cell line for the study of hepatocellular carcinoma. The cells were maintained in Petri dish by standard monolayer culture format. Culture medium of Dulbecco's modified eagle medium (DMEM, Invitrogen) was used and supplemented with 10% (v/v) fetal bovine serum (FBS; 10437028, Gibco-BRL Life Technologies) and 1% (v/v) penicillin-streptomycin (15140122, Gibco-BRL Lift Technologies). The cells were cultured in a 37 °C and 5% $CO_2$ humidified incubator (370, Thermoscientific) until confluence. To harvest the cells, 0.05% (15400054; Gibco-BRL Life Technologies) trypsin was added to the cells for 3 min. The cells were trypsinized and resuspended in the culture medium after centrifugation at 1200 rpm for 5 min.

Tumor spheroids were raised in standard 96-well microplates by liquid overlay technique [26]. The procedure is described here. A layer of hydrogel, i.e., non-adherent surface, was coated on the bottom surface of the culture well. The hydrogel was prepared by mixing 0.5% (w/v) agarose power (50004, Lonza) in culture medium. It was sterilized in autoclave at 121°C under 100 kPa for 20 min before coating. 40 $\mu$L hydrogel was applied to the culture well and maintained for 4 h. After hydrogel solidification, $3\times10^4$ cells suspended in 40 $\mu$L culture medium were respectively applied to each culture well. The cells were incubated in the incubator and gradually formed tumor spheroids on the hydrogel surface during the culture course. At preset schedule, the tumor spheroids were imaged using an inverted microscope (IX51, Olympus, Japan) mounted with a CCD camera. Subsequently, they were also scanned by the proposed OCT system. The OCT images were reconstructed and compared with the microscopic images as a reference.

### B. SETUP OF THE OPTICAL COHERENCE TOMOGRAPHY

A swept-source OCT system was developed for 3D imaging of tumor spheroids as an evaluation instrument for drug testing and gene therapy. Illustration of the system setup is shown in Fig. 1. The light source is a frequency-sweeping laser with a scan rate of 100 kHz, corresponding to an

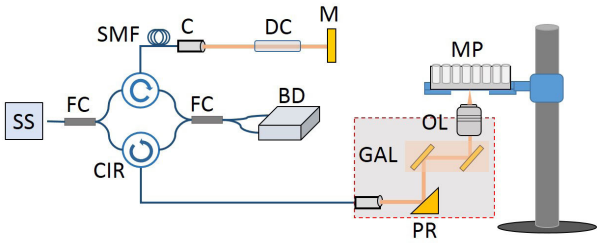


**FIGURE 1.** Illustration of the SS OCT setup with an inverted scanning head. SS: swept source, C: fiber coupler, CIR: fiber circulator, SMF: single-mode fiber, C: collimator, DC: dispersion compensator, BD: balanced detector, M mirror, PR: prism reflector, GAL: galvanometer, OL: objective lens, MP: microplate.

A-scan rate of 100 kHz. The light source was connected to a Mach-Zehnder interferometer consisting a reference and sample arms. In the sample arm, an inverted scanning head, consisting a collimator, prism reflector, and a two-axis galvanometer, was setup. A special-designed mounting platform was fabricated by a 3D printing machine for fixing standard 96-well microplates. The angle of the platform was adjustable for tilting the platform. The frame rate of the OCT system was 100 Hz and a transverse range of 3 mm was covered. The system took 5 seconds to scan a 3D image. The axial and transverse resolution of the system were 4.7 and 6.7 $\mu$m, respectively. The resolution could be further improved by using an objective lens with a higher magnification and a light source with a broader spectrum.

The tumor spheroids located on the hydrogel surface coated in the culture well. The diameter of the culture well is 6 mm for the standard 96-well microplate. The range of the OCT image was $3 \times 3 \times 3$ mm$^3$, which covered most of the volume of the culture well. A depth-dependent *en-face* image was extracted from 3D volumetric data composing of 500 B-scans. The edge of each tumor spheroid at specific depth was automatically determined by using morphological gradient method. Then, the area of each tumor spheroid at specific depth could be calculated and the volume of each tumor spheroid was obtained by summation of the areas over the entire depth range.

### C. EXPOSURE OF TUMOR SPHEROIDS TO ANTI-CANCER DRUG

To investigate the drug efficiency on tumor development, chemosensitivity of tumor spheroids is usually studied to obtain the response of drug-treated spheroids. In the current work, doxorubicin (D1515, Sigma) was used for inducing cell apoptosis and is a clinically proven drug for a wide range of cancers [27]–[29]. Tumor spheroids were raised in the culture well after 3 days of culture. Then, doxorubicin at different concentrations were respectively applied to the culture well on day 3. Depending on the drug concentration, tumor spheroids were inhibited and shrunk in the following 2 days of culture.

### D. TUMOR SPHEROIDS WITH GENE SILENCING

Tumor spheroids were raised by the cells with gene silencing in order to investigate the efficiency of gene therapy on

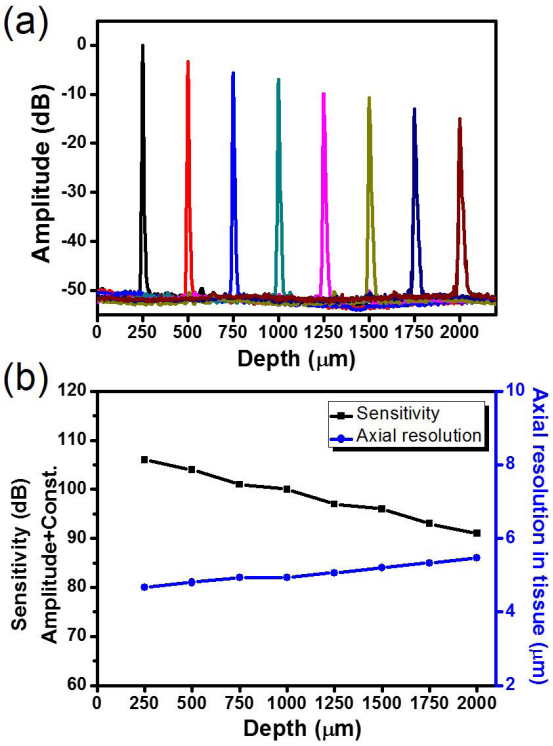


**FIGURE 2.** (a) Logarithmic plot of the measured point spread functions at the different depths. (b) Measured sensitivity and axial resolution in tissue against various OCT depths.

tumor development. siRNA targeting Glypican 3 (GPC3) (sense: 5'-CGUACUGCUUGGUCUCUUUTT-3' and antisense: 5'-AAAGAGACCAAGCAGUACGTT-3') and siRNA targeting SMARCA4 (sense: 5'-GGCUUGAUGGAACCAC GAATT-3' and antisense: 5'-UUCGUGGUUCCAUCAAGC CTG-3') were purchased from Life Technologies. Huh7 cells were seeded in 6-well microplates and cultured overnight. Then, the cells were transfected with siRNA at a concentration of 25 pmol/L using Lipofectamine®RNAiMAX Reagent according to the manufacturer's protocol. Then, the tumor spheroids were raised in the culture well up to 7 days. During the culture course, they were respectively observed by microscope and scanned by the OCT system.

## III. RESULTS AND DISCUSSION

### A. CHARACTERIZATION OF THE SWEPT-SOURCE OCT SYSTEM

The relationship between the system sensitivity and the imaging depth was investigated. The amplitude of the measured point spread functions (PSFs) at various depths is shown in Fig. 2(a). It indicated that the maximal amplitude decreased with the depth. Moreover, Fig. 2(b) represents the measured sensitives and resolutions at various OCT imaging depth. The result showed a total roll-off of 15 dB was measured over a depth range of 2 mm and the axial resolution degraded from 4.7 $\mu$m to 5.5 $\mu$m. Here, the refractive index of culture medium was assumed to be 1.5. The imaging contrast can be further improved when a culture medium with a higher refractive index is used.

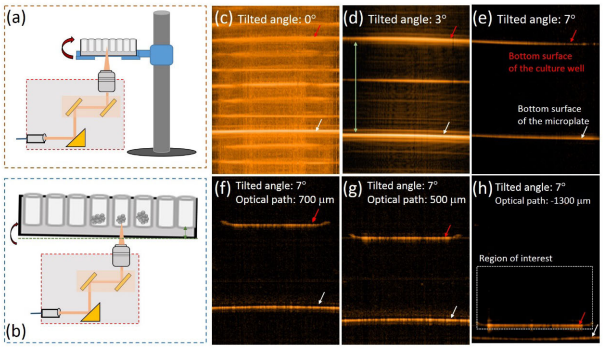


**FIGURE 3.** (a) Setup of the sample arm with a tiltable mount. (b) Schematic diagram for OCT imaging tumor spheroids in the culture wells. (c)-(e) 2D OCT images of microplate obtained at the angles of 0°, 3°, and 7°, respectively. (f)-(g) 2D OCT images of microplate by changing the optical path length in the reference arm with a tilted angle of 7°. The white and red arrows indicate the bottom surface of the microplate and the bottom surface of the culture well, respectively. The green arrow shows the thickness between both surfaces. The white square represents the region of interest.

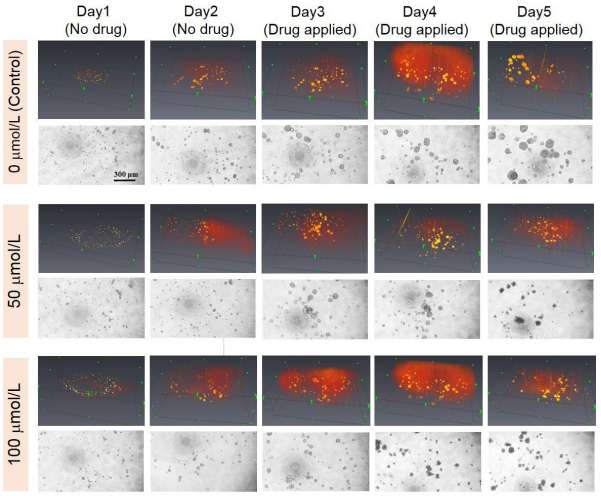


**FIGURE 4.** Microscopic images and OCT images of the tumor spheroids respectively exposed to doxorubicin at different concentrations of 0 (control), 50, and 100 $\mu$mol/L. The tumor spheroids were initially raised on the hydrogel surface in the beginning 3 days of culture. Then, doxorubicin was applied on day 3. The tumor spheroids were continuously cultured for the following 2 days.

In this study, the OCT system was implemented for evaluating the response of the tumor spheroids under tested condition. Since tumor spheroids were cultured with culture medium in the culture well of a microplate, the OCT beam was incident from the bottom surface of the microplate and penetrated into the well, as shown in Fig. 3(a). The thickness of our microplate is 1.2 mm, probably leading to a poor imaging sensitivity. From Fig. 2(b), it can be noted that the sensitivity was decreased to 97 dB at the depth of 1250 $\mu$m, which was not the best imaging sensitivity (106 dB). A strong backscattered intensity from the bottom surface of the microplate caused severe signal saturation, resulting in artifacts and blurring of the OCT images. To solve this problem, the specially designed mount for the microplate was fixed on another kinematic mount which can provide different tilted angles. The design is illustrated in Fig. 3(b). The results showing in Fig. 3(c)-3(h) are the 2D OCT results of the microplate without culture medium and cells. The white and red arrows indicate the bottom surface of the microplate and the bottom surface of the culture well, respectively. The green arrow represents the thickness between two surfaces. In Fig. 3(c), the optical beam was orthogonally incident on the bottom surface of the microplate. Signal saturation and image artifacts were resulted in the image. When the microplate was tilted by an angle of 3°, the artifacts were reduced much, as shown in Fig. 3(d). Until the angle of 7° was applied, the artifacts could be completely removed. Thus, the strong back reflection can be suppressed by tilting the microplate, as shown in Fig. 3(e). Moreover, to further improve the sensitivity degradation due to the thickness between two surfaces, the optical path length in the reference arm was adjusted to maximize the system sensitivity in the region of interest (above the bottom surface of the culture well). Fig. 3(f)-(h) represent the OCT images with different optical paths of 700, 500, and $-130$ $\mu$m in the reference arms with a tilted angle of 7°. In Fig. 3(h), the white arrow shows the mirror image of the bottom layer of the microplate. In this case, the system sensitivity could be maximized in the region of interest and the mirror image did not cause artifacts in the region of interest as indicated by the white square in Fig. 3(h). Therefore, a tilted angle of 7° and an optical path of $-1300$ $\mu$m applied in Fig. 3(h) were set for the following experiments.

### B. QUANTITATIVE MONITORING OF TUMOR SPHEROIDS EXPOSED TO ANTI-CANCER DRUG

Because the drug response of tumor spheroids was found to be drastically different compared to the cells in monolayer format [30], tumor spheroids were initially raised on the hydrogel surface in the beginning 3 days of culture. The cells with the diameter of around 10 $\mu$m were seeded on the hydrogel coated in the culture well on day 0. They proliferated and gradually formed tumor spheroids on the hydrogel surface. After 3-day culture, the diameter of partial tumor spheroids reached over 100 $\mu$m. Then, culture medium containing doxorubicin at different concentrations of 0 (control), 50, and 100 $\mu$mol/L were respectively applied to the culture wells and then continuously cultured for the following 2 days. Microscopic images and OCT images of the tumor spheroids were captured and are shown in Fig. 4. From the microscopic images, dose-dependent effect could be observed to show doxorubicin inhibited cell proliferation and induced cell apoptosis in the tumor spheroids. Basically, the efficacy of doxorubicin could be confirmed by the qualitative result. To investigate the dynamic response of the drug-treated tumor spheroids quantitatively, average volume of the spheroids could be extracted from the OCT images. The result is shown in Fig. 5. For the drug concentration of 50 $\mu$mol/L, the spheroid volume maintained similar level on day 3, 4 and 5. It indicated the growth of the tumor

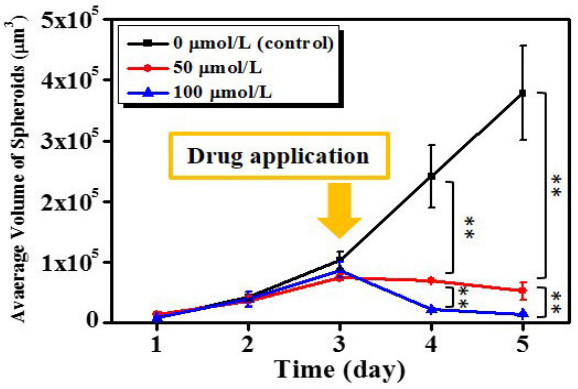


**FIGURE 5.** Dynamic response of the drug-treated tumor spheroids. The data were extracted from the OCT images and present as mean ± standard error from more than three repeated experiments. The *p*-values were calculated by unpaired *t*-test and represented by ** for $p < 0.01$.

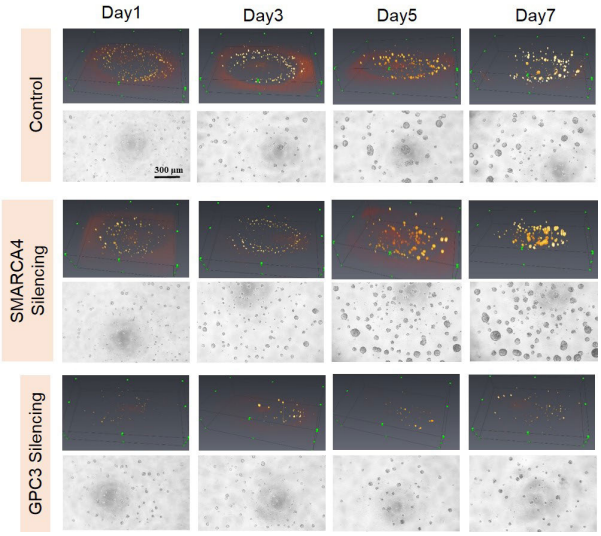


**FIGURE 6.** Microscopic images and OCT images of the tumor spheroids without gene silencing (control), with SMARCA4 silencing, and with GPC3 silencing, respectively. The tumor spheroids were cultured for 7 days.

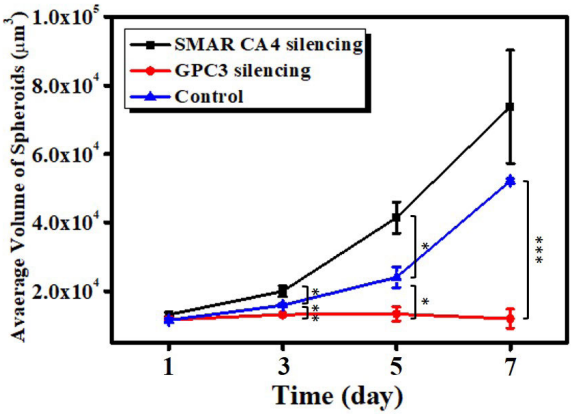


**FIGURE 7.** Dynamic response of the tumor spheroids with and without gene silencing. The data were extracted from the OCT images and present as mean ± standard error from more than three repeated experiments. The *p*-values were calculated by unpaired *t*-test and represented by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

spheroids was inhibited by such a drug dosage. On the other hand, the spheroid volume significantly decreased after the application of 100 $\mu$mol/L drug. Cell apoptosis was induced and the spheroids shrunk under high dosage of drug. The OCT technique can provide further quantitative and objective information to describe the dynamic response of the drug-treated tumor spheroids in a non-invasive and non-label approach.

### C. QUANTITATIVE MONITORING OF TUMOR SPHEROIDS WITH GENE SILENCING

To evaluate the efficacy of gene therapy, RNA interference is commonly used to examine the biological and molecular effects of specific gene suppression. For example, GPC3 is a member of the family of glypican heparin sulfate proteoglycans and is a potential therapeutic target in hepatocellular carcinoma. Some studies reported suppression of GPC3 reduces growth of hepatocellular carcinoma cells [31], [32]. Moreover, SMARCA4 gene was found to be a cancer suppressor and significantly correlates with cancer progression in hepatocellular carcinoma patients [33], [34]. In this study, cells with GPC3 and SMARCA4 silencing were respectively prepared and seeded on the hydrogel coated in the culture wells. The cells were cultured and gradually formed tumor spheroids in a 7-day culture course. Microscopic images and OCT images of the tumor spheroids were captured and are shown in Fig. 6. The experiment of the tumor spheroids without gene silencing was set as control group. Comparing to the control group, the tumor spheroids with SMARCA4 silencing had a faster proliferation rate and inhibition of the tumor spheroids with GPC3 silencing was observed obviously. Furthermore, the dynamic response of the tumor spheroids with gene silencing was quantitatively investigated by the OCT technique and the result is shown in Fig. 7. Starting from day 3, significant differences were found between the tumor spheroids with and without gene silencing. That indicated SMARCA4 and GPC3 genes highly influence to the tumor development. The proposed OCT system provides a quantitative tool for tumor spheroid assay in conventional biological laboratories.

## IV. CONCLUSION

A swept-source OCT system was developed and demonstrated on quantitative monitoring the response of tumor spheroids in 3D environment. The OCT system was with a scan rate of 100 kHz and took 5 seconds to scan a $3 \times 3 \times 3$ mm$^3$ image. Moreover, a special inverted probe was designed for mounting standard microwell plate. These developments targeted at achieving high throughput screening and fitting the conventional cell culture consumables. Investigations of the tumor spheroids exposed to anti-cancer drug and modified by gene silencing were respectively demonstrated to show the practicability of the proposed system. Dynamic response of the tumor spheroids under tested

condition could be quantitatively monitored and analyzed by the OCT technique. The proposed system provides an accurate and informative assessment for achieving greater clinical prediction and accelerating the drug development process.

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